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(54) Title: METHODS AND COMPOSITIONS OF USE OF CD8+ TUMOR INFILTRATING LYMPHOCYTE SUBTYPES AND GENE SIGNATURES THEREOF

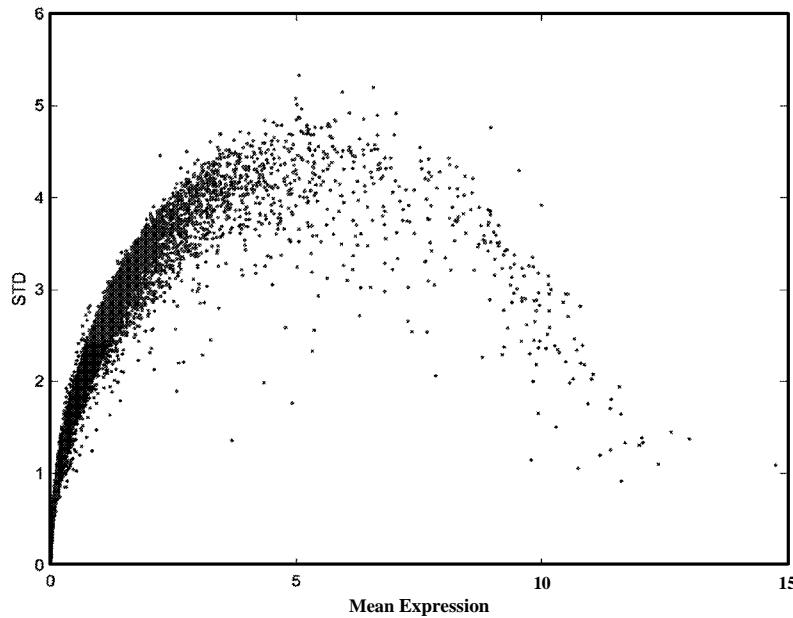


FIG. 1

(57) Abstract: The subject matter disclosed herein is generally directed to CD8+ tumor infiltrating lymphocytes comprising gene signatures associated with response to immunotherapy treatment. Moreover, the subject matter disclosed herein is generally directed to methods and compositions for use of the gene signatures. Specifically, disclosed herein are gene signatures associated with response to checkpoint blockade therapy and immune cell subtypes characterized by said gene signatures. Further disclosed are methods of using said gene signatures and immune cell subtypes. Further disclosed are pharmaceutical compositions comprising populations of CD8+ TILs enriched for a specific subtype.

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METHODS AND COMPOSITION OF USE OF CD8+ TUMOR INFILTRATING LYMPHOCYTE SUBTYPES AND GENE SIGNATURES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application Nos. 62/505,101, filed May 11, 2017 and 62/574,878, filed October 20, 2017. The entire contents of the above-identified applications are hereby fully incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under Grant No. CA208756 awarded by the National Institutes of Health. The government has certain rights in the invention.

TECHNICAL FIELD

[0003] The subject matter disclosed herein is generally directed to CD8+ tumor infiltrating lymphocytes comprising gene signatures associated with response to immunotherapy treatment and overall survival. Moreover, the subject matter disclosed herein is generally directed to methods and compositions for use of the signature genes.

BACKGROUND

[0004] The development of antibodies that effectively block the activities of immune checkpoint proteins, including CTLA4, PD-1 or its ligand, PD-L1¹, has led to their approval by the FDA for treating a wide variety of cancers, including melanoma, non-small-cell lung carcinoma (NSCLC), renal cell carcinoma (RCC), urothelial bladder cancer (UBC), head and neck squamous cell carcinoma (HNSCC), refractory Hodgkin's lymphoma (HL), and most recently hepatocellular carcinoma (HCC) and gastric cancer². In melanoma, despite the high response rate (-20% for anti-CTLA4, -45% for anti-PD-1, -60% for anti-PD-1+anti-CTLA4)³⁴, most patients are refractory to therapy or acquire resistance, and eventually succumb to disease. Thus, identification of the key components that drive or prevent effective responses to checkpoint therapy remains an urgent need for accelerating progress in the fields of cancer immunotherapy, and perhaps, medical oncology.

[0005] Checkpoint therapies are designed to overcome the inhibition of antigen-specific, effector T lymphocytes (T-cells) by the tumor or the immune microenvironment. Thus, the state and number of these cells, especially CD8⁺ cytotoxic T-cells are likely to determine the

clinical outcome. Indeed, the number of infiltrating CD8⁺ T-cells detected before ⁵ or during early treatment ⁶ have been shown to be associated with clinical outcome. The ability of these CD8⁺ T-cells to target the malignant cells is dampened by persistent exposure to stimulation and co-inhibition by checkpoint proteins, resulting in a state of exhaustion ^{7,8}, characterized by the expression of multiple co-inhibitory receptors on the T-cell surface (e.g. PD1, CTLA4, TIM3, TIGIT), unique regulators of gene expression (*BATF*, *PRDM1*), and most importantly, dysfunctional effector activity. Additionally, the efficiency of checkpoint therapy depends on CD8⁺ T-cell recognition of neoantigens presented on human leukocyte antigen (HLA) class I by tumor cells ^{9,10}. Hence, a deeper understanding of the cellular and molecular determinants of response are needed.

[0006] To date, several factors have been analyzed for their association with tumor growth and clinical outcome in patients. These include levels of PD-L1 protein ⁴⁻¹¹, load of tumor-derived neoantigens ¹², defects in antigen presentation and IFNg pathways ¹³⁻¹⁶, abundance of partially exhausted CD8⁺ T-cells in the tumor ¹⁷, proportion of suppressive myeloid cells in the blood ¹⁸, and the magnitude of T-cell reinvigoration in relation to pretreatment tumor burden ¹⁹. While these studies have collectively contributed to the model explaining the efficacy of checkpoint therapy, their major limitations include low predictive power and the use of pre-defined immune markers, limiting their ability to identify optimal and novel components that explain or predict clinical outcomes. Thus, there is a need to more systematically identify markers and mechanisms associated with response to therapy.

[0007] Citation or identification of any document in this application is not an admission that such document is available as prior art to the present invention.

SUMMARY

[0008] In one aspect, the present invention provides for a method of detecting a checkpoint blockade (CPB) therapy responder gene signature comprising, detecting in CD45+ cells obtained from a biological sample the expression of a gene signature comprising one or more genes or polypeptides selected from the group consisting of: TCF7; or TCF7, PLAC8, LTB, and CCR7; or TCF7, LEF1, S1PR1, PLAC8, LTB, CCR7, IGHD, PAX5, FCRL1, FCER2, CD19, CD22, BANK1, MS4A1, BLK, RALGPS2 and FAM129C; or TCF7, PLAC8, LTB, LY9, SELL, IGKC and CCR7.

[0009] In another aspect, the present invention provides for a method of detecting a checkpoint blockade (CPB) therapy responder gene signature comprising, detecting in CD8+

T cells obtained from a biological sample the expression of a gene signature comprising one or more genes or polypeptides selected from the group consisting of: TCF7; or TCF7 and IL7R; or TCF7, IL7R, FOSL2, REL, FOXPI, and STAT4; or TCF7, PLAC8, LTB, and CCR7; or TCF7, LEF1, S1PR1, PLAC8, LTB, and CCR7; or TCF7, IL7R, GPR183, and MGAT4A; or TCF7, IL7R, GPR183, LMNA, NR4A3, CD55, AFM1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N and CSRNP1; or TCF7, IL7R, GPR183, LMNA, NR4A3, CD55, AFM1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N, CSRNP1, FAM65B, PIK3R1, RGPD6, SKIL, TSC22D2, USP36, FOXPI, EGRI, MYADM, ZFP36L2, FAM102A, RGCC, PDE4B, PFKFB3, FOSB, DCTN6 and BTG2; or CD8_G genes listed in Table 6.

[0010] In certain embodiments, the CD8 T cells having a responder signature does not express ENTPD1 (CD39) and HAVCR2.

[0011] In another aspect, the present invention provides for a method of detecting a checkpoint blockade (CPB) therapy non-responder gene signature comprising, detecting in CD45+ cells obtained from a biological sample the expression of a gene signature comprising one or more genes or polypeptides selected from the group consisting of: ENTPD1 and HAVCR2; or CCL3, CD38 and HAVCR2; or CD38, PDCD1, CCL3, SNAP47, VCAM1, HAVCR2, FASLG, ENTPD1, SIRPG, MY07A, FABP5, NDUFB3, UBE2F, CLTA and SNRPD1; or FASLG, VCAM1, CCL3, LAG3, CXCR6, IFNG, PDCD1, KLRD1, HAVCR2, SIRPG, SNAP47, DTHD1, PRF1, GZMH, F2R, CD38, CXCL13, TNFRSF4, TNFRSF18, MAF, ETV7, CD4, CTLA4, FCRL6, SPON2, KLRG1, TRGC1, A2M, FCGR3A, GZMA, HOPX, NKG7, PXN, TNFRSF9, GEM, NABI, DFNB31, CADMI, CRTAM, GPR56, MY07A, DUSP4, METRNL and PHLDA1; or LAYN, GEM, VCAM1, RDH10, TNFRSF18, FAM3C, AFAP1L2, KIR2DL4, MTSS1, ETV1, CTLA4, MY07A, ENTPD1, TNFRSF9, CADMI, DFNB31, CXCL13, HAVCR2, GPR56, GOLFM4, NABI, PHLDA1, TGIF1, SEC14L1, IGFLR1, NAMPTL, PAM, HSPB1, TNIP3, BPGM, TP53INP1, TRPS1, UBE2F, NDFIP2, PON2, PELII, METRNL, SNAP47 and APLP2; or CCL3, LGALS1, CD38, EPSTI1, WARS, PLEK, HAVCR2, LGALS3, FABP5, MT2A, GBP1, PLSCR1, CCR5, GSTO1, ANXA5, GLUL, PYCARD, TYMP, IFI6, VAMP5, OASL, GZMB, TXN, SQRDL, RHOC, AP2S1, GZMH, CCL4L2, SNAP47, LAP3, ATP6V1B2, CCL4L1, LAMP2, PSMA4, SERPINB1, HIGD1A, UBE2F, TALDO1, CD63, CLTA, S100A11, PHPT1, GBP4, PRDX3, PSMB2, BST2, GBP5, CTSC, NDUFB3, NPC2, GALM, GLIPR2, CCL4, PRF1, IFNG, IFI30, CHST12, ISG15, MYD88, IDH2, MTHFD2, CHMP2A,

NDUFA9, CHMP5, CALM3, ANXA2, PPT1, GTF3C6, NDUFAB1, CXCR6, RNF181, LGALS9, COX5A, OAS2, PDCD1, SNRPC, BHLHE40, TWF2, SLAMF7, TXN2, CARD 16, ANAPCI 1, MRPL51, LIMS1, NDUFA12, RANBP1, GBP2, PSMC1, ACTR1A, CD2BP2, VDAC1, EMC7, MX1, GPS1, ATP5J2, USMG5, SHFM1, ATP5₁, FAM96A, CASP1, PARP9, NOP10, GNG5, CYC1, RAB1 1A, PGAM1, ENTPD1, PDIA6, PSMC3, TMBIM1, UBE2L6, PSMA6, EIF6, DCTN3, SEC1 1A, CSTB, ETFB, DBI, GRN, ELOVL1, UBE2L3, PSMB3, NDUFB7, DOK2, SEC61G, IGFLR1, ATP5H, COPZ1, ATP6V1F, BNIP3L, NUTF2, AKRIAI, MDH2, VAMP8, ROMO1, CXCR3, SAMHD1, NUCB1, ACTN4, ZYX, FLOT1, BLOC1S1, STAT1, VFMP, PAM, NUDT21, MYOIG, C17orf49, GTF2A2, HIST2H2AA4, C19orf10, ABI3, TRAPPC5, PSMC4, NDUFC2, HN1, SNRPD3, CMC1, RAB27A, NDUFA6, POMP, PFKP, ATP5G3, TMEM179B, PSMD9, IRF7, CNIH1, DYNLRB1, APOL2, TKT, DCTN2, GSDMD, STOM, CTSD, KDELR2, ATP5J, RPS27L, PSME2, DRAPI, NDUFBIO, DECR1, GSTP1, TMED9, MGAT1, HSPB1, COX8A, ZEB2, ILK, PSMB6, HK1, CD58, TMX1, GZMA, SRI, PSMG2, ARL8B, NKG7, GPX1, ACP5, CHP1, GPR171, ATP6V0B, KLRD1, H2AFY, PPM1G, PRDX5, PSMA5, FBXW5, ATP6AP1, CD4, SNRPD1, XAF1, LY6E, DYNLT1, AK2, PSMA2, YIPF3, S100A10, SCP2, MRPS34, PSMD4, CDC123, BTG3, TMEM258, TSPO, SDHB, TCEB1, WDR830S, HCST, NAA10, CTSB, YARS, GLRX, RBCK1, RBX1, LAMTOR1, UQCRCFS1, NDUFB4, CAPZA2, BRK1, ADRM1, NDUFB2, ETFA, VDAC3, NUDT5, IFITM3, BANF1, ZNHIT1, CAPG, NHP2, LASP1, TOMM5, MVP, CTSW, AURKAIP1, RARRES3, PSMB10, TMEM173, SLX1A, APOBEC3G, GFMAP4, EIF4E, CTLA4, NDUFS8, CYB5B, PIK3R5, HEXB, STXBP2, PSMD8, SEC61B, RGS10, PHB, ATP5C1, ARF5, SUM03, PRDX6, RNH1, ATP5F1, UQCRC1, SARNP, PLIN2, PIN1, SDHC, SF3B14, CAPRINI, POLR2G, COX7B, UQCR10, FBX07, NDUFB6, S100A4, PRELID1, TRPV2, SF3B5, MYOIF, SCAMP2, RNF7, CXCL13, RAB1B, SHKBP1, PET100, HM13, VTI1B, S100A6, ARPC5, FDPS, MINOS 1, RABIO, NEDD8, BATF, PHB2, ERH, NCOA4, PDIA4, PSMB9, C11orf48, TMEM50A, TIGIT, NDUFA1 1, NELFE, COX6C, SLA2, PSMB8, NDUFS7, RER1, RAB8A, CAPN1, MRPL20, COX5B, SEC13, FKBP1A, PRDMI, RABIA, RHOG, CYB5R3, AIP, ABRACL, PSMB7, COX6B1, PSMD7, PPA1, PCMT1, SURF4, ENY2, TCEB2, MAP2K3, AL353354.2, AKIRIN2, MAPRE1, GRSF1, DUSP4, ATG3, SRGAP2, ATP6V0D1, NELFCD, LRPAP1, C14orf166, SNRPB2, CHMP4A, SFT2D1, CASP4, NME1-NME2, FAM96B, FDFT1, SLC25A39, LMAN2, MDHI, RHBDD2, ARPC5L, TBCA, EBP, SEC14L1, EIF2S2, CST7, STARD7, SOD2, SPN, FAM32A, SEC1 1C,

TNFRSF1B, POLR2E, NDUFA13, OSTC, UFCI, C18orf32, SRP19, C14orf2, UQCR1 1, PDCD6, AP2M1, PPP1CA, ATP6AP2, SSR3, UNCI 3D, FERMT3, ARHGAPI, EIF3I, CECR1, MRPS6, DNPHI, DCXR, PSMF1, SNRPG, CNDP2, ANXA1 1, SLM02, C16orf13, CAPN2, BSG, LAMTOR5, SIVA1, TRAPPCL, TMCO1, PSMD13, PSMB1, RSU1, NDUFA1, TUBB, DCTN1, SH3GLB1, BCAP31, RTFDC1, UFD1L, GPI, DNAJB11, SNX17, SH2D2A, Clorf43, BUD31, PSTPIP1, CTSA, TPST2, MPV17, APMAP, CMC2, UQCRQ, TBCB, C9orf16, PARK7, ATP5EP2, SHISA5, SMC4, TAPI, SCANDI, SIRPG, HDLBP, EMC4, FIS1, TPI1, GOLGA7, POLR2J, EIF2S1, UBA3, P4HB, UQCRH, CSNK2B, SZRD1, NDUFA3, ATP50, DERL2, COPS6, COPE, SNX6, FLU and ERGIC3.

[0012] In another aspect, the present invention provides for a method of detecting a checkpoint blockade (CPB) therapy non-responder gene signature comprising, detecting in CD8+ T cells obtained from a biological sample the expression of a gene signature comprising one or more genes or polypeptides selected from the group consisting of: ENTPD1 and HAVCR2; or CCL3, CD38 and HAVCR2; or CD38, CCL3, VCAMI, GOLEVI4, HAVCR2, PRDX3, ENTPD1, PTTG1, CCR5, TRAFD1, PDCD1, CXCR6, BATF, PTPN6, LAG3 and CTLA4; or LAYN, GEM, VCAMI, RDH10, TNFRSF18, FAM3C, AFAP1L2, KIR2DL4, MTSSI, ETV1, CTLA4, MY07A, ENTPD1, TNFRSF9, CADMI, DFNB31, CXCL13, HAVCR2, GPR56, GOLIM4, NAB1, PHLDA1, TGIF1, SEC14L1, IGFLR1, NAMPTL, PAM, HSPB1, TNIP3, BPGM, TP53INP1, TRPS1, UBE2F, NDFIP2, PON2, PELII, METRNL, SNAP47 and APLP2; or CD38, EPSTI1, GOLFM4, WARS, PDCD1, CCL3, SNAP47, VCAMI, SKA2, HAVCR2, LGALS9, PRDX3, FASLG, ENTPD1, FABP5, SIRPG, LSM2, NDUFB3, TRAFD1, UBE2F, NMI, IFI35, CLTA, MTHFD1, MY07A, IFI27L2, MCM5, STMNI, ID3, RGS3, SNRPD1, PTTG1 and FIBP; or CD8_B genes listed in Table 6.

[0013] In certain embodiments, the biological sample is a tumor sample obtained from a subject in need thereof. In certain embodiments, the gene signature is detected in tumor infiltrating lymphocytes (TILs). In certain embodiments, the biological sample comprises *ex vivo* or *in vitro* immune cells, preferably CD8+ T cells. In certain embodiments, the gene signature is detected by deconvolution of bulk expression data such that gene expression in immune cells is detected.

[0014] In certain embodiments, detecting a higher proportion immune cells expressing a responder signature as compared to a non-responder signature indicates sensitivity to checkpoint blockade (CPB) therapy and an increased overall survival, and wherein detecting

a higher proportion immune cells expressing a non-responder signature indicates resistance to checkpoint blockade (CPB) therapy and a decreased overall survival. In certain embodiments, detecting a higher proportion of TCF7+CD8+ as compared to TCF7-CD8+ T cells indicates sensitivity to checkpoint blockade (CPB) therapy and an increased overall survival, and wherein detecting a higher proportion TCF7-CD8+ as compared to TCF7+CD8+ T cells indicates resistance to checkpoint blockade (CPB) therapy and a decreased overall survival. In certain embodiments, TCF7+CD8+ and TCF7-CD8+ T cells are detected by immunofluorescence. In certain embodiments, the checkpoint blockade (CPB) therapy comprises anti-CTLA4, anti-PD-L1, anti-PD1 therapy or combinations thereof.

[0015] In another aspect, the present invention provides for a method of predicting cancer clinical outcome in a subject in need thereof comprising detecting in a sample obtained from the subject the ratio of immune cells enriched for expression of a gene signature according to any of claims 1 to 3 as compared to immune cells enriched for expression of a gene signature according to claims 4 or 5, wherein a ratio greater than one indicates sensitivity to an immunotherapy and an increased overall survival, and wherein a ratio less than one indicates resistance to an immunotherapy and a decreased overall survival.

[0016] In another aspect, the present invention provides for a method of predicting cancer clinical outcome in a subject in need thereof comprising detecting in a sample obtained from the subject the ratio of TCF7+CD8+ to TCF7-CD8+ T cells, wherein a ratio greater than one indicates sensitivity to an immunotherapy and an increased overall survival and wherein a ratio less than one indicates resistance to an immunotherapy and a decreased overall survival. In certain embodiments, TCF7+CD8+ and TCF7-CD8+ T cells are detected by immunofluorescence.

[0017] In certain embodiments, the method further comprises detecting mutations associated with loss of antigen presentation in tumor cells obtained from the subject, wherein detecting a mutation associated with loss of antigen presentation indicates resistance to an immunotherapy and a decreased overall survival. In certain embodiments, the mutations result in the loss of one or more genes or polypeptides selected from the group consisting of B2M, ULA-A, ULA-B, and ULA-C. In certain embodiments, predicting cancer clinical outcome is performed before, after or during treatment with a checkpoint blockade (CPB) therapy.

[0018] In another aspect, the present invention provides for a method of enriching for memory/effector CD8+ T cells comprising sorting for CD8+ T cells lacking expression of ENTPD1 and HAVCR2 and/or lacking expression of CD38.

[0019] In another aspect, the present invention provides for a method of enriching for exhausted CD8+ T cells comprising sorting for CD8+ T cells that express ENTPD1 and HAVCR2 and/or express CD38.

[0020] In certain embodiments, the cells are sorted using antibodies specific to ENTPD1 and HAVCR2 and/or CD38.

[0021] In another aspect, the present invention provides for a population of CD8+ T cells, wherein the population of cells comprises CD8+ T cells that lack expression of ENTPD1 and HAVCR2 and/or CD38. The population of cells may be depleted for CD8+ T cells that express ENTPD1 and HAVCR2 and/or CD38. The population of cells may be enriched for CD8+ T cells that lack expression of ENTPD1 and HAVCR2 and/or CD38.

[0022] In another aspect, the present invention provides for a population of CD8+ T cells, wherein the population of cells comprises cells having a responder gene signature according to any of claims 1 to 3. The population of cells may be depleted for cells having a non-responder gene signature according to claims 4 or 5. The population of cells may be enriched for cells having a responder gene signature according to any of claims 1 to 3. The population of cells may express a chimeric antigen receptor (CAR) or an endogenous T cell receptor (TCR). The population of cells may comprise CD8+ T cells obtained from a subject suffering from cancer.

[0023] In certain embodiments, the population of CD8+ T cells are modulated to decrease activity or expression of one or more genes or polypeptides selected from the group consisting of: ENTPD1 and HAVCR2; or CCL3, CD38 and HAVCR2; or CD38, CCL3, VCAM1, GOLIM4, HAVCR2, PRDX3, ENTPD1, PTTG1, CCR5, TRAFD1, PDCD1, CXCR6, BATF, PTPN6, LAG3 and CTLA4; or CD38, EPSTI1, GOLIM4, WARS, PDCCD1, CCL3, SNAP47, VCAM1, SKA2, HAVCR2, LGALS9, PRDX3, FASLG, ENTPD1, FABP5, SIRPG, LSM2, NDUFB3, TRAFD1, UBE2F, NMI, IFI35, CLTA, MTHFD1, MY07A, IFI27L2, MCM5, STMN1, ID3, RGS3, SNRPD1, PTTG1 and FIBP; or CD8_B genes listed in Table 6.

[0024] In certain embodiments, the population of CD8+ T cells are modulated to increase activity or expression one or more genes or polypeptides selected from the group consisting of: TCF7; or TCF7 and IL7R; or TCF7, IL7R, FOSL2, REL, FOXP1, and STAT4; or TCF7,

PLAC8, LTB, and CCR7; or TCF7, LEF1, S1PR1, PLAC8, LTB, and CCR7; or TCF7, IL7R, GPR183, and MGAT4A; or TCF7, IL7R, GPR183, LMNA, NR4A3, CD55, AIM1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N and CSRNP1; or TCF7, IL7R, GPR183, LMNA, NR4A3, CD55, AIM1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N, CSRNP1, FAM65B, PIK3R1, RGPD6, SKIL, TSC22D2, USP36, FOXP1, EGR1, MYADM, ZFP36L2, FAM102A, RGCC, PDE4B, PFKFB3, FOSB, DCTN6 and BTG2; or CD8_G genes listed in Table 6.

[0025] In certain embodiments, the one or more genes are modulated with a genetic modifying agent. In certain embodiments, the population of cells comprises activated T cells. In certain embodiments, the population of cells comprises T cells activated with tumor specific antigens. In certain embodiments, the tumor specific antigens are subject specific antigens.

[0026] In another aspect, the present invention provides for a pharmaceutical composition comprising the population of cells according to any embodiment herein.

[0027] In another aspect, the present invention provides for a method of treating cancer in a subject in need thereof comprising administering an inhibitor of CD39 and an inhibitor of TFM3 or an inhibitor of CD39 and an inhibitor of PD1. The inhibitor of TIM3 may comprise anti-TIM3 antibodies or the inhibitor of PD1 may comprise anti-PD1 antibodies. The inhibitor of CD39 may comprise POM-1.

[0028] In another aspect, the present invention provides for a method of treating cancer in a subject in need thereof comprising: predicting cancer clinical outcome in the subject according to any of claims 14 to 19; and treating the subject, wherein responders are treated with an immunotherapy comprising checkpoint blockade (CPB) therapy, wherein non-responders are treated with: adoptive cell transfer and optionally checkpoint blockade (CPB) therapy; or an inhibitor of CD39 and an inhibitor of TIM3; or an inhibitor of CD39 and an inhibitor of PD1; or an agent capable of targeting, inhibiting or depleting CD8+ TILs having said non-responder signature and optionally checkpoint blockade (CPB) therapy; or an agent capable of activating, maintaining or increasing CD8+ TILs having said responder signature and optionally checkpoint blockade (CPB) therapy, or wherein non-responders comprising tumors not capable of presenting antigens are treated with a therapy other than checkpoint blockade (CPB) therapy.

[0029] In certain embodiments, the adoptive cell transfer comprises: autologous T cells having the responder signature; or autologous T cells specific against tumor antigens, having

the responder signature; or autologous T cells transduced with T cell receptors targeting tumor antigens, having the responder signature; or autologous CAR T cells having the responder gene signature; or allogenic T cells having the responder signature; or allogenic T cells specific against tumor antigens, having the responder signature; or allogenic T cells transduced with T cell receptors targeting tumor antigens, having the responder signature; or allogenic CAR T cells having the responder gene signature. In certain embodiments, the autologous T cells are obtained from the subject and cells having the non-responder signature are depleted and/or cells having the responder signature are expanded. In certain embodiments, CAR T cells are enriched for cells having a responder signature or depleted for cells having a non-responder signature. In certain embodiments, the agent capable of targeting, inhibiting or depleting CD8+ TILs having a non-responder signature comprises: an agent capable of binding to a cell surface or secreted CD8+ T cell non-responder signature gene; or an agent capable of reducing the expression or activity of the non-responder signature. In certain embodiments, the agent capable of activating, maintaining or increasing CD8+ TILs having a responder signature comprises an agent capable of increasing or activating the expression of the responder signature. In certain embodiments, checkpoint blockade (CPB) therapy comprises anti-CTLA4, anti-PD-L1, anti-PD1 therapy or combinations thereof.

[0030] In another aspect, the present invention provides for a method of treating cancer in a subject in need thereof comprising administering an agent capable of increasing the expression or activity of one or more genes or polypeptides selected from the group consisting of TCF7, IL7R, GPR183, LMNA, NR4A3, CD55, AIM1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N, CSRNP 1, FAM65B, PIK3R1, RGPD6, SKIL, TSC22D2, USP36, FOXP1, STAT4, PLAC8, LTB LEF1, S1PR1, EGR1, MYADM, ZFP36L2, FAM102A, RGCC, PDE4B, PFKFB3, FOSB, DCTN6 and BTG2 in combination with checkpoint blockade therapy.

[0031] In another aspect, the present invention provides for a method of treating cancer in a subject in need thereof comprising administering an agent capable of reducing the expression or activity of one or more genes or polypeptides selected from the group consisting of CD38, CCL3, VCAM1, GOLFM4, HAVCR2, PRDX3, ENTPD1, PTTG1, CCR5, TRAFD1, PDCD1, CXCR6, BATF, PTPN6, LAG3 and CTLA4 in combination with checkpoint blockade therapy.

[0032] In another aspect, the present invention provides for a method of treating cancer in a subject in need thereof comprising administering CD8+ T cells expressing a gene signature comprising of one or more genes selected from the group consisting of TCF7, IL7R, GPR183, LMNA, NR4A3, CD55, AEVI1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N, CSRNP1, FAM65B, PIK3R1, RGPD6, SKIL, TSC22D2, USP36, FOXP1, STAT4, PLAC8, LTB LEFI, SIPRI, EGRI, MYADM, ZFP36L2, FAM102A, RGCC, PDE4B, PFKFB3, FOSB, DCTN6 and BTG2 in combination with checkpoint blockade therapy.

[0033] In certain embodiments, agent comprises a therapeutic antibody, antibody fragment, antibody-like protein scaffold, aptamer, protein, genetic modifying agent or small molecule.

[0034] In another aspect, the present invention provides for a method of monitoring a subject in need thereof undergoing treatment with checkpoint blockade (CPB) therapy, said method comprising detecting in a tumor sample obtained from the subject the expression or activity of a gene signature comprising one or more genes or polypeptides selected from the group consisting of: ENTPD1 and HAVCR2; or CCL3, CD38 and HAVCR2; or CD38, CCL3, VCAM1, GOLIM4, HAVCR2, PRDX3, ENTPD1, PTTG1, CCR5, TRAFD1, PDCD1, CXCR6, BATF, PTPN6, LAG3 and CTLA4; or CD38, EPSTI1, GOLIM4, WARS, PDCD1, CCL3, SNAP47, VCAM1, SKA2, HAVCR2, LGALS9, PRDX3, FASLG, ENTPD1, FABP5, SIRPG, LSM2, NDUFB3, TRAFD1, UBE2F, NMI, IFI35, CLTA, MTHFD1, MY07A, IFI27L2, MCM5, STMNL, ID3, RGS3, SNRPD1, PTTG1 and FIBP; or CD8_B genes listed in Table 6, wherein the treatment is adjusted if the signature is increased in CD8+ TILs after treatment.

[0035] In another aspect, the present invention provides for a method of monitoring a subject in need thereof undergoing treatment with checkpoint blockade (CPB) therapy, said method comprising detecting in a tumor sample obtained from the subject the expression or activity of a gene signature comprising one or more genes or polypeptides selected from the group consisting of: TCF7; or TCF7 and IL7R; or TCF7, IL7R, FOSL2, REL, FOXP1, and STAT4; or TCF7, PLAC8, LTB, and CCR7; or TCF7, LEFI, SIPRI, PLAC8, LTB, and CCR7; or TCF7, IL7R, GPR183, and MGAT4A; or TCF7, IL7R, GPR183, LMNA, NR4A3, CD55, AIM1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N and CSRNP1; or TCF7, IL7R, GPR183, LMNA, NR4A3, CD55, AFM1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N, CSRNP1, FAM65B, PIK3R1, RGPD6,

SKIL, TSC22D2, USP36, FOXP1, EGRI, MYADM, ZFP36L2, FAM102A, RGCC, PDE4B, PFKFB3, FOSB, DCTN6 and BTG2; or CD8_G genes listed in Table 6, wherein the treatment is adjusted if the signature is decreased in CD8+ TILs after treatment.

[0036] In another aspect, the present invention provides for a method of manufacturing cells for use in adoptive cell transfer comprising: obtaining CD8+ T cells; and depleting cells having a non-responder signature as defined in claims 4 or 5 or selecting for cells having a responder signature as defined in any of claims 1 to 3. The method may further comprise expanding cells having a responder signature. The method may further comprise activating the cells. The method may further comprise expressing a chimeric antigen receptor (CAR) or an endogenous T cell receptor (TCR) in the cells.

[0037] In another aspect, the present invention provides for a kit comprising reagents to detect at least one gene or polypeptide according to a gene signature as defined in claims 1 or 5. The kit may comprise at least one antibody, antibody fragment, or aptamer. The kit may comprise primers and/or probes or fluorescently bar-coded oligonucleotide probes for hybridization to RNA.

[0038] These and other aspects, objects, features, and advantages of the example embodiments will become apparent to those having ordinary skill in the art upon consideration of the following detailed description of illustrated example embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

[0039] **FIG. 1** - illustrates the mean expression of genes in CD8+ T cells and the variability in expression. The most variable genes are selected for tSNE analysis based on genes with a var>6 and that are expressed in at least 5% of the cells (-4000 genes).

[0040] **FIG. 2** - illustrates tSNE analysis based on most variable genes in CD8 cells. tSNE1 is correlated with the number of expressed genes. As a control, expression of CD8A\CD8B is not correlated with tSNE score

[0041] **FIG. 3** - illustrates clustering by tSNE analysis of single cells and association with response to check point blockade therapy.

[0042] **FIG. 4** - illustrates a heatmap of genes in the clusters from figure 3.

[0043] **FIG. 5** - illustrates the percentage of cells having a non-responder (Bad) and responder (Good) signature in patients that responded (R) or did not respond (NR) to checkpoint blockade therapy.

[0044] **FIG. 6** - illustrates further cluster analysis showing the non-responder cluster can be split into two clusters.

[0045] **FIG. 7** - illustrates a heatmap of genes in the clusters from figure 6.

[0046] **FIG. 8** - illustrates cell cycle analysis of the single cells.

[0047] **FIG. 9** - illustrates a cell cycle cluster based on figure 8.

[0048] **FIG. 10** - illustrates further cluster analysis showing the T cells can be separated into 6 clusters.

[0049] **FIG. 11** - illustrates a heatmap of genes in the clusters from figure 10.

[0050] **FIG. 12** - illustrates the enrichment for metabolic functions in the 6 clusters from figure 10.

[0051] **FIG. 13** - illustrates transport reaction activity across the 6 clusters from figure 10. Heatmap shows metabolites associated with the transporter genes.

[0052] **FIG. 14** - illustrates transport reaction activity across the 6 clusters from figure 10. Heatmap shows transporter genes expressed in the different clusters.

[0053] **FIG. 15** - illustrates a pipeline for determining T cell receptors (TCR).

[0054] **FIG. 16** - illustrates TCR analysis in the single T cells. Left panel shows clonal expansion as determined by the same TCR being detected in the same patient in different time points. Right panel shows clonal enrichment as determined by the same TCR being detected in the same patient in single time points.

[0055] **FIG. 17** - illustrates that $\delta\gamma$ T-cells are enriched in CD4/CD8 double negative T cells (DN).

[0056] **FIG. 18** - illustrates analysis of V51, V δ 2 and V δ 3 T-cells.

[0057] **FIG. 19** - illustrates FACS analysis of CD39+Tim3+ (DP) cells and CD39-Tim3- (DN) cells sorted from patient samples using cluster specific markers.

[0058] **FIG. 20** - illustrates tSNE analysis of cells sorted in figure 19 using cluster specific markers.

[0059] **FIG. 21A-21E** - illustrates characterization of tumor infiltrating CD8 T cells. G1 refers to group 1, responder signature and G2 refers to group 2, non-responder signature in this figure. **Figure 21A** illustrates a tSNE analysis showing that tumor infiltrating CD8 T cells cluster into G1 or G2. **Figure 21B** illustrates a heat map showing expression of G1 and G2 genes in responders and non-responders. **Figure 21C** illustrates the ratio of G2/G1 expression in responders and non-responders. Patients positive for antigen presentation and the IFN gamma pathway and patients defective for antigen presentation and the IFN gamma

pathway are distinguished. **Figure 21D** illustrates a graph showing overall survival of patients with low or high expression of Gl. **Figure 21E** illustrates immunofluorescence images stained for CD8 and TCF7 in a responder and non-responder patient. The percentage of CD8+ cells and the ratio of TCF+/TCF- CD8+ cells are calculated for the responder and non-responder patient.

[0060] **FIG 22A-22C** - illustrate immunofluorescence imaging and calculation of TCF7 positive CD8 cells using CellProfiler (cellprofiler.org) and a novel pipeline.

[0061] **FIG. 23** - **The immune landscape of melanoma patients treated with checkpoint therapy.** **Figure23A.** Schematic of the studied cohort. Top panel describes the 32 patient cohort, number of samples taken relevant to treatment initiation (baseline-B, on/post treatment-P) along with the clinical status (responder-R, nonresponder-NR). Lower panel delineates the workflow used. WES- whole exome sequencing, QC- quality control, TCR- T cell receptor. **Figure23B.** tSNE plot of all CD45⁺ cells collected in this study. Cells are shaded based on 11 clusters identified by &-means clustering analysis (**Methods**). **Figure23C.** Heatmap describing scaled expression values ($\log_2(\text{TPM}+1)$) of discriminative gene sets for each cluster defined in **(B)**. A list of representative genes is shown for each cluster next to the right margin bars. Shading scheme is based on z-score distribution from -2.5 to 2.5. **Figure23D.** Box plots comparing the percentage of cells (out of CD45⁺) in Gl, G3, G4, G6 and G11 clusters as defined in **(B)**, between responder and non-responder lesions. Each symbol represents an individual sample. Two-sided Wilcoxon rank-sum *P*-value is shown ***P*=0.003; ***P*=0.003; **P*=0.01; ***P*=0.005; ***>=1.3x10⁻⁵. **Figure23E.** Box plots comparing the percentage of exhausted and activated CD45⁺CD3⁺ cells on the basis of a pre-defined list of known marker genes (**table 3**), between responder and non-responder lesions. Each symbol represents an individual sample. Two-sided Wilcoxon rank-sum *P*-value is shown ****P*=2x10⁻⁴; ***P*=0.002. **Figure23F.** Box plots comparing the percentage of B-cells and Myeloid cells on the basis of a pre-defined list of known marker genes (**table 3**) between responder and non-responder lesions. Each symbol represents an individual sample. Two-sided Wilcoxon rank-sum *P*-value is shown ***P*=0.004; ***P*=0.002. **Figure23G.** Box plots comparing the percentage of Memory CD8⁺ T-cells on the basis of a pre-defined list of known marker genes (**table 3**) between responder and non-responder lesions. Each symbol represents an individual sample. Two-sided Wilcoxon rank-sum *P*-value is shown ***P*=0.001. **Figure23H.** Heatmap describing scaled expression values ($\log_2(\text{TPM}+1)$) of discriminative

gene sets between responder and non-responder lesions. Marker genes are shown per cluster. Shading scheme is based on z-score distribution from -2.5 to 2.5.

[0062] FIG. 24 - Identification of CD8⁺ T-cell states associated with clinical outcome. **Figure 24A.** tSNE plot of all CD8⁺ T cells collected in this study. Cells are shaded based on 2 clusters identified by &-means clustering (**Methods**). **Figure 24B.** Heatmap showing scaled expression values ($\log_2(\text{TPM}+1)$) of discriminating genes for the clusters defined in (A). A list of representative genes are shown for each cluster next to the right margin bars. Shading scheme is based on z-score distribution from -2.5 to 2.5 . **Figure 24C.** Box plots comparing the percentage of CD8_G and CD8_B (out of CD8⁺ cells) clusters in responder and non-responder lesions . Each symbol represents an individual sample. Two-sided Wilcoxon rank-sum P-value is shown i^l^x10^responders; P=0.005 non-responders. **Figure 24D.** The logio ratio between the number of cells in CD8_B/CD8_G per sample is computed. A comparison of this measurement between responder and non-responder lesions is shown for all samples, baseline and post-treatment samples separately. Circles marked in white represents samples with defects in antigen presentation and the IFN γ pathway (Ag presentationVIFNy pathway $^-$) as inferred by WES, IHC and flow-cytometry analysis. Marked Circles represent samples without defects in those pathways. The significance score (one-sided Wilcoxon P-value) for each comparison is shown below. **Figure 24E.** Heatmap describing scaled expression values ($\log_2(\text{TPM}+1)$) of discriminative gene sets from CD8_G and CD8_B clusters between responder and non-responder lesions. Marker genes are shown per cluster. Shading scheme is based on z-score distribution from -2.5 to 2.5 . **Figure 24F.** Representative images from the multiplex immunofluorescence staining of tissue nuclei stained with DAPI, CD8 and TCF7 from a responder and non-responder patient prior to therapy with anti-PD1. Original Magnification X400. **G.** Box plots comparing the percentage of CD8⁺TCF7 $^+$ and CD8⁺TCF7 $^-$ cells as inferred by immunofluorescence staining, between responder and non-responder patients. Each symbol represents an individual sample. Two-sided Wilcoxon rank-sum P-value is shown, P=3.9x10 $^{-6}$ responders; R=1.1x10 $^{-8}$ non-responders. **Figure 24H-I.** Box plots showing a quantitative analysis (**Methods**) of TCF7 $^+$ CD8⁺/TCF7 $^-$ CD8⁺ ratio out of CD8⁺ cells (**H**) and of CD8⁺ cells out of all nuclei (**I**) between responder (n=20) and non-responder patients (n=23). n.s- not significant. One-sided Wilcoxon P-value is shown P=2.4x10 $^{-6}$. **J.** Kaplan-Meier survival curve, composed of data from 33 patients treated with anti-PD1 therapy. Patients were divided into two groups based on TCF7 $^+$ CD8⁺/TCF7 $^-$ CD8⁺ ratio (n=16 >1; n=17 <1) as inferred by immunofluorescence

staining. A ratio of $TCF7^+CD87TCF7^-CD8^+ > 1$ was associated with better overall survival (log-rank $P=0.03$) when compared to patients with $TCF7^+CD8^+/TCF7^-CD8^+ < 1$.

[0063] FIG. 25 - CD8⁺ T-cell state heterogeneity and its association with clinical response. **Figure 25A.** tSNE plot of all CD8⁺ T-cells collected in this study. Cells are shaded based on 6 clusters identified by &-means clustering (**Methods**). **Figure 25B.** Heatmap showing scaled expression values ($\log_2(\text{TPM}+1)$) of discriminative gene sets for each cluster defined in (A). A list of representative genes is shown for each cluster next to the right margin bars. Shading scheme is based on z-score distribution from -2.5 to 2.5 . **Figure 25C.** Box plots comparing the percentage of CD8_1 to 6 (out of CD8⁺ cells) clusters between responders and non-responders . Each symbol represents an individual sample. One-sided Wilcoxon P-values are shown, $^{**}P\text{-value}=0.001$ for CD8_1, $^*P\text{-value}=0.013$ for CD8_3, $^{**}P\text{-value}=0.003$, and ns-not significant. **D.** Trajectory analysis for the 6 CD8⁺ T-cells clusters identified in (A). Cell expression profiles in a two dimensional independent space. Solid black line indicates the main diameter path of the minimum spanning tree (MST) and provides the backbone of Monocle's pseudotime ordering of the cells. Each dot represents an individual cell shaded by cluster (upper plot) or by pseudotime (lower plot).

[0064] FIG. 26 - Discriminating exhausted from memory cells using TIM3 and ENTPD1/CD39. **Figure 26A.** Heatmap showing scaled expression values ($\log_2(\text{TPM}+1)$) of discriminative gene sets between CD8_2 (exhaustion) and CD8_4+6 (memory/effector) with original unsorted, and sorted (CD39⁺TIM3⁺ and CD39⁻TIM3⁺) cells. A list of representative genes are shown for each cluster next to the right margin bars. Shading scheme is based on z-score distribution from -2.5 to 2.5 . **Figure 26B.** Representative flow cytometric plots (upper part) of intracellular staining for IL-2, IFNy and TNF α in CD39⁺ and CD39⁻ cells (out of gated CD45⁺CD3⁺CD8⁺ cells). Flow cytometry quantification of cytokine-producing cells obtained from 12 metastatic melanoma patients (lower part). Bars indicate the mean values. Data were combined from 2 replicate experiments. Unpaired-student's *t*-test with $^{**}P\text{-value}=0.0016$ and $^{***}P\text{-value}=5\times 10^{-4}$ is shown. **Figure 26C.** A schematic summary of the therapy regimen used in the transplantable B16-F10 mouse model (described in **Methods**). Mice were divided into four groups (n=10 per group): untreated (vehicle control), anti-TIM3, CD39 inhibitor (CD39i, using POM-1 small molecule) and anti-TIM3 in combination with CD39L **Figure 26D.** Box plots showing the kinetics of tumor growth between the different groups of mice on days +4, +7, +11, +14 and +18 post tumor transplantation. Data in box plots are means+SEM. P-value was determined by unpaired-student's *t*-test. **Figure 26E.**

Mean tumor volumes for all 4 groups are shown, means \pm SEM. **Figure 26F.** Survival at day 30 of B16-F10 tumor-bearing mice for all 4 groups. Log-rank P-value is shown. Data shown for **C-F** represents one out of two independent experiments, n=10 for each group per experiment. ns- not significant.

[0065] FIG. 27 - Distinctive chromatin accessibility in CD39⁺TIM3⁺ and CD39⁻ TIM3⁻ cells. **Figure 27A.** Schematic of ATAC-seq analysis performed on sorted CD39⁺TFM3⁺ and CD39TFM3⁻ cells from 5 melanoma patients. **Figure 27B.** Heatmap describing averaged scaled expression values ($\log_2(\text{TPM}+1)$) of differentially expressed transcription factors for sorted CD39⁺TIM3⁺ and CD39TFM3⁻ cells (from n=5 patients). Shading scheme is based on z-score distribution from -0.6 to 0.6. **Figure 27C.** Heatmap describing patient specific (n=5) differentially accessible regions (FDR<0.01) in CD39⁺TFM3⁺ and CD39TFM3⁻ sorted populations. Shading scheme is based on z-score distribution from -3 to 3. **Figure 27D.** ATAC-seq traces for open chromatin regions near selected genes in CD39⁺TFM3⁺ and CD39TFM3⁻ is shown. **Figure 27E.** Graph depicting enrichment of TF motifs in open chromatin specific to CD39TFM3⁻ and CD39⁺TIM3⁺ cells is on the x-axis, and differential expression of TF on the y-axis. **Figure 27F.** Left, enhancer binding sites near the listed genes for *BATF* and *TCF7*. Significant genes associated with these sites are marked and non-significant genes are white. The corresponding genes are also differentially expressed between CD39⁺TFM3⁺ cells (enriched with *BATF*) and CD39TFM3⁻ cells (enriched with *TCF7*). Right, the number of genes that are differentially expressed with a corresponding differential peak containing *BATF* or *TCF7* is shown.

[0066] FIG. 28 - TCR analysis and its relationship with cell state and clinical outcome. **Figure 28A.** Schematic illustration of the TCR analysis pipeline. TCR reconstruction was done using the MixCR algorithm (**Methods**), and were classified into 4 groups: *Persistent*: TCRs found in matched baseline and post treatment samples; *Enriched*: Multiple TCRs found in a single time point of a given patient; *Singlets*: single TCRs that detected only once in our dataset; and *Common*: TCRs found in different patients. **Figure 28B.** tSNE plot (left panel) delineating the six identified CD8⁺ T-cell clusters and persistent TCRs in responder lesions (black triangles) and non-responder lesions (gray circles). Bar plot (right panel) summarizing the fraction of persistent TCRs per patient across the different clusters between responder (R) and non-responder (NR) samples. Two-sided Wilcoxon rank-sum P-value is shown *P=0.03; **P=0.0085. **Figure 28C-D.** Fraction of persistent TCRs per patient, aggregated for CD8_1 to 3 (CD8_1-3) and CD8_4 to 6 (CD8_4-6) clusters for R and

NR samples; ns- not significant. **Figure 28E.** tSNE plot (left panel) delineating the six identified clusters and enriched TCRs in responders (black triangles) and non-responders (gray circles). Bar plot (right panel) summarizing the fraction of enriched TCRs per patient across the different clusters and split into R and NR samples. **P=0.003; *P=0.03 for CD8_3; *P=0.02 for CD8_5. **Figure 28F-G.** Fraction of enriched TCR per patient, aggregated for CD8_1 to 3 (CD8_1-3; P=0.014) and CD8_4 to 6 (CD8_4-6; P=0.019) clusters for R and NR samples. **Figure 28H.** tSNE plot (left panel) delineating the six identified clusters and singlets TCRs in responders (black triangles) and non-responders (gray circles). Bar plot (right panel) summarizing the fraction of singlets TCRs per patient across the different clusters and split into R and NR samples. *V=0.009 for CD8_1; V=0.02; *V=0.004 for CD8_5. **Figure 28I-J.** Fraction of singlets TCR per patient, aggregated for CD8_1 to 3 (CD8_1-3; P=0.002) and CD8_4 to 6 (CD8_4-6; P=0.002) clusters for R and NR samples.

[0067] FIG. 29 - Association of the 11 CD45⁺clusters with clinical outcome during the course of checkpoint therapy. **Figure 29A.** For each patient, the percentage of cells (out of CD45⁺cells) classified to one of the 11 clusters identified by &-means clustering is shown. Pie chart on the right, summarizes the corresponding percentages across all cells collected in this study. **Figure 29B-C.** Box plots comparing the abundance of cells in the corresponding clusters between all baseline and post-treatment samples (**B**) and only in patients with matched longitudinal samples (**C**). Pie charts on the right, summarizes the corresponding percentages for each analysis. **Figure 29D.** Box plots comparing the abundance of cells in the corresponding clusters between responder and non-responder lesions. Pie chart on the right, summarizes the corresponding percentages for each cluster. B- baseline, P- post, R- responder, NR-non-responder, n.s- not significant.

[0068] FIG. 30 - Supervised analysis of T cell states. **Figure 30A.** The percentage of exhausted, activated or activated/exhausted CD45⁺CD3⁺ cells in each patient, on the basis of a pre-defined list of known marker genes is shown. Pie chart on the right summarizes the corresponding percentage across all CD45⁺CD3⁺ T-cells collected in this study **Figure 30B.** Comparison of the abundance of all three cell states as in (**A**) between baseline and post-treatment samples (left) and responder and non-responder lesions (right), on the basis of the pre-defined list of known genes as in (**A**). **Figure 30C-D.** Box plots comparing the abundance of cells in all three cell states between baseline and post-treatment samples (**C**) and for the activated/exhausted state between responder and non-responder lesions (**D**). B- baseline, P- post, R- responder, NR-non-responder, ns- not significant.

[0069] **FIG. 31 - Comparing the composition of known cell types with clinical outcome and checkpoint therapy.** **Figure 31A.** tSNE plot of all CD45⁺cells collected in this study. Cells are shaded by cell type on the basis of pre-defined markers (table 3). **Figure 31B.** The percentage of known immune cell types in each patient, on the basis of a pre-defined list of known marker genes. Pie chart (below bar graph) summarizes the corresponding percentage of known cell types across all CD45⁺cells collected in this cohort. **Figure 31C-D.** A comparison of the abundance of known cell types as in (B) between baseline and post-treatment samples (C) and responder and non-responder lesions (D). **Figure 31E-F.** Box plots comparing the abundance of known cell types between baseline and post-treatment samples (E) and between responder and non-responder lesions (F). B- baseline, P- post, R- responder, NR-non-responder, ns- not significant.

[0070] **FIG. 32 - Supervised analysis of CD4⁺ and CD8⁺ T-cells expressing effector, memory and regulatory genes.** **Figure 32A.** The percentage of effector, memory and regulatory CD45⁺CD3⁺cells in each patient, on the basis of pre-defined list of known marker genes is shown. Pie chart on the right summarizes the corresponding percentage across all CD45⁺CD3⁺ T-cells collected in this study. **Figure 32B-C.** Box plots and pie charts comparing the abundance of different T-cell types between baseline and post-treatment samples (B) and between responder and non-responder lesions (C). B- baseline, P- post, R- responder, NR-non-responder, ns- not significant.

[0071] **FIG. 33 - Comparing the supervised cell type classification to the unsupervised clustering of immune cells.** A comparison between the supervised classification of single cells to cell types (right) to the unsupervised clustering of immune cells identified by &-means clustering (left). For each one of the 11 unsupervised clusters identified, the percentage of cell types as defined by the supervised analysis is shown.

[0072] **FIG. 34 - Detection of cluster-specific genes differentially expressed between responder and non-responder samples.** **Figure 34A-B.** Heatmap showing scaled expression values ($\log_2(\text{TPM}+1)$) of genes that are significantly more expressed in responder (A) and non-responder (B) samples. The analysis was done on a specific set of genes (top 20 cluster-specific marker genes, **Table 2**). A list of all significant genes is shown for each cluster next to the left margin. Shading scheme is based on z-score distribution from -2.5 to 2.5 .

[0073] **FIG. 35 - Detection of genes differentially expressed between responder and non-responder samples.** Heatmap showing scaled expression values ($\log_2(\text{TPM}+1)$) of

genes that are differentially expressed between responder and non-responder samples. A list of representative genes is shown for each cluster next to the left margin. Shading scheme is based on z-score distribution from -2.5 to 2.5.

[0074] **FIG. 36 - Annotating CD8_G and CD8_B clusters to the whole immune cell population clusters.** tSNE plot of all CD45⁺ clustres (n=1 1) collected in this study (left) is shown. Cells are shaded based on 11 clusters identified by &-means clustering analysis (**Methods**). Right tSNE plot shows the distribution of CD8_G and CD8_B in relation to all immune cells analyzed in this study.

[0075] **FIG. 37 - Quantification of two CD8⁺ T cell states associated with clinical response.** **Figure 37A-B.** For each sample, the percentage of cells found in CD8_G and CD8_B (out of all CD8⁺T cells) in responder lesions (A) and non-responder lesions (B) is shown. * symbol marks samples with defects in antigen presentation and the IFNypathway as inferred from WES, IHC and flow-cytometry analysis. P# indicates patient number as described in **table 1**. **Figure 37C.** Pie charts summarize the average percentage of the 2 clusters in the responders and non-responders groups.

[0076] **FIG. 38 - Detection of defects in antigen presentation increases response prediction.** **Figure 38A.** Representative immunohistochemistry staining (1 out of 3) of sections from patient #3 with homozygote mutations in *B2M*(as inferred from WES). Sections were stained with an antibody cocktail for melanoma cells (mel. cocktail) using anti-melanosome (HMB45), anti-MART-1/melan A and anti-Tyrosinase, to discern melanoma cells from normal cells; or with an antibody specific for B2M. Original Magnification X100. **Figure 38B.** Flow-cytometry plot (left) and histogram (right), showing the expression of HLA-A,B,C in immune and tumor cells in patient #15. **Figure 38C-D.** Receiver operating characteristic (ROC) analysis was constructed to evaluate the prognostic power of the ratio between CD8_B/CD8_G as shown in Fig 24D between responder and non-responder lesions. The area under the ROC curve (AUC) was used to quantify response prediction, and one-sided Wilcoxon test was used to assess significance of the AUC results. The AUC value for all samples (C) was 0.87 ($P=1.1 \times 10^{-5}$) and (D) 0.95 ($P=3.8 \times 10^{-7}$) when excluding the 6 samples with defects in antigen presentation and the IFNypathway as inferred by WES, IHC and flow-cytometry analysis.

[0077] **FIG. 39 - Detection and quantitation of TCF7⁺CD8⁺and TCF7⁻CD8⁺ cells in a cohort of patients treated with anti-PDI.** **Figure 39A.** Schematic illustration for the immunofluorescence pipeline. Sections from an independent cohort of 33 patients (n=43

samples; responders=20, non-responders=23) treated with anti-PD1 were stained with DAPI, CD8 and TCF7 and analyzed with CellProfiler (**Methods**). For each sample the percentage of CD8⁺TCF7^(line) or CD8⁺TCF7^(white line) was calculated out of the total nuclei detected.

Figure 39B-C. Representative overlayed images from melanomas of 10 responder (**B**) and 10 non-responder patients (**C**) stained with DAPI, CD8 and TCF7. For each patient, 10 random fields were scanned and analyzed. The ratio of CD8⁺TCF7^{+/}/CD8⁺TCF7⁻detected in each patient is shown on the upper right corner of the imaged sections. Original Magnification X400. TCF7- transcription factor 7.

[0078] **FIG. 40 - Quantification of CD8⁺TCF7⁺ and CD8⁺TCF7⁻ protein levels in responder and non-responder lesions.** **Figure 40A-B.** For each sample, the percentage of CD8+TCF7⁺ and CD8+TCF7⁻ cells (out of all CD8⁺T cells) found in responder (n=20) lesions (**A**) and non-responder (n=23) lesions (**B**) is shown. P# indicates patient number as described in **table 8**. **Figure 40C.** Pie charts summarize the average percentage of the 2 cell phenotypes in the responders and non-responders groups.

[0079] **FIG. 41 - High frequencies of CD8⁺TCF7⁺ T cells are associated with and predict clinical response.** **Figure 41A.** Box plots showing the quantitative analysis of TCF7⁺CD8⁺/TCF7⁻CD8⁺ratio in baseline and post-treatment samples, between responder (n=20) and non-responder patients (n=23). One-sided Wilcoxon *P-value* is shown, **P=0.0011, ***P=0.00017. **Figure 41B-D.** Receiver operating characteristic (ROC) analysis was constructed to evaluate the prognostic power of the ratio between TCF7⁺CD8⁺/TCF7⁻CD8⁺ between responder and non-responder lesions. The area under the ROC curve (AUC) was used to quantify response prediction, and one-sided Wilcoxon test was used to assess significance of the AUC results. The AUC value was 0.9 CP=2.4x10⁻⁶) for all samples (**B**), 0.88 (P=1.1x10⁻³) for post-treatment samples (**C**) and 0.98 (P=1.7x10⁻⁴) for baseline samples (**D**).

[0080] **FIG. 42 - TCF7 kinetics between baseline and post-treatment samples.** **Figure 42A-B.** Representative overlayed images from patients with matched baseline and post-treatment samples from the second PD1 cohort (**A**) and from the initial single-cell RNAseq cohort (**B**) stained with DAPI, CD8 and TCF7. For each patient, 10 random fields were scanned and analyzed. The ratio of CD8⁺TCF7^{+/}/CD8⁺TCF7⁻detected in each patient is shown on the upper right corner of the imaged sections, left upper corner shows lesion status: regression (circles), progression (squares). Original Magnification X400. TCF7- transcription factor 7.

[0081] **FIG. 43 - Proportion of exhaustion and memory markers across the six clusters.** **Figure 43A.** tSNE plot of all CD8⁺T-cells profiled in the first cohort. Cells are shaded based on 6 clusters identified by &-means clustering as shown in Fig 25A. B-I. tSNE plot shaded such that single-cells with a high expression level ($\log_2(\text{TPM}+1)>2$) of *ENTPD1*(B), *HA VCR2*(C), *PDCD1*(D), *LAG3*(E), *TIGIT*(F), *TCF7*(G), *TNF*(H), and *IL7R*(I) are shaded, and those with a low expression level ($\log_2(\text{TPM}+1)<2$) are shaded in gray. Bar plots (to the right) summarize the corresponding percentages of each gene in the 6 identified.

[0082] **FIG. 44 - Hierarchical and trajectory analysis of CD8⁺ T-cell clusters.** **Figure 44A.** Hierarchical tree structure for the six CD8⁺T-cell clusters. In each split, gene markers up-regulated in the corresponding cluster are identified by comparing the corresponding cells to the rest of the cells found in the last common ancestor (Methods). **Figure 44B.** Violin plots showing organization of cells corresponding to the six CD8 clusters by pseudotime as inferred by Monocle. **Figure 44C.** Cell expression profiles in a two dimensional independent space for each cluster (CD8_1 to 6) is shown. Solid black line indicates the main diameter path of the minimum spanning tree (MST) and provides the backbone of Monocle's pseudotime ordering of the cells. Each dot represent an individual cell shaded by cluster.

[0083] **FIG. 45 - Characterization of CD39⁺CD8⁺cells in melanoma patients.** **Figure 45A.** Gene expression level distribution ($\log_2(\text{TPM}+1)$) of CD39 (*ENTPD1*, left) and TIM3 (*HAVCR2*, right) in the six CD8 clusters is shown. Each dot represents an individual cell. **Figure 45B.** Gating strategy that was used to isolate CD39⁺TIM⁺and CD39⁻TIM3⁻, CD8⁺T-cells from 4 melanoma patients. **Figure 45C.** Heatmap of scaled expression values ($\log_2(\text{TPM}+1)$) of discriminative gene sets between sorted CD39⁺TIM3⁺and CD39⁻TIM3⁻, CD8⁺T-cells. A list of representative genes are shown for each cell population. Shading scheme is based on z-score distribution from -1 to 1. **Figure 45D.** Flow-cytometry quantification of PD1 and TIM3 in CD39⁺, CD39⁻(CD8⁺T-cells), and CD39 expression in PD1⁺, PD1⁻, TIM3⁺and TIM3⁻(CD8⁺T-cells) from 10 metastatic melanomas from 10 patients is shown. Bar indicate the mean values. Data were combined from 2 replicate experiments. unpaired-student's t-test *P-value=0.03, ****P-value<0.0001, ns- not significant. **Figure 45E.** Individual tumor volumes of intradermal B16-F10 implants in the untreated (control), anti-TEVI3, CD39 inhibitor (CD39i), and anti-TIM3 in combination with CD39i groups is shown. Data shown represents one out of two independent experiments, n=10 for each group per experiment.

[0084] FIG. 46 - Dual inhibition of PD1 and CD39 synergistically reduces tumor growth and improves survival. **Figure 46A.** A schematic summary of the therapy regimen used in the transplantable B16-F10 mouse model (described in **Methods**). Mice were divided into four groups (n=10 per group): untreated (vehicle control), anti-PD1, CD39 inhibitor (CD39i, using POM-1 small molecule) and anti-PD1 in combination with CD39L **Figure 46B.** Box plots showing the kinetics of tumor growth between the different groups of mice on days +4, +7, +11, +14, +18 and +21 post tumor transplantation. Data in box plots are means+SEM. P-value was determined by unpaired-student's *t*-test. **Figure 46C.** Survival at day 40 of B16-F10 tumor-bearing mice for all 4 groups. Log-rank P-value is shown. Data shown for **A-C** represents one out of two independent experiments, n=10 for each group per experiment. ns- not significant.

[0085] FIG. 47 - CD39⁺TIM3⁺and CD39⁻TIM3⁻cells have a distinctive epigenetic landscape. **Figure 47A.** Heatmap of averaged scaled expression values ($\log_2(\text{TPM}+1)$) of discriminative transcription factors for non-sorted CD39⁺TIM3⁺and CD39⁻TIM3⁻ cells as defined by single cell RNA expression. Shading scheme is based on z-score distribution from -0.5 to 0.5. **Figure 47B.** Heatmap of averaged scaled expression values ($\log_2(\text{TPM}+1)$) of discriminative transcription factors for CD8_B and CD8_G cells as in Fig 24. Shading scheme is based on z-score distribution from -0.3 to 0.3. **Figure 47C.** ATAC-seq traces for open chromatin regions near selected genes in CD39⁻TIM3⁻ cells and CD39⁺TIM3⁺ cells in all 5 patients is shown. **Figure 47D.** Transcription factor (TF) enrichment graphs for BATF and TCF7 in CD39⁺TIM3⁺and CD39⁻TIM3⁻ sorted cells are shown. Each graph shows the enrichment peaks relative to background (x-axis). Black bars indicate CD39⁺TIM3⁺(top) or CD39⁻TIM3⁻(bottom) peaks, while white bars indicate background peaks. Motif enrichment was calculated using the minimum hypergeometric (minHG) test (**Methods**).

[0086] FIG. 48 - Coupling TCR clonality with T-cell states. **Figure 48A.** tSNE plot delineating the six clusters and persistent TCRs (black triangle). The fraction of persistent TCRs in each cluster out of total persistent TCRs is shown on the right. **Figure 48B.** tSNE plot delineating the six clusters and enriched TCRs (black triangle). The fraction of enriched TCRs in each cluster out of total enriched TCRs is shown on the right. C. The fraction of singlets TCRs in each cluster out of total singlets TCRs is presented D.tSNE plot (left panel) delineating the six identified clusters and common TCRs in responder lesions (black triangles) and non-responder lesions (gray circles). Bar plot (right panel) summarizing the fraction of common TCRs per patient across the different clusters between responder (R) and

non-responder (NR) samples. ns- not significant. E-F. Fraction of common TCRs per patient, aggregated for CD8_1 to 3 (CD8_1-3) and CD8_4 to 6 (CD8_4-6) clusters for R and NR samples. G. tSNE plot delineating the six clusters and common TCRs (black triangle). The fraction of common TCRs in each cluster out of total common TCRs is shown on the right.

[0087] **FIG. 49 - Gene Variance used for the unsupervised clustering.** Variance of each gene vs. the fraction of cells expressing each gene ($\log_2(\text{TPM}+1)>0$). Left panel: genes expressed in more than 10% of the cells and less than 90% are shaded. Right panel: genes with variance 6 are shaded. As the set of genes expressed in less than 10% of the cells are of less interest for clustering analysis, we set as a minimal threshold the maximal variance observed in this group of genes, as indicated by the black arrow.

[0088] **FIG. 50 - Determining an optimal number of clusters for all immune cells.** Variance explained by each k -means solution ranging from $k=3,\dots,15$, when applied to all analyzed single-cells. Percentage of variance explained is computed as described in the **Methods** section.

[0089] **FIG. 51 - Determining an optimal number of clusters for all CD8⁺ T-cells.** Variance explained by each k -means solution ranging from $k=2,\dots,13$, when applied to all analyzed CD8 T-cells. Percentage of variance explained is computed as described in the Methods section.

[0090] **FIG. 52 - Hierarchical structure of splitting clusters .** Clustering of CD8_B and CD8_G, separately, into two (upper panel) or three (lower panel) clusters. **Figure 52A.** Hierarchical structure is shown where CD8_B is split into 2 or 3 clusters, which correspond to the $k = 4$ and $k = 6$ solutions, respectively. **Figure 52B.** Hierarchical structure is shown where CD8_G is split into 2 and 3 clusters, corresponding to the $k = 4$ and $k = 6$ solutions, respectively.

DETAILED DESCRIPTION

[0091] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure pertains. Definitions of common terms and techniques in molecular biology may be found in Molecular Cloning: A Laboratory Manual, 2nd edition (1989) (Sambrook, Fritsch, and Maniatis); Molecular Cloning: A Laboratory Manual, 4th edition (2012) (Green and Sambrook); Current Protocols in Molecular Biology (1987) (F.M. Ausubel et al. eds.); the series Methods in Enzymology (Academic Press, Inc.); PCR 2: A Practical Approach (1995)

(M.J. MacPherson, B.D. Hames, and G.R. Taylor eds.): Antibodies, A Laboratory Manual (1988) (Harlow and Lane, eds.): Antibodies A Laboratory Manual, 2nd edition 2013 (E.A. Greenfield ed.); Animal Cell Culture (1987) (R.I. Freshney, ed.); Benjamin Lewin, Genes IX, published by Jones and Bartlet, 2008 (ISBN 0763752223); Kendrew *et al.* (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0632021829); Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 9780471185710); Singleton *et al.*, Dictionary of Microbiology and Molecular Biology 2nd ed., J. Wiley & Sons (New York, N.Y. 1994), March, Advanced Organic Chemistry Reactions, Mechanisms and Structure 4th ed., John Wiley & Sons (New York, N.Y. 1992); and Marten H. Hofker and Jan van Deursen, Transgenic Mouse Methods and Protocols, 2nd edition (2011).

[0092] As used herein, the singular forms "a", "an", and "the" include both singular and plural referents unless the context clearly dictates otherwise.

[0093] The term "optional" or "optionally" means that the subsequent described event, circumstance or substituent may or may not occur, and that the description includes instances where the event or circumstance occurs and instances where it does not.

[0094] The recitation of numerical ranges by endpoints includes all numbers and fractions subsumed within the respective ranges, as well as the recited endpoints.

[0095] The terms "about" or "approximately" as used herein when referring to a measurable value such as a parameter, an amount, a temporal duration, and the like, are meant to encompass variations of and from the specified value, such as variations of +/-10% or less, +/-5% or less, +/-1% or less, and +/-0.1% or less of and from the specified value, insofar such variations are appropriate to perform in the disclosed invention. It is to be understood that the value to which the modifier "about" or "approximately" refers is itself also specifically, and preferably, disclosed.

[0096] Reference throughout this specification to "one embodiment", "an embodiment," "an example embodiment," means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases "in one embodiment," "in an embodiment," or "an example embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment, but may. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner, as would be

apparent to a person skilled in the art from this disclosure, in one or more embodiments. Furthermore, while some embodiments described herein include some but not other features included in other embodiments, combinations of features of different embodiments are meant to be within the scope of the invention. For example, in the appended claims, any of the claimed embodiments can be used in any combination.

[0097] The terms "subject", "individual" or "patient" are used interchangeably throughout this specification, and typically and preferably denote humans, but may also encompass reference to non-human animals, preferably warm-blooded animals, even more preferably mammals, such as, e.g., non-human primates, rodents, canines, felines, equines, ovines, porcines, and the like. The term "non-human animals" includes all vertebrates, e.g., mammals, such as non-human primates, (particularly higher primates), sheep, dog, rodent (e.g. mouse or rat), guinea pig, goat, pig, cat, rabbits, cows, and non-mammals such as chickens, amphibians, reptiles etc. In one embodiment, the subject is a non-human mammal. In another embodiment, the subject is human. In another embodiment, the subject is an experimental animal or animal substitute as a disease model. The term does not denote a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are intended to be covered. Examples of subjects include humans, dogs, cats, cows, goats, and mice. The term subject is further intended to include transgenic species.

[0098] All publications, published patent documents, and patent applications cited in this application are indicative of the level of skill in the art(s) to which the application pertains. All publications, published patent documents, and patent applications cited herein are hereby incorporated by reference to the same extent as though each individual publication, published patent document, or patent application was specifically and individually indicated as being incorporated by reference.

Overview

[0099] Embodiments disclosed herein relate to cell products, substances, compositions, markers, marker signatures, molecular targets, kits of parts and methods useful in characterizing, evaluating and modulating the immune system and immune responses. Applicants used single-cell RNA sequencing (scRNA-seq) to gain a deeper understanding of the cellular and molecular components orchestrating immunity in melanoma patients treated with checkpoint therapy. Through this unbiased approach, Applicants defined the immune cell composition of melanoma tumors, identified unique cell states coupled with response, and developed a simple assay that can accurately predict clinical outcome in an independent

cohort. Moreover, Applicants assessed the identity and function of some of the newly identified cell states, delineated their epigenetic landscape, and tested a new therapeutic combination that enhanced immunity in a mouse melanoma model. The analysis demonstrates the utility of applying unbiased single-cell methods to uncover the principles that underlie the success or failure of immunotherapy.

[0100] Thus, it is an objective of the present invention to determine whether a patient should be treated with a checkpoint blockade (CPB) therapy. Applicants have identified that the ratio of CD8+ TILs expressing a non-responder signature and CD8+ TILs expressing a responder signature can predict sensitivity or resistance to CPB therapy as well as predicting overall survival. Applicants have further identified that detecting the quantity of CD8+ T cells expressing a single transcription factor that is part of the responder signature can be used to distinguish between CPB therapy responders and non-responders. It is another objective of the present invention to modulate the ratio of CD8+ TILs expressing a non-responder signature to CD8+ TILs expressing a responder signature. It is another objective of the present invention to provide for adoptive cell transfer methods for treatment of a cancer patient, wherein the cells are enriched for CD8+ TILs expressing a responder signature. It is another objective of the present invention to select patients for treatment with an immunotherapy. It is another object of the invention to target non-responder CD8+ T cells for cancer therapy.

[0101] The biomarkers of the present invention were discovered by analysis of expression profiles of single immune cells within populations of cells from freshly isolated tumors, thus allowing the discovery of novel gene signatures and immune cell subtypes that were previously unrecognized. Treatment of solid tumors has been revolutionized by immune checkpoint blockade therapies; yet even in melanoma, for which high response rates are observed, the majority of patients do not respond. Specifically, to identify key immunological components associated with success or failure of immunotherapy, Applicants profiled 16,291 immune cells from 48 tumor samples of melanoma patients treated with checkpoint inhibitors, using single-cell transcriptomics. Applicants obtained samples from melanoma patients receiving checkpoint blockade therapy both before they received treatment and after they received treatment with a checkpoint inhibitor. Applicants have identified a non-responder signature and a responder signature in the CD8+ TILs. Applicants have identified that the ratio of CD8+ TILs expressing a non-responder signature and CD8+ TILs expressing a responder signature can predict sensitivity or resistance to CPB therapy, as well as

predicting overall survival. Applicants identified unique exhaustion and memory/effector states of CD8⁺ T-cells associated with tumor regression, and found that the expression of a single transcription factor, *TCF7*, in CD8⁺ T-cells was sufficient to predict clinical outcome in an independent cohort. Specifically, Applicants show using immunofluorescence that responders have more CD8+ TCF7+ T cells than CD8+ TCF7- T cells and vice versa. Thus, detection of CD8+ TCF7+ T cells may be used to predict overall survival in cancer patients. Applicants delineated the epigenetic landscape and clonality of these T-cell states, and demonstrated enhanced anti-tumor immunity by targeting a novel combination of factors identified in exhausted cells. Applicants, show using a melanoma cancer model that targeting CD39 and TIM3 on non-responder cells results in a significant increase in survival. This study provides extensive unbiased data in human tumors for discovery of predictors, therapeutic targets and combination therapies for enhancing checkpoint immunotherapy.

[0102] The presence of CD8+ T cell subtypes may be determined by subtype specific signature biomarkers. It is generally recognized within the art, that tumors are a conglomeration of many cells that make up a tumor microenvironment, whereby the cells communicate and affect each other in specific ways. As such, specific immune cell types within this microenvironment may express certain gene products for this microenvironment.

[0103] In further aspects, the invention relates to a signature or set of biomarkers (e.g., responder and/or non-responder signature) that may be detected in combination with other signatures or set of biomarkers (e.g., malignant cell signatures). The signatures may be a gene signature, protein signature, and/or other genetic or epigenetic signature of particular tumor cell subpopulations, as defined herein (e.g., tumor cells with mutations in genes associated with antigen presentation or the IFN gamma pathway).

[0104] The invention hereto also further relates to particular immune cell subpopulations, which may be identified based on the methods according to the invention as discussed herein; as well as methods to obtain such cell subpopulations; use of such subpopulations in therapeutics; controlling therapeutic responses by targeting biomarkers relevant to the cell subpopulation; and screening methods to identify agents capable of inducing or suppressing particular immune cell (sub)populations.

[0105] In certain example embodiments, the immune cells comprise two sub-populations. A first subpopulation characterized by the expression of a number of inhibitory receptors (non-responder), and a second subpopulation characterized by the expression of a number of memory and/or differentiation genes (responder). In certain example embodiments, these

subpopulations may be used to determine responsiveness to various therapeutics. Particular advantageous uses include methods for identifying agents capable of inducing or suppressing particular immune cell (sub)populations based on the gene signatures, protein signature, and/or other genetic or epigenetic signature as defined herein.

[0106] The invention further relates to agents capable of inducing or suppressing particular immune cell (sub)populations based on the gene signatures, protein signature, and/or other genetic or epigenetic signature as defined herein, as well as their use for modulating, such as inducing or repressing, a particular gene signature, protein signature, and/or other genetic or epigenetic signature. In one embodiment, genes in one population of cells may be activated or suppressed in order to affect the cells of another population. In related aspects, modulating, such as inducing or repressing, a particular gene signature, protein signature, and/or other genetic or epigenetic signature may modify overall immune cell composition, such as immune cell composition, such as immune cell subpopulation composition or distribution, or functionality.

Biomarkers and Signatures

[0107] The invention further relates to various biomarkers for detecting immune cell (e.g., CD8+ T cell) subpopulations. In certain example embodiments, these CD8+ T cell populations are tumor infiltrating lymphocytes (TIL). The methods may comprise detecting a first population of CD8+ T cells as described further below, a second population of CD8+ T cells as described further below, or both. The first and second CD8+ T cell populations may be detected by detecting one or more biomarkers in a sample.

[0108] The term "biomarker" is widespread in the art and commonly broadly denotes a biological molecule, more particularly an endogenous biological molecule, and/or a detectable portion thereof, whose qualitative and/or quantitative evaluation in a tested object (e.g., in or on a cell, cell population, tissue, organ, or organism, e.g., in a biological sample of a subject) is predictive or informative with respect to one or more aspects of the tested object's phenotype and/or genotype. The terms "marker" and "biomarker" may be used interchangeably throughout this specification. Biomarkers as intended herein may be nucleic acid-based or peptide-, polypeptide- and/or protein-based. For example, a marker may be comprised of peptide(s), polypeptide(s) and/or protein(s) encoded by a given gene, or of detectable portions thereof. Further, whereas the term "nucleic acid" generally encompasses DNA, RNA and DNA/RNA hybrid molecules, in the context of markers the term may typically refer to heterogeneous nuclear RNA (hnRNA), pre-mRNA, messenger RNA

(mRNA), or complementary DNA (cDNA), or detectable portions thereof. Such nucleic acid species are particularly useful as markers, since they contain qualitative and/or quantitative information about the expression of the gene. Particularly preferably, a nucleic acid-based marker may encompass mRNA of a given gene, or cDNA made of the mRNA, or detectable portions thereof. Any such nucleic acid(s), peptide(s), polypeptide(s) and/or protein(s) encoded by or produced from a given gene are encompassed by the term "gene product(s)".

[0109] Preferably, markers as intended herein may be extracellular or cell surface markers, as methods to measure extracellular or cell surface marker(s) need not disturb the integrity of the cell membrane and may not require fixation / permeabilization of the cells.

[0110] Unless otherwise apparent from the context, reference herein to any marker, such as a peptide, polypeptide, protein, or nucleic acid, may generally also encompass modified forms of said marker, such as bearing post-expression modifications including, for example, phosphorylation, glycosylation, lipidation, methylation, cysteinylolation, sulphonation, glutathionylation, acetylation, oxidation of methionine to methionine sulphoxide or methionine sulphone, and the like.

[0111] The term "peptide" as used throughout this specification preferably refers to a polypeptide as used herein consisting essentially of 50 amino acids or less, e.g., 45 amino acids or less, preferably 40 amino acids or less, e.g., 35 amino acids or less, more preferably 30 amino acids or less, e.g., 25 or less, 20 or less, 15 or less, 10 or less or 5 or less amino acids.

[0112] The term "polypeptide" as used throughout this specification generally encompasses polymeric chains of amino acid residues linked by peptide bonds. Hence, insofar a protein is only composed of a single polypeptide chain, the terms "protein" and "polypeptide" may be used interchangeably herein to denote such a protein. The term is not limited to any minimum length of the polypeptide chain. The term may encompass naturally, recombinantly, semi-synthetically or synthetically produced polypeptides. The term also encompasses polypeptides that carry one or more co- or post-expression-type modifications of the polypeptide chain, such as, without limitation, glycosylation, acetylation, phosphorylation, sulfonation, methylation, ubiquitination, signal peptide removal, N-terminal Met removal, conversion of pro-enzymes or pre-hormones into active forms, etc. The term further also includes polypeptide variants or mutants which carry amino acid sequence variations vis-a-vis a corresponding native polypeptide, such as, e.g., amino acid deletions, additions and/or substitutions. The term contemplates both full-length polypeptides and

polypeptide parts or fragments, e.g., naturally-occurring polypeptide parts that ensue from processing of such full-length polypeptides.

[0113] The term "protein" as used throughout this specification generally encompasses macromolecules comprising one or more polypeptide chains, i.e., polymeric chains of amino acid residues linked by peptide bonds. The term may encompass naturally, recombinantly, semi-synthetically or synthetically produced proteins. The term also encompasses proteins that carry one or more co- or post-expression-type modifications of the polypeptide chain(s), such as, without limitation, glycosylation, acetylation, phosphorylation, sulfonation, methylation, ubiquitination, signal peptide removal, N-terminal Met removal, conversion of pro-enzymes or pre-hormones into active forms, etc. The term further also includes protein variants or mutants which carry amino acid sequence variations vis-a-vis a corresponding native proteins, such as, e.g., amino acid deletions, additions and/or substitutions. The term contemplates both full-length proteins and protein parts or fragments, e.g., naturally-occurring protein parts that ensue from processing of such full-length proteins.

[0114] The reference to any marker, including any peptide, polypeptide, protein, or nucleic acid, corresponds to the marker commonly known under the respective designations in the art. The terms encompass such markers of any organism where found, and particularly of animals, preferably warm-blooded animals, more preferably vertebrates, yet more preferably mammals, including humans and non-human mammals, still more preferably of humans. All gene name symbols refer to the gene as commonly known in the art. The examples described herein that refer to the human gene names are to be understood to also encompass genes in any other organism (e.g., homologous, orthologous genes). The term, homolog, may apply to the relationship between genes separated by the event of speciation (e.g., ortholog). Orthologs are genes in different species that evolved from a common ancestral gene by speciation. Normally, orthologs retain the same function in the course of evolution. Gene symbols may be those referred to by the HUGO Gene Nomenclature Committee (HGNC) or National Center for Biotechnology Information (NCBI). Any reference to the gene symbol is a reference made to the entire gene or variants of the gene. The signature as described herein may encompass any of the genes described herein.

[0115] The terms particularly encompass such markers, including any peptides, polypeptides, proteins, or nucleic acids, with a native sequence, i.e., ones of which the primary sequence is the same as that of the markers found in or derived from nature. A skilled person understands that native sequences may differ between different species due to

genetic divergence between such species. Moreover, native sequences may differ between or within different individuals of the same species due to normal genetic diversity (variation) within a given species. Also, native sequences may differ between or even within different individuals of the same species due to somatic mutations, or post-transcriptional or post-translational modifications. Any such variants or isoforms of markers are intended herein. Accordingly, all sequences of markers found in or derived from nature are considered "native". The terms encompass the markers when forming a part of a living organism, organ, tissue or cell, when forming a part of a biological sample, as well as when at least partly isolated from such sources. The terms also encompass markers when produced by recombinant or synthetic means.

[0116] In certain embodiments, markers, including any peptides, polypeptides, proteins, or nucleic acids, may be human, i.e., their primary sequence may be the same as a corresponding primary sequence of or present in a naturally occurring human markers. Hence, the qualifier "human" in this connection relates to the primary sequence of the respective markers, rather than to their origin or source. For example, such markers may be present in or isolated from samples of human subjects or may be obtained by other means (e.g., by recombinant expression, cell-free transcription or translation, or non-biological nucleic acid or peptide synthesis).

[0117] The reference herein to any marker, including any peptide, polypeptide, protein, or nucleic acid, also encompasses fragments thereof. Hence, the reference herein to measuring (or measuring the quantity of) any one marker may encompass measuring the marker and/or measuring one or more fragments thereof.

[0118] For example, any marker and/or one or more fragments thereof may be measured collectively, such that the measured quantity corresponds to the sum amounts of the collectively measured species. In another example, any marker and/or one or more fragments thereof may be measured each individually. The terms encompass fragments arising by any mechanism, *in vivo* and/or *in vitro*, such as, without limitation, by alternative transcription or translation, exo- and/or endo-proteolysis, exo- and/or endo-nucleolysis, or degradation of the peptide, polypeptide, protein, or nucleic acid, such as, for example, by physical, chemical and/or enzymatic proteolysis or nucleolysis.

[0119] The term "fragment" as used throughout this specification with reference to a peptide, polypeptide, or protein generally denotes a portion of the peptide, polypeptide, or protein, such as typically an N- and/or C-terminally truncated form of the peptide,

polypeptide, or protein. Preferably, a fragment may comprise at least about 30%, e.g., at least about 50% or at least about 70%, preferably at least about 80%, e.g., at least about 85%, more preferably at least about 90%, and yet more preferably at least about 95% or even about 99% of the amino acid sequence length of said peptide, polypeptide, or protein. For example, insofar not exceeding the length of the full-length peptide, polypeptide, or protein, a fragment may include a sequence of ≥ 5 consecutive amino acids, or ≥ 10 consecutive amino acids, or ≥ 20 consecutive amino acids, or ≥ 30 consecutive amino acids, e.g., >40 consecutive amino acids, such as for example ≥ 50 consecutive amino acids, e.g., ≥ 60 , ≥ 70 , ≥ 80 , ≥ 90 , ≥ 100 , ≥ 200 , ≥ 300 , ≥ 400 , ≥ 500 or ≥ 600 consecutive amino acids of the corresponding full-length peptide, polypeptide, or protein.

[0120] The term "fragment" as used throughout this specification with reference to a nucleic acid (polynucleotide) generally denotes a 5'- and/or 3'-truncated form of a nucleic acid. Preferably, a fragment may comprise at least about 30%, e.g., at least about 50% or at least about 70%, preferably at least about 80%, e.g., at least about 85%, more preferably at least about 90%, and yet more preferably at least about 95% or even about 99% of the nucleic acid sequence length of said nucleic acid. For example, insofar not exceeding the length of the full-length nucleic acid, a fragment may include a sequence of ≥ 5 consecutive nucleotides, or ≥ 10 consecutive nucleotides, or ≥ 20 consecutive nucleotides, or ≥ 30 consecutive nucleotides, e.g., >40 consecutive nucleotides, such as for example ≥ 50 consecutive nucleotides, e.g., ≥ 60 , ≥ 70 , ≥ 80 , ≥ 90 , ≥ 100 , ≥ 200 , ≥ 300 , ≥ 400 , ≥ 500 or ≥ 600 consecutive nucleotides of the corresponding full-length nucleic acid.

[0121] Cells such as immune cells as disclosed herein may in the context of the present specification be said to "comprise the expression" or conversely to "not express" one or more markers, such as one or more genes or gene products; or be described as "positive" or conversely as "negative" for one or more markers, such as one or more genes or gene products; or be said to "comprise" a defined "gene or gene product signature".

[0122] Such terms are commonplace and well-understood by the skilled person when characterizing cell phenotypes. By means of additional guidance, when a cell is said to be positive for or to express or comprise expression of a given marker, such as a given gene or gene product, a skilled person would conclude the presence or evidence of a distinct signal for the marker when carrying out a measurement capable of detecting or quantifying the marker in or on the cell. Suitably, the presence or evidence of the distinct signal for the

marker would be concluded based on a comparison of the measurement result obtained for the cell to a result of the same measurement carried out for a negative control (for example, a cell known to not express the marker) and/or a positive control (for example, a cell known to express the marker). Where the measurement method allows for a quantitative assessment of the marker, a positive cell may generate a signal for the marker that is at least 1.5-fold higher than a signal generated for the marker by a negative control cell or than an average signal generated for the marker by a population of negative control cells, e.g., at least 2-fold, at least 4-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 40-fold, at least 50-fold higher or even higher. Further, a positive cell may generate a signal for the marker that is 3.0 or more standard deviations, e.g., 3.5 or more, 4.0 or more, 4.5 or more, or 5.0 or more standard deviations, higher than an average signal generated for the marker by a population of negative control cells.

Use of Signature Genes

[0123] The present invention is also directed to signatures and uses thereof. As used herein a "signature" may encompass any gene or genes, protein or proteins, or epigenetic element(s) whose expression profile or whose occurrence is associated with a specific cell type, subtype, or cell state of a specific cell type or subtype within a population of cells (e.g., tumor infiltrating lymphocytes). In certain embodiments, the expression of the CD8+ TIL signatures are dependent on epigenetic modification of the genes or regulatory elements associated with the genes. Thus, in certain embodiments, use of signature genes includes epigenetic modifications that may be detected or modulated. For ease of discussion, when discussing gene expression, any of gene or genes, protein or proteins, or epigenetic element(s) may be substituted. As used herein, the terms "signature", "expression profile", or "expression program" may be used interchangeably. It is to be understood that also when referring to proteins (e.g. differentially expressed proteins), such may fall within the definition of "gene" signature. Levels of expression or activity or prevalence may be compared between different cells in order to characterize or identify for instance signatures specific for cell (sub)populations. Increased or decreased expression or activity of signature genes may be compared between different cells in order to characterize or identify for instance specific cell (sub)populations. The detection of a signature in single cells may be used to identify and quantitate for instance specific cell (sub)populations. A signature may include a gene or genes, protein or proteins, or epigenetic element(s) whose expression or occurrence is specific to a cell (sub)population, such that expression or occurrence is

exclusive to the cell (sub)population. A gene signature as used herein, may thus refer to any set of up- and down-regulated genes that are representative of a cell type or subtype. A gene signature as used herein, may also refer to any set of up- and down-regulated genes between different cells or cell (sub)populations derived from a gene-expression profile. For example, a gene signature may comprise a list of genes differentially expressed in a distinction of interest.

[0124] The signature as defined herein (being it a gene signature, protein signature or other genetic or epigenetic signature) can be used to indicate the presence of a cell type, a subtype of the cell type, the state of the microenvironment of a population of cells, a particular cell type population or subpopulation, and/or the overall status of the entire cell (sub)population. Furthermore, the signature may be indicative of cells within a population of cells *in vivo*. The signature may also be used to suggest for instance particular therapies, or to follow up treatment, or to suggest ways to modulate immune systems. The signatures of the present invention may be discovered by analysis of expression profiles of single-cells within a population of cells from isolated samples (e.g. tumor samples), thus allowing the discovery of novel cell subtypes or cell states that were previously invisible or unrecognized. The presence of subtypes or cell states may be determined by subtype specific or cell state specific signatures. The presence of these specific cell (sub)types or cell states may be determined by applying the signature genes to bulk sequencing data in a sample. Not being bound by a theory the signatures of the present invention may be microenvironment specific, such as their expression in a particular spatio-temporal context. Not being bound by a theory, signatures as discussed herein are specific to a particular pathological context. Not being bound by a theory, a combination of cell subtypes having a particular signature may indicate an outcome. Not being bound by a theory, the signatures can be used to deconvolute the network of cells present in a particular pathological condition. Not being bound by a theory the presence of specific cells and cell subtypes are indicative of a particular response to treatment, such as including increased or decreased susceptibility to treatment. The signature may indicate the presence of one particular cell type.

[0125] The signature according to certain embodiments of the present invention may comprise or consist of one or more genes, proteins and/or epigenetic elements, such as for instance 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more. In certain embodiments, the signature may comprise or consist of two or more genes, proteins and/or epigenetic elements, such as for instance 2, 3, 4, 5, 6, 7, 8, 9, 10 or more. In certain embodiments, the signature may comprise

or consist of three or more genes, proteins and/or epigenetic elements, such as for instance 3, 4, 5, 6, 7, 8, 9, 10 or more. In certain embodiments, the signature may comprise or consist of four or more genes, proteins and/or epigenetic elements, such as for instance 4, 5, 6, 7, 8, 9, 10 or more. In certain embodiments, the signature may comprise or consist of five or more genes, proteins and/or epigenetic elements, such as for instance 5, 6, 7, 8, 9, 10 or more. In certain embodiments, the signature may comprise or consist of six or more genes, proteins and/or epigenetic elements, such as for instance 6, 7, 8, 9, 10 or more. In certain embodiments, the signature may comprise or consist of seven or more genes, proteins and/or epigenetic elements, such as for instance 7, 8, 9, 10 or more. In certain embodiments, the signature may comprise or consist of eight or more genes, proteins and/or epigenetic elements, such as for instance 8, 9, 10 or more. In certain embodiments, the signature may comprise or consist of nine or more genes, proteins and/or epigenetic elements, such as for instance 9, 10 or more. In certain embodiments, the signature may comprise or consist of ten or more genes, proteins and/or epigenetic elements, such as for instance 10, 11, 12, 13, 14, 15, or more. It is to be understood that a signature according to the invention may for instance also include genes or proteins as well as epigenetic elements combined.

[0126] In certain embodiments, a signature is characterized as being specific for a particular immune cell or immune cell (sub)population if it is upregulated or only present, detected or detectable in that particular immune cell or immune cell (sub)population, or alternatively is downregulated or only absent, or undetectable in that particular immune cell or immune cell (sub)population. In this context, a signature consists of one or more differentially expressed genes/proteins or differential epigenetic elements when comparing different cells or cell (sub)populations, including comparing different immune cell or immune cell (sub)populations, as well as comparing immune cell or immune cell (sub)populations with non-immune cell or non-immune cell (sub)populations. It is to be understood that "differentially expressed" genes/proteins include genes/proteins which are up- or down-regulated as well as genes/proteins which are turned on or off. When referring to up- or down-regulation, in certain embodiments, such up- or down-regulation is preferably at least two-fold, such as two-fold, three-fold, four-fold, five-fold, or more, such as for instance at least ten-fold, at least 20-fold, at least 30-fold, at least 40-fold, at least 50-fold, or more. Alternatively, or in addition, differential expression may be determined based on common statistical tests, as is known in the art.

[0127] As discussed herein, differentially expressed genes/proteins, or differential epigenetic elements may be differentially expressed on a single cell level, or may be differentially expressed on a cell population level. Preferably, the differentially expressed genes/ proteins or epigenetic elements as discussed herein, such as constituting the gene signatures as discussed herein, when as to the cell population or subpopulation level, refer to genes that are differentially expressed in all or substantially all cells of the population or subpopulation (such as at least 80%, preferably at least 90%, such as at least 95% of the individual cells). This allows one to define a particular subpopulation of immune cells. As referred to herein, a "subpopulation" of cells preferably refers to a particular subset of cells of a particular cell type which can be distinguished or are uniquely identifiable and set apart from other cells of this cell type. The cell subpopulation may be phenotypically characterized, and is preferably characterized by the signature as discussed herein. A cell (sub)population as referred to herein may constitute of a (sub)population of cells of a particular cell type characterized by a specific cell state.

[0128] When referring to induction, or alternatively suppression of a particular signature, preferable is meant induction or alternatively suppression (or upregulation or downregulation) of at least one gene/protein and/or epigenetic element of the signature, such as for instance at least two, at least three, at least four, at least five, at least six, or all genes/proteins and/or epigenetic elements of the signature.

[0129] Signatures may be functionally validated as being uniquely associated with a particular immune responder phenotype. Induction or suppression of a particular signature may consequentially be associated with or causally drive a particular immune responder phenotype.

[0130] Various aspects and embodiments of the invention may involve analyzing gene signatures, protein signature, and/or other genetic or epigenetic signature based on single cell analyses (e.g. single cell RNA sequencing) or alternatively based on cell population analyses, as is defined herein elsewhere.

[0131] In certain example embodiments, the signature genes may be used to deconvolute the network of cells present in a tumor based on comparing them to data from bulk analysis of a tumor sample. In certain example embodiments, the presence of specific immune cells and immune cell subtypes may be indicative of tumor growth, invasiveness and/or resistance to treatment. In one example embodiment, detection of one or more signature genes may indicate the presence of a particular cell type or cell types. In certain example embodiments,

the presence of immune cell types within a tumor may indicate that the tumor will be resistant to a treatment. In one embodiment, the signature genes of the present invention are applied to bulk sequencing data from a tumor sample obtained from a subject, such that information relating to disease outcome and personalized treatments is determined.

[0132] In certain embodiments, cell type markers for immune cells present in a tumor (i.e., tumor microenvironment) may be used to deconvolute bulk expression data (see, e.g., Venteicher, A.S., Tirosh, L, Hebert, C , Yizhak, K., C , N., Filbin, M.G., Hoverstadt, V., Escalante, L.E., Saw, M.L., Rodman, C , et al. (2017). Decoupling genetics, lineages and tumor micro-environment in gliomas by single-cell RNA-seq. *Science* 355; Tirosh, L, Izar, B., Prakadan, S.M., Wadsworth, M.H., 2nd, Treacy, D., Trombetta, J.J., Rotem, A., Rodman, C , Lian, C , Murphy, G., et al. (2016a). Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. *Science* 352, 189-196; and Tirosh, L, Venteicher, A.S., Hebert, C , Escalante, L.E., Patel, A.P., Yizhak, K., Fisher, J.M., Rodman, C , Mount, C , Filbin, M.G., et al. (2016b). Single-cell RNA-seq supports a developmental hierarchy in human oligodendrogloma. *Nature* 539, 309-313).

[0133] In certain embodiments, mutations occur in individual cancers that may be used to detect cancer progression. These mutations may be used in conjunction with the responder and non-responder phenotypes described herein. Mutations related to T cell cytolytic activity against tumors have been characterized and may be detected as part of the present invention (see e.g., Rooney et al., Molecular and genetic properties of tumors associated with local immune cytolytic activity, *Cell*. 2015 January 15; 160(1-2): 48-61). In certain example embodiments, a patient may be selected for a check point blockade therapy based on detection of a gene signature as described herein in combination with mutations related to T cell cytolytic activity (see e.g., WO2016100975A1). In certain embodiments, cancer specific mutations associated with cytolytic activity may be a mutation in a gene selected from the group consisting of CASP8, B2M, PIK3CA, SMC1A, ARID5B, TET2, ALPK2, COL5A1, TP53, DNER, NCOR1, MORC4, CIC, IRF6, MYOCD, ANKLE 1, CNKSR1, NFI, SOS1, ARID2, CUL4B, DDX3X, FUBP1, TCP1 1L2, HLA-A, B or C, CSNK2A1, MET, ASXL1, PD-L1, PD-L2, IDO1, ID02, ALOX12B and ALOX15B, or copy number gain, excluding whole-chromosome events, impacting any of the following chromosomal bands: 6ql6.1-q21, 6q22.31-q24.1, 6q25.1-q26, 7pl 1.2-ql 1.1, 8p23.1, 8pl 1.23-pl 1.21 (containing IDO1, ID02), 9p24.2-p23 (containing PDL1, PDL2), 10pl5.3, 10pl5.1-pl3, 1lpl4.1, 12pl3.32-pl3.2, 17pl3.1 (containing ALOX12B, ALOX15B), and 22ql 1.1-ql 1.21. In

certain embodiments, the mutation is further associated with antigen presentation. Mutations associated with antigen presentation may comprises mutations in B2M and HLA-A, B or C.

Detection of "Non-Responder" Sub-Populations

[0134] In one embodiment, the method comprises detecting a first population of immune cells from a biological sample of a subject. Immune cells can be detected by sorting for CD45+ cells. CD45 is a pan-leukocyte protein. In one embodiment, the method comprises detecting a first population of CD8+ TIL from a biological sample of a subject. In certain example embodiments, detection of the first population in the biological sample indicates a likelihood that a subject will be non-responsive to a particular therapy. In certain example embodiments, detection of the first population indicates a likelihood the subject will be non-responsive to a checkpoint blockade therapy. The first population may generally be characterized by increased expression of inhibitory receptors.

[0135] A marker, for example a gene or gene product, for example a peptide, polypeptide, protein, or nucleic acid, or a group of two or more markers, is "detected" or "measured" in a tested object (e.g., in or on a cell, cell population, tissue, organ, or organism, e.g., in a biological sample of a subject) when the presence or absence and/or quantity of said marker or said group of markers is detected or determined in the tested object, preferably substantially to the exclusion of other molecules and analytes, e.g., other genes or gene products.

[0136] The terms "sample" or "biological sample" as used throughout this specification include any biological specimen obtained from a subject. Particularly useful samples are those known to comprise, or expected or predicted to comprise immune cells as taught herein. Preferably, a sample may be readily obtainable by minimally invasive methods, such as blood collection or tissue biopsy, allowing the removal / isolation / provision of the sample from the subject. Examples of particularly useful samples include without limitation whole blood or a cell-containing fraction of whole blood, such as serum, white blood cells, or peripheral blood mononuclear cells (PBMC), lymph, lymphatic tissue, inflammation fluid, tissue specimens, or tissue biopsies. The term "tissue" as used throughout this specification refers to any animal tissue types including, but not limited to, bone, bone marrow, neural tissue, fibrous connective tissue, cartilage, muscle, vasculature, skin, adipose tissue, blood and glandular tissue or other non-bone tissue. The tissue may be healthy or affected by pathological alterations, e.g., tumor tissue or tissue affected by a disease comprising an immune component. The tissue may be from a living subject or may be cadaveric tissue. The

tissue may be autologous tissue or syngeneic tissue or may be allograft or xenograft tissue. A biological sample may also include cells grown in tissue culture, such as cells used for screening drugs or primary cells grown in culture for expansion.

[0137] The term "immune cell" as used throughout this specification generally encompasses any cell derived from a hematopoietic stem cell that plays a role in the immune response. The term is intended to encompass immune cells both of the innate or adaptive immune system. The immune cell as referred to herein may be a leukocyte, at any stage of differentiation (e.g., a stem cell, a progenitor cell, a mature cell) or any activation stage. Immune cells include lymphocytes (such as natural killer cells, T-cells (including, e.g., thymocytes, Th or Tc; Th1, Th2, Th17, Th $\alpha\beta$, CD4+, CD8+, effector Th, memory Th, regulatory Th, CD4+/CD8+ thymocytes, CD4-/CD8- thymocytes, $\gamma\delta$ T cells, etc.) or B-cells (including, e.g., pro-B cells, early pro-B cells, late pro-B cells, pre-B cells, large pre-B cells, small pre-B cells, immature or mature B-cells, producing antibodies of any isotype, T1 B-cells, T2, B-cells, naive B-cells, GC B-cells, plasmablasts, memory B-cells, plasma cells, follicular B-cells, marginal zone B-cells, B-1 cells, B-2 cells, regulatory B cells, etc.), such as for instance, monocytes (including, e.g., classical, non-classical, or intermediate monocytes), (segmented or banded) neutrophils, eosinophils, basophils, mast cells, histiocytes, microglia, including various subtypes, maturation, differentiation, or activation stages, such as for instance hematopoietic stem cells, myeloid progenitors, lymphoid progenitors, myeloblasts, promyelocytes, myelocytes, metamyelocytes, monoblasts, promonocytes, lymphoblasts, prolymphocytes, small lymphocytes, macrophages (including, e.g., Kupffer cells, stellate macrophages, M1 or M2 macrophages), (myeloid or lymphoid) dendritic cells (including, e.g., Langerhans cells, conventional or myeloid dendritic cells, plasmacytoid dendritic cells, mDC-1, mDC-2, Mo-DC, HP-DC, veiled cells), granulocytes, polymorphonuclear cells, antigen-presenting cells (APC), etc.

[0138] The terms "quantity", "amount" and "level" are synonymous and generally well-understood in the art. The terms as used throughout this specification may particularly refer to an absolute quantification of a marker in a tested object (e.g., in or on a cell, cell population, tissue, organ, or organism, e.g., in a biological sample of a subject), or to a relative quantification of a marker in a tested object, i.e., relative to another value such as relative to a reference value, or to a range of values indicating a base-line of the marker. Such values or ranges may be obtained as conventionally known.

[0139] An absolute quantity of a marker may be advantageously expressed as weight or as molar amount, or more commonly as a concentration, e.g., weight per volume or mol per volume. A relative quantity of a marker may be advantageously expressed as an increase or decrease or as a fold-increase or fold-decrease relative to said another value, such as relative to a reference value. Performing a relative comparison between first and second variables (e.g., first and second quantities) may but need not require determining first the absolute values of said first and second variables. For example, a measurement method may produce quantifiable readouts (such as, e.g., signal intensities) for said first and second variables, wherein said readouts are a function of the value of said variables, and wherein said readouts may be directly compared to produce a relative value for the first variable vs. the second variable, without the actual need to first convert the readouts to absolute values of the respective variables.

[0140] The terms "diagnosis" and "monitoring" are commonplace and well-understood in medical practice. By means of further explanation and without limitation the term "diagnosis" generally refers to the process or act of recognising, deciding on or concluding on a disease or condition in a subject on the basis of symptoms and signs and/or from results of various diagnostic procedures (such as, for example, from knowing the presence, absence and/or quantity of one or more biomarkers characteristic of the diagnosed disease or condition).

[0141] The term "monitoring" generally refers to the follow-up of a disease or a condition in a subject for any changes which may occur over time.

[0142] The terms "prognosing" or "prognosis" generally refer to an anticipation on the progression of a disease or condition and the prospect (e.g., the probability, duration, and/or extent) of recovery. A good prognosis of the diseases or conditions taught herein may generally encompass anticipation of a satisfactory partial or complete recovery from the diseases or conditions, preferably within an acceptable time period. A good prognosis of such may more commonly encompass anticipation of not further worsening or aggravating of such, preferably within a given time period. A poor prognosis of the diseases or conditions as taught herein may generally encompass anticipation of a substandard recovery and/or unsatisfactorily slow recovery, or to substantially no recovery or even further worsening of such.

[0143] The terms also encompass prediction of a disease. The terms "predicting" or "prediction" generally refer to an advance declaration, indication or foretelling of a disease or

condition in a subject not (yet) having said disease or condition. For example, a prediction of a disease or condition in a subject may indicate a probability, chance or risk that the subject will develop said disease or condition, for example within a certain time period or by a certain age. Said probability, chance or risk may be indicated inter alia as an absolute value, range or statistics, or may be indicated relative to a suitable control subject or subject population (such as, e.g., relative to a general, normal or healthy subject or subject population). Hence, the probability, chance or risk that a subject will develop a disease or condition may be advantageously indicated as increased or decreased, or as fold-increased or fold-decreased relative to a suitable control subject or subject population. As used herein, the term "prediction" of the conditions or diseases as taught herein in a subject may also particularly mean that the subject has a 'positive' prediction of such, i.e., that the subject is at risk of having such (e.g., the risk is significantly increased vis-a-vis a control subject or subject population). The term "prediction of no" diseases or conditions as taught herein as described herein in a subject may particularly mean that the subject has a 'negative' prediction of such, i.e., that the subject's risk of having such is not significantly increased vis-a-vis a control subject or subject population.

[0144] Reference values may be established according to known procedures previously employed for other cell populations, biomarkers and gene or gene product signatures. For example, a reference value may be established in an individual or a population of individuals characterized by a particular diagnosis, prediction and/or prognosis of said disease or condition (i.e., for whom said diagnosis, prediction and/or prognosis of the disease or condition holds true). Such population may comprise without limitation 2 or more, 10 or more, 100 or more, or even several hundred or more individuals.

[0145] A "deviation" of a first value from a second value may generally encompass any direction (e.g., increase: first value > second value; or decrease: first value < second value) and any extent of alteration.

[0146] For example, a deviation may encompass a decrease in a first value by, without limitation, at least about 10% (about 0.9-fold or less), or by at least about 20% (about 0.8-fold or less), or by at least about 30% (about 0.7-fold or less), or by at least about 40% (about 0.6-fold or less), or by at least about 50% (about 0.5-fold or less), or by at least about 60% (about 0.4-fold or less), or by at least about 70% (about 0.3-fold or less), or by at least about 80% (about 0.2-fold or less), or by at least about 90% (about 0.1-fold or less), relative to a second value with which a comparison is being made.

[0147] For example, a deviation may encompass an increase of a first value by, without limitation, at least about 10% (about 1.1-fold or more), or by at least about 20% (about 1.2-fold or more), or by at least about 30% (about 1.3-fold or more), or by at least about 40% (about 1.4-fold or more), or by at least about 50% (about 1.5-fold or more), or by at least about 60% (about 1.6-fold or more), or by at least about 70% (about 1.7-fold or more), or by at least about 80% (about 1.8-fold or more), or by at least about 90% (about 1.9-fold or more), or by at least about 100% (about 2-fold or more), or by at least about 150% (about 2.5-fold or more), or by at least about 200% (about 3-fold or more), or by at least about 500% (about 6-fold or more), or by at least about 700% (about 8-fold or more), or like, relative to a second value with which a comparison is being made.

[0148] Preferably, a deviation may refer to a statistically significant observed alteration. For example, a deviation may refer to an observed alteration which falls outside of error margins of reference values in a given population (as expressed, for example, by standard deviation or standard error, or by a predetermined multiple thereof, e.g., $\pm 1 \times SD$ or $\pm 2 \times SD$ or $\pm 3 \times SD$, or $\pm 1 \times SE$ or $\pm 2 \times SE$ or $\pm 3 \times SE$). Deviation may also refer to a value falling outside of a reference range defined by values in a given population (for example, outside of a range which comprises $>40\%$, $\geq 50\%$, $>60\%$, $>70\%$, $>75\%$ or $>80\%$ or $>85\%$ or $>90\%$ or $>95\%$ or even $>100\%$ of values in said population).

[0149] In a further embodiment, a deviation may be concluded if an observed alteration is beyond a given threshold or cut-off. Such threshold or cut-off may be selected as generally known in the art to provide for a chosen sensitivity and/or specificity of the prediction methods, e.g., sensitivity and/or specificity of at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 85%, or at least 90%, or at least 95%.

[0150] For example, receiver-operating characteristic (ROC) curve analysis can be used to select an optimal cut-off value of the quantity of a given immune cell population, biomarker or gene or gene product signatures, for clinical use of the present diagnostic tests, based on acceptable sensitivity and specificity, or related performance measures which are well-known per se, such as positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LR+), negative likelihood ratio (LR-), Youden index, or similar.

[0151] In certain embodiments, subjects comprising CD8+ TILs having a non-responder gene signature as described herein are treated with a non-immunotherapy treatment. A non-immunotherapy treatment may involve a non-immunotherapy standard of care. Aspects of the invention involve modifying the therapy within a standard of care based on the detection of a

gene signature as described herein. The term "standard of care" as used herein refers to the current treatment that is accepted by medical experts as a proper treatment for a certain type of disease and that is widely used by healthcare professionals. Standard of care is also called best practice, standard medical care, and standard therapy. Standards of care for cancer generally include surgery, lymph node removal, radiation, chemotherapy, targeted therapies, antibodies targeting the tumor, and immunotherapy. The standards of care for the most common cancers can be found on the website of National Cancer Institute (www.cancer.gov/cancertopics). A treatment clinical trial is a research study meant to help improve current treatments or obtain information on new treatments for patients with cancer. When clinical trials show that a new treatment is better than the standard treatment, the new treatment may be considered the new standard treatment.

[0152] In certain example embodiments, a method for detecting the first CD8+ tumor infiltrating lymphocyte (TIL) sub-population comprises detecting increased expression of one or more biomarkers in a sample, wherein the one or more biomarkers are selected from a first group consisting of CD38, CCL3, VCAM1, GOLIM4, HAVCR2, PRDX3, ENTPD1, PTTG1, CCR5, TRAFD1, PDCD1, CXCR6, BATF, PTPN6, LAG3 and CTLA4 or any CD8_B gene listed in Table 6.

[0153] The terms "increased" or "increase" or "upregulated" or "upregulate" as used herein generally mean an increase by a statistically significant amount. For avoidance of doubt, "increased" means a statistically significant increase of at least 10% as compared to a reference level, including an increase of at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100% or more, including, for example at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold, at least 10-fold increase or greater as compared to a reference level, as that term is defined herein.

[0154] In one example embodiment, the method comprises detecting CD83 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0155] In another example embodiment, the method comprises detecting CCL3 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0156] In another example embodiment, the method comprises detecting VCAM1 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0157] In another example embodiment, the method comprises detecting GOLIM4 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0158] In another example embodiment, the method comprises detecting HAVCR2 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0159] In another example embodiment, the method comprises detecting PRDX3 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0160] In another example embodiment, the method comprises detecting ENTPD1 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0161] In another example embodiment, the method comprises detecting PTTG1 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0162] In another example embodiment, the method may comprise detecting CCR5 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0163] In another example embodiment, the method may comprise detecting TRAFD1 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0164] In another example embodiment, the method may comprise detecting PDCD1 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0165] In another example embodiment, the method may comprise detecting CXCR6, and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0166] In another example embodiment, the method may comprise detecting BATF and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0167] In another example embodiment, the method may comprise detecting PTPN6 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0168] In another example embodiment, the method may comprise detecting LAG3 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0169] In another example embodiment, the method may comprise detecting CTLA4 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0170] In certain example embodiments, the method comprises detecting a first population of CD45+ cells obtained from a biological sample. In certain example embodiments, detection of the population in the biological sample indicates a likelihood that a subject will be responsive to a particular therapy. In certain example embodiments, detection of the population indicates a likelihood the subject will be responsive to a checkpoint blockade therapy. The population may generally be characterized by increased expression of memory and differentiation genes.

[0171] In certain embodiments, the population is characterized by expression of a gene signature comprising one or more genes or polypeptides selected from the group consisting of: TCF7; TCF7, PLAC8, LTB, and CCR7; or TCF7, LEF1, S1PR1, PLAC8, LTB, CCR7, IGHD, PAX5, FCRL1, FCER2, CD19, CD22, BANK1, MS4A1, BLK, RALGPS2 and FAM129C; or TCF7, PLAC8, LTB, LY9, SELL, IGKC and CCR7 (Tables 4 and 5).

Detection of "Responder" Sub-Populations

[0172] In another embodiment, the method comprises detecting a population of CD8+ TIL from a biological sample of a subject. In certain example embodiments, detection of the population in the biological sample indicates a likelihood that a subject will be responsive to a particular therapy. In certain example embodiments, detection of the population indicates a likelihood the subject will be responsive to a checkpoint blockade therapy. The second population may generally be characterized by increased expression of memory and differentiation genes.

[0173] In certain example embodiments, a method for detecting the second CD8+ tumor infiltrating lymphocyte (TIL) sub-population comprises detecting increased expression of one or more biomarkers selected from a second group consisting of IL7R, GPR183, TCF7, LMNA, NR4A3, CD55, AIM1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N, CSRNP1, FAM65B, PIK3R1, RGPD6, SKIL, TSC22D2, USP36, FOXP1, EGRI, MYADM, ZFP36L2, FAM102A, RGCC, PDE4B, PFKFB3, FOSB, DCTN6 and BTG2. In certain embodiments, the method comprises detecting increased expression of one

or more biomarkers selected from a second group consisting of IL7R, GPR183, TCF7, LMNA, NR4A3, CD55, AIM1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N and CSRNP1. In certain embodiments, the method comprises detecting increased expression of TCF7. In certain embodiments, the method comprises detecting increased expression or any CD8_G gene listed in Table 6.

[0174] In one example embodiment, the method of detecting the second population may comprise detecting IL7R and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32

[0175] In another example embodiment, the method of detecting the second population may comprise GPR183 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32

[0176] In another example embodiment, the method of detecting the second population may comprise TCF7 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0177] In another example embodiment, the method of detecting the second population may comprise LMNA and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0178] In another example embodiment, the method of detecting the second population may comprise NR4A3 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0179] In another example embodiment, the method of detecting the second population may comprise CD55 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0180] In another example embodiment, the method of detecting the second population may comprise AIM1 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0181] In another example embodiment, the method of detecting the second population may comprise MGAT4A and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0182] In another example embodiment, the method of detecting the second population may comprise PERI and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0183] In another example embodiment, the method of detecting the second population may comprise FOSL2 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0184] In another example embodiment, the method of detecting the second population may comprise TSPYL2 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0185] In another example embodiment, the method of detecting the second population may comprise REL and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0186] In another example embodiment, the method of detecting the second population may comprise FAM177A1 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0187] In another example embodiment, the method of detecting the second population may comprise YPEL5 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0188] In another example embodiment, the method of detecting the second population may comprise TC2N and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0189] In another example embodiment, the method of detecting the second population may comprise CSRNP1 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0190] In another example embodiment, the method of detecting the second population may comprise FAM65B and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0191] In another example embodiment, the method of detecting the second population may comprise PIK3R1 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0192] In another example embodiment, the method of detecting the second population may comprise RGPD6 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0193] In another example embodiment, the method of detecting the second population may comprise SKIL and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0194] In another example embodiment, the method of detecting the second population may comprise TSC22D2 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0195] In another example embodiment, the method of detecting the second population may comprise USP36 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0196] In another example embodiment, the method of detecting the second population may comprise FOXP1 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0197] In another example embodiment, the method of detecting the second population may comprise EGR1 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0198] In another example embodiment, the method of detecting the second population may comprise MYADM and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0199] In another example embodiment, the method of detecting the second population may comprise ZP36L2 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0200] In another example embodiment, the method of detecting the second population may comprise FAM102A and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0201] In another example embodiment, the method of detecting the second population may comprise RGCC and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0202] In another example embodiment, the method of detecting the second population may comprise PDE4B and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0203] In another example embodiment, the method of detecting the second population may comprise PFKFB3 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0204] In another example embodiment, the method of detecting the second population may comprise FOSB and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0205] In another example embodiment, the method of detecting the second population may comprise DCTN6 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0206] In another example embodiment, the method of detecting the second population may comprise BTG2 and at least N additional biomarkers selected from the first group, wherein N equals 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, or 32.

[0207] In certain example embodiments, the method comprises detecting a second population of CD45+ cells obtained from a biological sample. In certain example embodiments, detection of the population in the biological sample indicates a likelihood that a subject will be nonresponsive to a particular therapy. In certain example embodiments, detection of the population indicates a likelihood the subject will be nonresponsive to a checkpoint blockade therapy.

[0208] In certain embodiments, the population is characterized by expression of a gene signature comprising one or more genes or polypeptides selected from the group consisting of: ENTPD1 and HAVCR2; or CCL3, CD38 and HAVCR2; or CD38, PDCD1, CCL3, SNAP47, VCAMI, HAVCR2, FASLG, ENTPD1, SIRPG, MY07A, FABP5, NDUFB3, UBE2F, CLTA and SNRPDI; or FASLG, VCAMI, CCL3, LAG3, CXCR6, IFNG, PDCD1, KLRD1, HAVCR2, SIRPG, SNAP47, DTHD1, PRF1, GZMH, F2R, CD38, CXCL13, TNFRSF4, TNFRSF18, MAF, ETV7, CD4, CTLA4, FCRL6, SPON2, KLRG1, TRGC1, A2M, FCGR3A, GZMA, HOPX, NKG7, PXN, TNFRSF9, GEM, NABI, DFNB31, CADMI, CRTAM, GPR56, MY07A, DUSP4, METRNL and PHLDA1; or CCL3, LGALS1, CD38, EPSTI1, WARS, PLEK, HAVCR2, LGALS3, FABP5, MT2A, GBP1, PLSCR1, CCR5, GSTO1, ANXA5, GLUL, PYCARD, TYMP, IFI6, VAMP5, OASL, GZMB, TXN, SQRDL, RHOC, AP2S1, GZMH, CCL4L2, SNAP47, LAP3, ATP6V1B2, CCL4L1, LAMP2, PSMA4, SERPINB1, HIGD1A, UBE2F, TALDO1, CD63, CLTA, S100A11, PHPT1, GBP4, PRDX3, PSMB2, BST2, GBP5, CTSC, NDUFB3, NPC2, GALM, GLIPR2, CCL4, PRF1, IFNG, IFI30, CHST12, ISG15, MYD88, IDH2, MTHFD2, CHMP2A, NDUFA9, CHMP5, CALM3, ANXA2, PPT1, GTF3C6, NDUFAB1, CXCR6, RNF181, LGALS9, COX5A, OAS2, PDCD1, SNRPC, BHLHE40, TWF2, SLAMF7, TXN2, CARD16, ANAPCI1, MRPL51, LIMS1, NDUFA12, RANBP1, GBP2, PSMC1, ACTR1A, CD2BP2, VDAC1, EMC7, MX1, GPS1, ATP5J2, USMG5, SHFM1, ATP51, FAM96A,

CASP1, PARP9, NOP10, GNG5, CYC1, RAB1 1A, PGAM1, ENTPD1, PDIA6, PSMC3, TMBIM1, UBE2L6, PSMA6, EIF6, DCTN3, SEC 11A, CSTB, ETFB, DBI, GRN, ELOVL1, UBE2L3, PSMB3, NDUFB7, DOK2, SEC61G, IGFLR1, ATP5H, COPZ1, ATP6V1F, BNIP3L, NUTF2, AKRIAI, MDH2, VAMP8, ROMO1, CXCR3, SAMHD1, NUCB1, ACTN4, ZYX, FLOT1, BLOC1S1, STAT1, VFMP, PAM, NUDT21, MYOIG, C17orf49, GTF2A2, HIST2H2AA4, C19orf10, ABI3, TRAPPC5, PSMC4, NDUFC2, HN1, SNRPD3, CMC1, RAB27A, NDUFA6, POMP, PFKP, ATP5G3, TMEM179B, PSMD9, IRF7, CNIH1, DYNLRB1, APOL2, TKT, DCTN2, GSDMD, STOM, CTSD, KDELR2, ATP5J, RPS27L, PSME2, DRAPI, NDUFBIO, DECR1, GSTP1, TMED9, MGAT1, HSPB1, COX8A, ZEB2, ILK, PSMB6, HK1, CD58, TMX1, GZMA, SRI, PSMG2, ARL8B, NKG7, GPX1, ACP5, CHP1, GPR171, ATP6V0B, KLRD1, H2AFY, PPM1G, PRDX5, PSMA5, FBXW5, ATP6AP1, CD4, SNRPD1, XAF1, LY6E, DYNLT1, AK2, PSMA2, YIPF3, S100A10, SCP2, MRPS34, PSMD4, CDC123, BTG3, TMEM258, TSPO, SDHB, TCEB1, WDR830S, HCST, NAA10, CTSB, YARS, GLRX, RBCK1, RBX1, LAMTOR1, UQCRC1, NDUFB4, CAPZA2, BRK1, ADRM1, NDUFB2, ETFA, VDAC3, NUDT5, IFITM3, BANF1, ZNHIT1, CAPG, NHP2, LASP1, TOMM5, MVP, CTSW, AURKAIP1, RARRES3, PSMB10, TMEM173, SLX1A, APOBEC3G, GFMAP4, EIF4E, CTLA4, NDUFS8, CYB5B, PIK3R5, HEXB, STXBP2, PSMD8, SEC61B, RGS10, PHB, ATP5C1, ARF5, SUMO3, PRDX6, RNHI, ATP5F1, UQCRC1, SARNP, PLIN2, PIN1, SDHC, SF3B14, CAPRINI, POLR2G, COX7B, UQCRC10, FBX07, NDUFB6, S100A4, PRELID1, TRPV2, SF3B5, MYOIF, SCAMP2, RNF7, CXCL13, RAB1B, SHKBP1, PET100, HM13, VTI1B, S100A6, ARPC5, FDPS, MINOS 1, RAB10, NEDD8, BATF, PHB2, ERH, NCOA4, PDIA4, PSMB9, C11orf48, TMEM50A, TIGIT, NDUFA1 1, NELFE, COX6C, SLA2, PSMB8, NDUFS7, RER1, RAB8A, CAPN1, MRPL20, COX5B, SEC13, FKBP1A, PRDM1, RAB1A, RHOG, CYB5R3, AIP, ABRACL, PSMB7, COX6B1, PSMD7, PPA1, PCMT1, SURF4, ENY2, TCEB2, MAP2K3, AL353354.2, AKIRIN2, MAPRE1, GRSF1, DUSP4, ATG3, SRGAP2, ATP6V0D1, NELFCD, LRPAPl, C14orf166, SNRPB2, CHMP4A, SFT2D1, CASP4, NME1-NME2, FAM96B, FDFT1, SLC25A39, LMAN2, MDH1, RHBDD2, ARPC5L, TBCA, EBP, SEC14L1, EIF2S2, CST7, STARD7, SOD2, SPN, FAM32A, SEC1 1C, TNFRSF1B, POLR2E, NDUFA13, OSTC, UFC1, C18orf32, SRP19, C14orf2, UQCRC1 1, PDCD6, AP2M1, PPP1CA, ATP6AP2, SSR3, UNCI 3D, FERMT3, ARHGAP1, EIF3I, CECR1, MRPS6, DNPH1, DCXR, PSMF1, SNRPG, CNDP2, ANXA1 1, SLM02, C16orf13, CAPN2, BSG, LAMTOR5, SIVA1, TRAPPC1, TMCO1, PSMD13, PSMB1, RSU1,

NDUFA1, TUBB, DCTN1, SH3GLB1, BCAP31, RTFDC1, UFDIL, GPI, DNAJB11, SNX17, SH2D2A, Clorf43, BUD31, PSTPIP1, CTSA, TPST2, MPV17, APMAP, CMC2, UQCRQ, TBCB, C9orf16, PARK7, ATP5EP2, SHISA5, SMC4, TAPI, SCAND1, SIRPG, HDLBP, EMC4, FIS1, TPI1, GOLGA7, POLR2J, EIF2S1, UBA3, P4HB, UQCRH, CSNK2B, SZRD1, NDUFA3, ATP50, DERL2, COPS6, COPE, SNX6, FLU and ERGIC3. (Tables 4 and 5).

Treatment Selection

[0209] In another aspect, the invention comprises determining a subject's responsiveness to a particular therapeutic, including a checkpoint blockade therapeutic, by determining a ratio of the first cell population to the second cell population, wherein if the ratio of the second population in a sample is lower relative to the first population the subject is classified as non-responsive to the therapeutic, and wherein if the ratio of the second cell population is higher in a sample relative to the first population the patient is classified as responsive to the therapeutic.

[0210] Thus, in certain example embodiments a method of treating a subject in need thereof may comprise detecting a first CD8+ cell population from a sample from the subject using any of the biomarkers or combination of biomarkers discussed above, detecting a second CD8+ cell population from a same or different sample from the subject using any of the biomarkers or combination of biomarkers discussed above, and determining a ratio of the first CD8+ population to the second CD8+ population, wherein the subject is treated with a first therapeutic or therapeutic combination if the ratio of the first population is higher relative to the second population, and wherein the subject is treated with a second therapeutic or therapeutic combination if the ratio of the second population is higher relative to the first population. In certain example embodiment, the first therapeutic or therapeutic combination is a non-checkpoint blockade therapeutic, and the second therapeutic or therapeutic combination is a checkpoint blockade therapeutic.

[0211] Thus, in certain example embodiments a method of treating a subject in need thereof may comprise detecting a first CD45+ cell population from a sample from the subject using any of the biomarkers or combination of biomarkers discussed above, detecting a second CD45+ cell population from a same or different sample from the subject using any of the biomarkers or combination of biomarkers discussed above, and determining a ratio of the first CD45+ population to the second CD45+ population, wherein the subject is treated with a first therapeutic or therapeutic combination if the ratio of the first population is higher

relative to the second population, and wherein the subject is treated with a second therapeutic or therapeutic combination if the ratio of the second population is higher relative to the first population. In certain example embodiment, the first therapeutic or therapeutic combination is a non-checkpoint blockade therapeutic, and the second therapeutic or therapeutic combination is a checkpoint blockade therapeutic.

Detection of Biomarkers

[0212] Depending on factors that can be evaluated and decided on by a skilled person, such as, inter alia, the type of a marker (e.g., peptide, polypeptide, protein, or nucleic acid), the type of the tested object (e.g., a cell, cell population, tissue, organ, or organism, e.g., the type of biological sample of a subject, e.g., whole blood, plasma, serum, tissue biopsy), the expected abundance of the marker in the tested object, the type, robustness, sensitivity and/or specificity of the detection method used to detect the marker, etc., the marker may be measured directly in the tested object, or the tested object may be subjected to one or more processing steps aimed at achieving an adequate measurement of the marker.

[0213] In one embodiment, the biomarkers are detected by immunofluorescence, immunohistochemistry, fluorescence activated cell sorting (FACS), mass cytometry (CyTOF), Drop-seq, RNA-seq, scRNA-seq, InDrop, single cell qPCR, MERFISH (multiplex (*in situ*) RNA FISH) and/or by *in situ* hybridization. Other methods including absorbance assays and colorimetric assays are known in the art and may be used herein. In certain embodiments, primers and/or probes or fluorescently bar-coded oligonucleotide probes for hybridization to RNA are used to detect biomarkers (see e.g., Geiss GK, et al., Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol. 2008 Mar;26(3):3 17-25).

[0214] In other example embodiments, detection of a mark may include immunological assay methods, wherein the ability of an assay to separate, detect and/or quantify a marker (such as, preferably, peptide, polypeptide, or protein) is conferred by specific binding between a separable, detectable and/or quantifiable immunological binding agent (antibody) and the marker. Immunological assay methods include without limitation immunohistochemistry, immunocytochemistry, flow cytometry, mass cytometry, fluorescence activated cell sorting (FACS), fluorescence microscopy, fluorescence based cell sorting using microfluidic systems, immunoaffinity adsorption based techniques such as affinity chromatography, magnetic particle separation, magnetic activated cell sorting or bead

based cell sorting using microfluidic systems, enzyme-linked immunosorbent assay (ELISA) and ELISPOT based techniques, radioimmunoassay (RIA), Western blot, etc.

[0215] In certain example embodiments, detection of a marker or signature may include biochemical assay methods, including *inter alia* assays of enzymatic activity, membrane channel activity, substance-binding activity, gene regulatory activity, or cell signalling activity of a marker, e.g., peptide, polypeptide, protein, or nucleic acid.

[0216] In other example embodiments, detection of a mark may include mass spectrometry analysis methods. Generally, any mass spectrometric (MS) techniques that are capable of obtaining precise information on the mass of peptides, and preferably also on fragmentation and/or (partial) amino acid sequence of selected peptides (e.g., in tandem mass spectrometry, MS/MS; or in post source decay, TOF MS), may be useful herein for separation, detection and/or quantification of markers (such as, preferably, peptides, polypeptides, or proteins). Suitable peptide MS and MS/MS techniques and systems are well-known *per se* (see, e.g., Methods in Molecular Biology, vol. 146: "Mass Spectrometry of Proteins and Peptides", by Chapman, ed., Humana Press 2000, ISBN 089603609x; Biemann 1990. Methods Enzymol 193: 455-79; or Methods in Enzymology, vol. 402: "Biological Mass Spectrometry", by Burlingame, ed., Academic Press 2005, ISBN 9780121828073) and may be used herein. MS arrangements, instruments and systems suitable for biomarker peptide analysis may include, without limitation, matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) MS; MALDI-TOF post-source-decay (PSD); MALDI-TOF/TOF; surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF) MS; electrospray ionization mass spectrometry (ESI-MS); ESI-MS/MS; ESI-MS/(MS)*n* (*n* is an integer greater than zero); ESI 3D or linear (2D) ion trap MS; ESI triple quadrupole MS; ESI quadrupole orthogonal TOF (Q-TOF); ESI Fourier transform MS systems; desorption/ionization on silicon (DIOS); secondary ion mass spectrometry (SIMS); atmospheric pressure chemical ionization mass spectrometry (APCI-MS); APCI-MS/MS; APCI- (MS)*n*; atmospheric pressure photoionization mass spectrometry (APPI-MS); APPI-MS/MS; and APPI- (MS)*n*. Peptide ion fragmentation in tandem MS (MS/MS) arrangements may be achieved using manners established in the art, such as, e.g., collision induced dissociation (CID). Detection and quantification of markers by mass spectrometry may involve multiple reaction monitoring (MRM), such as described among others by Kuhn et al. 2004 (Proteomics 4: 1175-86). MS peptide analysis methods may be advantageously

combined with upstream peptide or protein separation or fractionation methods, such as for example with the chromatographic and other methods.

[0217] In other example embodiments, detection of a marker may include chromatography methods. In a one example embodiment, chromatography refers to a process in which a mixture of substances (analytes) carried by a moving stream of liquid or gas ("mobile phase") is separated into components as a result of differential distribution of the analytes, as they flow around or over a stationary liquid or solid phase ("stationary phase"), between said mobile phase and said stationary phase. The stationary phase may be usually a finely divided solid, a sheet of filter material, or a thin film of a liquid on the surface of a solid, or the like. Chromatography may be columnar. While particulars of chromatography are well known in the art, for further guidance see, e.g., Meyer M., 1998, ISBN: 047198373X, and "Practical HPLC Methodology and Applications", Bidlingmeyer, B. A., John Wiley & Sons Inc., 1993. Exemplary types of chromatography include, without limitation, high-performance liquid chromatography (HPLC), normal phase HPLC (NP-HPLC), reversed phase HPLC (RP-HPLC), ion exchange chromatography (IEC), such as cation or anion exchange chromatography, hydrophilic interaction chromatography (HILIC), hydrophobic interaction chromatography (HIC), size exclusion chromatography (SEC) including gel filtration chromatography or gel permeation chromatography, chromatofocusing, affinity chromatography such as immunoaffinity, immobilised metal affinity chromatography, and the like.

[0218] In certain embodiments, further techniques for separating, detecting and/or quantifying markers may be used in conjunction with any of the above described detection methods. Such methods include, without limitation, chemical extraction partitioning, isoelectric focusing (IEF) including capillary isoelectric focusing (CIEF), capillary isotachophoresis (CITP), capillary electrochromatography (CEC), and the like, one-dimensional polyacrylamide gel electrophoresis (PAGE), two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), capillary gel electrophoresis (CGE), capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), free flow electrophoresis (FFE), etc.

[0219] In certain examples, such methods may include separating, detecting and/or quantifying markers at the nucleic acid level, more particularly RNA level, e.g., at the level of hnRNA, pre-mRNA, mRNA, or cDNA. Standard quantitative RNA or cDNA measurement tools known in the art may be used. Non-limiting examples include

hybridization-based analysis, microarray expression analysis, digital gene expression profiling (DGE), RNA-in-situ hybridization (RISH), Northern-blot analysis and the like; PCR, RT-PCR, RT-qPCR, end-point PCR, digital PCR or the like; supported oligonucleotide detection, pyrosequencing, polony cyclic sequencing by synthesis, simultaneous bi-directional sequencing, single-molecule sequencing, single molecule real time sequencing, true single molecule sequencing, hybridization-assisted nanopore sequencing, sequencing by synthesis, single-cell RNA sequencing (sc-RNA seq), or the like.

[0220] In certain embodiments, the invention involves plate based single cell RNA sequencing (see, e.g., Picelli, S. et al., 2014, "Full-length RNA-seq from single cells using Smart-seq2" Nature protocols 9, 171-181, doi:10.1038/nprot.2014.006).

[0221] In certain embodiments, the invention involves high-throughput single-cell RNA-seq and/or targeted nucleic acid profiling (for example, sequencing, quantitative reverse transcription polymerase chain reaction, and the like) where the RNAs from different cells are tagged individually, allowing a single library to be created while retaining the cell identity of each read. In this regard reference is made to Macosko et al., 2015, "Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets" Cell 161, 1202-1214; International patent application number PCT/US20 15/049 178, published as WO2016/040476 on March 17, 2016; Klein et al., 2015, "Droplet Barcoding for Single-Cell Transcriptomics Applied to Embryonic Stem Cells" Cell 161, 1187-1201; International patent application number PCT/US2016/027734, published as WO2016168584A1 on October 20, 2016; Zheng, et al., 2016, "Haplotyping germline and cancer genomes with high-throughput linked-read sequencing" Nature Biotechnology 34, 303-31 1; Zheng, et al., 2017, "Massively parallel digital transcriptional profiling of single cells" Nat. Commun. 8, 14049 doi: 10.1038/ncomms14049; International patent publication number WO2014210353A2; Zilionis, et al., 2017, "Single-cell barcoding and sequencing using droplet microfluidics" Nat Protoc. Jan;12(1):44-73; Cao et al., 2017, "Comprehensive single cell transcriptional profiling of a multicellular organism by combinatorial indexing" bioRxiv preprint first posted online Feb. 2, 2017, doi: dx.doi.org/10.1101/104844; Rosenberg et al., 2017, "Scaling single cell transcriptomics through split pool barcoding" bioRxiv preprint first posted online Feb. 2, 2017, doi: dx.doi.org/10.1101/105 163; Vitak, et al., "Sequencing thousands of single-cell genomes with combinatorial indexing" Nature Methods, 14(3):302-308, 2017; Cao, et al., Comprehensive single-cell transcriptional profiling of a multicellular organism. Science, 357(6352):661-667, 2017; and Gierahn et al., "Seq-Well: portable, low-cost RNA

sequencing of single cells at high throughput" Nature Methods 14, 395-398 (2017), all the contents and disclosure of each of which are herein incorporated by reference in their entirety.

[0222] In certain embodiments, the invention involves single nucleus RNA sequencing. In this regard reference is made to Swiech et al., 2014, "In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9" Nature Biotechnology Vol. 33, pp. 102-106; Habib et al., 2016, "Div-Seq: Single-nucleus RNA-Seq reveals dynamics of rare adult newborn neurons" Science, Vol. 353, Issue 6302, pp. 925-928; Habib et al., 2017, "Massively parallel single-nucleus RNA-seq with DroNc-seq" Nat Methods. 2017 Oct;14(10):955-958; and International patent application number PCT/US2016/059239, published as WO2017164936 on September 28, 2017, which are herein incorporated by reference in their entirety.

[0223] In one embodiment, immune cells are stained for immune cell subtype specific signature genes. In one embodiment, the cells are fixed. In another embodiment, the cells are formalin fixed and paraffin embedded. In another example embodiment, the immune cell subtypes may be quantitated in a section of a tumor.

[0224] The method may allow to detect or conclude the presence or absence of the specified immune cells in a tested object (e.g., in a cell population, tissue, organ, organism, or in a biological sample of a subject). The method may also allow to quantify the specified immune cells in a tested object (e.g., in a cell population, tissue, organ, organism, or in a biological sample of a subject). The quantity of the specified immune cells in the tested object such as the biological sample may be suitably expressed for example as the number (count) of the specified immune cells per standard unit of volume (e.g., ml, μ l or nl) or weight (e.g., g or mg or ng) of the tested object such as the biological sample. The quantity of the specified immune cells in the tested object such as the biological sample may also be suitably expressed as a percentage or fraction (by number) of all cells comprised in the tested object such as the biological sample, or as a percentage or fraction (by number) of a select subset of the cells comprised in the tested object such as the biological sample, e.g., as a percentage or fraction (by number) of white blood cells, peripheral blood mononuclear cells, immune cells, antigen presenting cells, or dendritic cells comprised in the tested object such as the biological sample. The quantity of the specified immune cells in the tested object such as the biological sample may also be suitably represented by an absolute or relative quantity

of a suitable surrogate analyte, such as a peptide, polypeptide, protein, or nucleic acid expressed or comprised by the specified immune cells.

[0225] Where a marker is detected in or on a cell, the cell may be conventionally denoted as positive (+) or negative (-) for the marker. Semi-quantitative denotations of marker expression in cells are also commonplace in the art, such as particularly in flow cytometry quantifications, for example, "dim" vs. "bright", or "low" vs. "medium" / "intermediate" vs. "high", or "-" vs. "+" vs. "++", commonly controlled in flow cytometry quantifications by setting of the gates. Where a marker is quantified in or on a cell, absolute quantity of the marker may also be expressed for example as the number of molecules of the marker comprised by the cell.

[0226] Where a marker is detected and/or quantified on a single cell level in a cell population, the quantity of the marker may also be expressed as a percentage or fraction (by number) of cells comprised in said population that are positive for said marker, or as percentages or fractions (by number) of cells comprised in said population that are "dim" or "bright", or that are "low" or "medium" / "intermediate" or "high", or that are "-" or "+" or "++". By means of an example, a sizeable proportion of the tested cells of the cell population may be positive for the marker, e.g., at least about 20%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, or up to 100%.

Isolated Cells

[0227] In one aspect, the invention is directed to isolated cell populations having the phenotypes described herein and/or as identified by the signatures defined herein. Accordingly, methods for detecting, quantifying or isolating the specified immune cells may be marker-based or gene or gene product signature-based, i.e., may involve isolation of cells expressing or not expressing marker(s) or combination(s) of markers the expression or lack of expression of which is taught herein as typifying or characterizing the specified immune cells, or may involve detection, quantification or isolation of cells comprising gene or gene product signature(s) taught herein as typifying or characterizing the specified immune cells.

[0228] The terms "isolating" or "purifying" as used throughout this specification with reference to a particular component of a composition or mixture (e.g., the tested object such as the biological sample) encompass processes or techniques whereby such component is separated from one or more or (substantially) all other components of the composition or mixture (e.g., the tested object such as the biological sample). The terms do not require

absolute purity. Instead, isolating or purifying the component will produce a discrete environment in which the abundance of the component relative to one or more or all other components is greater than in the starting composition or mixture (e.g., the tested object such as the biological sample). A discrete environment may denote a single medium, such as for example a single solution, dispersion, gel, precipitate, etc. Isolating or purifying the specified immune cells from the tested object such as the biological sample may increase the abundance of the specified immune cells relative to all other cells comprised in the tested object such as the biological sample, or relative to other cells of a select subset of the cells comprised in the tested object such as the biological sample, e.g., relative to other white blood cells, peripheral blood mononuclear cells, immune cells, antigen presenting cells, or dendritic cells comprised in the tested object such as the biological sample. By means of example, isolating or purifying the specified immune cells from the tested object such as the biological sample may yield a cell population, in which the specified immune cells constitute at least 40% (by number) of all cells of said cell population, for example, at least 45%, preferably at least 50%, at least 55%, more preferably at least 60%, at least 65%, still more preferably at least 70%, at least 75%, even more preferably at least 80%, at least 85%, and yet more preferably at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even 100% of all cells of said cell population.

[0229] Any existing, available or conventional separation, detection and/or quantification methods may be used to measure the presence or absence (e.g., readout being present vs. absent; or detectable amount vs. undetectable amount) and/or quantity (e.g., readout being an absolute or relative quantity) of the specified immune cells in, or to isolate the specified immune cells from, a tested object (e.g., a cell population, tissue, organ, organism, or a biological sample of a subject). Such methods allow to detect, quantify or isolate the specified immune cells in or from the tested object (e.g., a cell population, tissue, organ, organism, or a biological sample of a subject) substantially to the exclusion of other cells comprised in the tested object. Such methods may allow to detect, quantify or isolate the specified immune cells with sensitivity of at least 50%, at least 55%, at least 60%, at least 65%, preferably at least 70%, at least 75%, more preferably at least 80%, at least 85%, even more preferably at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even 100%, and/or with specificity of at least 50%, at least 55%, at least 60%, at least 65%, preferably at least 70%, at least 75%, more preferably at least 80%, at least 85%, even more preferably at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at

least 99%, or even 100%. By means of example, at least 40% (by number), for example at least 45%, preferably at least 50%, at least 55%, more preferably at least 60%, at least 65%, still more preferably at least 70%, at least 75%, even more preferably at least 80%, at least 85%, and yet more preferably at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even 100% of all cells detected, quantified or isolated by such methods may correspond to the specified immune cells.

[0230] The isolated immune cells or immune cell populations as disclosed throughout this specification may be suitably cultured or cultivated *in vitro*. The terms "culturing" or "cell culture" are common in the art and broadly refer to maintenance of cells and potentially expansion (proliferation, propagation) of cells *in vitro*. Typically, animal cells, such as mammalian cells, such as human cells, are cultured by exposing them to (i.e., contacting them with) a suitable cell culture medium in a vessel or container adequate for the purpose (e.g., a 96-, 24-, or 6-well plate, a T-25, T-75, T-150 or T-225 flask, or a cell factory), at art-known conditions conducive to *in vitro* cell culture, such as temperature of 37°C, 5% v/v CO₂ and > 95% humidity.

[0231] The term "medium" as used herein broadly encompasses any cell culture medium conducive to maintenance of cells, preferably conducive to proliferation of cells. Typically, the medium will be a liquid culture medium, which facilitates easy manipulation (e.g., decantation, pipetting, centrifugation, filtration, and such) thereof.

[0232] Typically, the medium will comprise a basal medium formulation as known in the art. Many basal media formulations (available, e.g., from the American Type Culture Collection, ATCC; or from Invitrogen, Carlsbad, California) can be used, including but not limited to Eagle's Minimum Essential Medium (MEM), Dulbecco's Modified Eagle's Medium (DMEM), alpha modified Minimum Essential Medium (alpha-MEM), Basal Medium Essential (BME), Iscove's Modified Dulbecco's Medium (IMDM), BGJb medium, F-12 Nutrient Mixture (Ham), Liebovitz L-15, DMEM/F-12, Essential Modified Eagle's Medium (EMEM), RPMI-1640, Medium 199, Waymouth's MB 752/1 or Williams Medium E, and modifications and/or combinations thereof. Compositions of basal media are generally known in the art and it is within the skill of one in the art to modify or modulate concentrations of media and/or media supplements as necessary for the cells cultured.

[0233] Such basal media formulations contain ingredients necessary for mammalian cell development, which are known per se. By means of illustration and not limitation, these ingredients may include inorganic salts (in particular salts containing Na, K, Mg, Ca, Cl, P

and possibly Cu, Fe, Se and Zn), physiological buffers (e.g., HEPES, bicarbonate), nucleotides, nucleosides and/or nucleic acid bases, ribose, deoxyribose, amino acids, vitamins, antioxidants (e.g., glutathione) and sources of carbon (e.g., glucose, sodium pyruvate, sodium acetate), etc.

[0234] For use in culture, basal media can be supplied with one or more further components. For example, additional supplements can be used to supply the cells with the necessary trace elements and substances for optimal growth and expansion. Furthermore, antioxidant supplements may be added, e.g., β -mercaptoethanol. While many basal media already contain amino acids, some amino acids may be supplemented later, e.g., L-glutamine, which is known to be less stable when in solution. A medium may be further supplied with antibiotic and/or antimycotic compounds, such as, typically, mixtures of penicillin and streptomycin, and/or other compounds, exemplified but not limited to, amphotericin, ampicillin, gentamicin, bleomycin, hygromycin, kanamycin, mitomycin, mycophenolic acid, nalidixic acid, neomycin, nystatin, paromomycin, polymyxin, puromycin, rifampicin, spectinomycin, tetracycline, tylosin, and zeocin.

[0235] Lipids and lipid carriers can also be used to supplement cell culture media. Such lipids and carriers can include, but are not limited to cyclodextrin, cholesterol, linoleic acid conjugated to albumin, linoleic acid and oleic acid conjugated to albumin, unconjugated linoleic acid, linoleic-oleic-arachidonic acid conjugated to albumin, oleic acid unconjugated and conjugated to albumin, among others. Albumin can similarly be used in fatty-acid free formulations.

[0236] Also contemplated is supplementation of cell culture media with mammalian plasma or sera. Plasma or sera often contain cellular factors and components that facilitate cell viability and expansion. Optionally, plasma or serum may be heat inactivated. Heat inactivation is used in the art mainly to remove the complement. Heat inactivation typically involves incubating the plasma or serum at 56°C for 30 to 60min, e.g., 30min, with steady mixing, after which the plasma or serum is allowed to gradually cool to ambient temperature. A skilled person will be aware of any common modifications and requirements of the above procedure. Optionally, plasma or serum may be sterilised prior to storage or use. Usual means of sterilisation may involve, e.g., filtration through one or more filters with pore size smaller than $1\mu\text{m}$, preferably smaller than $0.5\mu\text{m}$, e.g., smaller than $0.45\mu\text{m}$, $0.40\mu\text{m}$, $0.35\mu\text{m}$, $0.30\mu\text{m}$ or $0.25\mu\text{m}$, more preferably $0.2\mu\text{m}$ or smaller, e.g., $0.15\mu\text{m}$ or smaller, O.IQum or smaller. Suitable sera or plasmas for use in media as taught herein may include human serum

or plasma, or serum or plasma from non-human animals, preferably non-human mammals, such as, e.g., non-human primates (e.g., lemurs, monkeys, apes), foetal or adult bovine, horse, porcine, lamb, goat, dog, rabbit, mouse or rat serum or plasma, etc., or any combination of such. In certain preferred embodiments, a medium as taught herein may comprise bovine serum or plasma, preferably foetal bovine (calf) serum or plasma, more preferably foetal bovine (calf) serum (FCS or FBS). When culturing human cells, media may preferably comprise human serum or plasma, such as autologous or allogeneic human serum or plasma, preferably human serum, such as autologous or allogeneic human serum, more preferably autologous human serum or plasma, even more preferably autologous human serum.

[0237] In certain preferred embodiments, serum or plasma can be substituted in media by serum replacements, such as to provide for serum-free media (i.e., chemically defined media). The provision of serum-free media may be advantageous particularly with view to administration of the media or fraction(s) thereof to subjects, especially to human subjects (e.g., improved bio-safety). By the term "serum replacement" it is broadly meant any a composition that may be used to replace the functions (e.g., cell maintenance and growth supportive function) of animal serum in a cell culture medium. A conventional serum replacement may typically comprise vitamins, albumin, lipids, amino acids, transferrin, antioxidants, insulin and trace elements. Many commercialized serum replacement additives, such as KnockOut Serum Replacement (KOSR), N2, B27, Insulin-Transferrin-Selenium Supplement (ITS), and G5 are well known and are readily available to those skilled in the art.

[0238] Plasma or serum or serum replacement may be comprised in media as taught herein at a proportion (volume of plasma or serum or serum replacement /volume of medium) between about 0.5% v/v and about 40.0% v/v, preferably between about 5.0% v/v and about 20.0% v/v, e.g., between about 5.0% v/v and about 15.0 % v/v, more preferably between about 8.0% v/v and about 12.0% v/v, e.g., about 10.0% v/v.

[0239] In certain embodiments, methods for detecting, quantifying or isolating the specified immune cells may be single-cell-based, *i.e.*, may allow to discretely detect, quantify or isolate the specified immune cells as individual cells. In other embodiments, methods for detecting, quantifying or isolating the specified immune cells may be cell population-based, *i.e.*, may only allow to detect, quantify or isolate the specified immune cells as a group or collection of cells, without providing information on or allowing to isolate individual cells.

[0240] Methods for detecting, quantifying or isolating the specified immune cells may employ any of the above-described techniques for measuring markers, insofar the separation

or the qualitative and/or quantitative measurement of the marker(s) can be correlated with or translated into detection, quantification or isolation of the specified immune cells. For example, any of the above-described biochemical assay methods, immunological assay methods, mass spectrometry analysis methods, chromatography methods, or nucleic acid analysis method, or combinations thereof for measuring markers, may be employed for detecting, quantifying or isolating the specified immune cells.

[0241] In certain embodiments, the cells are detected, quantified or isolated using a technique selected from the group consisting of flow cytometry, fluorescence activated cell sorting, mass cytometry, fluorescence microscopy, affinity separation, magnetic cell separation, microfluidic separation, and combinations thereof.

[0242] Flow cytometry encompasses methods by which individual cells of a cell population are analyzed by their optical properties (e.g., light absorbance, light scattering and fluorescence properties, etc.) as they pass in a narrow stream in single file through a laser beam. Flow cytometry methods include fluorescence activated cell sorting (FACS) methods by which a population of cells having particular optical properties are separated from other cells.

[0243] Elemental mass spectrometry-based flow cytometry, or mass cytometry, offers an approach to analyze cells by replacing fluorochrome-labelled binding reagents with mass tagged binding reagents, i.e., tagged with an element or isotope having a defined mass. In these methods, labeled particles are introduced into a mass cytometer, where they are individually atomized and ionized. The individual particles are then subjected to elemental analysis, which identifies and measures the abundance of the mass tags used. The identities and the amounts of the isotopic elements associated with each particle are then stored and analyzed. Due to the resolution of elemental analysis and the number of elemental isotopes that can be used, it is possible to simultaneously measure up to 100 or more parameters on a single particle.

[0244] Fluorescence microscopy broadly encompasses methods by which individual cells of a cell population are microscopically analyzed by their fluorescence properties. Fluorescence microscopy approaches may be manual or preferably automated.

[0245] Affinity separation also referred to as affinity chromatography broadly encompasses techniques involving specific interactions of cells present in a mobile phase, such as a suitable liquid phase (e.g., cell population in an aqueous suspension) with, and thereby adsorption of the cells to, a stationary phase, such as a suitable solid phase; followed

by separation of the stationary phase from the remainder of the mobile phase; and recovery (e.g., elution) of the adsorbed cells from the stationary phase. Affinity separation may be columnar, or alternatively, may entail batch treatment, wherein the stationary phase is collected / separated from the liquid phases by suitable techniques, such as centrifugation or application of magnetic field (e.g., where the stationary phase comprises magnetic substrate, such as magnetic particles or beads). Accordingly, magnetic cell separation is also envisaged herein.

[0246] Microfluidic systems allow for accurate and high throughput cell detection, quantification and/or sorting, exploiting a variety of physical principles. Cell sorting on microchips provides numerous advantages by reducing the size of necessary equipment, eliminating potentially biohazardous aerosols, and simplifying the complex protocols commonly associated with cell sorting. The term "microfluidic system" as used throughout this specification broadly refers to systems having one or more fluid microchannels. Microchannels denote fluid channels having cross-sectional dimensions the largest of which are typically less than 1 mm, preferably less than 500 μm , more preferably less than 400 μm , more preferably less than 300 μm , more preferably less than 200 μm , e.g., 100 μm or smaller. Such microfluidic systems can be used for manipulating fluid and/or objects such as droplets, bubbles, capsules, particles, cells and the like. Microfluidic systems may allow for example for fluorescent label-based (e.g., employing fluorophore-conjugated binding agent(s), such as fluorophore-conjugated antibody(ies)), bead-based (e.g., bead-conjugated binding agent(s), such as bead-conjugated antibody(ies)), or label-free cell sorting (reviewed in Shields et al., Lab Chip. 2015, vol. 15: 1230-1249).

Use of Specific Binding Agents

[0247] In certain embodiments, the aforementioned methods and techniques may employ agent(s) capable of specifically binding to one or more gene products, e.g., peptides, polypeptides, proteins, or nucleic acids, expressed or not expressed by the immune cells as taught herein. In certain preferred embodiments, such one or more gene products, e.g., peptides, polypeptides, or proteins, may be expressed on the cell surface of the immune cells (i.e., cell surface markers, e.g., transmembrane peptides, polypeptides or proteins, or secreted peptides, polypeptides or proteins which remain associated with the cell surface). Hence, further disclosed are binding agents capable of specifically binding to markers, such as genes or gene products, e.g., peptides, polypeptides, proteins, or nucleic acids as taught herein. Binding agents as intended throughout this specification may include *inter alia* antibodies,

aptamers, spiegelmers (L-aptamers), photoaptamers, protein, peptides, peptidomimetics, nucleic acids such as oligonucleotides (e.g., hybridization probes or amplification or sequencing primers and primer pairs), small molecules, or combinations thereof.

[0248] The term "aptamer" refers to single-stranded or double-stranded oligo-DNA, oligo-RNA or oligo-DNA/RNA or any analogue thereof that specifically binds to a target molecule such as a peptide. Advantageously, aptamers display fairly high specificity and affinity (e.g., KA in the order 1x10⁹ M⁻¹) for their targets. Aptamer production is described inter alia in US 5,270,163; Ellington & Szostak 1990 (Nature 346: 818-822); Tuerk & Gold 1990 (Science 249: 505-510); or "The Aptamer Handbook: Functional Oligonucleotides and Their Applications", by Klussmann, ed., Wiley-VCH 2006, ISBN 3527310592, incorporated by reference herein. The term "photoaptamer" refers to an aptamer that contains one or more photoreactive functional groups that can covalently bind to or crosslink with a target molecule. The term "spiegelmer" refers to an aptamer which includes L-DNA, L-RNA, or other left-handed nucleotide derivatives or nucleotide-like molecules. Aptamers containing left-handed nucleotides are resistant to degradation by naturally occurring enzymes, which normally act on substrates containing right-handed nucleotides. The term "peptidomimetic" refers to a non-peptide agent that is a topological analogue of a corresponding peptide. Methods of rationally designing peptidomimetics of peptides are known in the art. For example, the rational design of three peptidomimetics based on the sulphated 8-mer peptide CCK26-33, and of two peptidomimetics based on the 11-mer peptide Substance P, and related peptidomimetic design principles, are described in Horwell 1995 (Trends Biotechnol 13: 132-134).

[0249] Binding agents may be in various forms, e.g., lyophilised, free in solution, or immobilised on a solid phase. They may be, e.g., provided in a multi-well plate or as an array or microarray, or they may be packaged separately, individually, or in combination.

[0250] The term "specifically bind" as used throughout this specification means that an agent (denoted herein also as "specific-binding agent") binds to one or more desired molecules or analytes (e.g., peptides, polypeptides, proteins, or nucleic acids) substantially to the exclusion of other molecules which are random or unrelated, and optionally substantially to the exclusion of other molecules that are structurally related. The term "specifically bind" does not necessarily require that an agent binds exclusively to its intended target(s). For example, an agent may be said to specifically bind to target(s) of interest if its affinity for such intended target(s) under the conditions of binding is at least about 2-fold greater,

preferably at least about 5-fold greater, more preferably at least about 10-fold greater, yet more preferably at least about 25-fold greater, still more preferably at least about 50-fold greater, and even more preferably at least about 100-fold, or at least about 1000-fold, or at least about 10^4 -fold, or at least about 10^5 -fold, or at least about 10^6 -fold or more greater, than its affinity for a non-target molecule, such as for a suitable control molecule (e.g., bovine serum albumin, casein).

[0251] Preferably, the specific binding agent may bind to its intended target(s) with affinity constant (KA) of such binding $KA \geq 1 \times 10^{-6} M^{-1}$, more preferably $KA \geq 1 \times 10^{-7} M^{-1}$, yet more preferably $KA \geq 1 \times 10^{-8} M^{-1}$, even more preferably $KA \geq 1 \times 10^{-9} M^{-1}$, and still more preferably $KA \geq 1 \times 10^{-10} M^{-1}$ or $KA \geq 1 \times 10^{-11} M^{-1}$ or $KA \geq 1 \times 10^{-12} M^{-1}$, wherein $KA = [SBA_T]/[SBA][T]$, SBA denotes the specific-binding agent, T denotes the intended target. Determination of KA can be carried out by methods known in the art, such as for example, using equilibrium dialysis and Scatchard plot analysis.

[0252] In certain embodiments, the one or more binding agents may be one or more antibodies. As used herein, the term "antibody" is used in its broadest sense and generally refers to any immunologic binding agent. The term specifically encompasses intact monoclonal antibodies, polyclonal antibodies, multivalent (e.g., 2-, 3- or more-valent) and/or multi-specific antibodies (e.g., bi- or more-specific antibodies) formed from at least two intact antibodies, and antibody fragments insofar they exhibit the desired biological activity (particularly, ability to specifically bind an antigen of interest, i.e., antigen-binding fragments), as well as multivalent and/or multi-specific composites of such fragments. The term "antibody" is not only inclusive of antibodies generated by methods comprising immunization, but also includes any polypeptide, e.g., a recombinantly expressed polypeptide, which is made to encompass at least one complementarity-determining region (CDR) capable of specifically binding to an epitope on an antigen of interest. Hence, the term applies to such molecules regardless whether they are produced *in vitro* or *in vivo*. Antibodies also encompasses chimeric, humanized and fully humanized antibodies.

[0253] An antibody may be any of IgA, IgD, IgE, IgG and IgM classes, and preferably IgG class antibody. An antibody may be a polyclonal antibody, e.g., an antiserum or immunoglobulins purified there from (e.g., affinity-purified). An antibody may be a monoclonal antibody or a mixture of monoclonal antibodies. Monoclonal antibodies can target a particular antigen or a particular epitope within an antigen with greater selectivity and reproducibility. By means of example and not limitation, monoclonal antibodies may be

made by the hybridoma method first described by Kohler et al. 1975 (Nature 256: 495), or may be made by recombinant DNA methods (e.g., as in US 4,816,567). Monoclonal antibodies may also be isolated from phage antibody libraries using techniques as described by Clackson et al. 1991 (Nature 352: 624-628) and Marks et al. 1991 (J Mol Biol 222: 581-597), for example.

[0254] Antibody binding agents may be antibody fragments. "Antibody fragments" comprise a portion of an intact antibody, comprising the antigen-binding or variable region thereof. Examples of antibody fragments include Fab, Fab', F(ab')2, Fv and scFv fragments, single domain (sd) Fv, such as VH domains, VL domains and VHH domains; diabodies; linear antibodies; single-chain antibody molecules, in particular heavy-chain antibodies; and multivalent and/or multispecific antibodies formed from antibody fragment(s), e.g., dibodies, tribodies, and multibodies. The above designations Fab, Fab', F(ab')2, Fv, scFv etc. are intended to have their art-established meaning.

[0255] The term antibody includes antibodies originating from or comprising one or more portions derived from any animal species, preferably vertebrate species, including, e.g., birds and mammals. Without limitation, the antibodies may be chicken, turkey, goose, duck, guinea fowl, quail or pheasant. Also without limitation, the antibodies may be human, murine (e.g., mouse, rat, etc.), donkey, rabbit, goat, sheep, guinea pig, camel (e.g., Camelus bactrianus and Camelus dromaderius), llama (e.g., Lama paccos, Lama glama or Lama vicugna) or horse.

[0256] A skilled person will understand that an antibody can include one or more amino acid deletions, additions and/or substitutions (e.g., conservative substitutions), insofar such alterations preserve its binding of the respective antigen. An antibody may also include one or more native or artificial modifications of its constituent amino acid residues (e.g., glycosylation, etc.).

[0257] Methods of producing polyclonal and monoclonal antibodies as well as fragments thereof are well known in the art, as are methods to produce recombinant antibodies or fragments thereof (see for example, Harlow and Lane, "Antibodies: A Laboratory Manual", Cold Spring Harbour Laboratory, New York, 1988; Harlow and Lane, "Using Antibodies: A Laboratory Manual", Cold Spring Harbour Laboratory, New York, 1999, ISBN 0879695447; "Monoclonal Antibodies: A Manual of Techniques", by Zola, ed., CRC Press 1987, ISBN 0849364760; "Monoclonal Antibodies: A Practical Approach", by Dean & Shepherd, eds., Oxford University Press 2000, ISBN 0199637229; Methods in Molecular Biology, vol. 248:

"Antibody Engineering: Methods and Protocols", Lo, ed., Humana Press 2004, ISBN 1588290921).

[0258] As used herein, a "blocking" antibody or an antibody "antagonist" is one which inhibits or reduces biological activity of the antigen(s) it binds. In certain embodiments, the blocking antibodies or antagonist antibodies or portions thereof described herein completely inhibit the biological activity of the antigen(s).

[0259] Antibodies may act as agonists or antagonists of the recognized polypeptides. For example, the present invention includes antibodies which disrupt receptor/ligand interactions either partially or fully. The invention features both receptor-specific antibodies and ligand-specific antibodies. The invention also features receptor-specific antibodies which do not prevent ligand binding but prevent receptor activation. Receptor activation (i.e., signaling) may be determined by techniques described herein or otherwise known in the art. For example, receptor activation can be determined by detecting the phosphorylation (e.g., tyrosine or serine/threonine) of the receptor or of one of its down-stream substrates by immunoprecipitation followed by western blot analysis. In specific embodiments, antibodies are provided that inhibit ligand activity or receptor activity by at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, or at least 50% of the activity in absence of the antibody.

[0260] The invention also features receptor-specific antibodies which both prevent ligand binding and receptor activation as well as antibodies that recognize the receptor-ligand complex. Likewise, encompassed by the invention are neutralizing antibodies which bind the ligand and prevent binding of the ligand to the receptor, as well as antibodies which bind the ligand, thereby preventing receptor activation, but do not prevent the ligand from binding the receptor. Further included in the invention are antibodies which activate the receptor. These antibodies may act as receptor agonists, i.e., potentiate or activate either all or a subset of the biological activities of the ligand-mediated receptor activation, for example, by inducing dimerization of the receptor. The antibodies may be specified as agonists, antagonists or inverse agonists for biological activities comprising the specific biological activities of the peptides disclosed herein. The antibody agonists and antagonists can be made using methods known in the art. See, e.g., PCT publication WO 96/40281; U.S. Pat. No. 5,811,097; Deng et al., Blood 92(6): 1981-1988 (1998); Chen et al., Cancer Res. 58(16):3668-3678 (1998); Harrop et al., J. Immunol. 161(4): 1786-1794 (1998); Zhu et al., Cancer Res. 58(15):3209-3214 (1998); Yoon et al., J. Immunol. 160(7):3170-3179 (1998); Prat et al., J. Cell. Sci. Ill

(Pt2):237-247 (1998); Pitard et al., J. Immunol. Methods 205(2): 177-190 (1997); Liautard et al., Cytokine 9(4):233-241 (1997); Carlson et al., J. Biol. Chem. 272(17): 11295-1 1301 (1997); Taryman et al., Neuron 14(4):755-762 (1995); Muller et al., Structure 6(9): 1153-1 167 (1998); Bartunek et al., Cytokine 8(1): 14-20 (1996).

[0261] The antibodies as defined for the present invention include derivatives that are modified, i.e., by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from generating an anti-idiotypic response. For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, e.g., by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.

[0262] Simple binding assays can be used to screen for or detect agents that bind to a target protein, or disrupt the interaction between proteins (e.g., a receptor and a ligand). Because certain targets of the present invention are transmembrane proteins, assays that use the soluble forms of these proteins rather than full-length protein can be used, in some embodiments. Soluble forms include, for example, those lacking the transmembrane domain and/or those comprising the IgV domain or fragments thereof which retain their ability to bind their cognate binding partners. Further, agents that inhibit or enhance protein interactions for use in the compositions and methods described herein, can include recombinant peptido-mimetics.

[0263] Detection methods useful in screening assays include antibody-based methods, detection of a reporter moiety, detection of cytokines as described herein, and detection of a gene signature as described herein.

[0264] Another variation of assays to determine binding of a receptor protein to a ligand protein is through the use of affinity biosensor methods. Such methods may be based on the piezoelectric effect, electrochemistry, or optical methods, such as ellipsometry, optical wave guidance, and surface plasmon resonance (SPR).

[0265] The term "antibody-like protein scaffolds" or "engineered protein scaffolds" broadly encompasses proteinaceous non-immunoglobulin specific-binding agents, typically obtained by combinatorial engineering (such as site-directed random mutagenesis in

combination with phage display or other molecular selection techniques). Usually, such scaffolds are derived from robust and small soluble monomeric proteins (such as Kunitz inhibitors or lipocalins) or from a stably folded extra-membrane domain of a cell surface receptor (such as protein A, fibronectin or the ankyrin repeat).

[0266] Such scaffolds have been extensively reviewed in Binz et al. (Engineering novel binding proteins from nonimmunoglobulin domains. *Nat Biotechnol* 2005, 23:1257-1268), Gebauer and Skerra (Engineered protein scaffolds as next-generation antibody therapeutics. *Curr Opin Chem Biol.* 2009, 13:245-55), Gill and Damle (Biopharmaceutical drug discovery using novel protein scaffolds. *Curr Opin Biotechnol* 2006, 17:653-658), Skerra (Engineered protein scaffolds for molecular recognition. *J Mol Recognit* 2000, 13:167-187), and Skerra (Alternative non-antibody scaffolds for molecular recognition. *Curr Opin Biotechnol* 2007, 18:295-304), and include without limitation affibodies, based on the Z-domain of staphylococcal protein A, a three-helix bundle of 58 residues providing an interface on two of its alpha-helices (Nygren, Alternative binding proteins: Affibody binding proteins developed from a small three-helix bundle scaffold. *FEBS J* 2008, 275:2668-2676); engineered Kunitz domains based on a small (ca. 58 residues) and robust, disulphide-crosslinked serine protease inhibitor, typically of human origin (e.g. LACI-D1), which can be engineered for different protease specificities (Nixon and Wood, Engineered protein inhibitors of proteases. *Curr Opin Drug Discov Dev* 2006, 9:261-268); monobodies or adnectins based on the 10th extracellular domain of human fibronectin III (10Fn3), which adopts an Ig-like beta-sandwich fold (94 residues) with 2-3 exposed loops, but lacks the central disulphide bridge (Koide and Koide, Monobodies: antibody mimics based on the scaffold of the fibronectin type III domain. *Methods Mol Biol* 2007, 352:95-109); anticalins derived from the lipocalins, a diverse family of eight-stranded beta-barrel proteins (ca. 180 residues) that naturally form binding sites for small ligands by means of four structurally variable loops at the open end, which are abundant in humans, insects, and many other organisms (Skerra, Alternative binding proteins: Anticalins—Harnessing the structural plasticity of the lipocalin ligand pocket to engineer novel binding activities. *FEBS J* 2008, 275:2677-2683); DARPins, designed ankyrin repeat domains (166 residues), which provide a rigid interface arising from typically three repeated beta-turns (Stumpp et al., DARPins: a new generation of protein therapeutics. *Drug Discov Today* 2008, 13:695-701); avimers (multimerized LDLR-A module) (Silverman et al., Multivalent avimer proteins evolved by exon shuffling of a family of human receptor domains. *Nat Biotechnol* 2005, 23:1556-1561); and cysteine-rich knottin

peptides (Kolmar, Alternative binding proteins: biological activity and therapeutic potential of cystine-knot miniproteins. FEBS J 2008, 275:2684-2690).

[0267] Nucleic acid binding agents, such as oligonucleotide binding agents, are typically at least partly antisense to a target nucleic acid of interest. The term "antisense" generally refers to an agent (e.g., an oligonucleotide) configured to specifically anneal with (hybridize to) a given sequence in a target nucleic acid, such as for example in a target DNA, hnRNA, pre-mRNA or mRNA, and typically comprises, consist essentially of or consist of a nucleic acid sequence that is complementary or substantially complementary to said target nucleic acid sequence. Antisense agents suitable for use herein, such as hybridization probes or amplification or sequencing primers and primer pairs) may typically be capable of annealing with (hybridizing to) the respective target nucleic acid sequences at high stringency conditions, and capable of hybridizing specifically to the target under physiological conditions. The terms "complementary" or "complementarity" as used throughout this specification with reference to nucleic acids, refer to the normal binding of single-stranded nucleic acids under permissive salt (ionic strength) and temperature conditions by base pairing, preferably Watson-Crick base pairing. By means of example, complementary Watson-Crick base pairing occurs between the bases A and T, A and U or G and C. For example, the sequence 5'-A-G-U-3' is complementary to sequence 5'-A-C-U-3'.

[0268] The reference to oligonucleotides may in particular but without limitation include hybridization probes and/or amplification primers and/or sequencing primers, etc., as commonly used in nucleic acid detection technologies.

[0269] Binding agents as discussed herein may suitably comprise a detectable label. The term "label" refers to any atom, molecule, moiety or biomolecule that may be used to provide a detectable and preferably quantifiable read-out or property, and that may be attached to or made part of an entity of interest, such as a binding agent. Labels may be suitably detectable by for example mass spectrometric, spectroscopic, optical, colourimetric, magnetic, photochemical, biochemical, immunochemical or chemical means. Labels include without limitation dyes; radiolabels such as ^{32}P , ^{33}P , ^{35}S , ^{125}I , ^{131}I ; electron-dense reagents; enzymes (e.g., horse-radish peroxidase or alkaline phosphatase as commonly used in immunoassays); binding moieties such as biotin-streptavidin; haptens such as digoxigenin; luminogenic, phosphorescent or fluorogenic moieties; mass tags; and fluorescent dyes alone or in combination with moieties that may suppress or shift emission spectra by fluorescence resonance energy transfer (FRET).

[0270] In some embodiments, binding agents may be provided with a tag that permits detection with another agent (e.g., with a probe binding partner). Such tags may be, for example, biotin, streptavidin, his-tag, myc tag, maltose, maltose binding protein or any other kind of tag known in the art that has a binding partner. Example of associations which may be utilised in the probe:binding partner arrangement may be any, and includes, for example biotin:streptavidin, his-tag:metal ion (e.g., Ni²⁺), maltose:maltose binding protein, etc.

[0271] The marker-binding agent conjugate may be associated with or attached to a detection agent to facilitate detection. Examples of detection agents include, but are not limited to, luminescent labels; colourimetric labels, such as dyes; fluorescent labels; or chemical labels, such as electroactive agents (e.g., ferrocyanide); enzymes; radioactive labels; or radiofrequency labels. The detection agent may be a particle. Examples of such particles include, but are not limited to, colloidal gold particles; colloidal sulphur particles; colloidal selenium particles; colloidal barium sulfate particles; colloidal iron sulfate particles; metal iodate particles; silver halide particles; silica particles; colloidal metal (hydrorous) oxide particles; colloidal metal sulfide particles; colloidal lead selenide particles; colloidal cadmium selenide particles; colloidal metal phosphate particles; colloidal metal ferrite particles; any of the above-mentioned colloidal particles coated with organic or inorganic layers; protein or peptide molecules; liposomes; or organic polymer latex particles, such as polystyrene latex beads. Preferable particles may be colloidal gold particles.

[0272] In certain embodiments, the one or more binding agents are configured for use in a technique selected from the group consisting of flow cytometry, fluorescence activated cell sorting, mass cytometry, fluorescence microscopy, affinity separation, magnetic cell separation, microfluidic separation, and combinations thereof.

Therapeutic Uses of Isolated Cells

[0273] In certain embodiments, the method may comprise: a) isolating from a biological sample of the subject an immune cell or immune cell population as disclosed herein; b) in vitro expanding the immune cell or immune cell population of a); and c) administering the in vitro expanded immune cell or immune cell population of b) to the subject.

[0274] In certain embodiments, the method may further comprise formulating the in vitro expanded immune cell or immune cell population of b) into a pharmaceutical composition.

[0275] A "pharmaceutical composition" refers to a composition that usually contains an excipient, such as a pharmaceutically acceptable carrier that is conventional in the art and that is suitable for administration to cells or to a subject.

[0276] The term "pharmaceutically acceptable" as used throughout this specification is consistent with the art and means compatible with the other ingredients of a pharmaceutical composition and not deleterious to the recipient thereof.

[0277] As used herein, "carrier" or "excipient" includes any and all solvents, diluents, buffers (such as, e.g., neutral buffered saline or phosphate buffered saline), solubilisers, colloids, dispersion media, vehicles, fillers, chelating agents (such as, e.g., EDTA or glutathione), amino acids (such as, e.g., glycine), proteins, disintegrants, binders, lubricants, wetting agents, emulsifiers, sweeteners, colorants, flavourings, aromatisers, thickeners, agents for achieving a depot effect, coatings, antifungal agents, preservatives, stabilisers, antioxidants, tonicity controlling agents, absorption delaying agents, and the like. The use of such media and agents for pharmaceutical active components is well known in the art. Such materials should be non-toxic and should not interfere with the activity of the cells or active components.

[0278] The precise nature of the carrier or excipient or other material will depend on the route of administration. For example, the composition may be in the form of a parenterally acceptable aqueous solution, which is pyrogen-free and has suitable pH, isotonicity and stability. For general principles in medicinal formulation, the reader is referred to Cell Therapy: Stem Cell Transplantation, Gene Therapy, and Cellular Immunotherapy, by G. Morstyn & W. Sheridan eds., Cambridge University Press, 1996; and Hematopoietic Stem Cell Therapy, E. D. Ball, J. Lister & P. Law, Churchill Livingstone, 2000.

[0279] The pharmaceutical composition can be applied parenterally, rectally, orally or topically. Preferably, the pharmaceutical composition may be used for intravenous, intramuscular, subcutaneous, peritoneal, peridural, rectal, nasal, pulmonary, mucosal, or oral application. In a preferred embodiment, the pharmaceutical composition according to the invention is intended to be used as an infuse. The skilled person will understand that compositions which are to be administered orally or topically will usually not comprise cells, although it may be envisioned for oral compositions to also comprise cells, for example when gastro-intestinal tract indications are treated. Each of the cells or active components (e.g., immunomodulants) as discussed herein may be administered by the same route or may be administered by a different route. By means of example, and without limitation, cells may be administered parenterally and other active components may be administered orally.

[0280] Liquid pharmaceutical compositions may generally include a liquid carrier such as water or a pharmaceutically acceptable aqueous solution. For example, physiological saline

solution, tissue or cell culture media, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

[0281] The composition may include one or more cell protective molecules, cell regenerative molecules, growth factors, anti-apoptotic factors or factors that regulate gene expression in the cells. Such substances may render the cells independent of their environment.

[0282] Such pharmaceutical compositions may contain further components ensuring the viability of the cells therein. For example, the compositions may comprise a suitable buffer system (e.g., phosphate or carbonate buffer system) to achieve desirable pH, more usually near neutral pH, and may comprise sufficient salt to ensure isoosmotic conditions for the cells to prevent osmotic stress. For example, suitable solution for these purposes may be phosphate-buffered saline (PBS), sodium chloride solution, Ringer's Injection or Lactated Ringer's Injection, as known in the art. Further, the composition may comprise a carrier protein, e.g., albumin (e.g., bovine or human albumin), which may increase the viability of the cells.

[0283] Further suitably pharmaceutically acceptable carriers or additives are well known to those skilled in the art and for instance may be selected from proteins such as collagen or gelatine, carbohydrates such as starch, polysaccharides, sugars (dextrose, glucose and sucrose), cellulose derivatives like sodium or calcium carboxymethylcellulose, hydroxypropyl cellulose or hydroxypropylmethyl cellulose, pregeletanized starches, pectin agar, carrageenan, clays, hydrophilic gums (acacia gum, guar gum, arabic gum and xanthan gum), alginic acid, alginates, hyaluronic acid, polyglycolic and polylactic acid, dextran, pectins, synthetic polymers such as water-soluble acrylic polymer or polyvinylpyrrolidone, proteoglycans, calcium phosphate and the like.

[0284] In certain embodiments, a pharmaceutical cell preparation as taught herein may be administered in a form of liquid composition. In embodiments, the cells or pharmaceutical composition comprising such can be administered systemically, topically, within an organ or at a site of organ dysfunction or lesion.

[0285] Preferably, the pharmaceutical compositions may comprise a therapeutically effective amount of the specified immune cells and/or other active components (e.g., immunomodulants). The term "therapeutically effective amount" refers to an amount which can elicit a biological or medicinal response in a tissue, system, animal or human that is being sought by a researcher, veterinarian, medical doctor or other clinician, and in particular can

prevent or alleviate one or more of the local or systemic symptoms or features of a disease or condition being treated.

[0286] In certain embodiments, a treatment or pharmaceutical composition that reduces a non-responder signature, increases a responder signature, modulates the ratio of responder to non-responder CD8+ TILs, depletes non-responder CD8+ TILs, or increases responder CD8+ TILs is co-administered with a check point blockade therapy or is administered before administration of a check point blockade therapy. The check point blockade therapy may be an inhibitor of any check point protein described herein. The checkpoint blockade therapy may comprise anti-TIM3, anti-CTLA4, anti-PD-L1, anti-PD1, anti-TIGIT, anti-LAG3, or combinations thereof. Specific check point inhibitors include, but are not limited to anti-CTLA4 antibodies (e.g., Ipilimumab), anti-PD-1 antibodies (e.g., Nivolumab, Pembrolizumab), and anti-PD-L1 antibodies (e.g., Atezolizumab).

[0287] In certain embodiments, a marker of non-responder cells is targeted as described herein. In certain embodiments, CD39 is targeted. In certain embodiments, CD39 is targeted in combination with one or more checkpoint inhibitors. In certain embodiments, CD39 is inhibited in combination with one or more checkpoint inhibitors. In certain embodiments, CD39 is inhibited in combination with anti-TIM3. In certain embodiments, CD39 is inhibited in combination with anti-PD1. In certain embodiments, CD39 is inhibited in combination with anti-CTLA4. In certain embodiments, CD39 is inhibited in combination with anti-PD-L1. In certain embodiments, CD39 is inhibited in combination with anti-TIM3 and anti-CTLA4. In certain embodiments, CD39 is inhibited in combination with anti-TIM3 and anti-PD-1. In certain embodiments, CD39 is inhibited in combination of any one or more of anti-TEV13, anti-CTLA4, anti-PD-L1, anti-PD1, anti-TIGIT, or anti-LAG3.

[0288] As used herein, the term "CD39" has its general meaning in the art and refers to the CD39 protein also named as ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD1). CD39 is an ectoenzyme that hydrolases ATP/UTP and ADP/UDP to the respective nucleosides such as AMP. Accordingly, the term "CD39 inhibitor" refers to a compound that inhibits the activity or expression of CD39. In some embodiments, the CD39 inhibitor is an antibody having specificity for CD39. In certain embodiments, the CD39 inhibitor is a small molecule. CD39 activity modulators are well known in the art. For example, 6-N,N-Diethyl-d-y?-y-dibromomethylene adenosine triphosphate (ARL 67156) (Levesque et al (2007) Br. J. Pharmacol. 152: 141-150; Crack et al. (1959) Br. J. Pharmacol. 114: 475-481; Kennedy et al. (1996) Semtn. Neurosci. 8: 195-199) and 8-thiobutyladenosine

5'-triphosphate (8-Bu-S- ATP) are small molecule CD39 inhibitors (Gendron et al. (2000) J Med Chem. 43:2239- 2247). Other small molecule CD39 inhibitors, such as polyoxymeteate-1 (POM- 1) and α,β -methylene ADP (APCP), are also well known in the art (see, U.S.20 10/204 182 and US2013/0123345; U.S. Pat. 6,617,439). In addition, nucleic acid and antibody inhibitors of CD39 are also well known in the art (see, e.g., US20130273062A1).

[0289] As used throughout this specification, "immune response" refers to a response by a cell of the immune system, such as a B cell, T cell (CD4+ or CD8+), regulatory T cell, antigen-presenting cell, dendritic cell, monocyte, macrophage, NKT cell, NK cell, basophil, eosinophil, or neutrophil, to a stimulus. In some embodiments, the response is specific for a particular antigen (an "antigen-specific response"), and refers to a response by a CD4 T cell, CD8 T cell, or B cell via their antigen-specific receptor. In some embodiments, an immune response is a T cell response, such as a CD4+ response or a CD8+ response. Such responses by these cells can include, for example, cytotoxicity, proliferation, cytokine or chemokine production, trafficking, or phagocytosis, and can be dependent on the nature of the immune cell undergoing the response.

[0290] T cell response refers more specifically to an immune response in which T cells directly or indirectly mediate or otherwise contribute to an immune response in a subject. T cell-mediated response may be associated with cell mediated effects, cytokine mediated effects, and even effects associated with B cells if the B cells are stimulated, for example, by cytokines secreted by T cells. By means of an example but without limitation, effector functions of MHC class I restricted Cytotoxic T lymphocytes (CTLs), may include cytokine and/or cytolytic capabilities, such as lysis of target cells presenting an antigen peptide recognised by the T cell receptor (naturally-occurring TCR or genetically engineered TCR, e.g., chimeric antigen receptor, CAR), secretion of cytokines, preferably IFN gamma, TNF alpha and/or one or more immunostimulatory cytokines, such as IL-2, and/or antigen peptide-induced secretion of cytotoxic effector molecules, such as granzymes, perforins or granulysin. By means of example but without limitation, for MHC class II restricted T helper (Th) cells, effector functions may be antigen peptide-induced secretion of cytokines, preferably, IFN gamma, TNF alpha, IL-4, IL5, IL-10, and/or IL-2. By means of example but without limitation, for T regulatory (Treg) cells, effector functions may be antigen peptide-induced secretion of cytokines, preferably, IL-10, IL-35, and/or TGF-beta. B cell response refers more specifically to an immune response in which B cells directly or indirectly mediate or otherwise contribute to an immune response in a subject. Effector functions of B cells may

include in particular production and secretion of antigen-specific antibodies by B cells (e.g., polyclonal B cell response to a plurality of the epitopes of an antigen (antigen-specific antibody response)), antigen presentation, and/or cytokine secretion.

[0291] The term "immune tolerance" as used throughout this specification refers to any mechanism by which a potentially injurious immune response is prevented, suppressed, delayed in the onset or progression, reduced in the risk of the onset or progression, or shifted to a non-injurious immune response. Specific immune tolerance occurs when immune tolerance is preferentially invoked against certain antigen(s) in comparison with others.

[0292] The term "antigen" as used throughout this specification refers to a molecule or a portion of a molecule capable of being bound by an antibody, or by a T cell receptor (TCR) when presented by MHC molecules. At the molecular level, an antigen is characterized by its ability to be bound at the antigen-binding site of an antibody. The specific binding denotes that the antigen will be bound in a highly selective manner by its cognate antibody and not by the multitude of other antibodies which may be evoked by other antigens. An antigen is additionally capable of being recognized by the immune system. In some instances, an antigen is capable of eliciting a humoral immune response in a subject. In some instances, an antigen is capable of eliciting a cellular immune response in a subject, leading to the activation of B- and/or T-lymphocytes. In some instances, an antigen is capable of eliciting a humoral and cellular immune response in a subject. Hence, an antigen may be preferably antigenic and immunogenic. Alternatively, an antigen may be antigenic and not immunogenic. Typically, an antigen may be a peptide, polypeptide, protein, nucleic acid, an oligo- or polysaccharide, or a lipid, or any combination thereof, a glycoprotein, proteoglycan, glycolipid, etc. In certain embodiments, an antigen may be a peptide, polypeptide, or protein. An antigen may have one or more than one epitope. The terms "antigenic determinant" or "epitope" generally refer to the region or part of an antigen that specifically reacts with or is recognized by the immune system, specifically by antibodies, B cells, or T cells.

[0293] An antigen as contemplated throughout this specification may be obtained by any means available to a skilled person, e.g., may be isolated from a naturally-occurring material comprising the antigen, or may be produced recombinantly by a suitable host or host cell expression system and optionally isolated therefrom (e.g., a suitable bacterial, yeast, fungal, plant or animal host or host cell expression system), or may be produced recombinantly by cell-free transcription or translation, or non-biological nucleic acid or peptide synthesis.

[0294] The term "tumor antigen" as used throughout this specification refers to an antigen that is uniquely or differentially expressed by a tumor cell, whether intracellular or on the tumor cell surface (preferably on the tumor cell surface), compared to a normal or non-neoplastic cell. By means of example, a tumor antigen may be present in or on a tumor cell and not typically in or on normal cells or non-neoplastic cells (e.g., only expressed by a restricted number of normal tissues, such as testis and/or placenta), or a tumor antigen may be present in or on a tumor cell in greater amounts than in or on normal or non-neoplastic cells, or a tumor antigen may be present in or on tumor cells in a different form than that found in or on normal or non-neoplastic cells. The term thus includes tumor-specific antigens (TSA), including tumor-specific membrane antigens, tumor-associated antigens (TAA), including tumor-associated membrane antigens, embryonic antigens on tumors, growth factor receptors, growth factor ligands, etc. The term further includes cancer/testis (CT) antigens. Examples of tumor antigens include, without limitation, β -human chorionic gonadotropin (PHCG), glycoprotein 100 (gp100/Pmel 17), carcinoembryonic antigen (CEA), tyrosinase, tyrosinase-related protein 1 (gp75/TRPI), tyrosinase-related protein 2 (TRP-2), NY-BR-1, NY-CO-58, NY-ESO-1, MN/gp250, idiotypes, telomerase, synovial sarcoma X breakpoint 2 (SSX2), mucin 1 (MUC-1), antigens of the melanoma-associated antigen (MAGE) family, high molecular weight-melanoma associated antigen (HMW-MAA), melanoma antigen recognized by T cells 1 (MART1), Wilms' tumor gene 1 (WT1), HER2/neu, mesothelin (MSLN), alphafetoprotein (AFP), cancer antigen 125 (CA-125), and abnormal forms of ras or p53 (see also, WO2016187508A2). Tumor antigens may also be subject specific (e.g., subject specific neoantigens; see, e.g., U.S. patent 9,115,402; and international patent application publication numbers WO2016100977A1, WO2014168874A2, WO2015085233A1, and WO201509581 1A2).

[0295] The term "in vitro" generally denotes outside, or external to, a body, e.g., an animal or human body. The term encompasses "ex vivo".

[0296] In certain embodiments, the immune cell or immune cell population is autologous to said subject, i.e., the immune cell or immune cell population is isolated from the same subject as the subject to which / whom the immune cell or immune cell population is to be administered. In certain further embodiments, the immune cell or immune cell population is syngeneic to said subject, i.e., the immune cell or immune cell population is isolated from an identical twin of the subject to which / whom the immune cell or immune cell population is to be administered. In certain further embodiments, the immune cell or immune cell population

is allogeneic to said subject, i.e., the immune cell or immune cell population is isolated from a different subject of the same species as the subject to which / whom the immune cell or immune cell population is to be administered. In certain embodiments, the immune cell or immune cell population may even be xenogeneic to said subject, i.e., the immune cell or immune cell population may be isolated from a subject of a different species than the subject to which / whom the immune cell or immune cell population is to be administered.

[0297] Preferably, non-autologous, such as allogeneic cells may be selected such as to maximize the tissue compatibility between the subject and the administered cells, thereby reducing the chance of rejection of the administered cells by patient's immune system or graft-vs.-host reaction. For example, advantageously the cells may be typically selected which have either identical HLA haplotypes (including one or preferably more HLA-A, HLA-B, HLA-C, HLA-D, HLA-DR, HLA-DP and HLA-DQ) to the subject, or which have the most HLA antigen alleles common to the subject and none or the least of HLA antigens to which the subject contains pre-existing anti-HLA antibodies.

Activated T Cell Compositions

[0298] A further aspect of the invention relates to a method for preparing a composition comprising activated T cells, the method comprising isolating T cells from a biological sample of a subject and contacting said T cells in vitro with an immune cell or immune cell population, wherein the immune cell or immune cell population has been loaded with an antigen.

[0299] "Activation" generally refers to the state of a cell, such as preferably T cell, following sufficient cell surface moiety ligation (e.g., interaction between the T cell receptor on the surface of a T cell (such as naturally-occurring TCR or genetically engineered TCR, e.g., chimeric antigen receptor, CAR) and MHC-bound antigen peptide presented on the surface of an antigen presenting cell (e.g., dendritic cell) to induce a noticeable biochemical or morphological change of the cell, such as preferably T cell. In particular, "activation" may refer to the state of a T cell that has been sufficiently stimulated to induce detectable cellular proliferation of the T cell. Activation can also encompass induced cytokine production, and detectable T cell effector functions, e.g., regulatory or cytolytic effector functions. The T cells and antigen presenting cells may be suitably contacted by admixing the T cells and antigen presenting cells in an aqueous composition, e.g., in a culture medium, in sufficient numbers and for a sufficient duration of time to produce the desired T cell activation.

[0300] A further aspect of the invention relates to a method for adoptive immunotherapy in a subject in need thereof comprising administering to said subject a composition comprising activated T cells prepared with the method as taught above.

[0301] In certain embodiments, said T cells are CD8+ T cells, i.e., T cells expressing the CD8+ cell surface marker. More preferably, said T cells may be CD8+ T cells and said subject is suffering from proliferative disease.

[0302] In certain embodiments, the T cell, preferably a CD8+ T cell, may display specificity to a desired antigen, such as specificity to a tumor antigen (tumor antigen specificity). By means of an example, the T cell, preferably a CD8+ T cell, may have been isolated from a tumor of a subject. More preferably, the immune cell may be a tumor infiltrating lymphocyte (TIL). Generally, "tumor infiltrating lymphocytes" or "TILs" refer to white blood cells that have left the bloodstream and migrated into a tumor. Such T cells typically endogenously express a T cell receptor having specificity to an antigen expressed by the tumor cells (tumor antigen specificity).

[0303] In alternative embodiments, a T cell, preferably a CD8+ T cell, may be engineered to express a T cell receptor having specificity to a desired antigen, such as specificity to a tumor antigen (tumor antigen specificity). For example, the T cell, preferably a CD8+ T cell, may comprise a chimeric antigen receptor (CAR) having specificity to a desired antigen, such as a tumor-specific chimeric antigen receptor (CAR).

Adoptive Cell Therapy

[0304] In certain embodiments, the immune cells or immune cell populations as taught herein may be used for adoptive cell transfer (ACT). In certain embodiments, responder T cells are used for adoptive transfer. The cells may be further modified as discussed herein. The cells may express an endogenous T cell receptor (TCR) or a chimeric antigen receptor (CAR). As used herein, "ACT", "adoptive cell therapy" and "adoptive cell transfer" may be used interchangeably. In certain embodiments, Adoptive cell therapy (ACT) can refer to the transfer of cells to a patient with the goal of transferring the functionality and characteristics into the new host by engraftment of the cells (see, e.g., Mettananda et al., Editing an α-globin enhancer in primary human hematopoietic stem cells as a treatment for β-thalassemia, Nat Commun. 2017 Sep 4;8(1):424). As used herein, the term "engraft" or "engraftment" refers to the process of cell incorporation into a tissue of interest *in vivo* through contact with existing cells of the tissue. Adoptive cell therapy (ACT) can refer to the transfer of cells, most commonly immune-derived cells, back into the same patient or into a new recipient host with

the goal of transferring the immunologic functionality and characteristics into the new host. If possible, use of autologous cells helps the recipient by minimizing GVHD issues. The adoptive transfer of autologous tumor infiltrating lymphocytes (TIL) (Besser et al., (2010) Clin. Cancer Res 16 (9) 2646-55; Dudley et al., (2002) Science 298 (5594): 850-4; and Dudley et al., (2005) Journal of Clinical Oncology 23 (10): 2346-57.) or genetically redirected peripheral blood mononuclear cells (Johnson et al., (2009) Blood 114 (3): 535-46; and Morgan et al., (2006) Science 314(5796) 126-9) has been used to successfully treat patients with advanced solid tumors, including melanoma and colorectal carcinoma, as well as patients with CD19-expressing hematologic malignancies (Kalos et al., (2011) Science Translational Medicine 3 (95): 95ra73). In certain embodiments, allogenic cells immune cells are transferred (see, e.g., Ren et al., (2017) Clin Cancer Res 23 (9) 2255-2266). As described further herein, allogenic cells can be edited to reduce alloreactivity and prevent graft-versus-host disease. Thus, use of allogenic cells allows for cells to be obtained from healthy donors and prepared for use in patients as opposed to preparing autologous cells from a patient after diagnosis.

[0305] Aspects of the invention involve the adoptive transfer of immune system cells, such as T cells, specific for selected antigens, such as tumor associated antigens or tumor specific neoantigens (see, e.g., Maus et al., 2014, Adoptive Immunotherapy for Cancer or Viruses, Annual Review of Immunology, Vol. 32: 189-225; Rosenberg and Restifo, 2015, Adoptive cell transfer as personalized immunotherapy for human cancer, Science Vol. 348 no. 6230 pp. 62-68; Restifo et al., 2015, Adoptive immunotherapy for cancer: harnessing the T cell response. Nat. Rev. Immunol. 12(4): 269-281; and Jenson and Riddell, 2014, Design and implementation of adoptive therapy with chimeric antigen receptor-modified T cells. Immunol Rev. 257(1): 127-144; and Rajasagi et al., 2014, Systematic identification of personal tumor-specific neoantigens in chronic lymphocytic leukemia. Blood. 2014 Jul 17;124(3):453-62).

[0306] In certain embodiments, an antigen (such as a tumor antigen) to be targeted in adoptive cell therapy (such as particularly CAR or TCR T-cell therapy) of a disease (such as particularly of tumor or cancer) may be selected from a group consisting of: B cell maturation antigen (BCMA) (see, e.g., Friedman et al., Effective Targeting of Multiple BCMA-Expressing Hematological Malignancies by Anti-BCMA CAR T Cells, Hum Gene Ther. 2018 Mar 8; Berdeja JG, et al. Durable clinical responses in heavily pretreated patients with relapsed/refractory multiple myeloma: updated results from a multicenter study of bb2121

anti-Bcma CAR T cell therapy. *Blood.* 2017; 130:740; and Mouhieddine and Ghobrial, Immunotherapy in Multiple Myeloma: The Era of CAR T Cell Therapy, *Hematologist*, May-June 2018, Volume 15, issue 3); PSA (prostate-specific antigen); prostate-specific membrane antigen (PSMA); PSCA (Prostate stem cell antigen); Tyrosine-protein kinase transmembrane receptor ROR1; fibroblast activation protein (FAP); Tumor-associated glycoprotein 72 (TAG72); Carcinoembryonic antigen (CEA); Epithelial cell adhesion molecule (EPCAM); Mesothelin; Human Epidermal growth factor Receptor 2 (ERBB2 (Her2/neu)); Prostase; Prostatic acid phosphatase (PAP); elongation factor 2 mutant (ELF2M); Insulin-like growth factor 1 receptor (IGF-1R); gplOO; BCR-ABL (breakpoint cluster region-Abelson); tyrosinase; New York esophageal squamous cell carcinoma 1 (NY-ESO-1); κ-light chain, LAGE (L antigen); MAGE (melanoma antigen); Melanoma-associated antigen 1 (MAGE-A1); MAGE A3; MAGE A6; legumain; Human papillomavirus (HPV) E6; HPV E7; prostein; survivin; PCTA1 (Galectin 8); Melan-A/MART-1; Ras mutant; TRP-1 (tyrosinase related protein 1, or gp75); Tyrosinase-related Protein 2 (TRP2); TRP-2/INT2 (TRP-2/intron 2); RAGE (renal antigen); receptor for advanced glycation end products 1 (RAGE1); Renal ubiquitous 1, 2 (RU1, RU2); intestinal carboxyl esterase (iCE); Heat shock protein 70-2 (HSP70-2) mutant; thyroid stimulating hormone receptor (TSHR); CD 123; CD171; CD 19; CD20; CD22; CD26; CD30; CD33; CD44v7/8 (cluster of differentiation 44, exons 7/8); CD53; CD92; CDIOO; CD148; CD150; CD200; CD261; CD262; CD362; CS-1 (CD2 subset 1, CRACC, SLAMF7, CD319, and 19A24); C-type lectin-like molecule-1 (CLL-1); ganglioside GD3 (aNeu5Ac(2-8)aNeu5Ac(2-3)bDGAlp(l-4)bDGlcp(l-l)Cer); Tn antigen (Tn Ag); Fms-Like Tyrosine Kinase 3 (FLT3); CD38; CD 138; CD44v6; B7H3 (CD276); KIT (CD117); Interleukin-13 receptor subunit alpha-2 (IL-13Ra2); Interleukin 11 receptor alpha (IL-1 lRa); prostate stem cell antigen (PSCA); Protease Serine 2 1 (PRSS21); vascular endothelial growth factor receptor 2 (VEGFR2); Lewis(Y) antigen; CD24; Platelet-derived growth factor receptor beta (PDGFR-beta); stage-specific embryonic antigen-4 (SSEA-4); Mucin 1, cell surface associated (MUC1); mucin 16 (MUC16); epidermal growth factor receptor (EGFR); epidermal growth factor receptor variant III (EGFRvIII); neural cell adhesion molecule (NCAM); carbonic anhydrase IX (CAIX); Proteasome (Prosome, Macropain) Subunit, Beta Type, 9 (LMP2); ephrin type-A receptor 2 (EphA2); Ephrin B2; Fucosyl GM1; sialyl Lewis adhesion molecule (sLe); ganglioside GM3 (aNeu5Ac(2-3)bDGAlp(l-4)bDGlcp(l-l)Cer); TGS5; high molecular weight-melanoma-associated antigen (HMWMAA); o-acetyl-GD2 ganglioside (OAcGD2); Folate receptor alpha; Folate receptor

beta; tumor endothelial marker 1 (TEM1/CD248); tumor endothelial marker 7-related (TEM7R); claudin 6 (CLDN6); G protein-coupled receptor class C group 5, member D (GPRC5D); chromosome X open reading frame 61 (CXORF61); CD97; CD 179a; anaplastic lymphoma kinase (ALK); Polysialic acid; placenta-specific 1 (PLAC1); hexasaccharide portion of globoH glycoceramide (GloboH); mammary gland differentiation antigen (NY-BR-1); uroplakin 2 (UPK2); Hepatitis A virus cellular receptor 1 (HAVCR1); adrenoceptor beta 3 (ADRB3); pannexin 3 (PANX3); G protein-coupled receptor 20 (GPR20); lymphocyte antigen 6 complex, locus K 9 (LY6K); Olfactory receptor 51E2 (OR51E2); TCR Gamma Alternate Reading Frame Protein (TARP); Wilms tumor protein (WT1); ETS translocation variant gene 6, located on chromosome 12p (ETV6-AML); sperm protein 17 (SPA17); X Antigen Family, Member 1A (XAGE1); angiopoietin-binding cell surface receptor 2 (Tie 2); CT (cancer/testis (antigen)); melanoma cancer testis antigen-1 (MAD-CT-1); melanoma cancer testis antigen-2 (MAD-CT-2); Fos-related antigen 1; p53; p53 mutant; human Telomerase reverse transcriptase (hTERT); sarcoma translocation breakpoints; melanoma inhibitor of apoptosis (ML-IAP); ERG (transmembrane protease, serine 2 (TMPRSS2) ETS fusion gene); N-Acetyl glucosaminyl-transferase V (NA17); paired box protein Pax-3 (PAX3); Androgen receptor; Cyclin Bl; Cyclin Dl; v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN); Ras Homolog Family Member C (RhoC); Cytochrome P450 1B1 (CYP1B1); CCCTC-Binding Factor (Zinc Finger Protein-Like (BORIS); Squamous Cell Carcinoma Antigen Recognized By T Cells- 1 or 3 (SART1, SART3); Paired box protein Pax-5 (PAX5); proacrosin binding protein sp32 (OY-TES1); lymphocyte-specific protein tyrosine kinase (LCK); A kinase anchor protein 4 (AKAP-4); synovial sarcoma, X breakpoint-1, -2, -3 or -4 (SSX1, SSX2, SSX3, SSX4); CD79a; CD79b; CD72; Leukocyte-associated immunoglobulin-like receptor 1 (LAIR1); Fc fragment of IgA receptor (FCAR); Leukocyte immunoglobulin-like receptor subfamily A member 2 (LILRA2); CD300 molecule-like family member f (CD300LF); C-type lectin domain family 12 member A (CLEC12A); bone marrow stromal cell antigen 2 (BST2); EGF-like module-containing mucin-like hormone receptor-like 2 (EMR2); lymphocyte antigen 75 (LY75); Glycan-3 (GPC3); Fc receptor-like 5 (FCRL5); mouse double minute 2 homolog (MDM2); livin; alphafetoprotein (AFP); transmembrane activator and CAML Interactor (TACI); B-cell activating factor receptor (BAFF-R); V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS); immunoglobulin lambda-like polypeptide 1 (IGLL1); 707-AP (707 alanine proline); ART-4 (adenocarcinoma antigen recognized by T4 cells); BAGE (B antigen; b-catenin/m, b-

catenin/mutated); CAMEL (CTL-recognized antigen on melanoma); CAP1 (carcinoembryonic antigen peptide 1); CASP-8 (caspase-8); CDC27m (cell-division cycle 27 mutated); CDK4/m (cycline-dependent kinase 4 mutated); Cyp-B (cyclophilin B); DAM (differentiation antigen melanoma); EGP-2 (epithelial glycoprotein 2); EGP-40 (epithelial glycoprotein 40); Erbb2, 3, 4 (erythroblastic leukemia viral oncogene homolog-2, -3, 4); FBP (folate binding protein); fAChR (Fetal acetylcholine receptor); G250 (glycoprotein 250); GAGE (G antigen); GnT-V (N-acetylglicosaminyltransferase V); HAGE (helicose antigen); ULA-A (human leukocyte antigen-A); HST2 (human signet ring tumor 2); KIAA0205; KDR (kinase insert domain receptor); LDLR/FUT (low density lipid receptor/GDP L-fucose: b-D-galactosidase 2-a-L fucosyltransferase); L1CAM (LI cell adhesion molecule); MC1R (melanocortin 1 receptor); Myosin/m (myosin mutated); MUM-1, -2, -3 (melanoma ubiquitous mutated 1, 2, 3); NA88-A (NA cDNA clone of patient M88); KG2D (Natural killer group 2, member D) ligands; oncofetal antigen (h5T4); p190 minor bcr-abl (protein of 190KD bcr-abl); Pml/RAR α (promyelocytic leukaemia/retinoic acid receptor α); PRAME (preferentially expressed antigen of melanoma); SAGE (sarcoma antigen); TEL/AML1 (translocation Ets-family leukemia/acute myeloid leukemia 1); TPI/m (triosephosphate isomerase mutated); CD70; and any combination thereof.

[0307] In certain embodiments, an antigen to be targeted in adoptive cell therapy (such as particularly CAR or TCR T-cell therapy) of a disease (such as particularly of tumor or cancer) is a tumor-specific antigen (TSA).

[0308] In certain embodiments, an antigen to be targeted in adoptive cell therapy (such as particularly CAR or TCR T-cell therapy) of a disease (such as particularly of tumor or cancer) is a neoantigen.

[0309] In certain embodiments, an antigen to be targeted in adoptive cell therapy (such as particularly CAR or TCR T-cell therapy) of a disease (such as particularly of tumor or cancer) is a tumor-associated antigen (TAA).

[0310] In certain embodiments, an antigen to be targeted in adoptive cell therapy (such as particularly CAR or TCR T-cell therapy) of a disease (such as particularly of tumor or cancer) is a universal tumor antigen. In certain preferred embodiments, the universal tumor antigen is selected from the group consisting of: a human telomerase reverse transcriptase (hTERT), survivin, mouse double minute 2 homolog (MDM2), cytochrome P450 1B 1 (CYP1B), HER2/neu, Wilms' tumor gene 1 (WT1), livin, alphafetoprotein (AFP),

carcinoembryonic antigen (CEA), mucin 16 (MUC16), MUC1, prostate-specific membrane antigen (PSMA), p53, cyclin (D1), and any combinations thereof.

[0311] In certain embodiments, an antigen (such as a tumor antigen) to be targeted in adoptive cell therapy (such as particularly CAR or TCR T-cell therapy) of a disease (such as particularly of tumor or cancer) may be selected from a group consisting of: CD19, BCMA, CD70, CLL-1, MAGE A3, MAGE A6, HPV E6, HPV E7, WT1, CD22, CD171, ROR1, MUC16, and SSX2. In certain preferred embodiments, the antigen may be CD19. For example, CD19 may be targeted in hematologic malignancies, such as in lymphomas, more particularly in B-cell lymphomas, such as without limitation in diffuse large B-cell lymphoma, primary mediastinal b-cell lymphoma, transformed follicular lymphoma, marginal zone lymphoma, mantle cell lymphoma, acute lymphoblastic leukemia including adult and pediatric ALL, non-Hodgkin lymphoma, indolent non-Hodgkin lymphoma, or chronic lymphocytic leukemia. For example, BCMA may be targeted in multiple myeloma or plasma cell leukemia (see, e.g., 2018 American Association for Cancer Research (AACR) Annual meeting Poster: Allogeneic Chimeric Antigen Receptor T Cells Targeting B Cell Maturation Antigen). For example, CLL1 may be targeted in acute myeloid leukemia. For example, MAGE A3, MAGE A6, SSX2, and/or KRAS may be targeted in solid tumors. For example, HPV E6 and/or HPV E7 may be targeted in cervical cancer or head and neck cancer. For example, WT1 may be targeted in acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), chronic myeloid leukemia (CML), non-small cell lung cancer, breast, pancreatic, ovarian or colorectal cancers, or mesothelioma. For example, CD22 may be targeted in B cell malignancies, including non-Hodgkin lymphoma, diffuse large B-cell lymphoma, or acute lymphoblastic leukemia. For example, CD171 may be targeted in neuroblastoma, glioblastoma, or lung, pancreatic, or ovarian cancers. For example, ROR1 may be targeted in ROR1+ malignancies, including non-small cell lung cancer, triple negative breast cancer, pancreatic cancer, prostate cancer, ALL, chronic lymphocytic leukemia, or mantle cell lymphoma. For example, MUC16 may be targeted in MUC16ecto+ epithelial ovarian, fallopian tube or primary peritoneal cancer. For example, CD70 may be targeted in both hematologic malignancies as well as in solid cancers such as renal cell carcinoma (RCC), gliomas (e.g., GBM), and head and neck cancers (HNSCC). CD70 is expressed in both hematologic malignancies as well as in solid cancers, while its expression in normal tissues is restricted to a subset of lymphoid cell types (see, e.g., 2018 American Association for Cancer Research (AACR) Annual meeting Poster: Allogeneic

CRISPR Engineered Anti-CD70 CAR-T Cells Demonstrate Potent Preclinical Activity Against Both Solid and Hematological Cancer Cells).

[0312] Various strategies may for example be employed to genetically modify T cells by altering the specificity of the T cell receptor (TCR) for example by introducing new TCR α and β chains with selected peptide specificity (see U.S. Patent No. 8,697,854; PCT Patent Publications: WO2003020763, WO2004033685, WO2004044004, WO20051 14215, WO2006000830, WO2008038002, WO2008039818, WO2004074322, WO20051 13595, WO2006125962, WO2013 166321, WO2013039889, WO2014018863, WO2014083173; U.S. Patent No. 8,088,379).

[0313] As an alternative to, or addition to, TCR modifications, chimeric antigen receptors (CARs) may be used in order to generate immunoresponsive cells, such as T cells, specific for selected targets, such as malignant cells, with a wide variety of receptor chimera constructs having been described (see U.S. Patent Nos. 5,843,728; 5,851,828; 5,912,170; 6,004,811; 6,284,240; 6,392,013; 6,410,014; 6,753,162; 8,211,422; and, PCT Publication WO9215322).

[0314] In general, CARs are comprised of an extracellular domain, a transmembrane domain, and an intracellular domain, wherein the extracellular domain comprises an antigen-binding domain that is specific for a predetermined target. While the antigen-binding domain of a CAR is often an antibody or antibody fragment (e.g., a single chain variable fragment, scFv), the binding domain is not particularly limited so long as it results in specific recognition of a target. For example, in some embodiments, the antigen-binding domain may comprise a receptor, such that the CAR is capable of binding to the ligand of the receptor. Alternatively, the antigen-binding domain may comprise a ligand, such that the CAR is capable of binding the endogenous receptor of that ligand.

[0315] The antigen-binding domain of a CAR is generally separated from the transmembrane domain by a hinge or spacer. The spacer is also not particularly limited, and it is designed to provide the CAR with flexibility. For example, a spacer domain may comprise a portion of a human Fc domain, including a portion of the CH3 domain, or the hinge region of any immunoglobulin, such as IgA, IgD, IgE, IgG, or IgM, or variants thereof. Furthermore, the hinge region may be modified so as to prevent off-target binding by FcRs or other potential interfering objects. For example, the hinge may comprise an IgG4 Fc domain with or without a S228P, L235E, and/or N297Q mutation (according to Kabat numbering) in

order to decrease binding to FcRs. Additional spacers/hinges include, but are not limited to, CD4, CD8, and CD28 hinge regions.

[0316] The transmembrane domain of a CAR may be derived either from a natural or from a synthetic source. Where the source is natural, the domain may be derived from any membrane bound or transmembrane protein. Transmembrane regions of particular use in this disclosure may be derived from CD8, CD28, CD3, CD45, CD4, CD5, CDS, CD9, CD 16, CD22, CD33, CD37, CD64, CD80, CD86, CD 134, CD137, CD 154, TCR. Alternatively, the transmembrane domain may be synthetic, in which case it will comprise predominantly hydrophobic residues such as leucine and valine. Preferably a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain. Optionally, a short oligo- or polypeptide linker, preferably between 2 and 10 amino acids in length may form the linkage between the transmembrane domain and the cytoplasmic signaling domain of the CAR. A glycine-serine doublet provides a particularly suitable linker.

[0317] Alternative CAR constructs may be characterized as belonging to successive generations. First-generation CARs typically consist of a single-chain variable fragment of an antibody specific for an antigen, for example comprising a VL linked to a VH of a specific antibody, linked by a flexible linker, for example by a CD8a hinge domain and a CD8a transmembrane domain, to the transmembrane and intracellular signaling domains of either CD3C or FcR γ (scFv-CD3C or scFv-FcR γ ; see U.S. Patent No. 7,741,465; U.S. Patent No. 5,912,172; U.S. Patent No. 5,906,936). Second-generation CARs incorporate the intracellular domains of one or more costimulatory molecules, such as CD28, OX40 (CD134), or 4-1BB (CD137) within the endodomain (for example scFv-CD28/OX40/4-1BB-CD3 ζ see U.S. Patent Nos. 8,911,993; 8,916,381; 8,975,071; 9,101,584; 9,102,760; 9,102,761). Third-generation CARs include a combination of costimulatory endodomains, such a CD3 ζ -chain, CD97, GDI la-CD18, CD2, ICOS, CD27, CD154, CDS, OX40, 4-1BB, CD2, CD7, LIGHT, LFA-1, NKG2C, B7-H3, CD30, CD40, PD-1, or CD28 signaling domains (for example scFv-CD28-4-1BB-CD3C or scFv-CD28-OX40-CD3Q see U.S. Patent No. 8,906,682; U.S. Patent No. 8,399,645; U.S. Pat. No. 5,686,281; PCT Publication No. WO2014134165; PCT Publication No. WO2012079000). In certain embodiments, the primary signaling domain comprises a functional signaling domain of a protein selected from the group consisting of CD3 zeta, CD3 gamma, CD3 delta, CD3 epsilon, common FcR gamma (FCERIG), FcR beta (Fc Epsilon Rib), CD79a, CD79b, Fc gamma RIIa, DAPI, and DAP12. In certain preferred

embodiments, the primary signaling domain comprises a functional signaling domain of CD3 ζ or FcRy. In certain embodiments, the one or more costimulatory signaling domains comprise a functional signaling domain of a protein selected, each independently, from the group consisting of: CD27, CD28, 4-1BB (CD137), OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen- 1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, CDS, ICAM-1, GITR, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), CD 160, CD 19, CD4, CD8 alpha, CD8 beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD1 ld, ITGAE, CD103, ITGAL, CD1 la, LFA-1, ITGAM, CD1 lb, ITGAX, CD 11c, ITGB1, CD29, ITGB2, CD 18, ITGB7, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD 150, IPO-3), BLAME (SLAMF8), SELPLG (CD 162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, NKp44, NKp30, NKp46, and NKG2D. In certain embodiments, the one or more costimulatory signaling domains comprise a functional signaling domain of a protein selected, each independently, from the group consisting of: 4-1BB, CD27, and CD28. In certain embodiments, a chimeric antigen receptor may have the design as described in U.S. Patent No. 7,446,190, comprising an intracellular domain of CD3 ζ chain (such as amino acid residues 52-163 of the human CD3 zeta chain, as shown in SEQ ID NO: 14 of US 7,446,190), a signaling region from CD28 and an antigen-binding element (or portion or domain; such as scFv). The CD28 portion, when between the zeta chain portion and the antigen-binding element, may suitably include the transmembrane and signaling domains of CD28 (such as amino acid residues 114-220 of SEQ ID NO: 10, full sequence shown in SEQ ID NO: 6 of US 7,446,190; these can include the following portion of CD28 as set forth in Genbank identifier NM_006139 (sequence version 1, 2 or 3): IEV MY PPPY LDNEKS NGTIIHV KGK HLCPSPL FPGPS KPFW VL VVGGVL ACYS LL VT VAFI IFW VVR SKRS RL LHSD YMNM TPR RGP PTR KH YQPY APP RDFA AYRS) (SEQ ID No. 1). Alternatively, when the zeta sequence lies between the CD28 sequence and the antigen-binding element, intracellular domain of CD28 can be used alone (such as amino sequence set forth in SEQ ID NO: 9 of US 7,446,190). Hence, certain embodiments employ a CAR comprising (a) a zeta chain portion comprising the intracellular domain of human CD3 ζ chain, (b) a costimulatory signaling region, and (c) an antigen-binding element (or portion or

domain), wherein the costimulatory signaling region comprises the amino acid sequence encoded by SEQ ID NO: 6 of US 7,446,190.

[0318] Alternatively, costimulation may be orchestrated by expressing CARs in antigen-specific T cells, chosen so as to be activated and expanded following engagement of their native aPTCR, for example by antigen on professional antigen-presenting cells, with attendant costimulation. In addition, additional engineered receptors may be provided on the immunoresponsive cells, for example to improve targeting of a T-cell attack and/or minimize side effects

[0319] By means of an example and without limitation, Kochenderfer et al., (2009) J Immunother. 32 (7): 689-702 described anti-CD19 chimeric antigen receptors (CAR). FMC63-28Z CAR contained a single chain variable region moiety (scFv) recognizing CD19 derived from the FMC63 mouse hybridoma (described in Nicholson et al., (1997) Molecular Immunology 34: 1157-1165), a portion of the human CD28 molecule, and the intracellular component of the human TCR- ζ molecule. FMC63-CD828BBZ CAR contained the FMC63 scFv, the hinge and transmembrane regions of the CD8 molecule, the cytoplasmic portions of CD28 and 4-IBB, and the cytoplasmic component of the TCR- ζ molecule. The exact sequence of the CD28 molecule included in the FMC63-28Z CAR corresponded to Genbank identifier NM_006139; the sequence included all amino acids starting with the amino acid sequence IEVMYPPPY and continuing all the way to the carboxy-terminus of the protein. To encode the anti-CD 19 scFv component of the vector, the authors designed a DNA sequence which was based on a portion of a previously published CAR (Cooper et al., (2003) Blood 101: 1637-1644). This sequence encoded the following components in frame from the 5' end to the 3' end: an Xhol site, the human granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor α -chain signal sequence, the FMC63 light chain variable region (as in Nicholson et al., *supra*), a linker peptide (as in Cooper et al., *supra*), the FMC63 heavy chain variable region (as in Nicholson et al., *supra*), and a NotI site. A plasmid encoding this sequence was digested with Xhol and NotI. To form the MSGV-FMC63-28Z retroviral vector, the Xhol and NotI-digested fragment encoding the FMC63 scFv was ligated into a second Xhol and NotI-digested fragment that encoded the MSGV retroviral backbone (as in Hughes et al., (2005) Human Gene Therapy 16: 457-472) as well as part of the extracellular portion of human CD28, the entire transmembrane and cytoplasmic portion of human CD28, and the cytoplasmic portion of the human TCR- ζ molecule (as in Maher et al., 2002) Nature Biotechnology 20: 70-75). The FMC63-28Z CAR is included in the KTE-C19 (axicabtagene

ciloleucel) anti-CD 19 CAR-T therapy product in development by Kite Pharma, Inc. for the treatment of *inter alia* patients with relapsed/refractory aggressive B-cell non-Hodgkin lymphoma (NHL). Accordingly, in certain embodiments, cells intended for adoptive cell therapies, more particularly immunoresponsive cells such as T cells, may express the FMC63-28Z CAR as described by Kochenderfer et al. (*supra*). Hence, in certain embodiments, cells intended for adoptive cell therapies, more particularly immunoresponsive cells such as T cells, may comprise a CAR comprising an extracellular antigen-binding element (or portion or domain; such as scFv) that specifically binds to an antigen, an intracellular signaling domain comprising an intracellular domain of a CD3 ζ chain, and a costimulatory signaling region comprising a signaling domain of CD28. Preferably, the CD28 amino acid sequence is as set forth in Genbank identifier NM_006139 (sequence version 1, 2 or 3) starting with the amino acid sequence IEVMYPPPY (SEQ. ID. No. 2) and continuing all the way to the carboxy-terminus of the protein. The sequence is reproduced herein: IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWVLVVVGVLACYSLLVT VAFIIFWVRSKRSRLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS (SEQ. ID. No. 3). Preferably, the antigen is CD 19, more preferably the antigen-binding element is an anti-CD 19 scFv, even more preferably the anti-CD 19 scFv as described by Kochenderfer et al. (*supra*).

[0320] Additional anti-CD 19 CARs are further described in WO2015187528. More particularly Example 1 and Table 1 of WO2015187528, incorporated by reference herein, demonstrate the generation of anti-CD 19 CARs based on a fully human anti-CD 19 monoclonal antibody (47G4, as described in US20100104509) and murine anti-CD 19 monoclonal antibody (as described in Nicholson et al. and explained above). Various combinations of a signal sequence (human CD8-alpha or GM-CSF receptor), extracellular and transmembrane regions (human CD8-alpha) and intracellular T-cell signalling domains (CD28-CD3Q 4-IBB-CD3Q CD27-CD3Q CD28-CD27-CD3C, 4-IBB-CD27-CD3Q CD27-4-1BB-CD3 ζ ; CD28-CD27-Fc ϵ RI gamma chain; or CD28-Fc ϵ RI gamma chain) were disclosed. Hence, in certain embodiments, cells intended for adoptive cell therapies, more particularly immunoresponsive cells such as T cells, may comprise a CAR comprising an extracellular antigen-binding element that specifically binds to an antigen, an extracellular and transmembrane region as set forth in Table 1 of WO2015187528 and an intracellular T-cell signalling domain as set forth in Table 1 of WO2015187528. Preferably, the antigen is CD 19, more preferably the antigen-binding element is an anti-CD 19 scFv, even more

preferably the mouse or human anti-CD 19 scFv as described in Example 1 of WO2015187528. In certain embodiments, the CAR comprises, consists essentially of or consists of an amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13 as set forth in Table 1 of WO2015187528.

[0321] By means of an example and without limitation, chimeric antigen receptor that recognizes the CD70 antigen is described in WO2012058460A2 (see also, Park et al., CD70 as a target for chimeric antigen receptor T cells in head and neck squamous cell carcinoma, Oral Oncol. 2018 Mar;78: 145-150; and Jin et al., CD70, a novel target of CAR T-cell therapy for gliomas, Neuro Oncol. 2018 Jan 10;20(1):55-65). CD70 is expressed by diffuse large B-cell and follicular lymphoma and also by the malignant cells of Hodgkins lymphoma, Waldenstrom's macroglobulinemia and multiple myeloma, and by HTLV-1- and EBV-associated malignancies. (Agathanggelou et al. Am.J.Pathol. 1995;147: 1152-1160; Hunter et al., Blood 2004; 104:4881-26; Lens et al., J Immunol. 2005;174:6212-6219; Baba et al., J Virol. 2008;82:3843-3852.) In addition, CD70 is expressed by non-hematological malignancies such as renal cell carcinoma and glioblastoma. (Junker et al., J Urol. 2005;173:2150-2153; Chahalvi et al., Cancer Res 2005;65:5428-5438) Physiologically, CD70 expression is transient and restricted to a subset of highly activated T, B, and dendritic cells.

[0322] By means of an example and without limitation, chimeric antigen receptor that recognizes BCMA has been described (see, e.g., US20160046724A1; WO2016014789A2; WO2017211900A1; WO2015158671A1; US20180085444A1; WO2018028647A1; US20170283504A1; and WO2013154760A1).

[0323] In certain embodiments, the immune cell may, in addition to a CAR or exogenous TCR as described herein, further comprise a chimeric inhibitory receptor (inhibitory CAR) that specifically binds to a second target antigen and is capable of inducing an inhibitory or immunosuppressive or repressive signal to the cell upon recognition of the second target antigen. In certain embodiments, the chimeric inhibitory receptor comprises an extracellular antigen-binding element (or portion or domain) configured to specifically bind to a target antigen, a transmembrane domain, and an intracellular immunosuppressive or repressive signaling domain. In certain embodiments, the second target antigen is an antigen that is not expressed on the surface of a cancer cell or infected cell or the expression of which is

downregulated on a cancer cell or an infected cell. In certain embodiments, the second target antigen is an MHC-class I molecule. In certain embodiments, the intracellular signaling domain comprises a functional signaling portion of an immune checkpoint molecule, such as for example PD-1 or CTLA4. Advantageously, the inclusion of such inhibitory CAR reduces the chance of the engineered immune cells attacking non-target (e.g., non-cancer) tissues.

[0324] Alternatively, T-cells expressing CARs may be further modified to reduce or eliminate expression of endogenous TCRs in order to reduce off-target effects. Reduction or elimination of endogenous TCRs can reduce off-target effects and increase the effectiveness of the T cells (U.S. 9,181,527). T cells stably lacking expression of a functional TCR may be produced using a variety of approaches. T cells internalize, sort, and degrade the entire T cell receptor as a complex, with a half-life of about 10 hours in resting T cells and 3 hours in stimulated T cells (von Essen, M. et al. 2004. J. Immunol. 173:384-393). Proper functioning of the TCR complex requires the proper stoichiometric ratio of the proteins that compose the TCR complex. TCR function also requires two functioning TCR zeta proteins with ITAM motifs. The activation of the TCR upon engagement of its MHC-peptide ligand requires the engagement of several TCRs on the same T cell, which all must signal properly. Thus, if a TCR complex is destabilized with proteins that do not associate properly or cannot signal optimally, the T cell will not become activated sufficiently to begin a cellular response.

[0325] Accordingly, in some embodiments, TCR expression may be eliminated using RNA interference (e.g., shRNA, siRNA, miRNA, etc.), CRISPR, or other methods that target the nucleic acids encoding specific TCRs (e.g., TCR- α and TCR- β) and/or CD3 chains in primary T cells. By blocking expression of one or more of these proteins, the T cell will no longer produce one or more of the key components of the TCR complex, thereby destabilizing the TCR complex and preventing cell surface expression of a functional TCR.

[0326] In some instances, CAR may also comprise a switch mechanism for controlling expression and/or activation of the CAR. For example, a CAR may comprise an extracellular, transmembrane, and intracellular domain, in which the extracellular domain comprises a target-specific binding element that comprises a label, binding domain, or tag that is specific for a molecule other than the target antigen that is expressed on or by a target cell. In such embodiments, the specificity of the CAR is provided by a second construct that comprises a target antigen binding domain (e.g., an scFv or a bispecific antibody that is specific for both the target antigen and the label or tag on the CAR) and a domain that is recognized by or binds to the label, binding domain, or tag on the CAR. See, e.g., WO 2013/044225, WO

2016/000304, WO 2015/057834, WO 2015/057852, WO 2016/070061, US 9,233,125, US 2016/0129109. In this way, a T-cell that expresses the CAR can be administered to a subject, but the CAR cannot bind its target antigen until the second composition comprising an antigen-specific binding domain is administered.

[0327] Alternative switch mechanisms include CARs that require multimerization in order to activate their signaling function (see, e.g., US 2015/0368342, US 2016/0175359, US 2015/0368360) and/or an exogenous signal, such as a small molecule drug (US 2016/0166613, Yung et al., *Science*, 2015), in order to elicit a T-cell response. Some CARs may also comprise a "suicide switch" to induce cell death of the CAR T-cells following treatment (Buddee et al., *PLoS One*, 2013) or to downregulate expression of the CAR following binding to the target antigen (WO 2016/01 1210).

[0328] Alternative techniques may be used to transform target immunoresponsive cells, such as protoplast fusion, lipofection, transfection or electroporation. A wide variety of vectors may be used, such as retroviral vectors, lentiviral vectors, adenoviral vectors, adeno-associated viral vectors, plasmids or transposons, such as a Sleeping Beauty transposon (see U.S. Patent Nos. 6,489,458; 7,148,203; 7,160,682; 7,985,739; 8,227,432), may be used to introduce CARs, for example using 2nd generation antigen-specific CARs signaling through CD3 ζ and either CD28 or CD137. Viral vectors may for example include vectors based on HIV, SV40, EBV, HSV or BPV.

[0329] Cells that are targeted for transformation may for example include T cells, Natural Killer (NK) cells, cytotoxic T lymphocytes (CTL), regulatory T cells, human embryonic stem cells, tumor-infiltrating lymphocytes (TIL) or a pluripotent stem cell from which lymphoid cells may be differentiated. T cells expressing a desired CAR may for example be selected through co-culture with γ -irradiated activating and propagating cells (AaPC), which co-express the cancer antigen and co-stimulatory molecules. The engineered CAR T-cells may be expanded, for example by co-culture on AaPC in presence of soluble factors, such as IL-2 and IL-21. This expansion may for example be carried out so as to provide memory CAR+ T cells (which may for example be assayed by non-enzymatic digital array and/or multi-panel flow cytometry). In this way, CAR T cells may be provided that have specific cytotoxic activity against antigen-bearing tumors (optionally in conjunction with production of desired chemokines such as interferon- γ). CAR T cells of this kind may for example be used in animal models, for example to treat tumor xenografts.

[0330] In certain embodiments, ACT includes co-transferring CD4+ Th1 cells and CD8+ CTLs to induce a synergistic antitumour response (see, e.g., Li et al., Adoptive cell therapy with CD4+ T helper 1 cells and CD8+ cytotoxic T cells enhances complete rejection of an established tumour, leading to generation of endogenous memory responses to non-targeted tumour epitopes. Clin Transl Immunology. 2017 Oct; 6(10): e160).

[0331] In certain embodiments, Th17 cells are transferred to a subject in need thereof. Th17 cells have been reported to directly eradicate melanoma tumors in mice to a greater extent than Th1 cells (Muranski P, et al., Tumor-specific Th17-polarized cells eradicate large established melanoma. Blood. 2008 Jul 15; 112(2):362-73; and Martin-Orozco N, et al., T helper 17 cells promote cytotoxic T cell activation in tumor immunity. Immunity. 2009 Nov 20; 31(5):787-98). Those studies involved an adoptive T cell transfer (ACT) therapy approach, which takes advantage of CD4⁺ T cells that express a TCR recognizing tyrosinase tumor antigen. Exploitation of the TCR leads to rapid expansion of Th17 populations to large numbers *ex vivo* for reinfusion into the autologous tumor-bearing hosts.

[0332] In certain embodiments, ACT may include autologous iPSC-based vaccines, such as irradiated iPSCs in autologous anti-tumor vaccines (see e.g., Kooreman, Nigel G. et al., Autologous iPSC-Based Vaccines Elicit Anti-tumor Responses In Vivo, Cell Stem Cell 22, 1-13, 2018, doi.org/10.1016/j.stem.2018.01.016).

[0333] Unlike T-cell receptors (TCRs) that are MHC restricted, CARs can potentially bind any cell surface-expressed antigen and can thus be more universally used to treat patients (see Irving et al., Engineering Chimeric Antigen Receptor T-Cells for Racing in Solid Tumors: Don't Forget the Fuel, Front. Immunol., 03 April 2017, doi.org/10.3389/fimmu.2017.00267). In certain embodiments, in the absence of endogenous T-cell infiltrate (e.g., due to aberrant antigen processing and presentation), which precludes the use of TIL therapy and immune checkpoint blockade, the transfer of CAR T-cells may be used to treat patients (see, e.g., Hinrichs CS, Rosenberg SA. Exploiting the curative potential of adoptive T-cell therapy for cancer. Immunol Rev (2014) 257(1):56-71. doi: 10.1111/imr.12132).

[0334] Approaches such as the foregoing may be adapted to provide methods of treating and/or increasing survival of a subject having a disease, such as a neoplasia, for example by administering an effective amount of an immunoresponsive cell comprising an antigen recognizing receptor that binds a selected antigen, wherein the binding activates the

immunoresponsive cell, thereby treating or preventing the disease (such as a neoplasia, a pathogen infection, an autoimmune disorder, or an allogeneic transplant reaction).

[0335] In certain embodiments, the treatment can be administered after lymphodepleting pretreatment in the form of chemotherapy (typically a combination of cyclophosphamide and fludarabine) or radiation therapy. Initial studies in ACT had short lived responses and the transferred cells did not persist *in vivo* for very long (Houot et al., T-cell-based immunotherapy: adoptive cell transfer and checkpoint inhibition. *Cancer Immunol Res* (2015) 3(10): 1115-22; and Kamta et al., Advancing Cancer Therapy with Present and Emerging Immuno-Oncology Approaches. *Front. Oncol.* (2017) 7:64). Immune suppressor cells like Tregs and MDSCs may attenuate the activity of transferred cells by outcompeting them for the necessary cytokines. Not being bound by a theory lymphodepleting pretreatment may eliminate the suppressor cells allowing the TILs to persist.

[0336] In one embodiment, the treatment can be administrated into patients undergoing an immunosuppressive treatment (e.g., glucocorticoid treatment). The cells or population of cells, may be made resistant to at least one immunosuppressive agent due to the inactivation of a gene encoding a receptor for such immunosuppressive agent. In certain embodiments, the immunosuppressive treatment provides for the selection and expansion of the immunoresponsive T cells within the patient.

[0337] In certain embodiments, the treatment can be administered before primary treatment (e.g., surgery or radiation therapy) to shrink a tumor before the primary treatment. In another embodiment, the treatment can be administered after primary treatment to remove any remaining cancer cells.

[0338] In certain embodiments, immunometabolic barriers can be targeted therapeutically prior to and/or during ACT to enhance responses to ACT or CAR T-cell therapy and to support endogenous immunity (see, e.g., Irving et al., Engineering Chimeric Antigen Receptor T-Cells for Racing in Solid Tumors: Don't Forget the Fuel, *Front. Immunol.*, 03 April 2017, doi.org/10.3389/fimmu.2017.00267).

[0339] The administration of cells or population of cells, such as immune system cells or cell populations, such as more particularly immunoresponsive cells or cell populations, as disclosed herein may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The cells or population of cells may be administered to a patient subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, intrathecally, by intravenous or

intralymphatic injection, or intraperitoneally. In some embodiments, the disclosed CARs may be delivered or administered into a cavity formed by the resection of tumor tissue (i.e. intracavity delivery) or directly into a tumor prior to resection (i.e. intratumoral delivery). In one embodiment, the cell compositions of the present invention are preferably administered by intravenous injection.

[0340] The administration of the cells or population of cells can consist of the administration of 10^4 - 10^9 cells per kg body weight, preferably 10^5 to 10^6 cells/kg body weight including all integer values of cell numbers within those ranges. Dosing in CAR T cell therapies may for example involve administration of from 10^6 to 10^9 cells/kg, with or without a course of lymphodepletion, for example with cyclophosphamide. The cells or population of cells can be administrated in one or more doses. In another embodiment, the effective amount of cells are administrated as a single dose. In another embodiment, the effective amount of cells are administrated as more than one dose over a period time. Timing of administration is within the judgment of managing physician and depends on the clinical condition of the patient. The cells or population of cells may be obtained from any source, such as a blood bank or a donor. While individual needs vary, determination of optimal ranges of effective amounts of a given cell type for a particular disease or conditions are within the skill of one in the art. An effective amount means an amount which provides a therapeutic or prophylactic benefit. The dosage administrated will be dependent upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment and the nature of the effect desired.

[0341] In another embodiment, the effective amount of cells or composition comprising those cells are administrated parenterally. The administration can be an intravenous administration. The administration can be directly done by injection within a tumor.

[0342] To guard against possible adverse reactions, engineered immunoresponsive cells may be equipped with a transgenic safety switch, in the form of a transgene that renders the cells vulnerable to exposure to a specific signal. For example, the herpes simplex viral thymidine kinase (TK) gene may be used in this way, for example by introduction into allogeneic T lymphocytes used as donor lymphocyte infusions following stem cell transplantation (Greco, et al., Improving the safety of cell therapy with the TK-suicide gene. Front. Pharmacol. 2015; 6: 95). In such cells, administration of a nucleoside prodrug such as ganciclovir or acyclovir causes cell death. Alternative safety switch constructs include inducible caspase 9, for example triggered by administration of a small-molecule dimerizer

that brings together two nonfunctional icasp9 molecules to form the active enzyme. A wide variety of alternative approaches to implementing cellular proliferation controls have been described (see U.S. Patent Publication No. 20130071414; PCT Patent Publication WO2011146862; PCT Patent Publication WO2014011987; PCT Patent Publication WO2013040371; Zhou et al. BLOOD, 2014, 123/25:3895 - 3905; Di Stasi et al., The New England Journal of Medicine 2011; 365:1673-1683; Sadelain M, The New England Journal of Medicine 2011; 365:1735-173; Ramos et al., Stem Cells 28(6):1107-15 (2010)).

[0343] In a further refinement of adoptive therapies, genome editing may be used to tailor immunoresponsive cells to alternative implementations, for example providing edited CAR T cells (see Poirot et al., 2015, Multiplex genome edited T-cell manufacturing platform for "off-the-shelf" adoptive T-cell immunotherapies, Cancer Res 75 (18): 3853; Ren et al., 2017, Multiplex genome editing to generate universal CAR T cells resistant to PD1 inhibition, Clin Cancer Res. 2017 May 1;23(9):2255-2266. doi: 10.1158/1078-0432.CCR-16-1300. Epub 2016 Nov 4; Qasim et al., 2017, Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells, Sci Transl Med. 2017 Jan 25;9(374); Legut, et al., 2018, CRISPR-mediated TCR replacement generates superior anticancer transgenic T cells. Blood, 131(3), 311-322; and Georgiadis et al., Long Terminal Repeat CRISPR-CAR-Coupled "Universal" T Cells Mediate Potent Anti-leukemic Effects, Molecular Therapy, In Press, Corrected Proof, Available online 6 March 2018). Cells may be edited using any CRISPR system and method of use thereof as described herein. CRISPR systems may be delivered to an immune cell by any method described herein. In preferred embodiments, cells are edited ex vivo and transferred to a subject in need thereof. Immunoresponsive cells, CAR T cells or any cells used for adoptive cell transfer may be edited. Editing may be performed for example to insert or knock-in an exogenous gene, such as an exogenous gene encoding a CAR or a TCR, at a preselected locus in a cell (e.g. TRAC locus); to eliminate potential alloreactive T-cell receptors (TCR) or to prevent inappropriate pairing between endogenous and exogenous TCR chains, such as to knock-out or knock-down expression of an endogenous TCR in a cell; to disrupt the target of a chemotherapeutic agent in a cell; to block an immune checkpoint, such as to knock-out or knock-down expression of an immune checkpoint protein or receptor in a cell; to knock-out or knock-down expression of other gene or genes in a cell, the reduced expression or lack of expression of which can enhance the efficacy of adoptive therapies using the cell; to knock-out or knock-down expression of an endogenous gene in a cell, said endogenous gene encoding an antigen targeted by an

exogenous CAR or TCR; to knock-out or knock-down expression of one or more MHC constituent proteins in a cell; to activate a T cell; to modulate cells such that the cells are resistant to exhaustion or dysfunction; and/or increase the differentiation and/or proliferation of functionally exhausted or dysfunctional CD8+ T-cells (see PCT Patent Publications: WO2013 176915, WO2014059173, WO2014172606, WO2014184744, and WO2014191 128).

[0344] In certain embodiments, editing may result in inactivation of a gene. By inactivating a gene, it is intended that the gene of interest is not expressed in a functional protein form. In a particular embodiment, the CRISPR system specifically catalyzes cleavage in one targeted gene thereby inactivating said targeted gene. The nucleic acid strand breaks caused are commonly repaired through the distinct mechanisms of homologous recombination or non-homologous end joining (NHEJ). However, NHEJ is an imperfect repair process that often results in changes to the DNA sequence at the site of the cleavage. Repair via non-homologous end joining (NHEJ) often results in small insertions or deletions (Indel) and can be used for the creation of specific gene knockouts. Cells in which a cleavage induced mutagenesis event has occurred can be identified and/or selected by well-known methods in the art. In certain embodiments, homology directed repair (HDR) is used to concurrently inactivate a gene (e.g., TRAC) and insert an endogenous TCR or CAR into the inactivated locus.

[0345] Hence, in certain embodiments, editing of cells (such as by CRISPR/Cas), particularly cells intended for adoptive cell therapies, more particularly immunoresponsive cells such as T cells, may be performed to insert or knock-in an exogenous gene, such as an exogenous gene encoding a CAR or a TCR, at a preselected locus in a cell. Conventionally, nucleic acid molecules encoding CARs or TCRs are transfected or transduced to cells using randomly integrating vectors, which, depending on the site of integration, may lead to clonal expansion, oncogenic transformation, variegated transgene expression and/or transcriptional silencing of the transgene. Directing of transgene(s) to a specific locus in a cell can minimize or avoid such risks and advantageously provide for uniform expression of the transgene(s) by the cells. Without limitation, suitable 'safe harbor' loci for directed transgene integration include CCR5 or AAVS1. Homology-directed repair (HDR) strategies are known and described elsewhere in this specification allowing to insert transgenes into desired loci (e.g., TRAC locus).

[0346] Further suitable loci for insertion of transgenes, in particular CAR or exogenous TCR transgenes, include without limitation loci comprising genes coding for constituents of endogenous T-cell receptor, such as T-cell receptor alpha locus (TRA) or T-cell receptor beta locus (TRB), for example T-cell receptor alpha constant (TRAC) locus, T-cell receptor beta constant 1 (TRBC1) locus or T-cell receptor beta constant 2 (TRBC2) locus. Advantageously, insertion of a transgene into such locus can simultaneously achieve expression of the transgene, potentially controlled by the endogenous promoter, and knock-out expression of the endogenous TCR. This approach has been exemplified in Eyquem et al., (2017) Nature 543: 113-117, wherein the authors used CRISPR/Cas9 gene editing to knock-in a DNA molecule encoding a CD19-specific CAR into the TRAC locus downstream of the endogenous promoter; the CAR-T cells obtained by CRISPR were significantly superior in terms of reduced tonic CAR signaling and exhaustion.

[0347] T cell receptors (TCR) are cell surface receptors that participate in the activation of T cells in response to the presentation of antigen. The TCR is generally made from two chains, α and β, which assemble to form a heterodimer and associates with the CD3-transducing subunits to form the T cell receptor complex present on the cell surface. Each α and β chain of the TCR consists of an immunoglobulin-like N-terminal variable (V) and constant (C) region, a hydrophobic transmembrane domain, and a short cytoplasmic region. As for immunoglobulin molecules, the variable region of the α and β chains are generated by V(D)J recombination, creating a large diversity of antigen specificities within the population of T cells. However, in contrast to immunoglobulins that recognize intact antigen, T cells are activated by processed peptide fragments in association with an MHC molecule, introducing an extra dimension to antigen recognition by T cells, known as MHC restriction. Recognition of MHC disparities between the donor and recipient through the T cell receptor leads to T cell proliferation and the potential development of graft versus host disease (GVHD). The inactivation of TCRA or TCRP can result in the elimination of the TCR from the surface of T cells preventing recognition of alloantigen and thus GVHD. However, TCR disruption generally results in the elimination of the CD3 signaling component and alters the means of further T cell expansion.

[0348] Hence, in certain embodiments, editing of cells (such as by CRISPR/Cas), particularly cells intended for adoptive cell therapies, more particularly immunoresponsive cells such as T cells, may be performed to knock-out or knock-down expression of an endogenous TCR in a cell. For example, NHEJ-based or HDR-based gene editing approaches

can be employed to disrupt the endogenous TCR alpha and/or beta chain genes. For example, gene editing system or systems, such as CRISPR/Cas system or systems, can be designed to target a sequence found within the TCR beta chain conserved between the beta 1 and beta 2 constant region genes (TRBC1 and TRBC2) and/or to target the constant region of the TCR alpha chain (TRAC) gene.

[0349] Allogeneic cells are rapidly rejected by the host immune system. It has been demonstrated that, allogeneic leukocytes present in non-irradiated blood products will persist for no more than 5 to 6 days (Boni, Muranski et al. 2008 Blood 11; 12(12):4746-54). Thus, to prevent rejection of allogeneic cells, the host's immune system usually has to be suppressed to some extent. However, in the case of adoptive cell transfer the use of immunosuppressive drugs also have a detrimental effect on the introduced therapeutic T cells. Therefore, to effectively use an adoptive immunotherapy approach in these conditions, the introduced cells would need to be resistant to the immunosuppressive treatment. Thus, in a particular embodiment, the present invention further comprises a step of modifying T cells to make them resistant to an immunosuppressive agent, preferably by inactivating at least one gene encoding a target for an immunosuppressive agent. An immunosuppressive agent is an agent that suppresses immune function by one of several mechanisms of action. An immunosuppressive agent can be, but is not limited to a calcineurin inhibitor, a target of rapamycin, an interleukin-2 receptor α-chain blocker, an inhibitor of inosine monophosphate dehydrogenase, an inhibitor of dihydrofolic acid reductase, a corticosteroid or an immunosuppressive antimetabolite. The present invention allows conferring immunosuppressive resistance to T cells for immunotherapy by inactivating the target of the immunosuppressive agent in T cells. As non-limiting examples, targets for an immunosuppressive agent can be a receptor for an immunosuppressive agent such as: CD52, glucocorticoid receptor (GR), a FKBP family gene member and a cyclophilin family gene member.

[0350] In certain embodiments, editing of cells (such as by CRISPR/Cas), particularly cells intended for adoptive cell therapies, more particularly immunoresponsive cells such as T cells, may be performed to block an immune checkpoint, such as to knock-out or knock-down expression of an immune checkpoint protein or receptor in a cell. Immune checkpoints are inhibitory pathways that slow down or stop immune reactions and prevent excessive tissue damage from uncontrolled activity of immune cells. In certain embodiments, the immune checkpoint targeted is the programmed death-1 (PD-1 or CD279) gene (PDCD1). In

other embodiments, the immune checkpoint targeted is cytotoxic T-lymphocyte-associated antigen (CTLA-4). In additional embodiments, the immune checkpoint targeted is another member of the CD28 and CTLA4 Ig superfamily such as BTLA, LAG3, ICOS, PDL1 or KIR. In further additional embodiments, the immune checkpoint targeted is a member of the TNFR superfamily such as CD40, OX40, CD137, GITR, CD27 or TIM-3.

[0351] Additional immune checkpoints include Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) (Watson HA, et al., SHP-1: the next checkpoint target for cancer immunotherapy? *Biochem Soc Trans.* 2016 Apr 15;44(2):356-62). SHP-1 is a widely expressed inhibitory protein tyrosine phosphatase (PTP). In T-cells, it is a negative regulator of antigen-dependent activation and proliferation. It is a cytosolic protein, and therefore not amenable to antibody-mediated therapies, but its role in activation and proliferation makes it an attractive target for genetic manipulation in adoptive transfer strategies, such as chimeric antigen receptor (CAR) T cells. Immune checkpoints may also include T cell immunoreceptor with Ig and ITEVI domains (TIGIT/Vstm3/WUCAM/VSIG9) and VISTA (Le Mercier I, et al., (2015) Beyond CTLA-4 and PD-1, the generation Z of negative checkpoint regulators. *Front. Immunol.* 6:418).

[0352] WO2014172606 relates to the use of MT1 and/or MT2 inhibitors to increase proliferation and/or activity of exhausted CD8+ T-cells and to decrease CD8+ T-cell exhaustion (e.g., decrease functionally exhausted or unresponsive CD8+ immune cells). In certain embodiments, metallothioneins are targeted by gene editing in adoptively transferred T cells.

[0353] In certain embodiments, targets of gene editing may be at least one targeted locus involved in the expression of an immune checkpoint protein. Such targets may include, but are not limited to CTLA4, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, ICOS (CD278), PDL1, KIR, LAG3, HAVCR2, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244 (2B4), TNFRSF10B, TNFRSF10A, CASP8, CASP 10, CASP3, CASP6, CASP7, FADD, FAS, TGFBRII, TGFRBRI, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, VISTA, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3, MT1, MT2, CD40, OX40, CD137, GITR, CD27, SHP-1, TIM-3, CEACAM-1, CEACAM-3, or CEACAM-5. In preferred embodiments, the gene locus involved in the expression of PD-1 or CTLA-4 genes is targeted. In other preferred embodiments, combinations of genes are targeted, such as but not limited to PD-1 and TIGIT.

[0354] By means of an example and without limitation, WO2016196388 concerns an engineered T cell comprising (a) a genetically engineered antigen receptor that specifically binds to an antigen, which receptor may be a CAR; and (b) a disrupted gene encoding a PD-L1, an agent for disruption of a gene encoding a PD-LI, and/or disruption of a gene encoding PD-L1, wherein the disruption of the gene may be mediated by a gene editing nuclease, a zinc finger nuclease (ZFN), CRISPR/Cas9 and/or TALEN. WO2015142675 relates to immune effector cells comprising a CAR in combination with an agent (such as CRISPR, TALEN or ZFN) that increases the efficacy of the immune effector cells in the treatment of cancer, wherein the agent may inhibit an immune inhibitory molecule, such as PDI, PD-L1, CTLA-4, TIM-3, LAG-3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, TGFR beta, CEACAM-1, CEACAM-3, or CEACAM-5. Ren et al., (2017) Clin Cancer Res 23 (9) 2255-2266 performed lentiviral delivery of CAR and electro-transfer of Cas9 mRNA and gRNAs targeting endogenous TCR, β-2 microglobulin (B2M) and PDI simultaneously, to generate gene-disrupted allogeneic CAR T cells deficient of TCR, ULA class I molecule and PDI.

[0355] In certain embodiments, cells may be engineered to express a CAR, wherein expression and/or function of methylcytosine dioxygenase genes (TET1, TET2 and/or TET3) in the cells has been reduced or eliminated, such as by CRISPR, ZNF or TALEN (for example, as described in WO201704916).

[0356] In certain embodiments, editing of cells (such as by CRISPR/Cas), particularly cells intended for adoptive cell therapies, more particularly immunoresponsive cells such as T cells, may be performed to knock-out or knock-down expression of an endogenous gene in a cell, said endogenous gene encoding an antigen targeted by an exogenous CAR or TCR, thereby reducing the likelihood of targeting of the engineered cells. In certain embodiments, the targeted antigen may be one or more antigen selected from the group consisting of CD38, CD138, CS-1, CD33, CD26, CD30, CD53, CD92, CD100, CD148, CD150, CD200, CD261, CD262, CD362, human telomerase reverse transcriptase (hTERT), survivin, mouse double minute 2 homolog (MDM2), cytochrome P450 1B1 (CYP1B), HER2/neu, Wilms' tumor gene 1 (WT1), livin, alphafetoprotein (AFP), carcinoembryonic antigen (CEA), mucin 16 (MUC16), MUC1, prostate-specific membrane antigen (PSMA), p53, cyclin (D1), B cell maturation antigen (BCMA), transmembrane activator and CAML Interactor (TACI), and B-cell activating factor receptor (BAFF-R) (for example, as described in WO2016011210 and WO2017011804).

[0357] In certain embodiments, editing of cells (such as by CRISPR/Cas), particularly cells intended for adoptive cell therapies, more particularly immunoresponsive cells such as T cells, may be performed to knock-out or knock-down expression of one or more MHC constituent proteins, such as one or more HLA proteins and/or beta-2 microglobulin (B2M), in a cell, whereby rejection of non-autologous (e.g., allogeneic) cells by the recipient's immune system can be reduced or avoided. In preferred embodiments, one or more HLA class I proteins, such as HLA-A, B and/or C, and/or B2M may be knocked-out or knocked-down. Preferably, B2M may be knocked-out or knocked-down. By means of an example, Ren et al., (2017) Clin Cancer Res 23 (9) 2255-2266 performed lentiviral delivery of CAR and electro-transfer of Cas9 mRNA and gRNAs targeting endogenous TCR, β-2 microglobulin (B2M) and PDI simultaneously, to generate gene-disrupted allogeneic CAR T cells deficient of TCR, HLA class I molecule and PDI.

[0358] In other embodiments, at least two genes are edited. Pairs of genes may include, but are not limited to PDI and TCRA, PDI and TCRp, CTLA-4 and TCRA, CTLA-4 and TCRp, LAG3 and TCRA, LAG3 and TCRp, Tim3 and TCRA, Tim3 and TCRp, BTLA and TCRA, BTLA and TCRp, BY55 and TCRA, BY55 and TCRp, TIGIT and TCRA, TIGIT and TCRp, B7H5 and TCRA, B7H5 and TCRp, LAIR1 and TCRA, LAIR1 and TCRp, SIGLEC10 and TCRA, SIGLEC10 and TCRp, 2B4 and TCRA, 2B4 and TCRp, B2M and TCRA, B2M and TCRp.

[0359] In certain embodiments, a cell may be multiply edited (multiplex genome editing) as taught herein to (1) knock-out or knock-down expression of an endogenous TCR (for example, TRBC1, TRBC2 and/or TRAC), (2) knock-out or knock-down expression of an immune checkpoint protein or receptor (for example PDI, PD-L1 and/or CTLA4); and (3) knock-out or knock-down expression of one or more MHC constituent proteins (for example, HLA-A, B and/or C, and/or B2M, preferably B2M).

[0360] Whether prior to or after genetic modification of the T cells, the T cells can be activated and expanded generally using methods as described, for example, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and 7,572,631. T cells can be expanded in vitro or in vivo.

[0361] Immune cells may be obtained using any method known in the art. In one embodiment, allogenic T cells may be obtained from healthy subjects. In one embodiment T cells that have infiltrated a tumor are isolated. T cells may be removed during surgery. T cells

may be isolated after removal of tumor tissue by biopsy. T cells may be isolated by any means known in the art. In one embodiment, T cells are obtained by apheresis. In one embodiment, the method may comprise obtaining a bulk population of T cells from a tumor sample by any suitable method known in the art. For example, a bulk population of T cells can be obtained from a tumor sample by dissociating the tumor sample into a cell suspension from which specific cell populations can be selected. Suitable methods of obtaining a bulk population of T cells may include, but are not limited to, any one or more of mechanically dissociating (e.g., mincing) the tumor, enzymatically dissociating (e.g., digesting) the tumor, and aspiration (e.g., as with a needle).

[0362] The bulk population of T cells obtained from a tumor sample may comprise any suitable type of T cell. Preferably, the bulk population of T cells obtained from a tumor sample comprises tumor infiltrating lymphocytes (TILs).

[0363] The tumor sample may be obtained from any mammal. Unless stated otherwise, as used herein, the term "mammal" refers to any mammal including, but not limited to, mammals of the order Logomorpha, such as rabbits; the order Carnivora, including Felines (cats) and Canines (dogs); the order Artiodactyla, including Bovines (cows) and Swines (pigs); or of the order Perssodactyla, including Equines (horses). The mammals may be non-human primates, e.g., of the order Primates, Ceboids, or Simoids (monkeys) or of the order Anthropoids (humans and apes). In some embodiments, the mammal may be a mammal of the order Rodentia, such as mice and hamsters. Preferably, the mammal is a non-human primate or a human. An especially preferred mammal is the human.

[0364] T cells can be obtained from a number of sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, spleen tissue, and tumors. In certain embodiments of the present invention, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as Ficoll separation. In one preferred embodiment, cells from the circulating blood of an individual are obtained by apheresis or leukapheresis. The apheresis product typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. In one embodiment, the cells collected by apheresis may be washed to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In one embodiment of the invention, the cells are washed with phosphate buffered saline (PBS). In an alternative embodiment, the wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations.

Initial activation steps in the absence of calcium lead to magnified activation. As those of ordinary skill in the art would readily appreciate a washing step may be accomplished by methods known to those in the art, such as by using a semi-automated "flow-through" centrifuge (for example, the Cobe 2991 cell processor) according to the manufacturer's instructions. After washing, the cells may be resuspended in a variety of biocompatible buffers, such as, for example, Ca-free, Mg-free PBS. Alternatively, the undesirable components of the apheresis sample may be removed and the cells directly resuspended in culture media.

[0365] In another embodiment, T cells are isolated from peripheral blood lymphocytes by lysing the red blood cells and depleting the monocytes, for example, by centrifugation through a PERCOLL™ gradient. A specific subpopulation of T cells, such as CD28+, CD4+, CDC, CD45RA+, and CD45RO+ T cells, can be further isolated by positive or negative selection techniques. For example, in one preferred embodiment, T cells are isolated by incubation with anti-CD3/anti-CD28 (i.e., 3x28)-conjugated beads, such as DYNABEADS® M-450 CD3/CD28 T, or XCYTE DYNABEADS™ for a time period sufficient for positive selection of the desired T cells. In one embodiment, the time period is about 30 minutes. In a further embodiment, the time period ranges from 30 minutes to 36 hours or longer and all integer values there between. In a further embodiment, the time period is at least 1, 2, 3, 4, 5, or 6 hours. In yet another preferred embodiment, the time period is 10 to 24 hours. In one preferred embodiment, the incubation time period is 24 hours. For isolation of T cells from patients with leukemia, use of longer incubation times, such as 24 hours, can increase cell yield. Longer incubation times may be used to isolate T cells in any situation where there are few T cells as compared to other cell types, such as in isolating tumor infiltrating lymphocytes (TIL) from tumor tissue or from immunocompromised individuals. Further, use of longer incubation times can increase the efficiency of capture of CD8+ T cells.

[0366] Enrichment of a T cell population by negative selection can be accomplished with a combination of antibodies directed to surface markers unique to the negatively selected cells. A preferred method is cell sorting and/or selection via negative magnetic immunoadherence or flow cytometry that uses a cocktail of monoclonal antibodies directed to cell surface markers present on the cells negatively selected. For example, to enrich for CD4+ cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CD1 lb, CD16, HLA-DR, and CD8.

[0367] Further, monocyte populations (i.e., CD14+ cells) may be depleted from blood preparations by a variety of methodologies, including anti-CD 14 coated beads or columns, or utilization of the phagocytotic activity of these cells to facilitate removal. Accordingly, in one embodiment, the invention uses paramagnetic particles of a size sufficient to be engulfed by phagocytotic monocytes. In certain embodiments, the paramagnetic particles are commercially available beads, for example, those produced by Life Technologies under the trade name Dynabeads™. In one embodiment, other non-specific cells are removed by coating the paramagnetic particles with "irrelevant" proteins (e.g., serum proteins or antibodies). Irrelevant proteins and antibodies include those proteins and antibodies or fragments thereof that do not specifically target the T cells to be isolated. In certain embodiments, the irrelevant beads include beads coated with sheep anti-mouse antibodies, goat anti-mouse antibodies, and human serum albumin.

[0368] In brief, such depletion of monocytes is performed by preincubating T cells isolated from whole blood, apheresed peripheral blood, or tumors with one or more varieties of irrelevant or non-antibody coupled paramagnetic particles at any amount that allows for removal of monocytes (approximately a 20:1 bead:cell ratio) for about 30 minutes to 2 hours at 22 to 37 degrees C, followed by magnetic removal of cells which have attached to or engulfed the paramagnetic particles. Such separation can be performed using standard methods available in the art. For example, any magnetic separation methodology may be used including a variety of which are commercially available, (e.g., DYNAL® Magnetic Particle Concentrator (DYNAL MPC®)). Assurance of requisite depletion can be monitored by a variety of methodologies known to those of ordinary skill in the art, including flow cytometric analysis of CD14 positive cells, before and after depletion.

[0369] For isolation of a desired population of cells by positive or negative selection, the concentration of cells and surface (e.g., particles such as beads) can be varied. In certain embodiments, it may be desirable to significantly decrease the volume in which beads and cells are mixed together (i.e., increase the concentration of cells), to ensure maximum contact of cells and beads. For example, in one embodiment, a concentration of 2 billion cells/ml is used. In one embodiment, a concentration of 1 billion cells/ml is used. In a further embodiment, greater than 100 million cells/ml is used. In a further embodiment, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In yet another embodiment, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further embodiments, concentrations of 125 or 150 million cells/ml can be used.

Using high concentrations can result in increased cell yield, cell activation, and cell expansion. Further, use of high cell concentrations allows more efficient capture of cells that may weakly express target antigens of interest, such as CD28-negative T cells, or from samples where there are many tumor cells present (i.e., leukemic blood, tumor tissue, etc). Such populations of cells may have therapeutic value and would be desirable to obtain. For example, using high concentration of cells allows more efficient selection of CD8+ T cells that normally have weaker CD28 expression.

[0370] In a related embodiment, it may be desirable to use lower concentrations of cells. By significantly diluting the mixture of T cells and surface (e.g., particles such as beads), interactions between the particles and cells is minimized. This selects for cells that express high amounts of desired antigens to be bound to the particles. For example, CD4+ T cells express higher levels of CD28 and are more efficiently captured than CD8+ T cells in dilute concentrations. In one embodiment, the concentration of cells used is 5×10^6 /ml. In other embodiments, the concentration used can be from about 1×10^5 /ml to 1×10^6 /ml, and any integer value in between.

[0371] T cells can also be frozen. Wishing not to be bound by theory, the freeze and subsequent thaw step provides a more uniform product by removing granulocytes and to some extent monocytes in the cell population. After a washing step to remove plasma and platelets, the cells may be suspended in a freezing solution. While many freezing solutions and parameters are known in the art and will be useful in this context, one method involves using PBS containing 20% DMSO and 8% human serum albumin, or other suitable cell freezing media, the cells then are frozen to -80° C at a rate of 1° per minute and stored in the vapor phase of a liquid nitrogen storage tank. Other methods of controlled freezing may be used as well as uncontrolled freezing immediately at -20° C. or in liquid nitrogen.

[0372] T cells for use in the present invention may also be antigen-specific T cells. For example, tumor-specific T cells can be used. In certain embodiments, antigen-specific T cells can be isolated from a patient of interest, such as a patient afflicted with a cancer or an infectious disease. In one embodiment, neoepitopes are determined for a subject and T cells specific to these antigens are isolated. Antigen-specific cells for use in expansion may also be generated in vitro using any number of methods known in the art, for example, as described in U.S. Patent Publication No. US 20040224402 entitled, Generation and Isolation of Antigen-Specific T Cells, or in U.S. Pat. Nos. 6,040,177. Antigen-specific cells for use in the present invention may also be generated using any number of methods known in the art, for

example, as described in Current Protocols in Immunology, or Current Protocols in Cell Biology, both published by John Wiley & Sons, Inc., Boston, Mass.

[0373] In a related embodiment, it may be desirable to sort or otherwise positively select (e.g. via magnetic selection) the antigen specific cells prior to or following one or two rounds of expansion. Sorting or positively selecting antigen-specific cells can be carried out using peptide-MHC tetramers (Altman, et al., Science. 1996 Oct. 4; 274(5284):94-6). In another embodiment, the adaptable tetramer technology approach is used (Andersen et al., 2012 Nat Protoc. 7:891-902). Tetramers are limited by the need to utilize predicted binding peptides based on prior hypotheses, and the restriction to specific HLAs. Peptide-MHC tetramers can be generated using techniques known in the art and can be made with any MHC molecule of interest and any antigen of interest as described herein. Specific epitopes to be used in this context can be identified using numerous assays known in the art. For example, the ability of a polypeptide to bind to MHC class I may be evaluated indirectly by monitoring the ability to promote incorporation of ¹²⁵I labeled P2-microglobulin ($\beta 2\mu$) into MHC class I/p2m/peptide heterotrimeric complexes (see Parker et al., J. Immunol. 152:163, 1994).

[0374] In one embodiment cells are directly labeled with an epitope-specific reagent for isolation by flow cytometry followed by characterization of phenotype and TCRs. In one embodiment, T cells are isolated by contacting with T cell specific antibodies. Sorting of antigen-specific T cells, or generally any cells of the present invention, can be carried out using any of a variety of commercially available cell sorters, including, but not limited to, MoFlo sorter (DakoCytomation, Fort Collins, Colo.), FACSariaTM, FACSArrayTM, FACS VantageTM, BDTM LSR II, and FACSCaliburTM (BD Biosciences, San Jose, Calif.).

[0375] In a preferred embodiment, the method comprises selecting cells that also express CD3. The method may comprise specifically selecting the cells in any suitable manner. Preferably, the selecting is carried out using flow cytometry. The flow cytometry may be carried out using any suitable method known in the art. The flow cytometry may employ any suitable antibodies and stains. Preferably, the antibody is chosen such that it specifically recognizes and binds to the particular biomarker being selected. For example, the specific selection of CD3, CD8, TIM-3, LAG-3, 4-1BB, or PD-1 may be carried out using anti-CD3, anti-CD8, anti-TIM-3, anti-LAG-3, anti-4-1BB, or anti-PD-1 antibodies, respectively. The antibody or antibodies may be conjugated to a bead (e.g., a magnetic bead) or to a fluorochrome. Preferably, the flow cytometry is fluorescence-activated cell sorting (FACS). TCRs expressed on T cells can be selected based on reactivity to autologous tumors.

Additionally, T cells that are reactive to tumors can be selected for based on markers using the methods described in patent publication Nos. WO2014133567 and WO2014133568, herein incorporated by reference in their entirety. Additionally, activated T cells can be selected for based on surface expression of CD 107a.

[0376] In one embodiment of the invention, the method further comprises expanding the numbers of T cells in the enriched cell population. Such methods are described in U.S. Patent No. 8,637,307 and is herein incorporated by reference in its entirety. The numbers of T cells may be increased at least about 3-fold (or 4-, 5-, 6-, 7-, 8-, or 9-fold), more preferably at least about 10-fold (or 20-, 30-, 40-, 50-, 60-, 70-, 80-, or 90-fold), more preferably at least about 100-fold, more preferably at least about 1,000 fold, or most preferably at least about 100,000-fold. The numbers of T cells may be expanded using any suitable method known in the art. Exemplary methods of expanding the numbers of cells are described in patent publication No. WO 2003057171, U.S. Patent No. 8,034,334, and U.S. Patent Application Publication No. 2012/0244133, each of which is incorporated herein by reference.

[0377] In one embodiment, *ex vivo* T cell expansion can be performed by isolation of T cells and subsequent stimulation or activation followed by further expansion. In one embodiment of the invention, the T cells may be stimulated or activated by a single agent. In another embodiment, T cells are stimulated or activated with two agents, one that induces a primary signal and a second that is a co-stimulatory signal. Ligands useful for stimulating a single signal or stimulating a primary signal and an accessory molecule that stimulates a second signal may be used in soluble form. Ligands may be attached to the surface of a cell, to an Engineered Multivalent Signaling Platform (EMSP), or immobilized on a surface. In a preferred embodiment both primary and secondary agents are co-immobilized on a surface, for example a bead or a cell. In one embodiment, the molecule providing the primary activation signal may be a CD3 ligand, and the co-stimulatory molecule may be a CD28 ligand or 4-1BB ligand.

[0378] In certain embodiments, T cells comprising a CAR or an exogenous TCR, may be manufactured as described in WO2015120096, by a method comprising: enriching a population of lymphocytes obtained from a donor subject; stimulating the population of lymphocytes with one or more T-cell stimulating agents to produce a population of activated T cells, wherein the stimulation is performed in a closed system using serum-free culture medium; transducing the population of activated T cells with a viral vector comprising a nucleic acid molecule which encodes the CAR or TCR, using a single cycle transduction to

produce a population of transduced T cells, wherein the transduction is performed in a closed system using serum-free culture medium; and expanding the population of transduced T cells for a predetermined time to produce a population of engineered T cells, wherein the expansion is performed in a closed system using serum-free culture medium. In certain embodiments, T cells comprising a CAR or an exogenous TCR, may be manufactured as described in WO2015120096, by a method comprising: obtaining a population of lymphocytes; stimulating the population of lymphocytes with one or more stimulating agents to produce a population of activated T cells, wherein the stimulation is performed in a closed system using serum-free culture medium; transducing the population of activated T cells with a viral vector comprising a nucleic acid molecule which encodes the CAR or TCR, using at least one cycle transduction to produce a population of transduced T cells, wherein the transduction is performed in a closed system using serum-free culture medium; and expanding the population of transduced T cells to produce a population of engineered T cells, wherein the expansion is performed in a closed system using serum-free culture medium. The predetermined time for expanding the population of transduced T cells may be 3 days. The time from enriching the population of lymphocytes to producing the engineered T cells may be 6 days. The closed system may be a closed bag system. Further provided is population of T cells comprising a CAR or an exogenous TCR obtainable or obtained by said method, and a pharmaceutical composition comprising such cells.

[0379] In certain embodiments, T cell maturation or differentiation in vitro may be delayed or inhibited by the method as described in WO2017070395, comprising contacting one or more T cells from a subject in need of a T cell therapy with an AKT inhibitor (such as, e.g., one or a combination of two or more AKT inhibitors disclosed in claim 8 of WO2017070395) and at least one of exogenous Interleukin-7 (IL-7) and exogenous Interleukin-15 (IL-15), wherein the resulting T cells exhibit delayed maturation or differentiation, and/or wherein the resulting T cells exhibit improved T cell function (such as, e.g., increased T cell proliferation; increased cytokine production; and/or increased cytolytic activity) relative to a T cell function of a T cell cultured in the absence of an AKT inhibitor.

[0380] **In certain embodiments, a patient in need of a T cell therapy may be conditioned by a method as described in WO2016191756 comprising administering to the patient a dose of cyclophosphamide between 200 mg/m²/day and 2000 mg/m²/day and a dose of fludarabine between 20 mg/m²/day and 900 mg/m²/day.**

[0381] In one embodiment, adoptive cell transfer may comprise: isolating from a biological sample of the subject a CD8⁺ T cell population; depleting from the CD8⁺ T cell population CD8⁺ T cells having a non-responder signature as described herein; *in vitro* expanding the depleted CD8⁺ T cell population; and administering the *in vitro* expanded CD8⁺ T cell population to the subject. In one embodiment, adoptive cell transfer may comprise: isolating from a biological sample of the subject a CD8⁺ T cell or CD8⁺ T cell population having a responder signature as described herein; *in vitro* expanding the CD8⁺ T cell or CD8⁺ T cell population; and administering the *in vitro* expanded CD8⁺ T cell or CD8⁺ T cell population to the subject. The method may further comprise enriching the expanded cells for CD8⁺ T cells having a responder signature as described herein. The method may further comprise depleting the expanded cells for CD8⁺ T cells having a non-responder signature as described herein. In certain embodiments, the method may further comprise formulating the *in vitro* expanded immune cell or immune cell population into a pharmaceutical composition.

Cancer

[0382] In certain example embodiments, the pharmaceutical compositions and adoptive cell transfer strategies may be used to treat various forms of cancer. Examples of cancer include but are not limited to carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include without limitation: squamous cell cancer (e.g., epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung and large cell carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioma, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial cancer or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, as well as CNS cancer, melanoma, head and neck cancer, bone cancer, bone marrow cancer, duodenum cancer, oesophageal cancer, thyroid cancer, or hematological cancer.

[0383] Other non-limiting examples of cancers or malignancies include, but are not limited to: Acute Childhood Lymphoblastic Leukemia, Acute Lymphoblastic Leukemia, Acute Lymphocytic Leukemia, Acute Myeloid Leukemia, Adrenocortical Carcinoma, Adult (Primary) Hepatocellular Cancer, Adult (Primary) Liver Cancer, Adult Acute Lymphocytic

Leukemia, Adult Acute Myeloid Leukemia, Adult Hodgkin's Disease, Adult Hodgkin's Lymphoma, Adult Lymphocytic Leukemia, Adult Non-Hodgkin's Lymphoma, Adult Primary Liver Cancer, Adult Soft Tissue Sarcoma, AIDS-Related Lymphoma, AIDS-Related Malignancies, Anal Cancer, Astrocytoma, Bile Duct Cancer, Bladder Cancer, Bone Cancer, Brain Stem Glioma, Brain Tumours, Breast Cancer, Cancer of the Renal Pelvis and Urethra, Central Nervous System (Primary) Lymphoma, Central Nervous System Lymphoma, Cerebellar Astrocytoma, Cerebral Astrocytoma, Cervical Cancer, Childhood (Primary) Hepatocellular Cancer, Childhood (Primary) Liver Cancer, Childhood Acute Lymphoblastic Leukemia, Childhood Acute Myeloid Leukemia, Childhood Brain Stem Glioma, Glioblastoma, Childhood Cerebellar Astrocytoma, Childhood Cerebral Astrocytoma, Childhood Extracranial Germ Cell Tumours, Childhood Hodgkin's Disease, Childhood Hodgkin's Lymphoma, Childhood Hypothalamic and Visual Pathway Glioma, Childhood Lymphoblastic Leukemia, Childhood Medulloblastoma, Childhood Non-Hodgkin's Lymphoma, Childhood Pineal and Supratentorial Primitive Neuroectodermal Tumours, Childhood Primary Liver Cancer, Childhood Rhabdomyosarcoma, Childhood Soft Tissue Sarcoma, Childhood Visual Pathway and Hypothalamic Glioma, Chronic Lymphocytic Leukemia, Chronic Myelogenous Leukemia, Colon Cancer, Cutaneous T-Cell Lymphoma, Endocrine Pancreas Islet Cell Carcinoma, Endometrial Cancer, Ependymoma, Epithelial Cancer, Esophageal Cancer, Ewing's Sarcoma and Related Tumours, Exocrine Pancreatic Cancer, Extracranial Germ Cell Tumour, Extragonadal Germ Cell Tumour, Extrahepatic Bile Duct Cancer, Eye Cancer, Female Breast Cancer, Gaucher's Disease, Gallbladder Cancer, Gastric Cancer, Gastrointestinal Carcinoid Tumour, Gastrointestinal Tumours, Germ Cell Tumours, Gestational Trophoblastic Tumour, Hairy Cell Leukemia, Head and Neck Cancer, Hepatocellular Cancer, Hodgkin's Disease, Hodgkin's Lymphoma, Hypergammaglobulinemia, Hypopharyngeal Cancer, Intestinal Cancers, Intraocular Melanoma, Islet Cell Carcinoma, Islet Cell Pancreatic Cancer, Kaposi's Sarcoma, Kidney Cancer, Laryngeal Cancer, Lip and Oral Cavity Cancer, Liver Cancer, Lung Cancer, Lymphoproliferative Disorders, Macroglobulinemia, Male Breast Cancer, Malignant Mesothelioma, Malignant Thymoma, Medulloblastoma, Melanoma, Mesothelioma, Metastatic Occult Primary Squamous Neck Cancer, Metastatic Primary Squamous Neck Cancer, Metastatic Squamous Neck Cancer, Multiple Myeloma, Multiple Myeloma/Plasma Cell Neoplasm, Myelodysplastic Syndrome, Myelogenous Leukemia, Myeloid Leukemia, Myeloproliferative Disorders, Nasal Cavity and Paranasal Sinus Cancer, Nasopharyngeal

Cancer, Neuroblastoma, Non-Hodgkin's Lymphoma During Pregnancy, Nonmelanoma Skin Cancer, Non-Small Cell Lung Cancer, Occult Primary Metastatic Squamous Neck Cancer, Oropharyngeal Cancer, Osteo-/Malignant Fibrous Sarcoma, Osteosarcoma/Malignant Fibrous Histiocytoma, Osteosarcoma/Malignant Fibrous Histiocytoma of Bone, Ovarian Epithelial Cancer, Ovarian Germ Cell Tumour, Ovarian Low Malignant Potential Tumour, Pancreatic Cancer, Paraproteinemias, Purpura, Parathyroid Cancer, Penile Cancer, Pheochromocytoma, Pituitary Tumour, Plasma Cell Neoplasm/Multiple Myeloma, Primary Central Nervous System Lymphoma, Primary Liver Cancer, Prostate Cancer, Rectal Cancer, Renal Cell Cancer, Renal Pelvis and Urethra Cancer, Retinoblastoma, Rhabdomyosarcoma, Salivary Gland Cancer, Sarcoidosis Sarcomas, Sezary Syndrome, Skin Cancer, Small Cell Lung Cancer, Small Intestine Cancer, Soft Tissue Sarcoma, Squamous Neck Cancer, Stomach Cancer, Supratentorial Primitive Neuroectodermal and Pineal Tumours, T-Cell Lymphoma, Testicular Cancer, Thymoma, Thyroid Cancer, Transitional Cell Cancer of the Renal Pelvis and Urethra, Transitional Renal Pelvis and Urethra Cancer, Trophoblastic Tumours, Urethra and Renal Pelvis Cell Cancer, Urethral Cancer, Uterine Cancer, Uterine Sarcoma, Vaginal Cancer, Visual Pathway and Hypothalamic Glioma, Vulvar Cancer, Waldenstrom's Macroglobulinemia, or Wilms' Tumour.

[0384] In further examples, any combinations of methods such as discussed herein may be employed.

Identifying Immunomodulators

[0385] A further aspect of the invention relates to a method for identifying an immunomodulant capable of modulating one or more phenotypic aspects of an immune cell or immune cell population as disclosed herein, comprising: a) applying a candidate immunomodulant to the immune cell or immune cell population; b) detecting modulation of one or more phenotypic aspects of the immune cell or immune cell population by the candidate immunomodulant, thereby identifying the immunomodulant.

[0386] The term "modulate" broadly denotes a qualitative and/or quantitative alteration, change or variation in that which is being modulated. Where modulation can be assessed quantitatively - for example, where modulation comprises or consists of a change in a quantifiable variable such as a quantifiable property of a cell or where a quantifiable variable provides a suitable surrogate for the modulation - modulation specifically encompasses both increase (e.g., activation) or decrease (e.g., inhibition) in the measured variable. The term encompasses any extent of such modulation, e.g., any extent of such increase or decrease, and

may more particularly refer to statistically significant increase or decrease in the measured variable. By means of example, modulation may encompass an increase in the value of the measured variable by at least about 10%, e.g., by at least about 20%, preferably by at least about 30%, e.g., by at least about 40%, more preferably by at least about 50%, e.g., by at least about 75%, even more preferably by at least about 100%, e.g., by at least about 150%, 200%, 250%, 300%, 400% or by at least about 500%, compared to a reference situation without said modulation; or modulation may encompass a decrease or reduction in the value of the measured variable by at least about 10%, e.g., by at least about 20%, by at least about 30%, e.g., by at least about 40%, by at least about 50%, e.g., by at least about 60%, by at least about 70%, e.g., by at least about 80%, by at least about 90%, e.g., by at least about 95%, such as by at least about 96%, 97%, 98%, 99% or even by 100%, compared to a reference situation without said modulation. Preferably, modulation may be specific or selective, hence, one or more desired phenotypic aspects of an immune cell or immune cell population may be modulated without substantially altering other (unintended, undesired) phenotypic aspect(s).

[0387] The term "immunomodulant" broadly encompasses any condition, substance or agent capable of modulating one or more phenotypic aspects of an immune cell or immune cell population as disclosed herein. Such conditions, substances or agents may be of physical, chemical, biochemical and/or biological nature. The term "candidate immunomodulant" refers to any condition, substance or agent that is being examined for the ability to modulate one or more phenotypic aspects of an immune cell or immune cell population as disclosed herein in a method comprising applying the candidate immunomodulant to the immune cell or immune cell population (e.g., exposing the immune cell or immune cell population to the candidate immunomodulant or contacting the immune cell or immune cell population with the candidate immunomodulant) and observing whether the desired modulation takes place.

[0388] Immunomodulants may include any potential class of biologically active conditions, substances or agents, such as for instance antibodies, proteins, peptides, nucleic acids, oligonucleotides, small molecules, or combinations thereof.

[0389] By means of example but without limitation, immunomodulants can include low molecular weight compounds, but may also be larger compounds, or any organic or inorganic molecule effective in the given situation, including modified and unmodified nucleic acids such as antisense nucleic acids, RNAi, such as siRNA or shRNA, CRISPR/Cas systems, peptides, peptidomimetics, receptors, ligands, and antibodies, aptamers, polypeptides, nucleic

acid analogues or variants thereof. Examples include an oligomer of nucleic acids, amino acids, or carbohydrates including without limitation proteins, oligonucleotides, ribozymes, DNAzymes, glycoproteins, siRNAs, lipoproteins, aptamers, and modifications and combinations thereof. Agents can be selected from a group comprising: chemicals; small molecules; nucleic acid sequences; nucleic acid analogues; proteins; peptides; aptamers; antibodies; or fragments thereof. A nucleic acid sequence can be RNA or DNA, and can be single or double stranded, and can be selected from a group comprising; nucleic acid encoding a protein of interest, oligonucleotides, nucleic acid analogues, for example peptide - nucleic acid (PNA), pseudo-complementary PNA (pc-PNA), locked nucleic acid (LNA), modified RNA (mod-RNA), single guide RNA etc. Such nucleic acid sequences include, for example, but are not limited to, nucleic acid sequence encoding proteins, for example that act as transcriptional repressors, antisense molecules, ribozymes, small inhibitory nucleic acid sequences, for example but are not limited to RNAi, shRNAi, siRNA, micro RNAi (mRNAi), antisense oligonucleotides, CRISPR guide RNA, for example that target a CRISPR enzyme to a specific DNA target sequence etc. A protein and/or peptide or fragment thereof can be any protein of interest, for example, but are not limited to: mutated proteins; therapeutic proteins and truncated proteins, wherein the protein is normally absent or expressed at lower levels in the cell. Proteins can also be selected from a group comprising; mutated proteins, genetically engineered proteins, peptides, synthetic peptides, recombinant proteins, chimeric proteins, antibodies, midibodies, minibodies, triabodies, humanized proteins, humanized antibodies, chimeric antibodies, modified proteins and fragments thereof. Alternatively, the agent can be intracellular within the cell as a result of introduction of a nucleic acid sequence into the cell and its transcription resulting in the production of the nucleic acid and/or protein modulator of a gene within the cell. In some embodiments, the agent is any chemical, entity or moiety, including without limitation synthetic and naturally-occurring non-proteinaceous entities. In certain embodiments, the agent is a small molecule having a chemical moiety. Agents can be known to have a desired activity and/or property, or can be selected from a library of diverse compounds.

[0390] In certain embodiments, an immunomodulant may be a hormone, a cytokine, a lymphokine, a growth factor, a chemokine, a cell surface receptor ligand such as a cell surface receptor agonist or antagonist, or a mitogen.

[0391] Non-limiting examples of hormones include growth hormone (GH), adrenocorticotropic hormone (ACTH), dehydroepiandrosterone (DHEA), Cortisol, epinephrine, thyroid hormone, estrogen, progesterone, testosterone, or combinations thereof.

[0392] Non-limiting examples of cytokines include lymphokines (e.g., interferon- γ , IL-2, IL-3, IL-4, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon- γ , leukocyte migration inhibitory factors (T-LIF, B-LIF), lymphotoxin-alpha, macrophage-activating factor (MAF), macrophage migration-inhibitory factor (MIF), neuroleukin, immunologic suppressor factors, transfer factors, or combinations thereof), monokines (e.g., IL-1, TNF-alpha, interferon- α , interferon- β , colony stimulating factors, e.g., CSF2, CSF3, macrophage CSF or GM-CSF, or combinations thereof), chemokines (e.g., beta-thromboglobulin, C chemokines, CC chemokines, CXC chemokines, CX3C chemokines, macrophage inflammatory protein (MIP), or combinations thereof), interleukins (e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-24, IL-25, IL-26, IL-27, IL-28, IL-29, IL-30, IL-31, IL-32, IL-33, IL-34, IL-35, IL-36, or combinations thereof), and several related signalling molecules, such as tumour necrosis factor (TNF) and interferons (e.g., interferon- α , interferon- β , interferon- γ , interferon- λ , or combinations thereof).

[0393] Non-limiting examples of growth factors include those of fibroblast growth factor (FGF) family, bone morphogenic protein (BMP) family, platelet derived growth factor (PDGF) family, transforming growth factor beta (TGF β) family, nerve growth factor (NGF) family, epidermal growth factor (EGF) family, insulin related growth factor (IGF) family, hepatocyte growth factor (HGF) family, hematopoietic growth factors (HeGFs), platelet-derived endothelial cell growth factor (PD-ECGF), angiopoietin, vascular endothelial growth factor (VEGF) family, glucocorticoids, or combinations thereof.

[0394] Non-limiting examples of mitogens include phytohaemagglutinin (PHA), concanavalin A (conA), lipopolysaccharide (LPS), pokeweed mitogen (PWM), phorbol ester such as phorbol myristate acetate (PMA) with or without ionomycin, or combinations thereof.

[0395] Non-limiting examples of cell surface receptors the ligands of which may act as immunomodulants include Toll-like receptors (TLRs) (e.g., TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11, TLR12 or TLR13), CD80, CD86, CD40, CCR7, or C-type lectin receptors.

Altering Expression Using Immunomodulants

[0396] In certain embodiments, an immunomodulant may comprise altering expression and/or activity of one or more endogenous genes of the immune cell. The term "altered expression" denotes that the modification of the immune cell alters, i.e., changes or modulates, the expression of the recited gene(s) or polypeptides(s). The term "altered expression" encompasses any direction and any extent of said alteration. Hence, "altered expression" may reflect qualitative and/or quantitative change(s) of expression, and specifically encompasses both increase (e.g., activation or stimulation) or decrease (e.g., inhibition) of expression.

[0397] In certain embodiments, the present invention provides for gene signature screening. The concept of signature screening was introduced by Stegmaier et al. (Gene expression-based high-throughput screening (GE-HTS) and application to leukemia differentiation. *Nature Genet.* 36, 257-263 (2004)), who realized that if a gene-expression signature was the proxy for a phenotype of interest, it could be used to find small molecules that effect that phenotype without knowledge of a validated drug target. The signature of the present may be used to screen for drugs that reduce the signature in immune cells as described herein. The signature may be used for GE-HTS. In certain embodiments, pharmacological screens may be used to identify drugs that are selectively toxic to immune cells having a non-responder signature. In certain embodiments, drugs selectively toxic to immune cells having a non-responder signature are used for treatment of a cancer patient.

[0398] The Connectivity Map (cmap) is a collection of genome-wide transcriptional expression data from cultured human cells treated with bioactive small molecules and simple pattern-matching algorithms that together enable the discovery of functional connections between drugs, genes and diseases through the transitory feature of common gene-expression changes (see, Lamb et al., The Connectivity Map: Using Gene-Expression Signatures to Connect Small Molecules, Genes, and Disease. *Science* 29 Sep 2006: Vol. 313, Issue 5795, pp. 1929-1935, DOI: 10.1126/science.1132939; and Lamb, J., The Connectivity Map: a new tool for biomedical research. *Nature Reviews Cancer* January 2007: Vol. 7, pp. 54-60). In certain embodiments, Cmap can be used to screen for small molecules capable of modulating a signature of the present invention in silico.

[0399] Any one or more of the several successive molecular mechanisms involved in the expression of a given gene or polypeptide may be targeted by the immune cell modification as intended herein. Without limitation, these may include targeting the gene sequence (e.g., targeting the polypeptide-encoding, non-coding and/or regulatory portions of the gene

sequence), the transcription of the gene into RNA, the polyadenylation and where applicable splicing and/or other post-transcriptional modifications of the RNA into mRNA, the localization of the mRNA into cell cytoplasm, where applicable other post-transcriptional modifications of the mRNA, the translation of the mRNA into a polypeptide chain, where applicable post-translational modifications of the polypeptide, and/or folding of the polypeptide chain into the mature conformation of the polypeptide. For compartmentalized polypeptides, such as secreted polypeptides and transmembrane polypeptides, this may further include targeting trafficking of the polypeptides, i.e., the cellular mechanism by which polypeptides are transported to the appropriate sub-cellular compartment or organelle, membrane, e.g. the plasma membrane, or outside the cell.

[0400] Hence, "altered expression" may particularly denote altered production of the recited gene products by the modified immune cell. As used herein, the term "gene product(s)" includes RNA transcribed from a gene (e.g., mRNA), or a polypeptide encoded by a gene or translated from RNA.

[0401] Also, "altered expression" as intended herein may encompass modulating the activity of one or more endogenous gene products. Accordingly, "altered expression", "altering expression", "modulating expression", or "detecting expression" or similar may be used interchangeably with respectively "altered expression or activity", "altering expression or activity", "modulating expression or activity", or "detecting expression or activity" or similar. As used herein, "modulating" or "to modulate" generally means either reducing or inhibiting the activity of a target or antigen, or alternatively increasing the activity of the target or antigen, as measured using a suitable in vitro, cellular or in vivo assay. In particular, "modulating" or "to modulate" can mean either reducing or inhibiting the (relevant or intended) activity of, or alternatively increasing the (relevant or intended) biological activity of the target or antigen, as measured using a suitable in vitro, cellular or in vivo assay (which will usually depend on the target or antigen involved), by at least 5%, at least 10%, at least 25%, at least 50%, at least 60%, at least 70%, at least 80%, or 90% or more, compared to activity of the target or antigen in the same assay under the same conditions but without the presence of the inhibitor/antagonist agents or activator/agonist agents described herein.

[0402] As will be clear to the skilled person, "modulating" can also involve effecting a change (which can either be an increase or a decrease) in affinity, avidity, specificity and/or selectivity of a target or antigen, for one or more of its targets compared to the same conditions but without the presence of a modulating agent. Again, this can be determined in

any suitable manner and/or using any suitable assay known per se, depending on the target. In particular, an action as an inhibitor/antagonist or activator/agonist can be such that an intended biological or physiological activity is increased or decreased, respectively, by at least 5%, at least 10%, at least 25%, at least 50%, at least 60%, at least 70%, at least 80%, or 90% or more, compared to the biological or physiological activity in the same assay under the same conditions but without the presence of the inhibitor/antagonist agent or activator/agonist agent. Modulating can also involve activating the target or antigen or the mechanism or pathway in which it is involved.

[0403] In certain embodiments, an immunomodulant may be or may result in a genetic modification (e.g., mutation, editing, transgenesis, or combinations thereof) of an immune cell, for example, a genetic perturbation, such as a knock-out (i.e., resulting in a complete absence of expression and/or activity) of one or more endogenous genes / gene products, or a knock-down (i.e., resulting in a partial absence of expression and/or activity) of one or more endogenous genes / gene products, or another type of genetic modification modulating the expression and/or activity of one or more endogenous genes / gene products, or for example, introduction of one or more transgenes, such as one or more transgenes encoding one or more gene products. Such transgene may be suitably operably linked to suitable regulatory sequences, e.g., may be comprised in an expression cassette or an expression vector comprising suitable regulatory sequences, or may be configured to become operably linked to suitable regulatory sequences once inserted into the genetic material (e.g., genome) of the immune cell.

[0404] Any types of mutations achieving the intended effects are contemplated herein. For example, suitable mutations may include deletions, insertions, and/or substitutions. The term "deletion" refers to a mutation wherein one or more nucleotides, typically consecutive nucleotides, of a nucleic acid are removed, i.e., deleted, from the nucleic acid. The term "insertion" refers to a mutation wherein one or more nucleotides, typically consecutive nucleotides, are added, i.e., inserted, into a nucleic acid. The term "substitution" refers to a mutation wherein one or more nucleotides of a nucleic acid are each independently replaced, i.e., substituted, by another nucleotide.

[0405] In certain embodiments, a mutation may introduce a premature in-frame stop codon into the open reading frame (ORF) encoding a gene product. Such premature stop codon may lead to production of a C-terminally truncated form of said polypeptide (this may preferably affect, such as diminish or abolish, some or all biological function(s) of the

polypeptide) or, especially when the stop codon is introduced close to (e.g., about 20 or less, or about 10 or less amino acids downstream of) the translation initiation codon of the ORF, the stop codon may effectively abolish the production of the polypeptide. Various ways of introducing a premature in-frame stop codon are apparent to a skilled person. For example but without limitation, a suitable insertion, deletion or substitution of one or more nucleotides in the ORF may introduce the premature in-frame stop codon.

[0406] In other embodiments, a mutation may introduce a frame shift (e.g., +1 or +2 frame shift) in the ORF encoding a gene product. Typically, such frame shift may lead to a previously out-of-frame stop codon downstream of the mutation becoming an in-frame stop codon. Hence, such frame shift may lead to production of a form of the polypeptide having an alternative C-terminal portion and/or a C-terminally truncated form of said polypeptide (this may preferably affect, such as diminish or abolish, some or all biological function(s) of the polypeptide) or, especially when the mutation is introduced close to (e.g., about 20 or less, or about 10 or less amino acids downstream of) the translation initiation codon of the ORF, the frame shift may effectively abolish the production of the polypeptide. Various ways of introducing a frame shift are apparent to a skilled person. For example but without limitation, a suitable insertion or deletion of one or more (not multiple of 3) nucleotides in the ORF may lead to a frame shift.

[0407] In further embodiments, a mutation may delete at least a portion of the ORF encoding a gene product. Such deletion may lead to production of an N-terminally truncated form, a C-terminally truncated form and/or an internally deleted form of said polypeptide (this may preferably affect, such as diminish or abolish, some or all biological function(s) of the polypeptide). Preferably, the deletion may remove about 20% or more, or about 50% or more of the ORF's nucleotides. Especially when the deletion removes a sizeable portion of the ORF (e.g., about 50% or more, preferably about 60% or more, more preferably about 70% or more, even more preferably about 80% or more, still more preferably about 90% or more of the ORF's nucleotides) or when the deletion removes the entire ORF, the deletion may effectively abolish the production of the polypeptide. The skilled person can readily introduce such deletions.

[0408] In further embodiments, a mutation may delete at least a portion of a gene promoter, leading to impaired transcription of the gene product.

[0409] In certain other embodiments, a mutation may be a substitution of one or more nucleotides in the ORF encoding a gene product resulting in substitution of one or more

amino acids of the polypeptide. Such mutation may typically preserve the production of the polypeptide, and may preferably affect, such as diminish or abolish, some or all biological function(s) of the polypeptide. The skilled person can readily introduce such substitutions.

[0410] In certain preferred embodiments, a mutation may abolish native splicing of a pre-mRNA encoding a gene product. In the absence of native splicing, the pre-mRNA may be degraded, or the pre-mRNA may be alternatively spliced, or the pre-mRNA may be spliced improperly employing latent splice site(s) if available. Hence, such mutation may typically effectively abolish the production of the polypeptide's mRNA and thus the production of the polypeptide. Various ways of interfering with proper splicing are available to a skilled person, such as for example but without limitation, mutations which alter the sequence of one or more sequence elements required for splicing to render them inoperable, or mutations which comprise or consist of a deletion of one or more sequence elements required for splicing. The terms "splicing", "splicing of a gene", "splicing of a pre-mRNA" and similar as used herein are synonymous and have their art-established meaning. By means of additional explanation, splicing denotes the process and means of removing intervening sequences (introns) from pre-mRNA in the process of producing mature mRNA. The reference to splicing particularly aims at native splicing such as occurs under normal physiological conditions. The terms "pre-mRNA" and "transcript" are used herein to denote RNA species that precede mature mRNA, such as in particular a primary RNA transcript and any partially processed forms thereof. Sequence elements required for splicing refer particularly to cis elements in the sequence of pre-mRNA which direct the cellular splicing machinery (spliceosome) towards correct and precise removal of introns from the pre-mRNA. Sequence elements involved in splicing are generally known per se and can be further determined by known techniques including inter alia mutation or deletion analysis. By means of further explanation, "splice donor site" or "5' splice site" generally refer to a conserved sequence immediately adjacent to an exon-intron boundary at the 5' end of an intron. Commonly, a splice donor site may contain a dinucleotide GU, and may involve a consensus sequence of about 8 bases at about positions +2 to -6. "Splice acceptor site" or "3' splice site" generally refers to a conserved sequence immediately adjacent to an intron-exon boundary at the 3' end of an intron. Commonly, a splice acceptor site may contain a dinucleotide AG, and may involve a consensus sequence of about 16 bases at about positions -14 to +2.

Genetic Modifying Agents

[0411] In certain embodiments, the one or more modulating agents may be a genetic modifying agent. The genetic modifying agent may comprise a CRISPR system, a zinc finger nuclease system, a TALEN, or a meganuclease.

[0412] In general, a CRISPR-Cas or CRISPR system as used in herein and in documents, such as WO 2014/093622 (PCT/US2013/074667), refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated ("Cas") genes, including sequences encoding a Cas gene, a tracr (trans-activating CRISPR) sequence (e.g. tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a "direct repeat" and a tracrRNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (also referred to as a "spacer" in the context of an endogenous CRISPR system), or "RNA(s)" as that term is herein used (e.g., RNA(s) to guide Cas, such as Cas9, e.g. CRISPR RNA and transactivating (tracr) RNA or a single guide RNA (sgRNA) (chimeric RNA)) or other sequences and transcripts from a CRISPR locus. In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence (also referred to as a protospacer in the context of an endogenous CRISPR system). See, e.g., Shmakov et al. (2015) "Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems", Molecular Cell, DOI: dx.doi.org/10.1016/j.molcel.2015. 10.008.

[0413] In certain embodiments, a protospacer adjacent motif (PAM) or PAM-like motif directs binding of the effector protein complex as disclosed herein to the target locus of interest. In some embodiments, the PAM may be a 5' PAM (i.e., located upstream of the 5' end of the protospacer). In other embodiments, the PAM may be a 3' PAM (i.e., located downstream of the 5' end of the protospacer). The term "PAM" may be used interchangeably with the term "PFS" or "protospacer flanking site" or "protospacer flanking sequence".

[0414] In a preferred embodiment, the CRISPR effector protein may recognize a 3' PAM. In certain embodiments, the CRISPR effector protein may recognize a 3' PAM which is 5'H, wherein H is A, C or U.

[0415] In the context of formation of a CRISPR complex, "target sequence" refers to a sequence to which a guide sequence is designed to have complementarity, where hybridization between a target sequence and a guide sequence promotes the formation of a CRISPR complex. A target sequence may comprise RNA polynucleotides. The term "target RNA" refers to a RNA polynucleotide being or comprising the target sequence. In other words, the target RNA may be a RNA polynucleotide or a part of a RNA polynucleotide to

which a part of the gRNA, i.e. the guide sequence, is designed to have complementarity and to which the effector function mediated by the complex comprising CRISPR effector protein and a gRNA is to be directed. In some embodiments, a target sequence is located in the nucleus or cytoplasm of a cell.

[0416] In certain example embodiments, the CRISPR effector protein may be delivered using a nucleic acid molecule encoding the CRISPR effector protein. The nucleic acid molecule encoding a CRISPR effector protein, may advantageously be a codon optimized CRISPR effector protein. An example of a codon optimized sequence, is in this instance a sequence optimized for expression in eukaryote, e.g., humans (i.e. being optimized for expression in humans), or for another eukaryote, animal or mammal as herein discussed; see, e.g., SaCas9 human codon optimized sequence in WO 2014/093622 (PCT/US2013/074667). Whilst this is preferred, it will be appreciated that other examples are possible and codon optimization for a host species other than human, or for codon optimization for specific organs is known. In some embodiments, an enzyme coding sequence encoding a CRISPR effector protein is a codon optimized for expression in particular cells, such as eukaryotic cells. The eukaryotic cells may be those of or derived from a particular organism, such as a plant or a mammal, including but not limited to human, or non-human eukaryote or animal or mammal as herein discussed, e.g., mouse, rat, rabbit, dog, livestock, or non-human mammal or primate. In some embodiments, processes for modifying the germ line genetic identity of human beings and/or processes for modifying the genetic identity of animals which are likely to cause them suffering without any substantial medical benefit to man or animal, and also animals resulting from such processes, may be excluded. In general, codon optimization refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cells of interest by replacing at least one codon (e.g. about or more than about 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more codons) of the native sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Various species exhibit particular bias for certain codons of a particular amino acid. Codon bias (differences in codon usage between organisms) often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, among other things, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given

organism based on codon optimization. Codon usage tables are readily available, for example, at the "Codon Usage Database" available at kazusa.or.jp/codon/ and these tables can be adapted in a number of ways. See Nakamura, Y., et al. "Codon usage tabulated from the international DNA sequence databases: status for the year 2000" Nucl. Acids Res. 28:292 (2000). Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell are also available, such as Gene Forge (Aptagen; Jacobus, PA), are also available. In some embodiments, one or more codons (e.g. 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more, or all codons) in a sequence encoding a Cas correspond to the most frequently used codon for a particular amino acid.

[0417] In certain embodiments, the methods as described herein may comprise providing a Cas transgenic cell in which one or more nucleic acids encoding one or more guide RNAs are provided or introduced operably connected in the cell with a regulatory element comprising a promoter of one or more gene of interest. As used herein, the term "Cas transgenic cell" refers to a cell, such as a eukaryotic cell, in which a Cas gene has been genomically integrated. The nature, type, or origin of the cell are not particularly limiting according to the present invention. Also the way the Cas transgene is introduced in the cell may vary and can be any method as is known in the art. In certain embodiments, the Cas transgenic cell is obtained by introducing the Cas transgene in an isolated cell. In certain other embodiments, the Cas transgenic cell is obtained by isolating cells from a Cas transgenic organism. By means of example, and without limitation, the Cas transgenic cell as referred to herein may be derived from a Cas transgenic eukaryote, such as a Cas knock-in eukaryote. Reference is made to WO 2014/093622 (PCT/US 13/74667), incorporated herein by reference. Methods of US Patent Publication Nos. 20120017290 and 20110265198 assigned to Sangamo Biosciences, Inc. directed to targeting the Rosa locus may be modified to utilize the CRISPR Cas system of the present invention. Methods of US Patent Publication No. 20130236946 assigned to Cellectis directed to targeting the Rosa locus may also be modified to utilize the CRISPR Cas system of the present invention. By means of further example reference is made to Piatt et. al. (Cell; 159(2):440-455 (2014)), describing a Cas9 knock-in mouse, which is incorporated herein by reference. The Cas transgene can further comprise a Lox-Stop-polyA-Lox(LSL) cassette thereby rendering Cas expression inducible by Cre recombinase. Alternatively, the Cas transgenic cell may be obtained by introducing the Cas transgene in an isolated cell. Delivery systems for transgenes are well known in the art. By means of example, the Cas transgene may be delivered in for instance eukaryotic cell

by means of vector (e.g., AAV, adenovirus, lentivirus) and/or particle and/or nanoparticle delivery, as also described herein elsewhere.

[0418] It will be understood by the skilled person that the cell, such as the Cas transgenic cell, as referred to herein may comprise further genomic alterations besides having an integrated Cas gene or the mutations arising from the sequence specific action of Cas when complexed with RNA capable of guiding Cas to a target locus.

[0419] In certain aspects the invention involves vectors, e.g. for delivering or introducing in a cell Cas and/or RNA capable of guiding Cas to a target locus (i.e. guide RNA), but also for propagating these components (e.g. in prokaryotic cells). As used herein, a "vector" is a tool that allows or facilitates the transfer of an entity from one environment to another. It is a replicon, such as a plasmid, phage, or cosmid, into which another DNA segment may be inserted so as to bring about the replication of the inserted segment. Generally, a vector is capable of replication when associated with the proper control elements. In general, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Vectors include, but are not limited to, nucleic acid molecules that are single-stranded, double-stranded, or partially double-stranded; nucleic acid molecules that comprise one or more free ends, no free ends (e.g. circular); nucleic acid molecules that comprise DNA, RNA, or both; and other varieties of polynucleotides known in the art. One type of vector is a "plasmid," which refers to a circular double stranded DNA loop into which additional DNA segments can be inserted, such as by standard molecular cloning techniques. Another type of vector is a viral vector, wherein virally-derived DNA or RNA sequences are present in the vector for packaging into a virus (e.g. retroviruses, replication defective retroviruses, adenoviruses, replication defective adenoviruses, and adeno-associated viruses (AAVs)). Viral vectors also include polynucleotides carried by a virus for transfection into a host cell. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g. bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively-linked. Such vectors are referred to herein as "expression vectors." Common expression vectors of utility in recombinant DNA techniques are often in the form of plasmids.

[0420] Recombinant expression vectors can comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory elements, which may be selected on the basis of the host cells to be used for expression, that is operatively-linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory element(s) in a manner that allows for expression of the nucleotide sequence (e.g. in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell). With regards to recombination and cloning methods, mention is made of U.S. patent application 10/815,730, published September 2, 2004 as US 2004-0171 156 A1, the contents of which are herein incorporated by reference in their entirety. Thus, the embodiments disclosed herein may also comprise transgenic cells comprising the CRISPR effector system. In certain example embodiments, the transgenic cell may function as an individual discrete volume. In other words samples comprising a masking construct may be delivered to a cell, for example in a suitable delivery vesicle and if the target is present in the delivery vesicle the CRISPR effector is activated and a detectable signal generated.

[0421] The vector(s) can include the regulatory element(s), e.g., promoter(s). The vector(s) can comprise Cas encoding sequences, and/or a single, but possibly also can comprise at least 3 or 8 or 16 or 32 or 48 or 50 guide RNA(s) (e.g., sgRNAs) encoding sequences, such as 1-2, 1-3, 1-4 1-5, 3-6, 3-7, 3-8, 3-9, 3-10, 3-8, 3-16, 3-30, 3-32, 3-48, 3-50 RNA(s) (e.g., sgRNAs). In a single vector there can be a promoter for each RNA (e.g., sgRNA), advantageously when there are up to about 16 RNA(s); and, when a single vector provides for more than 16 RNA(s), one or more promoter(s) can drive expression of more than one of the RNA(s), e.g., when there are 32 RNA(s), each promoter can drive expression of two RNA(s), and when there are 48 RNA(s), each promoter can drive expression of three RNA(s). By simple arithmetic and well established cloning protocols and the teachings in this disclosure one skilled in the art can readily practice the invention as to the RNA(s) for a suitable exemplary vector such as AAV, and a suitable promoter such as the U6 promoter. For example, the packaging limit of AAV is -4.7 kb. The length of a single U6-gRNA (plus restriction sites for cloning) is 361 bp. Therefore, the skilled person can readily fit about 12-16, e.g., 13 U6-gRNA cassettes in a single vector. This can be assembled by any suitable means, such as a golden gate strategy used for TALE assembly (genome-engineering.org/taleffectors/). The skilled person can also use a tandem guide strategy to

increase the number of U6-gRNAs by approximately 1.5 times, e.g., to increase from 12-16, e.g., 13 to approximately 18-24, e.g., about 19 U6-gRNAs. Therefore, one skilled in the art can readily reach approximately 18-24, e.g., about 19 promoter-RNAs, e.g., U6-gRNAs in a single vector, e.g., an AAV vector. A further means for increasing the number of promoters and RNAs in a vector is to use a single promoter (e.g., U6) to express an array of RNAs separated by cleavable sequences. And an even further means for increasing the number of promoter-RNAs in a vector, is to express an array of promoter-RNAs separated by cleavable sequences in the intron of a coding sequence or gene; and, in this instance it is advantageous to use a polymerase II promoter, which can have increased expression and enable the transcription of long RNA in a tissue specific manner. (see, e.g., nar.oxfordjournals.org/content/34/7/e53.short and nature.com/mt/journal/v16/n9/abs/mt2008144a.html). In an advantageous embodiment, AAV may package U6 tandem gRNA targeting up to about 50 genes. Accordingly, from the knowledge in the art and the teachings in this disclosure the skilled person can readily make and use vector(s), e.g., a single vector, expressing multiple RNAs or guides under the control or operatively or functionally linked to one or more promoters—especially as to the numbers of RNAs or guides discussed herein, without any undue experimentation.

[0422] The guide RNA(s) encoding sequences and/or Cas encoding sequences, can be functionally or operatively linked to regulatory element(s) and hence the regulatory element(s) drive expression. The promoter(s) can be constitutive promoter(s) and/or conditional promoter(s) and/or inducible promoter(s) and/or tissue specific promoter(s). The promoter can be selected from the group consisting of RNA polymerases, pol I, pol II, pol III, T7, U6, HI, retroviral Rous sarcoma virus (RSV) LTR promoter, the cytomegalovirus (CMV) promoter, the SV40 promoter, the dihydrofolate reductase promoter, the β-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EFla promoter. An advantageous promoter is the promoter is U6.

[0423] Additional effectors for use according to the invention can be identified by their proximity to casl genes, for example, though not limited to, within the region 20 kb from the start of the casl gene and 20 kb from the end of the casl gene. In certain embodiments, the effector protein comprises at least one HEPN domain and at least 500 amino acids, and wherein the C2c2 effector protein is naturally present in a prokaryotic genome within 20 kb upstream or downstream of a Cas gene or a CRISPR array. Non-limiting examples of Cas proteins include Casl, CaslB, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8, Cas9 (also known

as Csn1 and Csn12), CaslO, Csyl, Csyl2, Csyl3, Csel, Cse2, Cscl, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmrl, Cmr3, Cmr4, Cmr5, Cmr6, Csbl, Csb2, Csb3, Csxl7, Csxl4, CsxlO, Csxl6, CsaX, Csxl3, Csxl, Csxl5, Csfl, Csf2, Csf3, Csf4, homologues thereof, or modified versions thereof. In certain example embodiments, the C2c2 effector protein is naturally present in a prokaryotic genome within 20kb upstream or downstream of a Cas 1 gene. The terms "orthologue" (also referred to as "ortholog" herein) and "homologue" (also referred to as "homolog" herein) are well known in the art. By means of further guidance, a "homologue" of a protein as used herein is a protein of the same species which performs the same or a similar function as the protein it is a homologue of. Homologous proteins may but need not be structurally related, or are only partially structurally related. An "orthologue" of a protein as used herein is a protein of a different species which performs the same or a similar function as the protein it is an orthologue of. Orthologous proteins may but need not be structurally related, or are only partially structurally related.

[0424] In some embodiments, the Cas sequence is fused to one or more nuclear localization sequences (NLSs), such as about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs. In some embodiments, the Cas comprises about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs at or near the amino-terminus, about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs at or near the carboxy-terminus, or a combination of these (e.g. zero or at least one or more NLS at the amino-terminus and zero or at one or more NLS at the carboxy terminus). When more than one NLS is present, each may be selected independently of the others, such that a single NLS may be present in more than one copy and/or in combination with one or more other NLSs present in one or more copies. In a preferred embodiment of the invention, the Cas comprises at most 6 NLSs. In some embodiments, an NLS is considered near the N- or C-terminus when the nearest amino acid of the NLS is within about 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, or more amino acids along the polypeptide chain from the N- or C-terminus. Non-limiting examples of NLSs include an NLS sequence derived from: the NLS of the SV40 virus large T-antigen, having the amino acid sequence PKKKRKV(SEQ ID NO: 4); the NLS from nucleoplasm[^] (e.g. the nucleoplasm[^] bipartite NLS with the sequence KRPAATKKAGQAKKKK) (SEQ ID NO: 5); the c-myc NLS having the amino acid sequence PAAKRVKLD (SEQ ID NO: 6) or RQRRNELKRSP(SEQ ID NO: 7); the hRNPA1 M9 NLS having the sequence NQS SNFGPMKGGNFGGRS SGPYGGGGQYF AKPRNQGGY(SEQ ID NO: 8); the

sequence RMRIZFKNKGKDTAELRRRVEVSVELRKAKKDEQILKRRNV (SEQ ID NO: 9) of the IBB domain from importin-alpha; the sequences VSRKRPRP (SEQ ID NO: 10) and PPKKARED (SEQ ID NO: 11) of the myoma T protein; the sequence POPKKKPL (SEQ ID NO: 12) of human p53; the sequence SALIKKKKKMAP (SEQ ID NO: 13) of mouse c-abl IV; the sequences DRLRR (SEQ ID NO: 14) and PKQKKRK (SEQ ID NO: 15) of the influenza virus NS1; the sequence RKLKKKIKKL (SEQ ID NO: 16) of the Hepatitis virus delta antigen; the sequence REKKKFLKRR (SEQ ID NO: 17) of the mouse Mx1 protein; the sequence KRKGDEVGVDEVAKKKSKK (SEQ ID NO: 18) of the human poly(ADP-ribose) polymerase; and the sequence RKCLQAGMNLEARTKK (SEQ ID NO: 19) of the steroid hormone receptors (human) glucocorticoid. In general, the one or more NLSs are of sufficient strength to drive accumulation of the Cas in a detectable amount in the nucleus of a eukaryotic cell. In general, strength of nuclear localization activity may derive from the number of NLSs in the Cas, the particular NLS(s) used, or a combination of these factors. Detection of accumulation in the nucleus may be performed by any suitable technique. For example, a detectable marker may be fused to the Cas, such that location within a cell may be visualized, such as in combination with a means for detecting the location of the nucleus (e.g. a stain specific for the nucleus such as DAPI). Cell nuclei may also be isolated from cells, the contents of which may then be analyzed by any suitable process for detecting protein, such as immunohistochemistry, Western blot, or enzyme activity assay. Accumulation in the nucleus may also be determined indirectly, such as by an assay for the effect of CRISPR complex formation (e.g. assay for DNA cleavage or mutation at the target sequence, or assay for altered gene expression activity affected by CRISPR complex formation and/or Cas enzyme activity), as compared to a control no exposed to the Cas or complex, or exposed to a Cas lacking the one or more NLSs.

Guide Molecules

[0425] The methods described herein may be used to screen inhibition of CRISPR systems employing different types of guide molecules. As used herein, the term "guide sequence" and "guide molecule" in the context of a CRISPR-Cas system, comprises any polynucleotide sequence having sufficient complementarity with a target nucleic acid sequence to hybridize with the target nucleic acid sequence and direct sequence-specific binding of a nucleic acid-targeting complex to the target nucleic acid sequence. The guide sequences made using the methods disclosed herein may be a full-length guide sequence, a truncated guide sequence, a full-length sgRNA sequence, a truncated sgRNA sequence, or an

E+F sgRNA sequence. In some embodiments, the degree of complementarity of the guide sequence to a given target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. In certain example embodiments, the guide molecule comprises a guide sequence that may be designed to have at least one mismatch with the target sequence, such that a RNA duplex formed between the guide sequence and the target sequence. Accordingly, the degree of complementarity is preferably less than 99%. For instance, where the guide sequence consists of 24 nucleotides, the degree of complementarity is more particularly about 96% or less. In particular embodiments, the guide sequence is designed to have a stretch of two or more adjacent mismatching nucleotides, such that the degree of complementarity over the entire guide sequence is further reduced. For instance, where the guide sequence consists of 24 nucleotides, the degree of complementarity is more particularly about 96% or less, more particularly, about 92% or less, more particularly about 88% or less, more particularly about 84% or less, more particularly about 80% or less, more particularly about 76% or less, more particularly about 72% or less, depending on whether the stretch of two or more mismatching nucleotides encompasses 2, 3, 4, 5, 6 or 7 nucleotides, etc. In some embodiments, aside from the stretch of one or more mismatching nucleotides, the degree of complementarity, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. Optimal alignment may be determined with the use of any suitable algorithm for aligning sequences, non-limiting example of which include the Smith-Waterman algorithm, the Needleman-Wunsch algorithm, algorithms based on the Burrows-Wheeler Transform (e.g., the Burrows Wheeler Aligner), ClustalW, Clustal X, BLAT, Novoalign (Novocraft Technologies; available at www.novocraft.com), ELAND (Illumina, San Diego, CA), SOAP (available at soap.genomics.org.cn), and Maq (available at maq.sourceforge.net). The ability of a guide sequence (within a nucleic acid-targeting guide RNA) to direct sequence-specific binding of a nucleic acid -targeting complex to a target nucleic acid sequence may be assessed by any suitable assay. For example, the components of a nucleic acid-targeting CRISPR system sufficient to form a nucleic acid-targeting complex, including the guide sequence to be tested, may be provided to a host cell having the corresponding target nucleic acid sequence, such as by transfection with vectors encoding the components of the nucleic acid-targeting complex, followed by an assessment of preferential targeting (e.g., cleavage) within the target nucleic acid sequence, such as by Surveyor assay as described herein. Similarly, cleavage of a target

nucleic acid sequence (or a sequence in the vicinity thereof) may be evaluated in a test tube by providing the target nucleic acid sequence, components of a nucleic acid-targeting complex, including the guide sequence to be tested and a control guide sequence different from the test guide sequence, and comparing binding or rate of cleavage at or in the vicinity of the target sequence between the test and control guide sequence reactions. Other assays are possible, and will occur to those skilled in the art. A guide sequence, and hence a nucleic acid-targeting guide RNA may be selected to target any target nucleic acid sequence.

[0426] In certain embodiments, the guide sequence or spacer length of the guide molecules is from 15 to 50 nt. In certain embodiments, the spacer length of the guide RNA is at least 15 nucleotides. In certain embodiments, the spacer length is from 15 to 17 nt, e.g., 15, 16, or 17 nt, from 17 to 20 nt, e.g., 17, 18, 19, or 20 nt, from 20 to 24 nt, e.g., 20, 21, 22, 23, or 24 nt, from 23 to 25 nt, e.g., 23, 24, or 25 nt, from 24 to 27 nt, e.g., 24, 25, 26, or 27 nt, from 27-30 nt, e.g., 27, 28, 29, or 30 nt, from 30-35 nt, e.g., 30, 31, 32, 33, 34, or 35 nt, or 35 nt or longer. In certain example embodiment, the guide sequence is 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 nt.

[0427] In some embodiments, the guide sequence is an RNA sequence of between 10 to 50 nt in length, but more particularly of about 20-30 nt advantageously about 20 nt, 23-25 nt or 24 nt. The guide sequence is selected so as to ensure that it hybridizes to the target sequence. This is described more in detail below. Selection can encompass further steps which increase efficacy and specificity.

[0428] In some embodiments, the guide sequence has a canonical length (e.g., about 15-30 nt) is used to hybridize with the target RNA or DNA. In some embodiments, a guide molecule is longer than the canonical length (e.g., >30 nt) is used to hybridize with the target RNA or DNA, such that a region of the guide sequence hybridizes with a region of the RNA or DNA strand outside of the Cas-guide target complex. This can be of interest where additional modifications, such deamination of nucleotides is of interest. In alternative embodiments, it is of interest to maintain the limitation of the canonical guide sequence length.

[0429] In some embodiments, the sequence of the guide molecule (direct repeat and/or spacer) is selected to reduce the degree secondary structure within the guide molecule. In

some embodiments, about or less than about 75%, 50%, 40%, 30%, 25%, 20%, 15%, 10%, 5%, 1%, or fewer of the nucleotides of the nucleic acid-targeting guide RNA participate in self-complementary base pairing when optimally folded. Optimal folding may be determined by any suitable polynucleotide folding algorithm. Some programs are based on calculating the minimal Gibbs free energy. An example of one such algorithm is mFold, as described by Zuker and Stiegler (Nucleic Acids Res. 9 (1981), 133-148). Another example folding algorithm is the online webserver RNAfold, developed at Institute for Theoretical Chemistry at the University of Vienna, using the centroid structure prediction algorithm (see e.g., A.R. Gruber et al., 2008, Cell 106(1): 23-24; and PA Carr and GM Church, 2009, Nature Biotechnology 27(12): 1151-62).

[0430] In some embodiments, it is of interest to reduce the susceptibility of the guide molecule to RNA cleavage, such as to cleavage by Cas13. Accordingly, in particular embodiments, the guide molecule is adjusted to avoid cleavage by Cas13 or other RNA-cleaving enzymes.

[0431] In certain embodiments, the guide molecule comprises non-naturally occurring nucleic acids and/or non-naturally occurring nucleotides and/or nucleotide analogs, and/or chemically modifications. Preferably, these non-naturally occurring nucleic acids and non-naturally occurring nucleotides are located outside the guide sequence. Non-naturally occurring nucleic acids can include, for example, mixtures of naturally and non-naturally occurring nucleotides. Non-naturally occurring nucleotides and/or nucleotide analogs may be modified at the ribose, phosphate, and/or base moiety. In an embodiment of the invention, a guide nucleic acid comprises ribonucleotides and non-ribonucleotides. In one such embodiment, a guide comprises one or more ribonucleotides and one or more deoxyribonucleotides. In an embodiment of the invention, the guide comprises one or more non-naturally occurring nucleotide or nucleotide analog such as a nucleotide with phosphorothioate linkage, a locked nucleic acid (LNA) nucleotides comprising a methylene bridge between the 2' and 4' carbons of the ribose ring, or bridged nucleic acids (BNA). Other examples of modified nucleotides include 2'-**O**-methyl analogs, 2'-deoxy analogs, or 2'-fluoro analogs. Further examples of modified bases include, but are not limited to, 2-aminopurine, 5-bromo-uridine, pseudouridine, inosine, 7-methylguanosine. Examples of guide RNA chemical modifications include, without limitation, incorporation of 2' -O-methyl (M), 2' -O-methyl 3' phosphorothioate (MS), S-constrained ethyl(cEt), or 2' -O-

methyl 3' thioPACE (MSP) at one or more terminal nucleotides. Such chemically modified guides can comprise increased stability and increased activity as compared to unmodified guides, though on-target vs. off-target specificity is not predictable. (See, Hendel, 2015, *Nat Biotechnol.* 33(9):985-9, doi: 10.1038/nbt.3290, published online 29 June 2015 Ragdarm et al., 0215, *PNAS*, E71 10-E71 11; Allerson et al., *J. Med. Chem.* 2005, 48:901-904; Bramsen et al., *Front. Genet.*, 2012, 3:154; Deng et al., *PNAS*, 2015, 112:1 1870-1 1875; Sharma et al., *MedChemComm.*, 2014, 5:1454-1471; Hendel et al., *Nat. Biotechnol.* (2015) 33(9): 985-989; Li et al., *Nature Biomedical Engineering*, 2017, 1, 0066 DOI:10.1038/s41551-017-0066). In some embodiments, the 5' and/or 3' end of a guide RNA is modified by a variety of functional moieties including fluorescent dyes, polyethylene glycol, cholesterol, proteins, or detection tags. (See Kelly et al., 2016, *J. Biotech.* 233:74-83). In certain embodiments, a guide comprises ribonucleotides in a region that binds to a target RNA and one or more deoxyribonucleotides and/or nucleotide analogs in a region that binds to Cas13. In an embodiment of the invention, deoxyribonucleotides and/or nucleotide analogs are incorporated in engineered guide structures, such as, without limitation, stem-loop regions, and the seed region. For Cas13 guide, in certain embodiments, the modification is not in the 5'-handle of the stem-loop regions. Chemical modification in the 5'-handle of the stem-loop region of a guide may abolish its function (see Li, et al., *Nature Biomedical Engineering*, 2017, 1:0066). In certain embodiments, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, or 75 nucleotides of a guide is chemically modified. In some embodiments, 3-5 nucleotides at either the 3' or the 5' end of a guide is chemically modified. In some embodiments, only minor modifications are introduced in the seed region, such as 2'-F modifications. In some embodiments, 2'-F modification is introduced at the 3' end of a guide. In certain embodiments, three to five nucleotides at the 5' and/or the 3' end of the guide are chemically modified with 2'-0-methyl (M), 2'-0-methyl 3' phosphorothioate (MS), S-constrained ethyl(cEt), or 2'-0-methyl 3' thioPACE (MSP). Such modification can enhance genome editing efficiency (see Hendel et al., *Nat. Biotechnol.* (2015) 33(9): 985-989). In certain embodiments, all of the phosphodiester bonds of a guide are substituted with phosphorothioates (PS) for enhancing levels of gene disruption. In certain embodiments, more than five nucleotides at the 5' and/or the 3' end of the guide are chemically modified with 2'-0-Me, 2'-F or ^-constrained ethyl(cEt). Such chemically modified guide can mediate enhanced levels of gene disruption (see Ragdarm et al., 0215, *PNAS*, E71 10-E71 11). In an embodiment of the invention, a guide

is modified to comprise a chemical moiety at its 3' and/or 5' end. Such moieties include, but are not limited to amine, azide, alkyne, thio, dibenzocyclooctyne (DBCO), or Rhodamine. In certain embodiment, the chemical moiety is conjugated to the guide by a linker, such as an alkyl chain. In certain embodiments, the chemical moiety of the modified guide can be used to attach the guide to another molecule, such as DNA, RNA, protein, or nanoparticles. Such chemically modified guide can be used to identify or enrich cells genetically edited by a CRISPR system (see Lee et al., *eLife*, 2017, 6:e25312, DOI: 10.7554).

[0432] In some embodiments, the modification to the guide is a chemical modification, an insertion, a deletion or a split. In some embodiments, the chemical modification includes, but is not limited to, incorporation of 2'-**0**-methyl (M) analogs, 2'-deoxy analogs, 2-thiouridine analogs, N6-methyladenosine analogs, 2'-fluoro analogs, 2-aminopurine, 5-bromo-uridine, pseudouridine (Ψ), N1-methylpseudouridine ($\text{m}1\Psi$), 5-methoxyuridine(5moU), inosine, 7-methylguanosine, 2'-**0**-methyl 3'phosphorothioate (MS), S-constrained ethyl(cEt), phosphorothioate (PS), or 2'-**0**-methyl 3'thioPACE (MSP). In some embodiments, the guide comprises one or more of phosphorothioate modifications. In certain embodiments, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 25 nucleotides of the guide are chemically modified. In certain embodiments, one or more nucleotides in the seed region are chemically modified. In certain embodiments, one or more nucleotides in the 3'-terminus are chemically modified. In certain embodiments, none of the nucleotides in the 5'-handle is chemically modified. In some embodiments, the chemical modification in the seed region is a minor modification, such as incorporation of a 2'-fluoro analog. In a specific embodiment, one nucleotide of the seed region is replaced with a 2'-fluoro analog. In some embodiments, 5 to 10 nucleotides in the 3'-terminus are chemically modified. Such chemical modifications at the 3'-terminus of the Cas13 CrRNA may improve Cas13 activity. In a specific embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 nucleotides in the 3'-terminus are replaced with 2'-fluoro analogues. In a specific embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 nucleotides in the 3'-terminus are replaced with 2'- O-methyl (M) analogs.

[0433] In some embodiments, the loop of the 5'-handle of the guide is modified. In some embodiments, the loop of the 5'-handle of the guide is modified to have a deletion, an insertion, a split, or chemical modifications. In certain embodiments, the modified loop comprises 3, 4, or 5 nucleotides. In certain embodiments, the loop comprises the sequence of UCUU, UUUU, UAUU, or UGUU.

[0434] In some embodiments, the guide molecule forms a stemloop with a separate non-covalently linked sequence, which can be DNA or RNA. In particular embodiments, the sequences forming the guide are first synthesized using the standard phosphoramidite synthetic protocol (Herdewijn, P., ed., Methods in Molecular Biology Col 288, Oligonucleotide Synthesis: Methods and Applications, Humana Press, New Jersey (2012)). In some embodiments, these sequences can be functionalized to contain an appropriate functional group for ligation using the standard protocol known in the art (Hermanson, G. T., Bioconjugate Techniques, Academic Press (2013)). Examples of functional groups include, but are not limited to, hydroxyl, amine, carboxylic acid, carboxylic acid halide, carboxylic acid active ester, aldehyde, carbonyl, chlorocarbonyl, imidazolylcarbonyl, hydrozide, semicarbazide, thio semicarbazide, thiol, maleimide, haloalkyl, sulfonyl, ally, propargyl, diene, alkyne, and azide. Once this sequence is functionalized, a covalent chemical bond or linkage can be formed between this sequence and the direct repeat sequence. Examples of chemical bonds include, but are not limited to, those based on carbamates, ethers, esters, amides, imines, amidines, aminotrizines, hydrozone, disulfides, thioethers, thioesters, phosphorothioates, phosphorodithioates, sulfonamides, sulfonates, fulfones, sulfoxides, ureas, thioureas, hydrazide, oxime, triazole, photolabile linkages, C-C bond forming groups such as Diels-Alder cyclo-addition pairs or ring-closing metathesis pairs, and Michael reaction pairs.

[0435] In some embodiments, these stem-loop forming sequences can be chemically synthesized. In some embodiments, the chemical synthesis uses automated, solid-phase oligonucleotide synthesis machines with 2'-acetoxyethyl orthoester (2'-ACE) (Scaringe et al., J. Am. Chem. Soc. (1998) 120: 11820-1 1821; Scaringe, Methods Enzymol. (2000) 317: 3-18) or 2'-thionocarbamate (2'-TC) chemistry (Dellinger et al., J. Am. Chem. Soc. (2011) 133: 11540-1 1546; Hendel et al., Nat. Biotechnol. (2015) 33:985-989).

[0436] In certain embodiments, the guide molecule comprises (1) a guide sequence capable of hybridizing to a target locus and (2) a tracr mate or direct repeat sequence whereby the direct repeat sequence is located upstream (i.e., 5') from the guide sequence. In a particular embodiment the seed sequence (i.e. the sequence essential critical for recognition and/or hybridization to the sequence at the target locus) of the guide sequence is approximately within the first 10 nucleotides of the guide sequence.

[0437] In a particular embodiment the guide molecule comprises a guide sequence linked to a direct repeat sequence, wherein the direct repeat sequence comprises one or more stem loops or optimized secondary structures. In particular embodiments, the direct repeat has a

minimum length of 16 nts and a single stem loop. In further embodiments the direct repeat has a length longer than 16 nts, preferably more than 17 nts, and has more than one stem loops or optimized secondary structures. In particular embodiments the guide molecule comprises or consists of the guide sequence linked to all or part of the natural direct repeat sequence. A typical Type V or Type VI CRISPR-cas guide molecule comprises (in 3' to 5' direction or in 5' to 3' direction): a guide sequence a first complimentary stretch (the "repeat"), a loop (which is typically 4 or 5 nucleotides long), a second complimentary stretch (the "anti-repeat" being complimentary to the repeat), and a poly A (often poly U in RNA) tail (terminator). In certain embodiments, the direct repeat sequence retains its natural architecture and forms a single stem loop. In particular embodiments, certain aspects of the guide architecture can be modified, for example by addition, subtraction, or substitution of features, whereas certain other aspects of guide architecture are maintained. Preferred locations for engineered guide molecule modifications, including but not limited to insertions, deletions, and substitutions include guide termini and regions of the guide molecule that are exposed when complexed with the CRISPR-Cas protein and/or target, for example the stemloop of the direct repeat sequence.

[0438] In particular embodiments, the stem comprises at least about 4bp comprising complementary X and Y sequences, although stems of more, e.g., 5, 6, 7, 8, 9, 10, 11 or 12 or fewer, e.g., 3, 2, base pairs are also contemplated. Thus, for example X2-10 and Y2-10 (wherein X and Y represent any complementary set of nucleotides) may be contemplated. In one aspect, the stem made of the X and Y nucleotides, together with the loop will form a complete hairpin in the overall secondary structure; and, this may be advantageous and the amount of base pairs can be any amount that forms a complete hairpin. In one aspect, any complementary X:Y basepairing sequence (e.g., as to length) is tolerated, so long as the secondary structure of the entire guide molecule is preserved. In one aspect, the loop that connects the stem made of X:Y basepairs can be any sequence of the same length (e.g., 4 or 5 nucleotides) or longer that does not interrupt the overall secondary structure of the guide molecule. In one aspect, the stemloop can further comprise, e.g. an MS2 aptamer. In one aspect, the stem comprises about 5-7bp comprising complementary X and Y sequences, although stems of more or fewer basepairs are also contemplated. In one aspect, non-Watson Crick basepairing is contemplated, where such pairing otherwise generally preserves the architecture of the stemloop at that position.

[0439] In particular embodiments the natural hairpin or stemloop structure of the guide molecule is extended or replaced by an extended stemloop. It has been demonstrated that extension of the stem can enhance the assembly of the guide molecule with the CRISPR-Cas protein (Chen et al. Cell. (2013); 155(7): 1479-1491). In particular embodiments the stem of the stemloop is extended by at least 1, 2, 3, 4, 5 or more complementary basepairs (i.e. corresponding to the addition of 2,4, 6, 8, 10 or more nucleotides in the guide molecule). In particular embodiments these are located at the end of the stem, adjacent to the loop of the stemloop.

[0440] In particular embodiments, the susceptibility of the guide molecule to RNases or to decreased expression can be reduced by slight modifications of the sequence of the guide molecule which do not affect its function. For instance, in particular embodiments, premature termination of transcription, such as premature transcription of U6 Pol-III, can be removed by modifying a putative Pol-III terminator (4 consecutive U's) in the guide molecules sequence. Where such sequence modification is required in the stemloop of the guide molecule, it is preferably ensured by a basepair flip.

[0441] In a particular embodiment, the direct repeat may be modified to comprise one or more protein-binding RNA aptamers. In a particular embodiment, one or more aptamers may be included such as part of optimized secondary structure. Such aptamers may be capable of binding a bacteriophage coat protein as detailed further herein.

[0442] In some embodiments, the guide molecule forms a duplex with a target RNA comprising at least one target cytosine residue to be edited. Upon hybridization of the guide RNA molecule to the target RNA, the cytidine deaminase binds to the single strand RNA in the duplex made accessible by the mismatch in the guide sequence and catalyzes deamination of one or more target cytosine residues comprised within the stretch of mismatching nucleotides.

[0443] A guide sequence, and hence a nucleic acid-targeting guide RNA may be selected to target any target nucleic acid sequence. The target sequence may be mRNA.

[0444] In certain embodiments, the target sequence should be associated with a PAM (protospacer adjacent motif) or PFS (protospacer flanking sequence or site); that is, a short sequence recognized by the CRISPR complex. Depending on the nature of the CRISPR-Cas protein, the target sequence should be selected such that its complementary sequence in the DNA duplex (also referred to herein as the non-target sequence) is upstream or downstream of the PAM. In the embodiments of the present invention where the CRISPR-Cas protein is a

Cas13 protein, the complementary sequence of the target sequence is downstream or 3' of the PAM or upstream or 5' of the PAM. The precise sequence and length requirements for the PAM differ depending on the Cas13 protein used, but PAMs are typically 2-5 base pair sequences adjacent the protospacer (that is, the target sequence). Examples of the natural PAM sequences for different Cas13 orthologues are provided herein below and the skilled person will be able to identify further PAM sequences for use with a given Cas13 protein.

[0445] Further, engineering of the PAM Interacting (PI) domain may allow programming of PAM specificity, improve target site recognition fidelity, and increase the versatility of the CRISPR-Cas protein, for example as described for Cas9 in Kleinstiver BP et al. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature*. 2015 Jul 23;523(7561):481-5. doi: 10.1038/nature14592. As further detailed herein, the skilled person will understand that Cas13 proteins may be modified analogously.

[0446] In particular embodiment, the guide is an escorted guide. By "escorted" is meant that the CRISPR-Cas system or complex or guide is delivered to a selected time or place within a cell, so that activity of the CRISPR-Cas system or complex or guide is spatially or temporally controlled. For example, the activity and destination of the CRISPR-Cas system or complex or guide may be controlled by an escort RNA aptamer sequence that has binding affinity for an aptamer ligand, such as a cell surface protein or other localized cellular component. Alternatively, the escort aptamer may for example be responsive to an aptamer effector on or in the cell, such as a transient effector, such as an external energy source that is applied to the cell at a particular time.

[0447] The escorted CRISPR-Cas systems or complexes have a guide molecule with a functional structure designed to improve guide molecule structure, architecture, stability, genetic expression, or any combination thereof. Such a structure can include an aptamer.

[0448] Aptamers are biomolecules that can be designed or selected to bind tightly to other ligands, for example using a technique called systematic evolution of ligands by exponential enrichment (SELEX; Tuerk C, Gold L: "Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase." *Science* 1990, 249:505-510). Nucleic acid aptamers can for example be selected from pools of random-sequence oligonucleotides, with high binding affinities and specificities for a wide range of biomedically relevant targets, suggesting a wide range of therapeutic utilities for aptamers (Keefe, Anthony D., Supriya Pai, and Andrew Ellington. "Aptamers as therapeutics." *Nature Reviews Drug Discovery* 9.7 (2010): 537-550). These characteristics also suggest a wide

range of uses for aptamers as drug delivery vehicles (Levy-Nissenbaum, Etgar, et al. "Nanotechnology and aptamers: applications in drug delivery." Trends in biotechnology 26.8 (2008): 442-449; and, Hicke BJ, Stephens AW. "Escort aptamers: a delivery service for diagnosis and therapy." J Clin Invest 2000, 106:923-928.). Aptamers may also be constructed that function as molecular switches, responding to a cue by changing properties, such as RNA aptamers that bind fluorophores to mimic the activity of green fluorescent protein (Paige, Jeremy S., Karen Y. Wu, and Sarnie R. Jaffrey. "RNA mimics of green fluorescent protein." Science 333.6042 (2011): 642-646). It has also been suggested that aptamers may be used as components of targeted siRNA therapeutic delivery systems, for example targeting cell surface proteins (Zhou, Jiehua, and John J. Rossi. "Aptamer-targeted cell-specific RNA interference." Silence 1.1 (2010): 4).

[0449] Accordingly, in particular embodiments, the guide molecule is modified, e.g., by one or more aptamer(s) designed to improve guide molecule delivery, including delivery across the cellular membrane, to intracellular compartments, or into the nucleus. Such a structure can include, either in addition to the one or more aptamer(s) or without such one or more aptamer(s), moiety(ies) so as to render the guide molecule deliverable, inducible or responsive to a selected effector. The invention accordingly comprehends an guide molecule that responds to normal or pathological physiological conditions, including without limitation pH, hypoxia, O_2 concentration, temperature, protein concentration, enzymatic concentration, lipid structure, light exposure, mechanical disruption (e.g. ultrasound waves), magnetic fields, electric fields, or electromagnetic radiation.

[0450] Light responsiveness of an inducible system may be achieved via the activation and binding of cryptochrome-2 and CIB1. Blue light stimulation induces an activating conformational change in cryptochrome-2, resulting in recruitment of its binding partner CIB1. This binding is fast and reversible, achieving saturation in <15 sec following pulsed stimulation and returning to baseline <15 min after the end of stimulation. These rapid binding kinetics result in a system temporally bound only by the speed of transcription/translation and transcript/protein degradation, rather than uptake and clearance of inducing agents. Cryptochrome-2 activation is also highly sensitive, allowing for the use of low light intensity stimulation and mitigating the risks of phototoxicity. Further, in a context such as the intact mammalian brain, variable light intensity may be used to control the size of a stimulated region, allowing for greater precision than vector delivery alone may offer.

[0451] The invention contemplates energy sources such as electromagnetic radiation, sound energy or thermal energy to induce the guide. Advantageously, the electromagnetic radiation is a component of visible light. In a preferred embodiment, the light is a blue light with a wavelength of about 450 to about 495 nm. In an especially preferred embodiment, the wavelength is about 488 nm. In another preferred embodiment, the light stimulation is via pulses. The light power may range from about 0-9 mW/cm². In a preferred embodiment, a stimulation paradigm of as low as 0.25 sec every 15 sec should result in maximal activation.

[0452] The chemical or energy sensitive guide may undergo a conformational change upon induction by the binding of a chemical source or by the energy allowing it act as a guide and have the Casl3 CRISPR-Cas system or complex function. The invention can involve applying the chemical source or energy so as to have the guide function and the Casl3 CRISPR-Cas system or complex function; and optionally further determining that the expression of the genomic locus is altered.

[0453] There are several different designs of this chemical inducible system: 1. ABI-PYL based system inducible by Abscisic Acid (ABA) (see, e.g., stke.sciencemag.org/cgi/content/abstract/sigtrans;4/164/rs2), 2. FKBP-FRB based system inducible by rapamycin (or related chemicals based on rapamycin) (see, e.g., www.nature.com/nmeth/journal/v2/n6/full/nmeth763.html), 3. GID1-GAI based system inducible by Gibberellin (GA) (see, e.g., www.nature.com/ncembio/journal/v8/n5/full/ncembio.922.html).

[0454] A chemical inducible system can be an estrogen receptor (ER) based system inducible by 4-hydroxytamoxifen (40HT) (see, e.g., [www.pnas.org/content/104/3/1027. abstract](http://www.pnas.org/content/104/3/1027.abstract)). A mutated ligand-binding domain of the estrogen receptor called ERT2 translocates into the nucleus of cells upon binding of 4-hydroxytamoxifen. In further embodiments of the invention any naturally occurring or engineered derivative of any nuclear receptor, thyroid hormone receptor, retinoic acid receptor, estrogen receptor, estrogen-related receptor, glucocorticoid receptor, progesterone receptor, androgen receptor may be used in inducible systems analogous to the ER based inducible system.

[0455] Another inducible system is based on the design using Transient receptor potential (TRP) ion channel based system inducible by energy, heat or radio-wave (see, e.g., www.sciencemag.org/content/336/6081/604). These TRP family proteins respond to different stimuli, including light and heat. When this protein is activated by light or heat, the ion

channel will open and allow the entering of ions such as calcium into the plasma membrane. This influx of ions will bind to intracellular ion interacting partners linked to a polypeptide including the guide and the other components of the Cas13 CRISPR-Cas complex or system, and the binding will induce the change of sub-cellular localization of the polypeptide, leading to the entire polypeptide entering the nucleus of cells. Once inside the nucleus, the guide protein and the other components of the Cas13 CRISPR-Cas complex will be active and modulating target gene expression in cells.

[0456] While light activation may be an advantageous embodiment, sometimes it may be disadvantageous especially for *in vivo* applications in which the light may not penetrate the skin or other organs. In this instance, other methods of energy activation are contemplated, in particular, electric field energy and/or ultrasound which have a similar effect.

[0457] Electric field energy is preferably administered substantially as described in the art, using one or more electric pulses of from about 1 Volt/cm to about 10 kVolts/cm under *in vivo* conditions. Instead of or in addition to the pulses, the electric field may be delivered in a continuous manner. The electric pulse may be applied for between 1 μ s and 500 milliseconds, preferably between 1 μ s and 100 milliseconds. The electric field may be applied continuously or in a pulsed manner for 5 about minutes.

[0458] As used herein, 'electric field energy' is the electrical energy to which a cell is exposed. Preferably the electric field has a strength of from about 1 Volt/cm to about 10 kVolts/cm or more under *in vivo* conditions (see WO97/49450).

[0459] As used herein, the term "electric field" includes one or more pulses at variable capacitance and voltage and including exponential and/or square wave and/or modulated wave and/or modulated square wave forms. References to electric fields and electricity should be taken to include reference the presence of an electric potential difference in the environment of a cell. Such an environment may be set up by way of static electricity, alternating current (AC), direct current (DC), etc, as known in the art. The electric field may be uniform, non-uniform or otherwise, and may vary in strength and/or direction in a time dependent manner.

[0460] Single or multiple applications of electric field, as well as single or multiple applications of ultrasound are also possible, in any order and in any combination. The ultrasound and/or the electric field may be delivered as single or multiple continuous applications, or as pulses (pulsatile delivery).

[0461] Electroporation has been used in both *in vitro* and *in vivo* procedures to introduce foreign material into living cells. With *in vitro* applications, a sample of live cells is first mixed with the agent of interest and placed between electrodes such as parallel plates. Then, the electrodes apply an electrical field to the cell/implant mixture. Examples of systems that perform *in vitro* electroporation include the Electro Cell Manipulator ECM600 product, and the Electro Square Porator T820, both made by the BTX Division of Genetronics, Inc (see U.S. Pat. No 5,869,326).

[0462] The known electroporation techniques (both *in vitro* and *in vivo*) function by applying a brief high voltage pulse to electrodes positioned around the treatment region. The electric field generated between the electrodes causes the cell membranes to temporarily become porous, whereupon molecules of the agent of interest enter the cells. In known electroporation applications, this electric field comprises a single square wave pulse on the order of 1000 V/cm, of about 100 .mu.s duration. Such a pulse may be generated, for example, in known applications of the Electro Square Porator T820.

[0463] Preferably, the electric field has a strength of from about 1 V/cm to about 10 kV/cm under *in vitro* conditions. Thus, the electric field may have a strength of 1 V/cm, 2 V/cm, 3 V/cm, 4 V/cm, 5 V/cm, 6 V/cm, 7 V/cm, 8 V/cm, 9 V/cm, 10 V/cm, 20 V/cm, 50 V/cm, 100 V/cm, 200 V/cm, 300 V/cm, 400 V/cm, 500 V/cm, 600 V/cm, 700 V/cm, 800 V/cm, 900 V/cm, 1 kV/cm, 2 kV/cm, 5 kV/cm, 10 kV/cm, 20 kV/cm, 50 kV/cm or more. More preferably from about 0.5 kV/cm to about 4.0 kV/cm under *in vitro* conditions. Preferably the electric field has a strength of from about 1 V/cm to about 10 kV/cm under *in vivo* conditions. However, the electric field strengths may be lowered where the number of pulses delivered to the target site are increased. Thus, pulsatile delivery of electric fields at lower field strengths is envisaged.

[0464] Preferably the application of the electric field is in the form of multiple pulses such as double pulses of the same strength and capacitance or sequential pulses of varying strength and/or capacitance. As used herein, the term "pulse" includes one or more electric pulses at variable capacitance and voltage and including exponential and/or square wave and/or modulated wave/square wave forms.

[0465] Preferably the electric pulse is delivered as a waveform selected from an exponential wave form, a square wave form, a modulated wave form and a modulated square wave form.

[0466] A preferred embodiment employs direct current at low voltage. Thus, Applicants disclose the use of an electric field which is applied to the cell, tissue or tissue mass at a field strength of between 1V/cm and 20V/cm, for a period of 100 milliseconds or more, preferably 15 minutes or more.

[0467] Ultrasound is advantageously administered at a power level of from about 0.05 W/cm² to about 100 W/cm². Diagnostic or therapeutic ultrasound may be used, or combinations thereof.

[0468] As used herein, the term "ultrasound" refers to a form of energy which consists of mechanical vibrations the frequencies of which are so high they are above the range of human hearing. Lower frequency limit of the ultrasonic spectrum may generally be taken as about 20 kHz. Most diagnostic applications of ultrasound employ frequencies in the range 1 and 15 MHz' (From Ultrasonics in Clinical Diagnosis, P. N. T. Wells, ed., 2nd. Edition, Publ. Churchill Livingstone [Edinburgh, London & NY, 1977]).

[0469] Ultrasound has been used in both diagnostic and therapeutic applications. When used as a diagnostic tool ("diagnostic ultrasound"), ultrasound is typically used in an energy density range of up to about 100 mW/cm² (FDA recommendation), although energy densities of up to 750 mW/cm² have been used. In physiotherapy, ultrasound is typically used as an energy source in a range up to about 3 to 4 W/cm² (WHO recommendation). In other therapeutic applications, higher intensities of ultrasound may be employed, for example, HIFU at 100 W/cm up to 1 kW/cm² (or even higher) for short periods of time. The term "ultrasound" as used in this specification is intended to encompass diagnostic, therapeutic and focused ultrasound.

[0470] Focused ultrasound (FUS) allows thermal energy to be delivered without an invasive probe (see Morocz et al 1998 Journal of Magnetic Resonance Imaging Vol.8, No. 1, pp. 136-142. Another form of focused ultrasound is high intensity focused ultrasound (HIFU) which is reviewed by Moussatov et al in Ultrasonics (1998) Vol.36, No. 8, pp.893-900 and TranHuuHue et al in Acustica (1997) Vol.83, No.6, pp. 1103-1 106.

[0471] Preferably, a combination of diagnostic ultrasound and a therapeutic ultrasound is employed. This combination is not intended to be limiting, however, and the skilled reader will appreciate that any variety of combinations of ultrasound may be used. Additionally, the energy density, frequency of ultrasound, and period of exposure may be varied.

[0472] Preferably the exposure to an ultrasound energy source is at a power density of from about 0.05 to about 100 Wcm-2. Even more preferably, the exposure to an ultrasound energy source is at a power density of from about 1 to about 15 Wcm-2.

[0473] Preferably the exposure to an ultrasound energy source is at a frequency of from about 0.015 to about 10.0 MHz. More preferably the exposure to an ultrasound energy source is at a frequency of from about 0.02 to about 5.0 MHz or about 6.0 MHz. Most preferably, the ultrasound is applied at a frequency of 3 MHz.

[0474] Preferably the exposure is for periods of from about 10 milliseconds to about 60 minutes. Preferably the exposure is for periods of from about 1 second to about 5 minutes. More preferably, the ultrasound is applied for about 2 minutes. Depending on the particular target cell to be disrupted, however, the exposure may be for a longer duration, for example, for 15 minutes.

[0475] Advantageously, the target tissue is exposed to an ultrasound energy source at an acoustic power density of from about 0.05 Wcm-2 to about 10 Wcm-2 with a frequency ranging from about 0.015 to about 10 MHz (see WO 98/52609). However, alternatives are also possible, for example, exposure to an ultrasound energy source at an acoustic power density of above 100 Wcm-2, but for reduced periods of time, for example, 1000 Wcm-2 for periods in the millisecond range or less.

[0476] Preferably the application of the ultrasound is in the form of multiple pulses; thus, both continuous wave and pulsed wave (pulsatile delivery of ultrasound) may be employed in any combination. For example, continuous wave ultrasound may be applied, followed by pulsed wave ultrasound, or vice versa. This may be repeated any number of times, in any order and combination. The pulsed wave ultrasound may be applied against a background of continuous wave ultrasound, and any number of pulses may be used in any number of groups.

[0477] Preferably, the ultrasound may comprise pulsed wave ultrasound. In a highly preferred embodiment, the ultrasound is applied at a power density of 0.7 Wcm-2 or 1.25 Wcm-2 as a continuous wave. Higher power densities may be employed if pulsed wave ultrasound is used.

[0478] Use of ultrasound is advantageous as, like light, it may be focused accurately on a target. Moreover, ultrasound is advantageous as it may be focused more deeply into tissues unlike light. It is therefore better suited to whole-tissue penetration (such as but not limited to a lobe of the liver) or whole organ (such as but not limited to the entire liver or an entire muscle, such as the heart) therapy. Another important advantage is that ultrasound is a non-

invasive stimulus which is used in a wide variety of diagnostic and therapeutic applications. By way of example, ultrasound is well known in medical imaging techniques and, additionally, in orthopedic therapy. Furthermore, instruments suitable for the application of ultrasound to a subject vertebrate are widely available and their use is well known in the art.

[0479] In particular embodiments, the guide molecule is modified by a secondary structure to increase the specificity of the CRISPR-Cas system and the secondary structure can protect against exonuclease activity and allow for 5' additions to the guide sequence also referred to herein as a protected guide molecule.

[0480] In one aspect, the invention provides for hybridizing a "protector RNA" to a sequence of the guide molecule, wherein the "protector RNA" is an RNA strand complementary to the 3' end of the guide molecule to thereby generate a partially double-stranded guide RNA. In an embodiment of the invention, protecting mismatched bases (i.e. the bases of the guide molecule which do not form part of the guide sequence) with a perfectly complementary protector sequence decreases the likelihood of target RNA binding to the mismatched basepairs at the 3' end. In particular embodiments of the invention, additional sequences comprising an extented length may also be present within the guide molecule such that the guide comprises a protector sequence within the guide molecule. This "protector sequence" ensures that the guide molecule comprises a "protected sequence" in addition to an "exposed sequence" (comprising the part of the guide sequence hybridizing to the target sequence). In particular embodiments, the guide molecule is modified by the presence of the protector guide to comprise a secondary structure such as a hairpin. Advantageously there are three or four to thirty or more, e.g., about 10 or more, contiguous base pairs having complementarity to the protected sequence, the guide sequence or both. It is advantageous that the protected portion does not impede thermodynamics of the CRISPR-Cas system interacting with its target. By providing such an extension including a partially double stranded guide molecule, the guide molecule is considered protected and results in improved specific binding of the CRISPR-Cas complex, while maintaining specific activity.

[0481] In particular embodiments, use is made of a truncated guide (tru-guide), i.e. a guide molecule which comprises a guide sequence which is truncated in length with respect to the canonical guide sequence length. As described by Nowak et al. (Nucleic Acids Res (2016) 44 (20): 9555-9564), such guides may allow catalytically active CRISPR-Cas enzyme to bind its target without cleaving the target RNA. In particular embodiments, a truncated

guide is used which allows the binding of the target but retains only nickase activity of the CRISPR-Cas enzyme.

CRISPR RNA-Targeting Effector Proteins

[0482] In one example embodiment, the CRISPR system effector protein is an RNA-targeting effector protein. In certain embodiments, the CRISPR system effector protein is a Type VI CRISPR system targeting RNA (e.g., Casl3a, Casl3b, Casl3c or Casl3d). Example RNA-targeting effector proteins include Casl3b and C2c2 (now known as Casl3a). It will be understood that the term "C2c2" herein is used interchangeably with "Casl3a". "C2c2" is now referred to as "Casl3a", and the terms are used interchangeably herein unless indicated otherwise. As used herein, the term "Casl3" refers to any Type VI CRISPR system targeting RNA (e.g., Casl3a, Casl3b, Casl3c or Casl3d). When the CRISPR protein is a C2c2 protein, a tracrRNA is not required. C2c2 has been described in Abudayyeh et al. (2016) "C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector"; Science; DOI: 10.1126/science.aaf5573; and Shmakov et al. (2015) "Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems", Molecular Cell, DOI: dx.doi.org/10.1016/j.molcel.2015.10.008; which are incorporated herein in their entirety by reference. Casl3b has been described in Smargon et al. (2017) "Casl3b Is a Type VI-B CRISPR-Associated RNA-Guided RNases Differentially Regulated by Accessory Proteins Csx27 and Csx28," Molecular Cell. 65, 1-13; dx.doi.org/10.1016/j.molcel.2016.12.023., which is incorporated herein in its entirety by reference.

[0483] In some embodiments, one or more elements of a nucleic acid-targeting system is derived from a particular organism comprising an endogenous CRISPR RNA-targeting system. In certain example embodiments, the effector protein CRISPR RNA-targeting system comprises at least one HEPN domain, including but not limited to the HEPN domains described herein, HEPN domains known in the art, and domains recognized to be HEPN domains by comparison to consensus sequence motifs. Several such domains are provided herein. In one non-limiting example, a consensus sequence can be derived from the sequences of C2c2 or Casl3b orthologs provided herein. In certain example embodiments, the effector protein comprises a single HEPN domain. In certain other example embodiments, the effector protein comprises two HEPN domains.

[0484] In one example embodiment, the effector protein comprise one or more HEPN domains comprising a RxxxxH motif sequence. The RxxxxH motif sequence can be, without limitation, from a HEPN domain described herein or a HEPN domain known in the art.

RxxxxH motif sequences further include motif sequences created by combining portions of two or more HEPN domains. As noted, consensus sequences can be derived from the sequences of the orthologs disclosed in U.S. Provisional Patent Application 62/432,240 entitled "Novel CRISPR Enzymes and Systems," U.S. Provisional Patent Application 62/471,710 entitled "Novel Type VI CRISPR Orthologs and Systems" filed on March 15, 2017, and U.S. Provisional Patent Application entitled "Novel Type VI CRISPR Orthologs and Systems," labeled as attorney docket number 47627-05-2133 and filed on April 12, 2017.

[0485] In certain other example embodiments, the CRISPR system effector protein is a C2c2 nuclease. The activity of C2c2 may depend on the presence of two HEPN domains. These have been shown to be RNase domains, *i.e.* nuclease (in particular an endonuclease) cutting RNA. C2c2 HEPN may also target DNA, or potentially DNA and/or RNA. On the basis that the HEPN domains of C2c2 are at least capable of binding to and, in their wild-type form, cutting RNA, then it is preferred that the C2c2 effector protein has RNase function. Regarding C2c2 CRISPR systems, reference is made to U.S. Provisional 62/351,662 filed on June 17, 2016 and U.S. Provisional 62/376,377 filed on August 17, 2016. Reference is also made to U.S. Provisional 62/351,803 filed on June 17, 2016. Reference is also made to U.S. Provisional entitled "Novel Crispr Enzymes and Systems" filed December 8, 2016 bearing Broad Institute No. 10035.PA4 and Attorney Docket No. 47627.03.2133. Reference is further made to East-Seletsky *et al.* "Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection" Nature doi: 10.1038/nature19802 and Abudayyeh *et al.* "C2c2 is a single-component programmable RNA-guided RNA targeting CRISPR effector" bioRxiv doi: 10.1101/054742.

[0486] In certain embodiments, the C2c2 effector protein is from an organism of a genus selected from the group consisting of: Leptotrichia, Listeria, Corynebacter, Sutterella, Legionella, Treponema, Filifactor, Eubacterium, Streptococcus, Lactobacillus, Mycoplasma, Bacteroides, Flaviivola, Flavobacterium, Sphaerochaeta, Azospirillum, Gluconacetobacter, Neisseria, Roseburia, Parvibaculum, Staphylococcus, Nitratifractor, Mycoplasma, Campylobacter, and Lachnospira, or the C2c2 effector protein is an organism selected from the group consisting of: Leptotrichia shahii, Leptotrichia wadei, Listeria seeligeri, Clostridium aminophilum, Carnobacterium gallinarum, Paludibacter propionicigenes, Listeria weihenstephanensis, or the C2c2 effector protein is a L. wadei F0279 or L. wadei F0279 (Lw2) C2C2 effector protein. In another embodiment, the one or more guide RNAs are

designed to detect a single nucleotide polymorphism, splice variant of a transcript, or a frameshift mutation in a target RNA or DNA.

[0487] In certain example embodiments, the RNA-targeting effector protein is a Type VI-B effector protein, such as Casl3b and Group 29 or Group 30 proteins. In certain example embodiments, the RNA-targeting effector protein comprises one or more HEPN domains. In certain example embodiments, the RNA-targeting effector protein comprises a C-terminal HEPN domain, a N-terminal HEPN domain, or both. Regarding example Type VI-B effector proteins that may be used in the context of this invention, reference is made to US Application No. 15/331,792 entitled "Novel CRISPR Enzymes and Systems" and filed October 21, 2016, International Patent Application No. PCT/US2016/058302 entitled "Novel CRISPR Enzymes and Systems", and filed October 21, 2016, and Smargon *et al.* "Casl3b is a Type VI-B CRISPR-associated RNA-Guided RNase differentially regulated by accessory proteins Csx27 and Csx28" Molecular Cell, 65, 1-13 (2017); dx.doi.org/10.1016/j.molcel.2016.12.023, and U.S. Provisional Application No. to be assigned, entitled "Novel Casl3b Orthologues CRISPR Enzymes and System" filed March 15, 2017. In particular embodiments, the Casl3b enzyme is derived from *Bergeyella zoohelcum*.

[0488] In certain example embodiments, the RNA-targeting effector protein is a Casl3c effector protein as disclosed in U.S. Provisional Patent Application No. 62/525,165 filed June 26, 2017, and PCT Application No. US 2017/047193 filed August 16, 2017.

[0489] In some embodiments, one or more elements of a nucleic acid-targeting system is derived from a particular organism comprising an endogenous CRISPR RNA-targeting system. In certain embodiments, the CRISPR RNA-targeting system is found in *Eubacterium* and *Ruminococcus*. In certain embodiments, the effector protein comprises targeted and collateral ssRNA cleavage activity. In certain embodiments, the effector protein comprises dual HEPN domains. In certain embodiments, the effector protein lacks a counterpart to the Helical-1 domain of Casl3a. In certain embodiments, the effector protein is smaller than previously characterized class 2 CRISPR effectors, with a median size of 928 aa. This median size is 190 aa (17%) less than that of Casl3c, more than 200 aa (18%) less than that of Casl3b, and more than 300 aa (26%) less than that of Casl3a. In certain embodiments, the effector protein has no requirement for a flanking sequence (e.g., PFS, PAM).

[0490] In certain embodiments, the effector protein locus structures include a WYL domain containing accessory protein (so denoted after three amino acids that were conserved

in the originally identified group of these domains; see, e.g., WYL domain IPR026881). In certain embodiments, the WYL domain accessory protein comprises at least one helix-turn-helix (HTH) or ribbon-helix-helix (RHH) DNA-binding domain. In certain embodiments, the WYL domain containing accessory protein increases both the targeted and the collateral ssRNA cleavage activity of the RNA-targeting effector protein. In certain embodiments, the WYL domain containing accessory protein comprises an N-terminal RHH domain, as well as a pattern of primarily hydrophobic conserved residues, including an invariant tyrosine-leucine doublet corresponding to the original WYL motif. In certain embodiments, the WYL domain containing accessory protein is WYL1. WYL1 is a single WYL-domain protein associated primarily with *Ruminococcus*.

[0491] In other example embodiments, the Type VI RNA-targeting Cas enzyme is Casl3d. In certain embodiments, Casl3d is *Eubacterium siraeum* DSM 15702 (EsCasl3d) or *Ruminococcus* sp. N15.MGS-57 (RspCasl3d) (see, e.g., Yan et al., Casl3d Is a Compact RNA-Targeting Type VI CRISPR Effector Positively Modulated by a WYL-Domain-Containing Accessory Protein, Molecular Cell (2018), doi.org/10.1016/j.molcel.2018.02.028). RspCasl3d and EsCasl3d have no flanking sequence requirements (e.g., PFS, PAM).

[00100] Casl3 RNA Editing

[0492] In one aspect, the invention provides a method of modifying or editing a target transcript in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR-Cas effector module complex to bind to the target polynucleotide to effect RNA base editing, wherein the CRISPR-Cas effector module complex comprises a Cas effector module complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a direct repeat sequence. In some embodiments, the Cas effector module comprises a catalytically inactive CRISPR-Cas protein. In some embodiments, the guide sequence is designed to introduce one or more mismatches to the RNA/RNA duplex formed between the target sequence and the guide sequence. In particular embodiments, the mismatch is an A-C mismatch. In some embodiments, the Cas effector may associate with one or more functional domains (e.g. via fusion protein or suitable linkers). In some embodiments, the effector domain comprises one or more cytidine or adenosine deaminases that mediate endogenous editing of via hydrolytic deamination. In particular embodiments, the effector domain comprises the adenosine deaminase acting on RNA (ADAR) family of enzymes. In particular embodiments, the

adenosine deaminase protein or catalytic domain thereof capable of deaminating adenosine or cytidine in RNA or is an RNA specific adenosine deaminase and/or is a bacterial, human, cephalopod, or Drosophila adenosine deaminase protein or catalytic domain thereof, preferably TadA, more preferably ADAR, optionally huADAR, optionally (hu)ADAR1 or (hu)ADAR2, preferably huADAR2 or catalytic domain thereof.

[0493] The present application relates to modifying a target RNA sequence of interest (see, e.g., Cox et al., *Science*. 2017 Nov 24;358(6366): 1019-1027). Using RNA-targeting rather than DNA targeting offers several advantages relevant for therapeutic development. First, there are substantial safety benefits to targeting RNA: there will be fewer off-target events because the available sequence space in the transcriptome is significantly smaller than the genome, and if an off-target event does occur, it will be transient and less likely to induce negative side effects. Second, RNA-targeting therapeutics will be more efficient because they are cell-type independent and not have to enter the nucleus, making them easier to deliver.

[0494] A further aspect of the invention relates to the method and composition as envisaged herein for use in prophylactic or therapeutic treatment, preferably wherein said target locus of interest is within a human or animal and to methods of modifying an Adenine or Cytidine in a target RNA sequence of interest, comprising delivering to said target RNA, the composition as described herein. In particular embodiments, the CRISPR system and the adenonsine deaminase, or catalytic domain thereof, are delivered as one or more polynucleotide molecules, as a ribonucleoprotein complex, optionally via particles, vesicles, or one or more viral vectors. In particular embodiments, the invention thus comprises compositions for use in therapy. This implies that the methods can be performed in vivo, ex vivo or in vitro. In particular embodiments, when the target is a human or animal target, the method is carried out ex vivo or in vitro.

[0495] A further aspect of the invention relates to the method as envisaged herein for use in prophylactic or therapeutic treatment, preferably wherein said target of interest is within a human or animal and to methods of modifying an Adenine or Cytidine in a target RNA sequence of interest, comprising delivering to said target RNA, the composition as described herein. In particular embodiments, the CRISPR system and the adenonsine deaminase, or catalytic domain thereof, are delivered as one or more polynucleotide molecules, as a ribonucleoprotein complex, optionally via particles, vesicles, or one or more viral vectors.

[0496] In one aspect, the invention provides a method of generating a eukaryotic cell comprising a modified or edited gene. In some embodiments, the method comprises (a)

introducing one or more vectors into a eukaryotic cell, wherein the one or more vectors drive expression of one or more of: Cas effector module, and a guide sequence linked to a direct repeat sequence, wherein the Cas effector module associate one or more effector domains that mediate base editing, and (b) allowing a CRISPR-Cas effector module complex to bind to a target polynucleotide to effect base editing of the target polynucleotide within said disease gene, wherein the CRISPR-Cas effector module complex comprises a Cas effector module complexed with the guide sequence that is hybridized to the target sequence within the target polynucleotide, wherein the guide sequence may be designed to introduce one or more mismatches between the RNA/RNA duplex formed between the guide sequence and the target sequence. In particular embodiments, the mismatch is an A-C mismatch. In some embodiments, the Cas effector may associate with one or more functional domains (e.g. via fusion protein or suitable linkers). In some embodiments, the effector domain comprises one or more cytidine or adenosine deaminases that mediate endogenous editing of via hydrolytic deamination. In particular embodiments, the effector domain comprises the adenosine deaminase acting on RNA (ADAR) family of enzymes. In particular embodiments, the adenosine deaminase protein or catalytic domain thereof capable of deaminating adenosine or cytidine in RNA or is an RNA specific adenosine deaminase and/or is a bacterial, human, cephalopod, or Drosophila adenosine deaminase protein or catalytic domain thereof, preferably TadA, more preferably ADAR, optionally huADAR, optionally (hu)ADAR1 or (hu)ADAR2, preferably huADAR2 or catalytic domain thereof.

[0497] A further aspect relates to an isolated cell obtained or obtainable from the methods described herein comprising the composition described herein or progeny of said modified cell, preferably wherein said cell comprises a hypoxanthine or a guanine in replace of said Adenine in said target RNA of interest compared to a corresponding cell not subjected to the method. In particular embodiments, the cell is a eukaryotic cell, preferably a human or non-human animal cell, optionally a therapeutic T cell or an antibody-producing B-cell.

[0498] In some embodiments, the modified cell is a therapeutic T cell, such as a T cell suitable for adoptive cell transfer therapies (e.g., CAR-T therapies). The modification may result in one or more desirable traits in the therapeutic T cell, as described further herein.

[0499] The invention further relates to a method for cell therapy, comprising administering to a patient in need thereof the modified cell described herein, wherein the presence of the modified cell remedies a disease in the patient.

[0500] The present invention may be further illustrated and extended based on aspects of CRISPR-Cas development and use as set forth in the following articles and particularly as relates to delivery of a CRISPR protein complex and uses of an RNA guided endonuclease in cells and organisms:

- Multiplex genome engineering using CRISPR-Cas systems. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., & Zhang, F. *Science* Feb 15;339(6121):819-23 (2013);
- RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Jiang W., Bikard D., Cox D., Zhang F, Marraffini LA. *Nat Biotechnol* Mar;31(3):233-9 (2013);
- One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR-Cas-Mediated Genome Engineering. Wang H., Yang H., Shivalila CS., Dawlaty MM., Cheng AW., Zhang F., Jaenisch R. *Cell* May 9;153(4):910-8 (2013);
- Optical control of mammalian endogenous transcription and epigenetic states. Konermann S, Brigham MD, Trevino AE, Hsu PD, Heidenreich M, Cong L, Piatt RJ, Scott DA, Church GM, Zhang F. *Nature*. Aug 22;500(7463):472-6. doi: 10.1038/Naturel2466. Epub 2013 Aug 23 (2013);
- > Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity. Ran, FA., Hsu, PD., Lin, CY., Gootenberg, JS., Konermann, S., Trevino, AE., Scott, DA., Inoue, A., Matoba, S., Zhang, Y., & Zhang, F. *Cell* Aug 28. pii: S0092-8674(13)01015-5 (2013-A);
- DNA targeting specificity of RNA-guided Cas9 nucleases. Hsu, P., Scott, D., Weinstein, J., Ran, FA., Konermann, S., Agarwala, V., Li, Y., Fine, E., Wu, X., Shalem, O., Cradick, TJ., Marraffini, LA., Bao, G., & Zhang, F. *Nat Biotechnol* doi:10.1038/nbt.2647 (2013);
- Genome engineering using the CRISPR-Cas9 system. Ran, FA., Hsu, PD., Wright, J., Agarwala, V., Scott, DA., Zhang, F. *Nature Protocols* Nov;8(1 1):2281-308 (2013-B);
- Genome-Scale CRISPR-Cas9 Knockout Screening in Human Cells. Shalem, O., Sanjana, NE., Hartenian, E., Shi, X., Scott, DA., Mikkelsen, T., Heckl, D., Ebert, BL., Root, DE., Doench, JG, Zhang, F. *Science* Dec 12. (2013);
- Crystal structure of cas9 in complex with guide RNA and target DNA. Nishimasu, H., Ran, FA., Hsu, PD., Konermann, S., Shehata, SI., Dohmae, N., Ishitani, R., Zhang, F., Nureki, O. *Cell* Feb 27, 156(5):935-49 (2014);

- Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. Wu X., Scott DA., Kriz AJ., Chiu AC, Hsu PD., Dadon DB., Cheng AW., Trevino AE., Konermann S., Chen S., Jaenisch R., Zhang F., Sharp PA. *Nat Biotechnol.* Apr 20. doi: 10.1038/nbt.2889 (2014);
- > CRISPR-Cas9 Knockin Mice for Genome Editing and Cancer Modeling. Piatt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, Kempton HR, Dahlman JE, Parnas O, Eisenhaure TM, Jovanovic M, Graham DB, Jhunjhunwala S, Heidenreich M, Xavier RJ, Langer R, Anderson DG, Hacohen N, Regev A, Feng G, Sharp PA, Zhang F. *Cell* 159(2): 440-455 DOI: 10.1016/j.cell.2014.09.014(2014);
- Development and Applications of CRISPR-Cas9 for Genome Engineering, Hsu PD, Lander ES, Zhang F., *Cell*. Jun 5;157(6):1262-78 (2014).
- Genetic screens in human cells using the CRISPR-Cas9 system, Wang T, Wei JJ, Sabatini DM, Lander ES., *Science*. January 3; 343(6166): 80-84. doi:10.1126/science.1246981 (2014);
- Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation, Doench JG, Hartenian E, Graham DB, Tothova Z, Hegde M, Smith I, Sullender M, Ebert BL, Xavier RJ, Root DE., (published online 3 September 2014) *Nat Biotechnol.* Dec;32(12): 1262-7 (2014);
- *In vivo* interrogation of gene function in the mammalian brain using CRISPR-Cas9, Swiech L, Heidenreich M, Banerjee A, Habib N, Li Y, Trombetta J, Sur M, Zhang F., (published online 19 October 2014) *Nat Biotechnol.* Jan;33(1): 102-6 (2015);
- Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex, Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H, Nureki O, Zhang F., *Nature*. Jan 29;517(7536):583-8 (2015).
- A split-Cas9 architecture for inducible genome editing and transcription modulation, Zetsche B, Volz SE, Zhang F., (published online 02 February 2015) *Nat Biotechnol.* Feb;33(2): 139-42 (2015);
- Genome-wide CRISPR Screen in a Mouse Model of Tumor Growth and Metastasis, Chen S, Sanjana NE, Zheng K, Shalem O, Lee K, Shi X, Scott DA, Song J, Pan JQ, Weissleder R, Lee H, Zhang F, Sharp PA. *Cell* 160, 1246-1260, March 12, 2015 (multiplex screen in mouse), and

- In vivo genome editing using *Staphylococcus aureus* Cas9, Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, Zhang F., (published online 01 April 2015), Nature. Apr 9;520(7546): 186-91 (2015).
- Shalem et al., "High-throughput functional genomics using CRISPR-Cas9," Nature Reviews Genetics 16, 299-311 (May 2015).
- Xu et al., "Sequence determinants of improved CRISPR sgRNA design," Genome Research 25, 1147-1157 (August 2015).
- Parnas et al., "A Genome-wide CRISPR Screen in Primary Immune Cells to Dissect Regulatory Networks," Cell 162, 675-686 (July 30, 2015).
- Ramanan et al., CRISPR-Cas9 cleavage of viral DNA efficiently suppresses hepatitis B virus," Scientific Reports 5:10833. doi: 10.1038/srep10833 (June 2, 2015)
- Nishimasu et al., Crystal Structure of *Staphylococcus aureus* Cas9," Cell 162, 1113-1126 (Aug. 27, 2015)
- BCL1 1A enhancer dissection by Cas9-mediated in situ saturating mutagenesis, Canver et al., Nature 527(7577): 192-7 (Nov. 12, 2015) doi: 10.1038/nature15521. Epub 2015 Sep 16.
- *Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System*, Zetsche et al., Cell 163, 759-71 (Sep 25, 2015).
- *Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems*, Shmakov et al., Molecular Cell, 60(3), 385-397 doi: 10.1016/j.molcel.2015.10.008 Epub October 22, 2015.
- *Rationally engineered Cas9 nucleases with improved specificity*, Slaymaker et al., Science 2016 Jan 1 351(6268): 84-88 doi: 10.1126/science.aad5227. Epub 2015 Dec 1.
- Gao et al, "Engineered Cpf1 Enzymes with Altered PAM Specificities," bioRxiv 091611; doi: http://dx.doi.org/10.1101/091611 (Dec. 4, 2016).
- > Cox et al., "RNA editing with CRISPR-Cas 13," Science. 2017 Nov 24;358(6366):1019-1027. doi: 10.1126/science.aaq0180. Epub 2017 Oct 25.

[0501] each of which is incorporated herein by reference, may be considered in the practice of the instant invention, and discussed briefly below:

- Cong et al. engineered type II CRISPR-Cas systems for use in eukaryotic cells based on both *Streptococcus thermophilus* Cas9 and also *Streptococcus pyogenes* Cas9 and

demonstrated that Cas9 nucleases can be directed by short RNAs to induce precise cleavage of DNA in human and mouse cells. Their study further showed that Cas9 as converted into a nicking enzyme can be used to facilitate homology-directed repair in eukaryotic cells with minimal mutagenic activity. Additionally, their study demonstrated that multiple guide sequences can be encoded into a single CRISPR array to enable simultaneous editing of several at endogenous genomic loci sites within the mammalian genome, demonstrating easy programmability and wide applicability of the RNA-guided nuclease technology. This ability to use RNA to program sequence specific DNA cleavage in cells defined a new class of genome engineering tools. These studies further showed that other CRISPR loci are likely to be transplantable into mammalian cells and can also mediate mammalian genome cleavage. Importantly, it can be envisaged that several aspects of the CRISPR-Cas system can be further improved to increase its efficiency and versatility.

- Jiang *et al.* used the clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated Cas9 endonuclease complexed with dual-RNAs to introduce precise mutations in the genomes of *Streptococcus pneumoniae* and *Escherichia coli*. The approach relied on dual-RNA:Cas9-directed cleavage at the targeted genomic site to kill unmutated cells and circumvents the need for selectable markers or counter-selection systems. The study reported reprogramming dual-RNA:Cas9 specificity by changing the sequence of short CRISPR RNA (crRNA) to make single- and multinucleotide changes carried on editing templates. The study showed that simultaneous use of two crRNAs enabled multiplex mutagenesis. Furthermore, when the approach was used in combination with recombineering, in *S. pneumoniae*, nearly 100% of cells that were recovered using the described approach contained the desired mutation, and in *E. coli*, 65% that were recovered contained the mutation.
- Wang *etal.* (2013) used the CRISPR-Cas system for the one-step generation of mice carrying mutations in multiple genes which were traditionally generated in multiple steps by sequential recombination in embryonic stem cells and/or time-consuming intercrossing of mice with a single mutation. The CRISPR-Cas system will greatly accelerate the *in vivo* study of functionally redundant genes and of epistatic gene interactions.

- Konermann *et al.* (2013) addressed the need in the art for versatile and robust technologies that enable optical and chemical modulation of DNA-binding domains based CRISPR Cas9 enzyme and also Transcriptional Activator Like Effectors
- Ran *et al.* (2013-A) described an approach that combined a Cas9 nickase mutant with paired guide RNAs to introduce targeted double-strand breaks. This addresses the issue of the Cas9 nuclease from the microbial CRISPR-Cas system being targeted to specific genomic loci by a guide sequence, which can tolerate certain mismatches to the DNA target and thereby promote undesired off-target mutagenesis. Because individual nicks in the genome are repaired with high fidelity, simultaneous nicking *via* appropriately offset guide RNAs is required for double-stranded breaks and extends the number of specifically recognized bases for target cleavage. The authors demonstrated that using paired nicking can reduce off-target activity by 50- to 1,500-fold in cell lines and to facilitate gene knockout in mouse zygotes without sacrificing on-target cleavage efficiency. This versatile strategy enables a wide variety of genome editing applications that require high specificity.
- Hsu *et al.* (2013) characterized SpCas9 targeting specificity in human cells to inform the selection of target sites and avoid off-target effects. The study evaluated >700 guide RNA variants and SpCas9-induced indel mutation levels at >100 predicted genomic off-target loci in 293T and 293FT cells. The authors that SpCas9 tolerates mismatches between guide RNA and target DNA at different positions in a sequence-dependent manner, sensitive to the number, position and distribution of mismatches. The authors further showed that SpCas9-mediated cleavage is unaffected by DNA methylation and that the dosage of SpCas9 and guide RNA can be titrated to minimize off-target modification. Additionally, to facilitate mammalian genome engineering applications, the authors reported providing a web-based software tool to guide the selection and validation of target sequences as well as off-target analyses.
- Ran *et al.* (2013-B) described a set of tools for Cas9-mediated genome editing *via* non-homologous end joining (NHEJ) or homology-directed repair (HDR) in mammalian cells, as well as generation of modified cell lines for downstream functional studies. To minimize off-target cleavage, the authors further described a double-nicking strategy using the Cas9 nickase mutant with paired guide RNAs. The protocol provided by the authors experimentally derived guidelines for the selection of target sites, evaluation of cleavage efficiency and analysis of off-target activity.

The studies showed that beginning with target design, gene modifications can be achieved within as little as 1-2 weeks, and modified clonal cell lines can be derived within 2-3 weeks.

- Shalem *et al.* described a new way to interrogate gene function on a genome-wide scale. Their studies showed that delivery of a genome-scale CRISPR-Cas9 knockout (GeCKO) library targeted 18,080 genes with 64,751 unique guide sequences enabled both negative and positive selection screening in human cells. First, the authors showed use of the GeCKO library to identify genes essential for cell viability in cancer and pluripotent stem cells. Next, in a melanoma model, the authors screened for genes whose loss is involved in resistance to vemurafenib, a therapeutic that inhibits mutant protein kinase BRAF. Their studies showed that the highest-ranking candidates included previously validated genes NF1 and MED12 as well as novel hits NF2, CUL3, TADA2B, and TADA1. The authors observed a high level of consistency between independent guide RNAs targeting the same gene and a high rate of hit confirmation, and thus demonstrated the promise of genome-scale screening with Cas9.
- Nishimasu *et al.* reported the crystal structure of *Streptococcus pyogenes* Cas9 in complex with sgRNA and its target DNA at 2.5 Å° resolution. The structure revealed a bilobed architecture composed of target recognition and nuclease lobes, accommodating the sgRNA:DNA heteroduplex in a positively charged groove at their interface. Whereas the recognition lobe is essential for binding sgRNA and DNA, the nuclease lobe contains the HNH and RuvC nuclease domains, which are properly positioned for cleavage of the complementary and non-complementary strands of the target DNA, respectively. The nuclease lobe also contains a carboxyl-terminal domain responsible for the interaction with the protospacer adjacent motif (PAM). This high-resolution structure and accompanying functional analyses have revealed the molecular mechanism of RNA-guided DNA targeting by Cas9, thus paving the way for the rational design of new, versatile genome-editing technologies.
- Wu *et al.* mapped genome-wide binding sites of a catalytically inactive Cas9 (dCas9) from *Streptococcus pyogenes* loaded with single guide RNAs (sgRNAs) in mouse embryonic stem cells (mESCs). The authors showed that each of the four sgRNAs tested targets dCas9 to between tens and thousands of genomic sites, frequently characterized by a 5-nucleotide seed region in the sgRNA and an NGG protospacer

adjacent motif (PAM). Chromatin inaccessibility decreases dCas9 binding to other sites with matching seed sequences; thus 70% of off-target sites are associated with genes. The authors showed that targeted sequencing of 295 dCas9 binding sites in mESCs transfected with catalytically active Cas9 identified only one site mutated above background levels. The authors proposed a two-state model for Cas9 binding and cleavage, in which a seed match triggers binding but extensive pairing with target DNA is required for cleavage.

- Piatt *et al.* established a Cre-dependent Cas9 knockin mouse. The authors demonstrated *in vivo* as well as *ex vivo* genome editing using adeno-associated virus (AAV)-, lentivirus-, or particle-mediated delivery of guide RNA in neurons, immune cells, and endothelial cells.
- Hsu *et al.* (2014) is a review article that discusses generally CRISPR-Cas9 history from yogurt to genome editing, including genetic screening of cells.
- Wang *et al.* (2014) relates to a pooled, loss-of-function genetic screening approach suitable for both positive and negative selection that uses a genome-scale lentiviral single guide RNA (sgRNA) library.
- Doench *et al.* created a pool of sgRNAs, tiling across all possible target sites of a panel of six endogenous mouse and three endogenous human genes and quantitatively assessed their ability to produce null alleles of their target gene by antibody staining and flow cytometry. The authors showed that optimization of the PAM improved activity and also provided an on-line tool for designing sgRNAs.
- Swiech *et al.* demonstrate that AAV-mediated SpCas9 genome editing can enable reverse genetic studies of gene function in the brain.
- Konermann *et al.* (2015) discusses the ability to attach multiple effector domains, e.g., transcriptional activator, functional and epigenomic regulators at appropriate positions on the guide such as stem or tetraloop with and without linkers.
- Zetsche *et al.* demonstrates that the Cas9 enzyme can be split into two and hence the assembly of Cas9 for activation can be controlled.
- Chen *et al.* relates to multiplex screening by demonstrating that a genome-wide *in vivo* CRISPR-Cas9 screen in mice reveals genes regulating lung metastasis.
- Ran *et al.* (2015) relates to SaCas9 and its ability to edit genomes and demonstrates that one cannot extrapolate from biochemical assays.

- Shalem *et al.* (2015) described ways in which catalytically inactive Cas9 (dCas9) fusions are used to synthetically repress (CRISPRi) or activate (CRISPRa) expression, showing advances using Cas9 for genome-scale screens, including arrayed and pooled screens, knockout approaches that inactivate genomic loci and strategies that modulate transcriptional activity.
- Xu *et al.* (2015) assessed the DNA sequence features that contribute to single guide RNA (sgRNA) efficiency in CRISPR-based screens. The authors explored efficiency of CRISPR-Cas9 knockout and nucleotide preference at the cleavage site. The authors also found that the sequence preference for CRISPRi/a is substantially different from that for CRISPR-Cas9 knockout.
- Parnas *et al.* (2015) introduced genome-wide pooled CRISPR-Cas9 libraries into dendritic cells (DCs) to identify genes that control the induction of tumor necrosis factor (Tnf) by bacterial lipopolysaccharide (LPS). Known regulators of Tlr4 signaling and previously unknown candidates were identified and classified into three functional modules with distinct effects on the canonical responses to LPS.
- Ramanan *et al.* (2015) demonstrated cleavage of viral episomal DNA (cccDNA) in infected cells. The HBV genome exists in the nuclei of infected hepatocytes as a 3.2kb double-stranded episomal DNA species called covalently closed circular DNA (cccDNA), which is a key component in the HBV life cycle whose replication is not inhibited by current therapies. The authors showed that sgRNAs specifically targeting highly conserved regions of HBV robustly suppresses viral replication and depleted cccDNA.
- Nishimasu *et al.* (2015) reported the crystal structures of SaCas9 in complex with a single guide RNA (sgRNA) and its double-stranded DNA targets, containing the 5'-TTGAAT-3' PAM and the 5'-TTGGGT-3' PAM. A structural comparison of SaCas9 with SpCas9 highlighted both structural conservation and divergence, explaining their distinct PAM specificities and orthologous sgRNA recognition.
- Canver *et al.* (2015) demonstrated a CRISPR-Cas9-based functional investigation of non-coding genomic elements. The authors developed pooled CRISPR-Cas9 guide RNA libraries to perform *in situ* saturating mutagenesis of the human and mouse BCL1 1A enhancers which revealed critical features of the enhancers.
- Zetsche et al. (2015) reported characterization of Cpf1, a class 2 CRISPR nuclease from Francisella novicida U112 having features distinct from Cas9. Cpf1 is a single

RNA-guided endonuclease lacking tracrRNA, utilizes a T-rich protospacer-adjacent motif, and cleaves DNA via a staggered DNA double-stranded break.

- Shmakov et al. (2015) reported three distinct Class 2 CRISPR-Cas systems. Two system CRISPR enzymes (C2c1 and C2c3) contain RuvC-like endonuclease domains distantly related to Cpf1. Unlike Cpf1, C2c1 depends on both crRNA and tracrRNA for DNA cleavage. The third enzyme (C2c2) contains two predicted HEPN RNase domains and is tracrRNA independent.
- Slaymaker et al (2016) reported the use of structure-guided protein engineering to improve the specificity of *Streptococcus pyogenes* Cas9 (SpCas9). The authors developed "enhanced specificity" SpCas9 (eSpCas9) variants which maintained robust on-target cleavage with reduced off-target effects.
- Cox et al., (2017) reported the use of catalytically inactive Casl3 (dCasl3) to direct adenosine-to-inosine deaminase activity by ADAR2 (adenosine deaminase acting on RNA type 2) to transcripts in mammalian cells. The system, referred to as RNA Editing for Programmable A to I Replacement (REPAIR), has no strict sequence constraints and can be used to edit full-length transcripts. The authors further engineered the system to create a high-specificity variant and minimized the system to facilitate viral delivery.

[0502] The methods and tools provided herein are may be designed for use with or Casl3, a type II nuclease that does not make use of tracrRNA. Orthologs of Casl3 have been identified in different bacterial species as described herein. Further type II nucleases with similar properties can be identified using methods described in the art (Shmakov et al. 2015, 60:385-397; Abudayeh et al. 2016, Science, 5;353(6299)). In particular embodiments, such methods for identifying novel CRISPR effector proteins may comprise the steps of selecting sequences from the database encoding a seed which identifies the presence of a CRISPR Cas locus, identifying loci located within 10 kb of the seed comprising Open Reading Frames (ORFs) in the selected sequences, selecting therefrom loci comprising ORFs of which only a single ORF encodes a novel CRISPR effector having greater than 700 amino acids and no more than 90% homology to a known CRISPR effector. In particular embodiments, the seed is a protein that is common to the CRISPR-Cas system, such as Casl. In further embodiments, the CRISPR array is used as a seed to identify new effector proteins.

[0503] Also, "Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing", Shengdar Q. Tsai, Nicolas Wyveldens, Cyd Khayter, Jennifer A. Foden, Vishal

Thapar, Deepak Reyon, Mathew J. Goodwin, Martin J. Aryee, J. Keith Joung Nature Biotechnology 32(6): 569-77 (2014), relates to dimeric RNA-guided FokI Nucleases that recognize extended sequences and can edit endogenous genes with high efficiencies in human cells.

[0504] With respect to general information on CRISPR/Cas Systems, components thereof, and delivery of such components, including methods, materials, delivery vehicles, vectors, particles, and making and using thereof, including as to amounts and formulations, as well as CRISPR-Cas-expressing eukaryotic cells, CRISPR-Cas expressing eukaryotes, such as a mouse, reference is made to: US Patents Nos. 8,999,641, 8,993,233, 8,697,359, 8,771,945, 8,795,965, 8,865,406, 8,871,445, 8,889,356, 8,889,418, 8,895,308, 8,906,616, 8,932,814, and 8,945,839; US Patent Publications US 2014-0310830 (US App. Ser. No. 14/105,031), US 2014-0287938 A1 (U.S. App. Ser. No. 14/213,991), US 2014-0273234 A1 (U.S. App. Ser. No. 14/293,674), US2014-0273232 A1 (U.S. App. Ser. No. 14/290,575), US 2014-0273231 (U.S. App. Ser. No. 14/259,420), US 2014-0256046 A1 (U.S. App. Ser. No. 14/226,274), US 2014-0248702 A1 (U.S. App. Ser. No. 14/258,458), US 2014-0242700 A1 (U.S. App. Ser. No. 14/222,930), US 2014-0242699 A1 (U.S. App. Ser. No. 14/183,512), US 2014-0242664 A1 (U.S. App. Ser. No. 14/104,990), US 2014-0234972 A1 (U.S. App. Ser. No. 14/183,471), US 2014-0227787 A1 (U.S. App. Ser. No. 14/256,912), US 2014-0189896 A1 (U.S. App. Ser. No. 14/105,035), US 2014-0186958 (U.S. App. Ser. No. 14/105,017), US 2014-0186919 A1 (U.S. App. Ser. No. 14/104,977), US 2014-0186843 A1 (U.S. App. Ser. No. 14/104,900), US 2014-0179770 A1 (U.S. App. Ser. No. 14/104,837) and US 2014-0179006 A1 (U.S. App. Ser. No. 14/183,486), US 2014-0170753 (US App Ser No 14/183,429); US 2015-0184139 (U.S. App. Ser. No. 14/324,960); 14/054,414 European Patent Applications EP 2 771 468 (EP13818570.7), EP 2 764 103 (EP13824232.6), and EP 2 784 162 (EP 14 1703 83.5); and PCT Patent Publications WO20 14/093 661 (PCT/US20 13/074743), WO2014/093694 (PCT/US20 13/074790), WO2014/093595 (PCT/US20 13/0746 11), WO20 14/0937 18 (PCT/US20 13/074825), WO20 14/093 709 (PCT/US20 13/0748 12), WO20 14/093 622 (PCT/US20 13/074667), WO2014/093635 (PCT/US20 13/074691), WO2014/093655 (PCT/US20 13/07473 6), WO20 14/0937 12 (PCT/US20 13/0748 19), WO20 14/093 701 (PCT/US20 13/074800), WO20 14/0 18423 (PCT/US2013/051418) , WO20 14/204723 (PCT/US20 14/04 1790), WO20 14/204724 (PCT/US20 14/04 1800), WO20 14/204725 (PCT/US2014/041803), WO20 14/204726 (PCT/US20 14/04 1804), WO20 14/204727 (PCT/US20 14/04 1806), WO20 14/204728

(PCT/US20 14/04 1808), WO20 14/204729 (PCT/US20 14/04 1809), WO20 15/0893 51
 (PCT/US20 14/069897), WO20 15/0893 54 (PCT/US20 14/069902), WO2015/089364
 (PCT/US20 14/069925), WO20 15/089427 (PCT/US20 14/070068), WO20 15/089462
 (PCT/US20 14/070 127), WO20 15/0894 19 (PCT/US2014/070057) , WO20 15/089465
 (PCT/US2014/070135) , WO20 15/089486 (PCT/US20 14/070 175), WO2015/058052
 (PCT/US20 14/06 1077), WO20 15/070083 (PCT/US2014/064663) , WO20 15/0893 54
 (PCT/US20 14/069902), WO20 15/0893 51 (PCT/US20 14/069897), WO2015/089364
 (PCT/US20 14/069925), WO20 15/089427 (PCT/US20 14/070068), WO20 15/089473
 (PCT/US20 14/070 152), WO20 15/089486 (PCT/US20 14/070 175), WO20 16/04925 8
 (PCT/US20 15/05 1830), WO20 16/094867 (PCT/US20 15/0653 85), WO20 16/094872
 (PCT/US2015/065393) , WO20 16/094874 (PCT/US2015/065396) , WO20 16/1 06244
 (PCT/US20 15/067 177).

[0505] Mention is also made of US application 62/180,709, 17-Jun-15, PROTECTED GUIDE RNAS (PGRNAS); US application 62/091,455, filed, 12-Dec-14, PROTECTED GUIDE RNAS (PGRNAS); US application 62/096,708, 24-Dec-14, PROTECTED GUIDE RNAS (PGRNAS); US applications 62/091,462, 12-Dec-14, 62/096,324, 23-Dec-14, 62/180,681, 17-Jun-2015, and 62/237,496, 5-Oct-2015, DEAD GUIDES FOR CRISPR TRANSCRIPTION FACTORS; US application 62/091,456, 12-Dec-14 and 62/180,692, 17-Jun-2015, ESCORTED AND FUNCTION ALIZED GUIDES FOR CRISPR-CAS SYSTEMS; US application 62/091,461, 12-Dec-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR GENOME EDITING AS TO HEMATOPOETIC STEM CELLS (HSCs); US application 62/094,903, 19-Dec-14, UNBIASED IDENTIFICATION OF DOUBLE-STRAND BREAKS AND GENOMIC REARRANGEMENT BY GENOME-WISE INSERT CAPTURE SEQUENCING; US application 62/096,761, 24-Dec-14, ENGINEERING OF SYSTEMS, METHODS AND OPTIMIZED ENZYME AND GUIDE SCAFFOLDS FOR SEQUENCE MANIPULATION; US application 62/098,059, 30-Dec-14, 62/181,641, 18-Jun-2015, and 62/181,667, 18-Jun-2015, RNA-TARGETING SYSTEM; US application 62/096,656, 24-Dec-14 and 62/181,151, 17-Jun-2015, CRISPR HAVING OR ASSOCIATED WITH DESTABILIZATION DOMAINS; US application 62/096,697, 24-Dec-14, CRISPR HAVING OR ASSOCIATED WITH AAV; US application 62/098,158, 30-Dec-14, ENGINEERED CRISPR COMPLEX INSERTIONAL TARGETING SYSTEMS; US application 62/151,052, 22-Apr-15, CELLULAR TARGETING FOR

EXTRACELLULAR EXOSOMAL REPORTING; US application 62/054,490, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR TARGETING DISORDERS AND DISEASES USING PARTICLE DELIVERY COMPONENTS; US application 61/939,154, 12-FEB-14, SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/055,484, 25-Sep-14, SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/087,537, 4-Dec-14, SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/054,651, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR MODELING COMPETITION OF MULTIPLE CANCER MUTATIONS IN VIVO; US application 62/067,886, 23-Oct-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR MODELING COMPETITION OF MULTIPLE CANCER MUTATIONS IN VIVO; US applications 62/054,675, 24-Sep-14 and 62/181,002, 17-Jun-2015, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS IN NEURONAL CELLS/TISSUES; US application 62/054,528, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS IN IMMUNE DISEASES OR DISORDERS; US application 62/055,454, 25-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR TARGETING DISORDERS AND DISEASES USING CELL PENETRATION PEPTIDES (CPP); US application 62/055,460, 25-Sep-14, MULTIFUNCTIONAL-CRISPR COMPLEXES AND/OR OPTIMIZED ENZYME LINKED FUNCTIONAL-CRISPR COMPLEXES; US application 62/087,475, 4-Dec-14 and 62/181,690, 18-Jun-2015, FUNCTIONAL SCREENING WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/055,487, 25-Sep-14, FUNCTIONAL SCREENING WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/087,546, 4-Dec-14 and 62/181,687, 18-Jun-2015, MULTIFUNCTIONAL CRISPR COMPLEXES AND/OR OPTIMIZED ENZYME LINKED FUNCTIONAL-CRISPR COMPLEXES; and US application 62/098,285, 30-Dec-14,

SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/054,651, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR MODELING COMPETITION OF MULTIPLE CANCER MUTATIONS IN VIVO; US application 62/067,886, 23-Oct-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR MODELING COMPETITION OF MULTIPLE CANCER MUTATIONS IN VIVO; US applications 62/054,675, 24-Sep-14 and 62/181,002, 17-Jun-2015, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS IN NEURONAL CELLS/TISSUES; US application 62/054,528, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS IN IMMUNE DISEASES OR DISORDERS; US application 62/055,454, 25-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR TARGETING DISORDERS AND DISEASES USING CELL PENETRATION PEPTIDES (CPP); US application 62/055,460, 25-Sep-14, MULTIFUNCTIONAL-CRISPR COMPLEXES AND/OR OPTIMIZED ENZYME LINKED FUNCTIONAL-CRISPR COMPLEXES; US application 62/087,475, 4-Dec-14 and 62/181,690, 18-Jun-2015, FUNCTIONAL SCREENING WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/055,487, 25-Sep-14, FUNCTIONAL SCREENING WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/087,546, 4-Dec-14 and 62/181,687, 18-Jun-2015, MULTIFUNCTIONAL CRISPR COMPLEXES AND/OR OPTIMIZED ENZYME LINKED FUNCTIONAL-CRISPR COMPLEXES; and US application 62/098,285, 30-Dec-14,

CRISPR MEDIATED IN VIVO MODELING AND GENETIC SCREENING OF TUMOR GROWTH AND METASTASIS.

[0506] Mention is made of US applications 62/181,659, 18-Jun-2015 and 62/207,318, 19-Aug-2015, ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS, ENZYME AND GUIDE SCAFFOLDS OF CAS9 ORTHOLOGS AND VARIANTS FOR SEQUENCE MANIPULATION. Mention is made of US applications 62/181,663, 18-Jun-2015 and 62/245,264, 22-Oct-2015, NOVEL CRISPR ENZYMES AND SYSTEMS, US applications 62/181,675, 18-Jun-2015, 62/285,349, 22-Oct-2015, 62/296,522, 17-Feb-2016, and 62/320,231, 8-Apr-2016, NOVEL CRISPR ENZYMES AND SYSTEMS, US application 62/232,067, 24-Sep-2015, US Application 14/975,085, 18-Dec-2015, European application No. 16150428.7, US application 62/205,733, 16-Aug-2015, US application 62/201,542, 5-Aug-2015, US application 62/193,507, 16-M-2015, and US application 62/181,739, 18-Jun-2015, each entitled NOVEL CRISPR ENZYMES AND SYSTEMS and of US application 62/245,270, 22-Oct-2015, NOVEL CRISPR ENZYMES AND SYSTEMS. Mention is also made of US application 61/939,256, 12-Feb-2014, and WO 2015/089473 (PCT/US2014/070152), 12-Dec-2014, each entitled ENGINEERING OF SYSTEMS, METHODS AND OPTIMIZED GUIDE COMPOSITIONS WITH NEW ARCHITECTURES FOR SEQUENCE MANIPULATION. Mention is also made of PCT/US2015/045504, 15-Aug-2015, US application 62/180,699, 17-Jun-2015, and US application 62/038,358, 17-Aug-2014, each entitled GENOME EDITING USING CAS9 NICKASES.

[0507] Each of these patents, patent publications, and applications, and all documents cited therein or during their prosecution ("appln cited documents") and all documents cited or referenced in the appln cited documents, together with any instructions, descriptions, product specifications, and product sheets for any products mentioned therein or in any document therein and incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention. All documents (e.g., these patents, patent publications and applications and the appln cited documents) are incorporated herein by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

[0508] In particular embodiments, pre-complexed guide RNA and CRISPR effector protein, (optionally, adenosine deaminase fused to a CRISPR protein or an adaptor) are delivered as a ribonucleoprotein (RNP). RNPs have the advantage that they lead to rapid

editing effects even more so than the RNA method because this process avoids the need for transcription. An important advantage is that both RNP delivery is transient, reducing off-target effects and toxicity issues. Efficient genome editing in different cell types has been observed by Kim et al. (2014, *Genome Res.* 24(6): 1012-9), Paix et al. (2015, *Genetics* 204(1):47-54), Chu et al. (2016, *BMC Biotechnol.* 16:4), and Wang et al. (2013, *Cell.* 9;153(4):910-8).

[0509] In particular embodiments, the ribonucleoprotein is delivered by way of a polypeptide-based shuttle agent as described in WO2016161516. WO2016161516 describes efficient transduction of polypeptide cargos using synthetic peptides comprising an endosome leakage domain (ELD) operably linked to a cell penetrating domain (CPD), to a histidine-rich domain and a CPD. Similarly these polypeptides can be used for the delivery of CRISPR-effector based RNPs in eukaryotic cells.

ZN-Finger Nucleases

[0510] Other preferred tools for genome editing for use in the context of this invention include zinc finger systems and TALE systems. One type of programmable DNA-binding domain is provided by artificial zinc-finger (ZF) technology, which involves arrays of ZF modules to target new DNA-binding sites in the genome. Each finger module in a ZF array targets three DNA bases. A customized array of individual zinc finger domains is assembled into a ZF protein (ZFP).

[0511] ZFPs can comprise a functional domain. The first synthetic zinc finger nucleases (ZFNs) were developed by fusing a ZF protein to the catalytic domain of the Type IIS restriction enzyme FokI. (Kim, Y. G. et al., 1994, Chimeric restriction endonuclease, *Proc. Natl. Acad. Sci. U.S.A.* 91, 883-887; Kim, Y. G. et al., 1996, Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc. Natl. Acad. Sci. U.S.A.* 93, 1156-1 160). Increased cleavage specificity can be attained with decreased off target activity by use of paired ZFN heterodimers, each targeting different nucleotide sequences separated by a short spacer. (Doyon, Y. et al., 2011, Enhancing zinc-finger-nuclease activity with improved obligate heterodimeric architectures. *Nat. Methods* 8, 74-79). ZFPs can also be designed as transcription activators and repressors and have been used to target many genes in a wide variety of organisms. Exemplary methods of genome editing using ZFNs can be found for example in U.S. Patent Nos. 6,534,261, 6,607,882, 6,746,838, 6,794,136, 6,824,978, 6,866,997, 6,933,113, 6,979,539, 7,013,219, 7,030,215, 7,220,719, 7,241,573, 7,241,574,

7,585,849, 7,595,376, 6,903,185, and 6,479,626, all of which are specifically incorporated by reference.

Tale Systems

[0512] As disclosed herein editing can be made by way of the transcription activator-like effector nucleases (TALENs) system. Transcription activator-like effectors (TALEs) can be engineered to bind practically any desired DNA sequence. Exemplary methods of genome editing using the TALEN system can be found for example in Cermak T. Doyle EL. Christian M. Wang L. Zhang Y. Schmidt C, et al. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res.* 2011;39:e82; Zhang F. Cong L. Lodato S. Kosuri S. Church GM. Arlotta P. Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. *Nat Biotechnol.* 2011;29:149-153 and US Patent Nos. 8,450,471, 8,440,431 and 8,440,432, all of which are specifically incorporated by reference.

[0513] In advantageous embodiments of the invention, the methods provided herein use isolated, non-naturally occurring, recombinant or engineered DNA binding proteins that comprise TALE monomers as a part of their organizational structure that enable the targeting of nucleic acid sequences with improved efficiency and expanded specificity.

[0514] Naturally occurring TALEs or "wild type TALEs" are nucleic acid binding proteins secreted by numerous species of proteobacteria. TALE polypeptides contain a nucleic acid binding domain composed of tandem repeats of highly conserved monomer polypeptides that are predominantly 33, 34 or 35 amino acids in length and that differ from each other mainly in amino acid positions 12 and 13. In advantageous embodiments the nucleic acid is DNA. As used herein, the term "polypeptide monomers", or "TALE monomers" will be used to refer to the highly conserved repetitive polypeptide sequences within the TALE nucleic acid binding domain and the term "repeat variable di-residues" or "RVD" will be used to refer to the highly variable amino acids at positions 12 and 13 of the polypeptide monomers. As provided throughout the disclosure, the amino acid residues of the RVD are depicted using the IUPAC single letter code for amino acids. A general representation of a TALE monomer which is comprised within the DNA binding domain is X₁-I-1-(X₁₂X₁₃)-X₁₄-33 or 34 or 35, where the subscript indicates the amino acid position and X represents any amino acid. X₁₂X₁₃ indicate the RVDs. In some polypeptide monomers, the variable amino acid at position 13 is missing or absent and in such polypeptide monomers, the RVD consists of a single amino acid. In such cases the RVD may

be alternatively represented as X*, where X represents X12 and (*) indicates that X13 is absent. The DNA binding domain comprises several repeats of TALE monomers and this may be represented as (X1-1 1-(X12X13)-X14-33 or 34 or 35)z, where in an advantageous embodiment, z is at least 5 to 40. In a further advantageous embodiment, z is at least 10 to 26.

[0515] The TALE monomers have a nucleotide binding affinity that is determined by the identity of the amino acids in its RVD. For example, polypeptide monomers with an RVD of NI preferentially bind to adenine (A), polypeptide monomers with an RVD of NG preferentially bind to thymine (T), polypeptide monomers with an RVD of HD preferentially bind to cytosine (C) and polypeptide monomers with an RVD of NN preferentially bind to both adenine (A) and guanine (G). In yet another embodiment of the invention, polypeptide monomers with an RVD of IG preferentially bind to T. Thus, the number and order of the polypeptide monomer repeats in the nucleic acid binding domain of a TALE determines its nucleic acid target specificity. In still further embodiments of the invention, polypeptide monomers with an RVD of NS recognize all four base pairs and may bind to A, T, G or C. The structure and function of TALEs is further described in, for example, Moscou et al., *Science* 326:1501 (2009); Boch et al., *Science* 326:1509-1512 (2009); and Zhang et al., *Nature Biotechnology* 29:149-153 (2011), each of which is incorporated by reference in its entirety.

[0516] The TALE polypeptides used in methods of the invention are isolated, non-naturally occurring, recombinant or engineered nucleic acid-binding proteins that have nucleic acid or DNA binding regions containing polypeptide monomer repeats that are designed to target specific nucleic acid sequences.

[0517] As described herein, polypeptide monomers having an RVD of HN or NH preferentially bind to guanine and thereby allow the generation of TALE polypeptides with high binding specificity for guanine containing target nucleic acid sequences. In a preferred embodiment of the invention, polypeptide monomers having RVDs RN, NN, NK, SN, NH, KN, HN, NQ, HH, RG, KH, RH and SS preferentially bind to guanine. In a much more advantageous embodiment of the invention, polypeptide monomers having RVDs RN, NK, NQ, HH, KH, RH, SS and SN preferentially bind to guanine and thereby allow the generation of TALE polypeptides with high binding specificity for guanine containing target nucleic acid sequences. In an even more advantageous embodiment of the invention, polypeptide monomers having RVDs HH, KH, NH, NK, NQ, RH, RN and SS preferentially bind to guanine and thereby allow the generation of TALE polypeptides with high binding specificity

for guanine containing target nucleic acid sequences. In a further advantageous embodiment, the RVDs that have high binding specificity for guanine are RN, NH RH and KH. Furthermore, polypeptide monomers having an RVD of NV preferentially bind to adenine and guanine. In more preferred embodiments of the invention, polypeptide monomers having RVDs of H*, HA, KA, N*, NA, NC, NS, RA, and S* bind to adenine, guanine, cytosine and thymine with comparable affinity.

[0518] The predetermined N-terminal to C-terminal order of the one or more polypeptide monomers of the nucleic acid or DNA binding domain determines the corresponding predetermined target nucleic acid sequence to which the TALE polypeptides will bind. As used herein the polypeptide monomers and at least one or more half polypeptide monomers are "specifically ordered to target" the genomic locus or gene of interest. In plant genomes, the natural TALE-binding sites always begin with a thymine (T), which may be specified by a cryptic signal within the non-repetitive N-terminus of the TALE polypeptide; in some cases this region may be referred to as repeat 0. In animal genomes, TALE binding sites do not necessarily have to begin with a thymine (T) and TALE polypeptides may target DNA sequences that begin with T, A, G or C. The tandem repeat of TALE monomers always ends with a half-length repeat or a stretch of sequence that may share identity with only the first 20 amino acids of a repetitive full length TALE monomer and this half repeat may be referred to as a half-monomer (FIG. 8), which is included in the term "TALE monomer". Therefore, it follows that the length of the nucleic acid or DNA being targeted is equal to the number of full polypeptide monomers plus two.

[0519] As described in Zhang et al., Nature Biotechnology 29:149-153 (2011), TALE polypeptide binding efficiency may be increased by including amino acid sequences from the "capping regions" that are directly N-terminal or C-terminal of the DNA binding region of naturally occurring TALEs into the engineered TALEs at positions N-terminal or C-terminal of the engineered TALE DNA binding region. Thus, in certain embodiments, the TALE polypeptides described herein further comprise an N-terminal capping region and/or a C-terminal capping region.

[0520] An exemplary amino acid sequence of a N-terminal capping region is:

M D P I R S R T P S P A R E L L S G P Q P D G V Q P T A D R G V S P
P A G G P L D G L P A R R T M S R T R L P S P P A P S P A F S A D S
F S D L L R Q F D P S L F N T S L F D S L P P F G A H H T E A A T G

E W D E V Q S G L R A A D A P P P T M R V A V T A A R P P R A K
P A

P R R R A A Q P S D A S P A A Q V D L R T L G Y S Q Q Q E K I K
P

K V R S T V A Q H H E A L V G H G F T H A H I V A L S Q H P A A L
G

T V A V K Y Q D M I A A L P E A T H E A I V G V G K Q W S G A R
A L

E A L L T V A G E L R G P P L Q L D T G Q L L K I A K R G G V T A
V

E A V H A W R N A L T G A P L N (SEQ ID NO:20)

[0521] An exemplary amino acid sequence of a C-terminal capping region is:

R P A L E S I V A Q L S R P D P A L A A L T N D H L V A L A C L G
G R P A L D A V K K G L P H A P A L I K R T N R R I P E R T S H R
V A D H A Q V V R V L G F F Q C H S H P A Q A F D D A M T Q F G
M
S R H G L L Q L F R R V G V T E L E A R S G T L P P A S Q R W D R
I L Q A S G M K R A K P S P T S T Q T P D Q A S L H A F A D S L E
R D L D A P S P M H E G D Q T R A S (SEQ ID NO:21)

[0522] As used herein the predetermined "N-terminus" to "C terminus" orientation of the N-terminal capping region, the DNA binding domain comprising the repeat TALE monomers and the C-terminal capping region provide structural basis for the organization of different domains in the d-TALEs or polypeptides of the invention.

[0523] The entire N-terminal and/or C-terminal capping regions are not necessary to enhance the binding activity of the DNA binding region. Therefore, in certain embodiments, fragments of the N-terminal and/or C-terminal capping regions are included in the TALE polypeptides described herein.

[0524] In certain embodiments, the TALE polypeptides described herein contain a N-terminal capping region fragment that included at least 10, 20, 30, 40, 50, 54, 60, 70, 80, 87, 90, 94, 100, 102, 110, 117, 120, 130, 140, 147, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260 or 270 amino acids of an N-terminal capping region. In certain embodiments, the N-terminal capping region fragment amino acids are of the C-terminus (the DNA-binding

region proximal end) of an N-terminal capping region. As described in Zhang et al., *Nature Biotechnology* 29:149-153 (2011), N-terminal capping region fragments that include the C-terminal 240 amino acids enhance binding activity equal to the full length capping region, while fragments that include the C-terminal 147 amino acids retain greater than 80% of the efficacy of the full length capping region, and fragments that include the C-terminal 117 amino acids retain greater than 50% of the activity of the full-length capping region.

[0525] In some embodiments, the TALE polypeptides described herein contain a C-terminal capping region fragment that included at least 6, 10, 20, 30, 37, 40, 50, 60, 68, 70, 80, 90, 100, 110, 120, 127, 130, 140, 150, 155, 160, 170, 180 amino acids of a C-terminal capping region. In certain embodiments, the C-terminal capping region fragment amino acids are of the N-terminus (the DNA-binding region proximal end) of a C-terminal capping region. As described in Zhang et al., *Nature Biotechnology* 29:149-153 (2011), C-terminal capping region fragments that include the C-terminal 68 amino acids enhance binding activity equal to the full length capping region, while fragments that include the C-terminal 20 amino acids retain greater than 50% of the efficacy of the full length capping region.

[0526] In certain embodiments, the capping regions of the TALE polypeptides described herein do not need to have identical sequences to the capping region sequences provided herein. Thus, in some embodiments, the capping region of the TALE polypeptides described herein have sequences that are at least 50%, 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical or share identity to the capping region amino acid sequences provided herein. Sequence identity is related to sequence homology. Homology comparisons may be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs may calculate percent (%) homology between two or more sequences and may also calculate the sequence identity shared by two or more amino acid or nucleic acid sequences. In some preferred embodiments, the capping region of the TALE polypeptides described herein have sequences that are at least 95% identical or share identity to the capping region amino acid sequences provided herein.

[0527] Sequence homologies may be generated by any of a number of computer programs known in the art, which include but are not limited to BLAST or FASTA. Suitable computer program for carrying out alignments like the GCG Wisconsin Bestfit package may also be used. Once the software has produced an optimal alignment, it is possible to calculate

% homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

[0528] In advantageous embodiments described herein, the TALE polypeptides of the invention include a nucleic acid binding domain linked to the one or more effector domains. The terms "effector domain" or "regulatory and functional domain" refer to a polypeptide sequence that has an activity other than binding to the nucleic acid sequence recognized by the nucleic acid binding domain. By combining a nucleic acid binding domain with one or more effector domains, the polypeptides of the invention may be used to target the one or more functions or activities mediated by the effector domain to a particular target DNA sequence to which the nucleic acid binding domain specifically binds.

[0529] In some embodiments of the TALE polypeptides described herein, the activity mediated by the effector domain is a biological activity. For example, in some embodiments the effector domain is a transcriptional inhibitor (i.e., a repressor domain), such as an mSin interaction domain (SID). SID4X domain or a Krippel-associated box (KRAB) or fragments of the KRAB domain. In some embodiments the effector domain is an enhancer of transcription (i.e. an activation domain), such as the VP16, VP64 or p65 activation domain. In some embodiments, the nucleic acid binding is linked, for example, with an effector domain that includes but is not limited to a transposase, integrase, recombinase, resolvase, invertase, protease, DNA methyltransferase, DNA demethylase, histone acetylase, histone deacetylase, nuclease, transcriptional repressor, transcriptional activator, transcription factor recruiting, protein nuclear-localization signal or cellular uptake signal.

[0530] In some embodiments, the effector domain is a protein domain which exhibits activities which include but are not limited to transposase activity, integrase activity, recombinase activity, resolvase activity, invertase activity, protease activity, DNA methyltransferase activity, DNA demethylase activity, histone acetylase activity, histone deacetylase activity, nuclease activity, nuclear-localization signaling activity, transcriptional repressor activity, transcriptional activator activity, transcription factor recruiting activity, or cellular uptake signaling activity. Other preferred embodiments of the invention may include any combination the activities described herein.

Meganucleases

[0531] As disclosed herein editing can be made by way of meganucleases, which are endodeoxyribonucleases characterized by a large recognition site (double-stranded DNA sequences of 12 to 40 base pairs). Exemplary method for using meganucleases can be found

in US Patent Nos: 8,163,514; 8,133,697; 8,021,867; 8,119,361; 8,119,381; 8,124,369; and 8,129,134, which are specifically incorporated by reference.

Transcriptional Activation/Repression

[0532] In certain embodiments, an immunomodulant may comprise (i) a DNA-binding portion configured to specifically bind to the endogenous gene and (ii) an effector domain mediating a biological activity.

[0533] In certain embodiments, the DNA-binding portion may comprises a zinc finger protein or DNA-binding domain thereof, a transcription activator-like effector (TALE) protein or DNA-binding domain thereof, or an RNA-guided protein or DNA-binding domain thereof.

[0534] In certain embodiments, the DNA-binding portion may comprise (i) Cas9 or Cpf1 or any Cas protein described herein modified to eliminate its nuclease activity, or (ii) DNA-binding domain of Cas9 or Cpf1 or any Cas protein described herein.

[0535] In some embodiments, the effector domain may be a transcriptional inhibitor (i.e., a repressor domain), such as an mSin interaction domain (SID). SID4X domain or a Kriippel-associated box (KRAB) or fragments of the KRAB domain. In some embodiments the effector domain may be an enhancer of transcription (i.e. an activation domain), such as the VP16, VP64 or p65 activation domain. In some embodiments, the nucleic acid binding portion may be linked, for example, with an effector domain that includes but is not limited to a transposase, integrase, recombinase, resolvase, invertase, protease, DNA methyltransferase, DNA demethylase, histone acetylase, histone deacetylase, nuclease, transcriptional repressor, transcriptional activator, transcription factor recruiting, protein nuclear-localization signal or cellular uptake signal. In some embodiments, the effector domain may be a protein domain which exhibits activities which include but are not limited to transposase activity, integrase activity, recombinase activity, resolvase activity, invertase activity, protease activity, DNA methyltransferase activity, DNA demethylase activity, histone acetylase activity, histone deacetylase activity, nuclease activity, nuclear-localization signaling activity, transcriptional repressor activity, transcriptional activator activity, transcription factor recruiting activity, or cellular uptake signaling activity. Other preferred embodiments of the invention may include any combination the activities described herein.

[0536] In certain embodiments, the agent capable of specifically binding to a gene product expressed on the cell surface of the immune cell is an antibody.

[0537] By means of an example, an agent, such as an antibody, capable of specifically binding to a gene product expressed on the cell surface of the immune cells may be conjugated with a therapeutic or effector agent for targeted delivery of the therapeutic or effector agent to the immune cells.

[0538] Examples of such therapeutic or effector agents include immunomodulatory classes as discussed herein, such as without limitation a toxin, drug, radionuclide, cytokine, lymphokine, chemokine, growth factor, tumor necrosis factor, hormone, hormone antagonist, enzyme, oligonucleotide, siRNA, RNAi, photoactive therapeutic agent, anti-angiogenic agent and pro-apoptotic agent.

[0539] Example toxins include ricin, abrin, alpha toxin, saporin, ribonuclease (RNase), DNase I, Staphylococcal enterotoxin-A, pokeweed antiviral protein, gelonin, diphtheria toxin, Pseudomonas exotoxin, or Pseudomonas endotoxin.

[0540] Example radionuclides include ^{103m}Rh , ^{103}Ru , ^{105}Rh , ^{105}Ru , ^{107}Hg , ^{109}Pd , ^{109}Pt , ^{111}Ag , ^{111}In , ^{113m}In , ^{119}Sb , ^{11}C , ^{121m}Te , ^{122m}Te , ^{125}I , ^{125m}Te , ^{126}I , ^{131}I , ^{133}I , ^{13}N , ^{142}Pr , ^{143}Pr , ^{149}Pm , ^{152}Dy , ^{153}Sm , ^{15}O , ^{161}Ho , ^{161}Tb , ^{165}Tm , ^{166}Dy , ^{166}Ho , ^{167}Tm , ^{168}Tm , ^{169}Er , ^{169}Yb , ^{177}Lu , ^{186}Re , ^{188}Re , ^{189m}Os , ^{189}Re , ^{192}Ir , ^{194}Ir , ^{197}Pt , ^{198}Au , ^{199}Au , ^{201}Tl , ^{203}Hg , ^{211}At , ^{211}Bi , ^{211}Pb , ^{212}Bi , ^{212}Pb , ^{213}Bi , ^{215}Po , ^{217}At , ^{219}Rn , ^{221}Fr , ^{223}Ra , ^{224}Ac , ^{225}Ac , ^{225}Fm , ^{32}P , ^{33}P , ^{47}Sc , ^{51}Cr , ^{57}Co , ^{58}Co , ^{59}Fe , ^{62}Cu , ^{67}Cu , ^{67}Ga , ^{75}Br , ^{75}Se , ^{76}Br , ^{77}As , ^{77}Br , ^{80m}Br , ^{89}Sr , ^{90}Y , ^{95}Ru , ^{97}Ru , "Mo or ^{99m}Tc . Preferably, the radionuclide may be an alpha-particle-emitting radionuclide.

[0541] Example enzymes include malate dehydrogenase, staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase or acetylcholinesterase. Such enzymes may be used, for example, in combination with prodrugs that are administered in relatively non-toxic form and converted at the target site by the enzyme into a cytotoxic agent. In other alternatives, a drug may be converted into less toxic form by endogenous enzymes in the subject but may be reconverted into a cytotoxic form by the therapeutic enzyme.

Kits

[0542] In another aspect, the invention is directed to kit and kit of parts. The terms "kit of parts" and "kit" as used throughout this specification refer to a product containing components necessary for carrying out the specified methods (e.g., methods for detecting, quantifying or isolating immune cells as taught herein), packed so as to allow their transport

and storage. Materials suitable for packing the components comprised in a kit include crystal, plastic (e.g., polyethylene, polypropylene, polycarbonate), bottles, flasks, vials, ampules, paper, envelopes, or other types of containers, carriers or supports. Where a kit comprises a plurality of components, at least a subset of the components (e.g., two or more of the plurality of components) or all of the components may be physically separated, e.g., comprised in or on separate containers, carriers or supports. The components comprised in a kit may be sufficient or may not be sufficient for carrying out the specified methods, such that external reagents or substances may not be necessary or may be necessary for performing the methods, respectively. Typically, kits are employed in conjunction with standard laboratory equipment, such as liquid handling equipment, environment (e.g., temperature) controlling equipment, analytical instruments, etc. In addition to the recited binding agents(s) as taught herein, such as for example, antibodies, hybridization probes, amplification and/or sequencing primers, optionally provided on arrays or microarrays, the present kits may also include some or all of solvents, buffers (such as for example but without limitation histidine-buffers, citrate-buffers, succinate-buffers, acetate-buffers, phosphate-buffers, formate buffers, benzoate buffers, TRIS (Tris(hydroxymethyl)-aminomethan) buffers or maleate buffers, or mixtures thereof), enzymes (such as for example but without limitation thermostable DNA polymerase), detectable labels, detection reagents, and control formulations (positive and/or negative), useful in the specified methods. Typically, the kits may also include instructions for use thereof, such as on a printed insert or on a computer readable medium. The terms may be used interchangeably with the term "article of manufacture", which broadly encompasses any man-made tangible structural product, when used in the present context.

[0543] The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1 - Identification of CD8+ TIL sub-types in melanoma patients treated with checkpoint blockade therapy

[0544] Applicants obtained samples from melanoma patients receiving checkpoint blockade therapy both before they received treatment and after they received treatment with a checkpoint inhibitor (3 patients were treated with anti-CTLA4, 2 were treated with a combination of anti-CTLA4 + anti-PD1 and the rest were treated with anti-PD1).

[0545] Single immune cells from these patient samples were each sequenced by RNA-seq and computational analysis was performed on each immune cell type to determine whether there was a correlation between immune cells and how well or not well the patient responded to the treatment. Figure 1 shows the mean expression of genes in the single cells and shows the variability of gene expression between single cells. A threshold was established, such that genes with $(\text{var}|\text{mean}) > 6$ and genes that were expressed in at least 5% of the cells were selected. About 4000 genes were selected and the results were robust to this threshold. Dimension reduction is performed such that the genes with the most variance are used to further cluster the cells (e.g., tSNE analysis). Applicants performed tSNE analysis using tSNE1 based on the most variable genes in CD8+ cells (Figure 2). tSNE1 is correlated with the number of expressed genes (Figure 2, left). As a control, expression of CD8A|CD8B is not correlated with tSNE score (Figure 2, middle and right). In other words, the level of expression of the genes in tSNE1 did not correlate to expression of CD8A|CD8B.

[0546] CD8+ TILs were clustered into two groups (dark - G1; light - G2) according to tSNE1 and tSNE2 (Figure 3). Starting with two clusters, applicants clearly see one cluster (G1) enriched with inhibitory receptors and the other one (G2) enriched with memory and differentiation genes (Figure 4). Group 2 CD8+ TILs are not functional.

Example 2 - The ratio of CD8+ TIL subtypes correlates to response to checkpoint blockade therapy

[0547] Applicants discovered a correlation in the CD8+ T cell context in that group 1 was enriched in non-responders to checkpoint blockade therapy and group 2 was enriched in responders to checkpoint blockade therapy (Figure 5). There are some intermediate cells between the two clusters, but in this analysis Applicants divided the cells only into two clusters. For each patient Applicants measured the ratio between responder and non-responder cells. Overall there are more responder cells in responders and vice versa. Outliers in the non-responder group were deficient in expression of genes associated with antigen presentation (e.g., B2M and ULA).

[0548] Applicants determined combinations of genes corresponding to the non-responder gene signature, such that the combinations of genes detected in the "non-responder" subpopulation can be used to distinguish "non-responder" from "responder" CD8+ T Cells. One combination comprises LAYN, GEM, VCAM1, RDH10, TNFRSF18, FAM3C, AFAP1L2, KTR2DL4, MTSS1, ETV1, CTLA4, MY07A, ENTPD1, TNFRSF9, CADM1, DFNB31, CXCL13, HAVCR2, GPR56, GOLEVI4, NABI, PHLDA1, TGIF1, SEC14L1,

IGFLR1, NAMPTL, PAM, HSPB1, TNIP3, BPGM, TP53INP1, TRPS1, UBE2F, NDFIP2, PON2, PELII, METRNL, SNAP47, APLP2 and/or PDCD1. Another combination comprises the genes in ranked order CD38, CCL3, VCAM1, MY07A, GOLFM4, HAVCR2, MCM5, NDFIP2, WARS, STMN1, LSM2, PRDX3, MTHFD1, SKA2, ENTPD1, SNAP47, FASLG, IFI35, PTTG1, DNPH1, EPST11, UBE2F, NMI, ACP5, CCR5, TRAFD1 and PDCD1. In preferred embodiments, the combination of non-responder signature comprises the genes in ranked order CD38, CCL3, VCAM1, GOLIM4, HAVCR2, PRDX3, ENTPD1, PTTG1, CCR5, TRAFD1, PDCD1, CXCR6, BATF, PTPN6, LAG3 and CTLA4. In certain embodiments, the non-responder signature comprises one or more genes, starting at the first gene and ending at the last gene, according to the ranked order.

[0549] Applicants determined that the ratio of CD8+ TILs having a non-responder signature and responder signature could be used to predict the response to checkpoint blockade therapy. Applicants measured CD8+ TIL populations in pre-treatment and post treatment samples (Figure 5). The responder subpopulation was enriched in samples from patients who responded to checkpoint blockade therapy and the non-responder subpopulation was enriched in samples from patients who did not respond. More than one or two known inhibitory receptors are expressed on the non-responder cells. In the non-responder signature, there are receptors that could be used as potential targets (e.g., KIR2DL4 and ENTPD1). Targeting more than one receptor can be more effective than a single checkpoint blockade therapy.

Example 3 - Further cluster analysis of CD8+ TIL sub-types

[0550] Applicants performed further clustering analysis and determined that the "non-responder" cluster can be split into two clusters (G2 & G3) (Figure 6). Both clusters express co-inhibitory receptors, but a subset of the cells (G2) also have a high expression of cell cycle genes (Figure 7)

[0551] Applicants performed cell cycle analysis of CD8+ TILs (Figure 8, 9). G1\S and G2\M scores are based on the average expression of genes from Tirosh, L, et al. (2016, Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. Science 352, 189-196). Cells with a high G1\S and G2\M score correspond to the cell cycle cluster.

[0552] Further clustering analysis indicated that the CD8+ TILs can be divided into 6 clusters (Figure 10). Expression of genes in each cluster is shown in a heatmap (Figure 11). Expression of genes in G1 related to activation (response to stimulation), G2 related to

exhaustion genes, G4 related to cell cycle genes, G5 related to memory\differentiation genes, and G6 related to heatshock\stress response genes.

[0553] Exemplary gene lists include "JCI" related to exhaustion in CD8 cells melanoma patients (Baitsch, et al., J Clin Invest. 2011;121(6):2350-2360. doi:10.1172/JCI46102), "Smith" related to autoimmunity (McKinney et al., Nat Med. 2010 May; 16(5): 586-91, 1p following 591. doi: 10.1038/nm.2130) and "Held" related to TCF7 and memory (Utzschneider et al., Immunity. 2016 Aug 16;45(2):415-27. doi: 10.1016/j.immuni.2016.07.021). Focusing on the gene lists: the JCI list is enriched with cluster #2 ($P=4.3e-11$) and the Smith list is enriched with cluster #4 ($P=1.7e-9$). The MSigDB includes 4872 gene sets associated with immunity. Dozens to hundreds of them correspond with the clusters described herein.

[0554] Applicants analyzed the enrichment for metabolic functions. Applicants analyzed the fraction of cells in each cluster that were significantly enriched with a metabolic pathway (Figure 12). The exhaustion (#2) and cell cycle (#4) clusters had the highest degree of metabolic activity with many ophox expressed genes. Applicants analyzed transport reaction activity across the clusters. Figure 13 shows levels of metabolites associated with the transporter genes. Figure 14 shows expression of transporter genes expressed in the different clusters.

Example 4 - Isolation, depletion and enrichment of CD8+ TIL sub-types from melanoma samples.

[0555] Modulation of the ratio of responder to non-responder CD8+TILs may be used in the treatment of cancer patients (e.g., adoptive cell therapy, CAR T cells). Applicants isolated the non-responder and responder CD8+ TIL populations from melanoma tumors. The isolation may be used to enrich responder populations or deplete non-responder populations, preferably in adoptive T cell transfer and CAR-T cells therapies. Isolation may be performed using population specific surface markers.

[0556] Specifically, Applicants isolated the different populations of cells from patient samples, performed scRNASeq and mapped them back to the clusters originally found using tSNE analysis (Figure 19 and 20). For isolation of cells from cluster G2 Applicants used CD45+CD3+CD8+CD39+Tim3+ antibodies and for cluster G5 Applicants used CD45+CD3+CD8+PD1+Tim3+ antibodies. In this tSNE plot G2 and G5 represent cells from the two exhaustion clusters. Applicants sorted for cells using CD45+CD3+CD8+CD39-Tim3- markers and CD45+CD3+CD8+PD1-Tim3- markers and obtained cells from cluster

G1 in this tSNE plot. G1 in this tSNE analysis represents effector/memory cells (e.g., responder). Thus, cells from the exhaustion cluster (non-responder gene signature) and effector/memory cluster (responder gene signature) can be isolated and enriched. These results were validated in 3 different patients. In other words, Applicants sorted cells that should be in clusters G2, G5 and G1 based on discriminative markers identified in the original scRNAseq. Applicants then used scRNA-seq to show that the sorted cells map back to the original G2, G5 and G1 clusters, demonstrating that the markers work and that the clusters are reproducible.

Example 5 - Identifying marker genes

[0557] In an exemplary embodiment, Applicants perform the following steps for identifying marker genes:

1. Given a cluster i and a gene j, Applicants apply a fisher test using the number of cells expressing gene j in cluster i vs. the number of cells expressing gene j in cells that do not belong to cluster i.
2. Removing genes that have a low expression in both groups.
3. Considering only genes that pass Bonferroni correction and $\log(FC) > 0.5$.
4. Sorting by log Fold-change (genes that are highly expressed in cluster i are ranked the highest).

Example 6 - T cell receptor (TCR) analysis

[0558] Figure 15 shows a TCR pipeline for identifying TCRs. Figure 16 shows clonal expansion of TCRs pre- and post-treatment with checkpoint blockade therapy (left) and clonal enrichment of TCRs detected in the same patient at single time points (right).

Example 7 - $\delta\gamma$ T-cell analysis

[0559] Applicants determined that $\delta\gamma$ T-cells are Enriched in CD4/CD8 double negative (DN) T cells (Figure 17). Applicants observed no difference between responders and non-responders in terms of V1 and V2 (Figure 18). V δ 1 T-cells have a high expression of inhibitory receptors and V δ 2 T-cells have a higher expression of KLRB1 and other genes.

Example 8 - TCF7 expression distinguishes between Responder and Non-Responder Patients

[0560] Tcf7 is also known as TCF-1 (encoded by *Tcf7*), and as used herein Tcf7 refers to the human gene, mouse gene and all other orthologues. Tcf7 may refer to the genes identified by the accession numbers NM_009331.4, NM_001313981.1, NM_003202.4, NM_213648.4, NM_201634.4, NM_00134851.3, NM_201632.4, NM_001346425.1, and

NM_001346450.1. TCF-1 is known as a signal-dependent transducer of environmental signals from the Wnt pathway via β -catenin (Rothenberg, Curr Opin Immunol. 2012 Apr;24(2): 132-8).

[0561] Applicants show that tumor infiltrating CD8 T cells corresponding to CPB therapy responders and non-responders cluster into two distinct groups (G1 or G2 in this plot) (Fig. 21a). Applicants identified a responder gene signature (G1 in this figure) and a non-responder gene signature (G2 in this figure). The expression of G1 and G2 genes in CD8 T cells are mutually exclusive, such that high expression of G1 is associated with low expression of G2, while high expression of G2 is associated with low expression of G1 (Fig. 21b). The ratio of G2/G1 expression on CD8 T cells can distinguish responders and non-responders before and after treatment (Fig. 21c). All of the responder patients had functional antigen presentation and the IFN gamma pathway, thus showing that these pathways are required to have a response to CPB therapy. The non-responders in the base line samples with a G2/G1 below 1 (i.e., G1 expression is higher than G2) both had defective antigen presentation and IFN gamma pathways. All of the patients with defective antigen presentation and IFN gamma pathways were non-responders and had G2/G1 ratios below 1. The responder and non-responder signatures can predict overall survival in cancer. Patients with low or high expression of G1 (responder signature) have shorter or longer overall survival (Fig. 21d). TCF7 is a transcription factor expressed in G1 and can be used alone to predict outcomes in melanoma. Immunofluorescence images stained for CD8 and TCF7 show more TCF7+ cells in a responder patient than in a non-responder patient (Fig. 21e). The percentage of CD8+ cells and the ratio of TCF+/TCF- CD8+ cells are calculated for the responder and non-responder patient.

[0562] Figure 22 shows immunofluorescence imaging and calculation of TCF7 positive CD8 cells using CellProfiler and a novel pipeline (see, e.g., Carpenter et al., (2006) CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biology 7:R100. PMID: 17076895; and Kamentsky et al., (2011) Improved structure, function, and compatibility for CellProfiler: modular high-throughput image analysis software. Bioinformatics 2011/doi. PMID: 21349861 PMCID: PMC3072555). Responders (Fig. 22b) and non-responders (Fig. 22c) were assayed. The responders had consistently more TCF7+ cells as indicated by the CellProfiler scores.

[0563] **Immunofluorescence assay and analysis.** Multiplex staining was performed on 4 μ m formalin-fixed paraffin-embedded sections using the Opal multiplex IHC system

(PerkinElmer; NEL800001KT) according to the manufacturer's instructions. Briefly, slides were baked for 1 hour at 65C followed by deparaffinization with xylene and a graded series of ethanol dilutions (100%, 95% and 70%), fixation with 10% neutral buffered formalin for 30 minutes, microwave antigen retrieval using the AR9 buffer (PerkinElmer; AR900250ML), and blocking. Primary antibodies used for staining were: CD8a (Biolegend; C8/144B; 372902; 1:100) detected with OPAL520 (1:100; Cy2); TCF7 (Cell Signaling; #2203; 1:100) detected with OPAL690 (1:100; Cy5.5). Counterstain was done using DAPI (1:1000) and subsequently mounted using Vectashield (Vectra; H-1000) fluorescence media. Slides were imaged using the Olympus 1X83 confocal microscope by scanning 10 random fields on each sample at 40X magnification, and analyzed with CellProfiler 2.2.0 (ref-PMID: 17076895) to detect the total number of nuclei, CD8⁺, TCF7⁺, and CD8⁺TCF7⁺ cells. Due to cellular heterogeneity between different slides/patients, in each sample the percentage of CD8⁺TCF7⁻ or CD8⁺TCF7⁺ was calculated out of the total nuclei detected. For the analysis, a new pipeline was made for detection of cells positive for CD8 and TCF7 (see below).

Example 9 -Single cell profiling of immune cells in patients treated with checkpoint inhibitors

[0564] To analyze the properties of immune cells associated with successful or failed checkpoint therapies, Applicants performed scRNA-seq on 48 tumor biopsies from 32 metastatic melanoma patients treated with checkpoint therapy (with 37 anti-PD1; and 11 anti-CTLA4+PD1 samples). This cohort included 11 patients with longitudinal biopsies taken at baseline and during treatment, 1 patient with 2 biopsies taken at one time point, and 20 patients with 1 sample each, taken at baseline or during treatment (**Figure 23A** and **Table 1**). Applicants used the following patient response categories defined by RECIST criteria: complete response (CR) and partial response (PR) for responders, or stable disease (SD) and progressive disease (PD) for non-responders ²⁰. However, to relate molecular and cellular variables with responses of the 48 lesions to therapy, Applicants focused on individual samples and classified them based on radiologic tumor assessments into two categories: progression/non-responder (NR, n=31, including SD/PD samples) or regression/responder (R, n=17, including CR/PR samples), which enabled us to associate response with molecular signatures at the single sample level (**Table 1**). Profiling was performed on 19,392 sorted CD45⁺ cells using an optimized version of the full length Smart-seq2 protocol ²¹, with a median of ~1.4 million paired-end reads per cell. A total of 16,291 sequenced cells passed quality control with a median of 2,588 genes detected per cell, and were used for downstream

analysis (**Methods**). Whole exome sequencing (WES) was available for 20 patients, with 4 that had mutations in *B2M*, *JAK1*, *STAT1* and *IFNGR1* (**Table 1**), recently reported as mechanisms for primary or acquired resistance to checkpoint therapy in melanoma¹³⁻¹⁵.

Example 10 - The immune cell composition of melanoma tumors and their association with clinical outcome

[0565] To define the immune landscape in an unbiased manner, Applicants initially performed unsupervised clustering of cells (on 16,291 cells that passed quality control, based on the ~4,000 most variable genes across all cells) using k-means clustering with a correlation distance metric (**Methods**). After testing for the robustness of this clustering solution and relationships to previously known cell types, Applicants identified 11 clusters that included, 2 B-cell clusters (G1- B-cells; G2 - plasma cells), 2 myeloid clusters (G3 - monocytes/macrophages; G4 - dendritic cells) and 7 clusters enriched for T/NK/NKT cells (G5-11), accounting for most of the immune infiltrate detected within the cohort (**Figure 23B-C** and **Table 2**). Applicants then tested whether these clusters change between baseline and post-therapy samples, or between responder and non-responder tumors. While differences in cluster frequencies were detected when looking at the single patient level between baseline and post-treatment samples (**Figure 29A**), no statistically significant changes in cluster frequencies were seen when Applicants compared all aggregated baseline to all aggregated on-treatment samples, or when only looking at patients with matched longitudinal samples (**Figure 29B-C**). However, when analyzing by clinical outcome, Applicants found that one cluster is significantly enriched in responder lesions while 4 clusters are enriched in non-responder lesions. Specifically, those include G1 (B-cells; Two-sided Wilcoxon P-value P=0.003), G3 (monocytes/macrophages, P-value=0.003), G4 (dendritic cells, P-value=0.015) G6 (Exhausted CD8⁺ T-cells, P-value=0.005) and G11(Lymphocytes exhausted/cell cycle, P-value=1.33x10⁻⁵; **Figure 23D** and **Figure 29D**). While both G6 and G11 clusters were enriched for genes linked to T-cell exhaustion, with differentially higher expression of co-inhibitory receptors {*LAG3*, *PDCD1*, *CD38*, *HAVCR2*, *TIGIT* and *ENTPD1*}; G11 was also enriched for cell cycle genes (negative regulators: *CASP3*, *CDK2*, *BRCA2*, *RBI* and *TP53*; positive regulators: *CDK1*, *CCNB1*, *MKI67*, *CDK4*, *CDCA5* and *TOP2A*) (**Table 2**). Consistent with these results, when using previously defined signatures for T-cell exhaustion^{7,22} (**Table 3**), Applicants observed a significant enrichment of T-cells with an exhausted signature in non-responder lesions (two-sided Wilcoxon P-value=0.002) and with an activated signature in responder lesions (R-value=2x10⁻⁴), but not

when these signatures were compared between baseline and post-therapy samples (**Figure 23E** and **Figure 30**).

[0566] Since the clusters (G5-G1 1) from the unsupervised analysis did not separate specific cell types, but mostly cell states, likely due to the shared transcriptional programs between T, NK and NKT cells^{23,24}, Applicants also determined the composition of known cell types using pre-defined markers (**Table 3** and **Figure 31** and **32**). In agreement with the unsupervised analysis, Applicants found a significant enrichment of B-cells in responder lesions (i^2 -value=0.004) and of myeloid cells in non-responder lesions (i^2 -value=0.002; **Figure 23F**). Moreover, Applicants observed a significant enrichment of CD8⁺ memory T-cells in responder lesions (P-value=0.001, **Figure 23G**). No significant differences in the composition of known cell types were detected between baseline and post-treatment samples when samples were aggregated together. However, changes were observed when looking at the single patient level (**Figure 31** and **32**). Next, when comparing all 11 clusters identified in the unsupervised analysis to the pre-defined markers, Applicants found very high correspondence between clusters G1-G4 and the pre-defined B/myeloid cell markers. In contrast, clusters G5-G1 1 did not show strong correspondence to a specific cell type, suggesting that different lymphocytes (e.g. T, NK and NKT cells) share similar cell states of exhaustion, activation, cytotoxicity and memory (**Figure 33**). Finally, Applicants wanted to leverage the unbiased approach to identify not only clusters but also specific markers associated with response. To that end, Applicants used two different strategies : (1). focusing on cluster discriminating genes, and examining whether they are differentially expressed between responder and non-responder samples (**Table 4**; **Figure 23H** and **Figure 34**); and (2). examining all genes in an unbiased manner and searching for differentially expressed genes (from all cells) between responder and non-responder samples (**Table 5** and **Figure 35**). Using these two different approaches (cluster specific and non-specific) Applicants identified an overlap for markers significantly enriched in responders (*PLAC8*, *LTB*, *TCF7* and *CCR7*) and non-responder (*CCL3*, *CD38* and *HAVCR2*) samples. Applicants conclude that the methods used here , reveal novel cell states and markers that associate with the clinical outcome of individual tumors to therapy, demonstrating the power of unbiased approaches to find molecular correlates of response.

Example 11 - Unbiased definition of CD8⁺ T-cell states and their association with response to therapy

[0567] Based on the significant association of T-cell states and markers with clinical response using either unsupervised or supervised analyses, their highest abundance within the cohort, and due to the dependency of checkpoint therapies on CD8⁺ T-cell recognition of tumor antigens presented by human leukocyte antigen (HLA) class-I complexes ^{5,25}, Applicants next focused the analysis on CD8⁺ T-cells. Clustering of all CD8⁺ T-cells (n=6,350) by k-means clustering revealed 2 cell states:CD8_G with increased expression of genes linked to memory, activation and cell survival (*IL7R*, *TCF7*, *REL*, *FOXP1*, *FOSL2* and *STAT4*) ²⁶ and reduced expression of co-inhibitory molecules; and CD8_B enriched for genes linked to cell exhaustion (*CD38*, *HAVCR2*, *ENTPD1*, *PDCD1*, *BATF*, *LAG3*, *CTLA4* and *PTPN6*) (**Figure 24A-B** and **Table 6**). When annotating these two clusters to the 11 clusters identified by the unsupervised analysis (**Figure 23B**), CD8_G cells were distributed primarily in G10 (memory T-cells), G5 (lymphocytes) and G8 (cytotoxicity) clusters, and CD8_B cells were localized mainly in clusters G11 (lymphocytes exhausted/cell-cycle), G6 (exhausted CD8⁺ T-cells) and G9 (exhausted/HS CD8⁺ T-cells; **Figure 36**). A central question is whether these states are associated with clinical outcome and what is their predictive power to distinguish responding from non-responding tumors. When comparing between these two cell states, Applicants found a significant enrichment for CD8_G in responding lesions (two-sided Wilcoxon *P-value*= $\backslash .4\times 10^{-6}$) and CD8_B in non-responding lesions (*P-value*=0.0058; **Figure 24C**). While cells with both states coexist in each of the responder and non-responder lesions, an overall higher proportion of CD8_G cells is found in responders, and CD8_B cells in non-responders (**Figure 37**). Thus, Applicants decided to calculate the ratio between the number of cells in these 2 clusters and observed a significant separation between responders (CD8_B/CD8_G<1) and non-responders (CD8_B/CD8_G>1) when looking at all samples, as well as baseline or post-treatment samples separately (**Figure 24D**). However, 9 non-responding lesions had unexpected ratios (CD8_B/CD8_G<1), and were more enriched for CD8_G. Applicants hypothesized that although these patients might have productive immunity, they had developed *de novo* resistance to checkpoint therapy. To dissect the genetic alterations associated with resistance, Applicants performed WES followed by immunohistochemistry and flow cytometry, and observed that 6 out of 9 samples (no DNA or slides were available for the 3 remaining lesions) showed complete loss of *B2M* or *HLA-A,B,C* (class-I), recently reported as a mechanism of resistance to checkpoint inhibition therapy in melanoma ^{14,15} (**Table 1** and **Figure 38A-B**). An analysis of predictive performance for the identified signatures demonstrated excellent predictive power when

considering all samples (AUC of ROC= 0.87; one-sided Wilcoxon P-value=1.1x10⁻⁵). However, when excluding the 6 samples deficient for *B2M* or *HLA-A,B,C*, the predictive power was increased significantly (AUC of ROC=0.96; P-value = 3.8x10⁻⁷, **Figure 38C-D**).

[0568] Similarly to the analysis performed on all immune cells, Applicants sought to identify specific CD8⁺ markers associated with clinical outcome. To that end Applicants focused on CD8_G and CD8_B top discriminating genes and examined whether they are differentially expressed between responder and non-responder samples. Applicants identified *TCF7* and *IL7R* as the top two CD8⁺ markers to be significantly associated with response, the first being also significant in the initial marker analysis when looking at all CD45⁺ cells. Moreover, Applicants found a -45% overlap between markers associated with non-responder lesions in CD8⁺ T-cells and all immune cells (*CD38*, *PDCD1*, *CCL3*, *SNAP47*, *VCAM1*, *HAVCR2*, *FASLG*, *ENTPD1*, *SIRPG*, *MYO 7A*, *FABP5*, *NDUFB3*, *UBE2F*, *CLTA* and *SNRPDL*; **Figure 24E** and **Table 7**). Overall, the results suggest that the abundance of CD8_G and CD8_B cellular states could be critical to the success of checkpoint therapy.

Example 12 - Elevated frequencies of CD8⁺TCF7⁺ T-cells are associated with outcome in a second independent anti-PD1-treated cohort

[0569] Since the ratio between CD8_G and CD8_B was significantly associated with response, , Applicants considered whether any of the specific markers identified in these clusters could be used to predict response to treatment in a second cohort, using a different approach that could easily be applied in the clinic. Applicants selected the proteins CD8a and transcription factor 7 (*TCF7*) for this analysis, because *TCF7* was the only top marker linked to response when analyzing all immune or only CD8⁺ T-cells (**Figure 23H**; **Figure 35** and **Figure 24E**), differentially expressed in CD8_G (4-fold higher expression than in CD8_B), and since all lymphocytes related clusters that were associated with response were either enriched for or completely composed of CD8⁺ T-cells (**Figure 23** and **24** and **Figure 33**). *TCF7* is part of the Wnt/p-catenin signaling pathway ²⁷ and has been shown to be crucial for differentiation, self-renewal and persistence of memory CD8⁺ T-cells ²⁸, as well as reinvigoration and effective immunity of CD8⁺ T-cells against chronic LCMV infection upon anti-PD1 treatment ²⁹³⁰. Thus, Applicants considered its association with response in a second cohort of 33 patients (n=43 samples) treated with anti-PD1 (**Table 8**). Using immunofluorescence staining followed by automated image analysis with CellProfiler ³¹ (**Figure 24F**, **Figure 39** and **pipeline**), Applicants calculated the ratio of TCF7⁺CD8⁺ to TCF7⁻CD8⁺ cells and its association with response. When comparing between these two cell

phenotypes Applicants found a significant enrichment for TCF7⁺CD8⁺ in responding patients (two-sided Wilcoxon $P\text{-value}=3.9\times 10^{-6}$) and TCF7⁻CD8⁺ in non-responder patients ($P\text{-value}=1.1\times 10^{-8}$; **Figure 24G**), and saw that cells with both states coexist in each of the responder and non-responder lesions (**Figure 40**). Analogous to the CD8⁺ single-cell RNAseq analysis Applicants performed (**Fig 24D**), Applicants found that a ratio >1 of TCF7⁺CD8⁺ to TCF7⁻CD8⁺ is typically associated with clinical response and a ratio <1 with lack of response when looking at all (n=43; one-sided Wilcoxon $P\text{-value}=2.4\times 10^{-6}$; **Figure 24H**), baseline (n=24; $P\text{-value}=0.001$) or post-treatment (n=19; $P\text{-value}=1\times 10^{-4}$; **Figure 41A**) samples. In contrast, no significant difference was observed when looking only at the percentage of tumor-associated CD8⁺ T-cells between responding and non-responding patients (**Figure 24I**). Consistent with the ability to predict lesion-level responses using the single-cell RNAseq-derived signatures in the first cohort, the power to predict response was similar in this independent cohort for all (AUC=0.91; **Figure 41B**), baseline (AUC=0.88; **Figure 41C**) or post-treatment samples (AUC=0.98; **Figure 41D**). Additionally, , when performing a Kaplan-Meier survival analysis Applicants found that patients with a ratio >1 have a significantly higher survival rate as compared to those with a ratio <1 (logrank $P\text{-value}=0.03$, **Figure 24J**).

[0570] Finally, Applicants asked if TCF7 protein levels changes overtime between matched baseline and post-treatment samples in the same patient. 8 patients out of 33 in the second cohort had matched baseline and post-treatment samples (**Table 8**). Although minor differences were detected in TCF7⁺CD8⁺ levels between baseline and post-treatment samples, the main change was observed when classifying samples by their response or lack of response to therapy, regardless to the fact if the sample was taken at baseline or during/after treatment (**Figure 40** and **Figure 42A**). Moreover, immunofluorescence staining of additional 7 samples (n=4 patients), initially analyzed in the single-cell RNAseq cohort revealed similar pattern between the TCF7⁺CD8⁺/TCF7⁻CD8⁺ ratio detected when performing the immunofluorescence staining pipeline to the CD8_B/CD8_G ratio Applicants identified when analyzing the single-cell RNAseq data (**Figure S9**, **Figure 42B** and **Table 8**). Collectively, the results suggest that the ratio between CD8⁺ T-cell subsets with distinctive phenotypes is a predictor of clinical outcome and survival.

Example 13 - Higher resolution analysis of CD8⁺ T-cells discovers novel exhausted and memory subsets

[0571] While the two CD8⁺ T-cell clusters were able to separate responders from non-responders, Applicants wondered if a greater heterogeneity in cell states could be observed in the single cell dataset. Using k-means clustering with a correlation distance metric on all CD8⁺ T-cells that passed quality control, and after testing for the robustness of this clustering solution (0.89; **Methods**) and relationships to previously known cell states, Applicants found 6 clusters, with CD8_G and CD8_B, each splitting into 3 clusters (**Figure 25A-B**). To identify cluster-specific gene markers, Applicants compared the expression level of genes associated with cells within a given cluster, to that of cells outside this cluster (**Methods**). Applicants found that these 6 new clusters included a mixture of known and novel exhaustion and memory/effector markers and were linked to treatment outcome (**Figure 25B**). CD8_1 expressed multiple markers of exhaustion (*HAVCR2*, *ENTPD1*, *PDCD1* and *PTPN11*) and negative (*CASP3*, *CDK2*, and *TP53*) or positive (*CDKI*, *CCNB1* and *MKI67*) regulators of cell cycle (**Table 9**), similar to the pattern observed in cluster G12 when analyzing all immune cells (**Table 2**), and to the transcriptional phenotype of terminally exhausted CD39⁺ (*ENTPD1*) CD8⁺ T-cells, detected during chronic infection with hepatitis C virus (HCV) ³². While CD8_2 expressed many exhaustion markers together with heat shock proteins (*HAVCR2*, *CTLA4*, *TIGIT*, *PDCD1*, *HSPB1*, *HSPA1A* and *HSPA4*) along with additional inhibitory receptors (*ENTPD1* and *KIR2DL4*), CD8_3 expressed several known exhaustion markers (*HAVCR2*, *CD38*, *PDCD1* and *PTPN6*), but lacked the expression of heat shock proteins and cell-cycle genes. In contrast, CD8_4 (*CCR7*, *IL7R*, *TCF7*, *TNF* and *S100A10*), and CD8_6 (*SELL*, *TCF7*, *LTB*, *IL7R*, *FLT3LG*, *IL16*) had a memory/effector like phenotype, while CD8_5 had the phenotype of early activated and more differentiated cells (*IL6ST*, *CXCL13*, *IL7R* and *CTLA4*), but was more enriched for *HAVCR2* and *PDCD1* when compared to CD8_4 and 6, but not CD8_1 to 3 (**Figure 43**). Interestingly, *GZMA*, *GZMB* and *PRF1* (coding for granzyme A, B and perforin 1), had much higher expression in the exhausted clusters CD8_1 to 3 (**Table 9**) and resembled exhaustion programs previously reported in melanoma ³³ and in a mouse model of chronic LCMV infection ⁷. Interestingly, when examining the clustering results when using fewer clusters, Applicants found that the clusters formed a hierarchy, that is, clusters split and did not mix as the number of clusters increased. To further examine the robustness and unique biological function of each of these clusters, Applicants took another approach, in which Applicants identified gene markers that are differentially expressed between the cluster and an "ancestor" cluster found in a lower-resolution cluster analysis, thus creating a functional annotation along the hierarchical

branching for the identified clusters (**Figure 44A**). Using this approach, similar marker genes were identified for the 6 different clusters, suggesting that these markers indeed highlight distinct biological functions (**Table 10**). Next, Applicants sought to associate these clusters with clinical outcome. When comparing between these 6 cell states Applicants found CD8_1 and CD8_3 to be significantly enriched in non-responder lesions (one-sided Wilcoxon *P-value*=0.001 for CD8_1 and *P-value*=0.013 for CD8_3) and CD8_5 in responder lesions (*P-value*=0.003; **Figure 25C**); analogous to the earlier observation showing an increased exhausted phenotype in non-responders and a memory/effectector phenotype in responders (**Figure 24**).

[0572] During cancer or chronic infections, T-cells in response to continuous stimuli undergo many transitions dictating cell fate, from initial activation and proliferation, to differentiation, exhaustion and in some cases reinvigoration, depending on the appropriate stimulation and state of the cells^{8,30}. To determine the potential trajectories underlying transitions between the identified cell states, Applicants performed a trajectory analysis using the Monocle tool³⁴. Although Applicants could not ascertain the direction of differentiation, Monocle ordered individual cells in pseudotime, placing them along a primary trajectory branch corresponding to the six clusters identified, along with 2 side branches (**Figure 25D**). While some overlap between the six clusters was observed, the continuous hierarchical ordering was very clear. One end of the main branch was enriched for clusters with a memory/effectector phenotype, starting with CD8_4, CD8_6 and then CD8_5, while the other end was enriched for clusters related to exhaustion, starting with CD8_1 and then moving to CD8_2 and 3, that overlapped each other and shared many transcriptional programs (**Figure 25D** and **Figure 44B-C**). Overall, the six states and their relationships represent novel phenotypic diversity for exhausted and memory/effectector CD8⁺ T-cells subsets associated with clinical response, and suggest that the developmental transitions could be identified among them.

Example 14 - TIM3 and ENTPD1 segregate exhausted from memory cells

[0573] Since a significant enrichment for *TCF7*⁺ cells with a memory/effectector phenotype was found in responder patients, Applicants asked if the single cell data could be used to find markers that discriminate memory/effectector from exhausted clusters, and could aid in defining new surface markers of CD8⁺ T-cell exhaustion in melanoma. One of the targetable proteins, CD39 (*ENTPD1*), found on some of the exhausted subsets was significantly enriched in clusters associated with non-responding tumors. CD39 is an ectonucleotidase that plays an

important role in the adenosine pathway, which in turn modulates the tumor microenvironment by reducing cytotoxicity function of effector (T and NK) cells and by increasing the abundance of suppressive cells (e.g. M2 macrophages, myeloid derived suppressor cells and regulatory T-cells)³⁵. Since TIM3 was co-expressed with CD39, with both having the highest expression level in CD8_2 (**Table 9** and **Figure 45A**) and minimal expression in CD8_4 and CD8_6 (-3% of cells, **Figure 43**) when compared to other exhaustion markers (*LAG3*, *TIGIT*, *PDCD1*), Applicants wanted to test if these markers could be used to isolate and validate the identity of cells corresponding to specific clusters. Applicants used scRNA-seq to profile expression of sorted CD39⁺TIM3⁺ (DP - double positive) and CD39TIM3⁻ (DN - double negative) CD8⁺ T-cells from 4 melanoma patients (**Figure 45B**) and found that the profiles recapitulated the original unsorted clusters (**Figure 26A** and **Figure 45C**). DN shared many attributes with memory cells (CD8_4 and 6), and DP cells appeared similar to CD8_2 exhausted cells. CD39 has been shown to be a marker for terminally exhausted CD8⁺ T-cells in patients with chronic HCV and HIV infections³². However, unlike PDI, CTLA4 and TIM3, CD39 in the context of CD8⁺ T-cell exhaustion in cancer is not well studied. While CD39⁺ and CD39⁻ cells had equal expression of PDI, CD39 turned out to be a key marker that separates all TIM3⁺ from TIM3⁻ cells (**Figure 45D**), the latter being reported as a marker of T-cell dysfunction in cancer and chronic infections³⁶. To determine the functional properties of CD39⁺ and CD39⁻ CD8⁺ T-cells, Applicants prepared single cell suspensions from 12 metastatic melanoma tumors and assessed their ability to produce cytokines in response to TCR stimulation. While CD39⁻ and CD39⁺ cells contained equivalent percentages of IL-2 producing cells, CD39⁺ cells had a significant reduction in both TNF α (unpaired-student's *t*-test p -value=0.0016) and IFNy-producing cells (P -value=5x10⁻⁴; **Figure 26B**). Thus, CD39 delimits a population of exhausted CD8⁺ T-cells in melanoma, and in conjunction with TIM3 can be used to discriminate exhausted from memory/effector cells.

Example 15 - Dual inhibition of TIM3 and CD39 synergistically reduces tumor growth and improves survival

[0574] Although it has been demonstrated that inhibition of the adenosine pathway through targeting of CD73, in combination with checkpoint therapy using anti-PDI and CTLA4 antibodies, can enhance anti-tumor immunity and reduce tumor growth³⁷, inhibition of CD39 in combination with co-inhibitors has not been evaluated. The identification of CD39 and TIM3 as highly expressed genes in exhausted clusters associated with non-

responding lesions, led us to examine the combined effect of CD39 and TIM3 blockade. To that end Applicants used the aggressive B16-F 10 melanoma mouse model. Mice were treated with the small molecule POM-1 to block CD39 activity³⁸, alone or in combination with anti-TIM3 blocking antibodies (**Figure 26C**). While monotherapy with either POM-1 or anti-TIM3 transiently reduced tumor growth until day 14 (with CD39 inhibition having a stronger effect), combination of both caused a dramatic reduction in tumor size, and more importantly, significantly increased survival (50% survival vs. 10% for CD39 inhibition or 0% for anti-TEVI3 and the untreated group) on day 30 (**Figure 26D-F** and **Figure 45E**). Since anti-PD1 treatment is currently the standard of care in metastatic melanoma patients, and since PD1 similarly to TIM3 was one of the top markers associated with lack of response, Applicants asked whether dual inhibition of PD1 and CD39 will increase response and improve survival. Similar to the observations when targeting both CD39 and TIM3 (although to a lesser extent), dual inhibition of CD39 and PD1 significantly reduced tumor burden on day 21 post transplantation, and improved survival when compared to untreated and mono therapy treated mice (**Figure 46**). Hence, through the single cell analysis of human melanoma tumors, Applicants identified new putative immunotherapeutic targets and combinations to enhance immunity.

Example 16 - Chromatin accessibility of melanoma-associated exhausted and memory cells

[0575] Since prospectively isolated CD39⁺TIM3⁺ (DP) and CD39⁻TIM3⁻ (DN) cells recapitulated phenotypes of cells within the previously unrecognized CD8_2 and CD8_4+6 clusters respectively, Applicants asked what are the epigenetic programs that govern these distinctive cell states. To dissect differential regions of open chromatin that corresponded to differences in gene expression, Applicants isolated DP and DN cells from 5 metastatic melanoma patients treated with the checkpoint inhibitor PD-1 and performed assays for transposase-accessible chromatin with next generation sequencing (ATAC-seq) and scRNA-seq³⁹ (**Figure 27A**). scRNA-seq analysis identified exclusive patterns of transcription factor (TF) expression for each of the sorted populations. Applicants found that DN cells had higher expression for several TFs, including *TCF7*, *STAT4*, *FOXP1* and *FOSB*, previously shown to be enriched in stem-cell memory CD8⁺ T-cells²⁶, while DP cells were enriched for *BATF*, *PRDM1*, *TOX*, *HMG2* and *IRF2*, as previously described in exhausted CD8⁺ T-cells^{7,40} (**Figure 27B**). Furthermore, similar patterns of TF expression were identified when compared to the original unsorted single cells separated computationally by expression of CD39 and

TEVI3 at the RNA level, and also in the original CD8_G and CD8_B clusters (**Figure 47A-B**). Analysis of ATAC-seq profiles identified unique patterns of open chromatin regions (OCRs) between DP and DN cells (**Figure 27C**); DP and DN cells sorted from all 5 patients displayed opposite patterns of increased and decreased ATAC-seq peaks openness in specific gene loci (*CTLA4*, *TIGIT*, *PTPRJ*, *IL2*, *ILR7* and *TCF7*) related to exhaustion and memory (**Figure 27D** and **Figure 47C**). OCRs were unequal between the two different cell states; a smaller fraction of OCRs were detected in DN cells (425; Benjamini-Hochberg FDR<0.01) when compared to DP cells (859; Benjamini-Hochberg FDR<0.01), consistent with a previous study showing an increase in CD8⁺ T-cell OCRs as cells differentiate in response to chronic LCMV infection ⁴¹. Next, Applicants sought to identify enrichment for TF motifs that distinguish DP from DN cells using the GOMER approach (**Methods**), comparing differential expression of specific TFs that bind to these motifs. Applicants identified differential peak motif enrichment coupled with high expression for *BATF*, *PRDMI*, *IRF4* and *NFAT5* in the DP cells, and *TCF7* and *FOXP1* in the DN cells (**Figure 27E**). Interestingly, Applicants found that *EOMES*, previously shown to be high in terminally differentiated exhausted cells ^{7,42}, had differential peak motif enrichment in the DN cells, but higher expression in DP cells. Since *BATF* and *TCF7* were the two TFs that had the highest peak motif enrichment and the highest expression in DP and DN respectively (**Figure 27E** and **Figure 47D**), and due to the strong association of *TCF7* with clinical responses, Applicants sought to identify genes that could be regulated by these TFs. To that end, Applicants compared whether differentially expressed genes near significant (Benjamini-Hochberg FDR<0.01) OCRs in DP or DN cells (as defined by GREAT ⁴³) contain enhancers with *BATF* or *TCF7* motifs. Applicants identified 95 genes in DP (16%; including *CXCL13*, *ENTPD1*, *CD38*, *CTLA4* and *HAVCR2*) and 6 genes in DN cells (20%; including *IL7R*, *PLAC8* and *SELL*), out of the total differentially expressed genes (584 for DP and 30 for DN), that meet these criteria (**Figure 27F**), suggesting that both *BATF* and *TCF7* control the expression of some of the key markers unique to each cell state. Overall, the analysis defined epigenetic programs and key TFs controlling two distinct and novel states of CD8⁺ T-cells associated with clinical outcome in humans, which could have implications for new therapeutic strategies that could increase the chance of durable responses.

Example 17 - TCR analysis identifies different patterns of expansion associated with cell states and clinical outcome.

[0576] Finally, Applicants interrogated the relationship between the clinical response, cell states and T-cell clonality. To do so, Applicants reconstructed T-cell receptor (TCR) sequences from the transcriptomic data using the MiXCR tool⁴⁴ for all identified CD8⁺ T-cells. Applicants defined 4 patterns of TCR clonality based on the CDR3 sequence identified in both α and β chains (**Figure 28A**): 1. persistent- TCRs that were detected in pre- and post-therapy samples from the same patient; 2. enriched- TCRs detected in multiple T-cells at a single time point; 3. singlets- TCRs found in only one T-cell at one time point, and 4. common- TCRs that were shared across patients.

[0577] Since the overall number of persistent TCRs was very low, especially in responders, Applicants could not make many conclusions about their relationships to clinical response. However, Applicants detected a significant enrichment for persistent TCRs in non-responders in clusters CD8_3 (two-sided Wilcoxon *P*-value=0.03) and CD8_6 (*P*-value=0.008) (**Figure 28B**), but not when aggregating exhausted clusters (CD8_1-3) or memory/effector ones (CD8_4-6) (**Figure 28C-D**). Interestingly, very few persistent TCRs were detected in the CD8_5 cluster (which was present predominantly in post-therapy samples) when looking at all patient CD8⁺ cells (**Figure 48A**), suggesting that these T cell clones did not exist prior to therapy.

[0578] While enriched and singlets TCRs had different patterns of distribution across the 6 clusters (with more enriched TCRs in exhausted clusters and more singlet TCRs in effector/memory clusters, **Figure 48B-C**), both were significantly enriched in the same direction for each cluster when comparing responder to non-responder lesions. Applicants detected a significant enrichment for non-responder lesions in CD8_1 (*P*-value=0.003 for enriched and χ^2 -value=0.009 for singlets) and CD8_3 (χ^2 -value=0.03 for enriched and *P*-value=0.02 for singlets), and a significant enrichment for responder lesions in CD8_5 (*P*-value=0.02 for enriched and χ^2 -value=0.004 for singlets) (**Figure 28E-J**). Applicants hypothesize that enriched TCRs are likely to have been exposed to persistent stimulation, explaining their higher proportions in the exhausted than effector/memory clusters, while singlet TCRs are more likely to be newly generated T-cells with lower exhaustion properties.

[0579] Although common TCRs were predominantly present in clusters CD8_2 and 3, no significant association was found with clinical outcome (**Figure 48D-G**). Collectively, this analysis allowed us to connect the transcriptional phenotype of cells and therapeutic outcomes to therapy with TCR clonality, and could aid in investigating T-cell dynamics and cell state plasticity. Indeed, when looking at the transitions of T-cell states (CD8_1-6) within

a specific clone (based on identical TCR sequence) across longitudinal samples in the same patient, Applicants discovered bilateral transitions between exhausted and memory/effectector states.

Example 18 - Discussion

[0580] Although immune checkpoint blockade leads to durable responses in patients with metastatic melanoma, refractory disease and progression after initial response remain major causes of mortality^{3,4}. While many studies have identified different components associated with clinical outcome, the principles of the immune system that underlie the success or failure to checkpoint therapy in humans remain relatively unexplored. To address this issue Applicants performed an unbiased analysis of immune cells using single-cell transcriptomics, to dissect the cellular and molecular determinants of response. Within the tumor, Applicants found specific CD8⁺ T-cell states associated with clinical outcome in melanoma patients treated with checkpoint therapy. The association between the cellular states (as determined by gene expression or antibody staining) and treatment outcome was observed in both baseline and on-therapy samples. Applicants validated these associations in two independent cohorts: one cohort of anti-PD1 patients (n=30) using a signature of memory/effectector T-cells, and a second anti-PD1 cohort (n=21) using the fraction of TCF7⁺CD8⁺ T-cells based on immunofluorescence staining. The analyses also identified novel sub-states of exhausted and memory/effectector T-cells. Among the identified markers of the exhausted sub-states, CD39 (*ENTPD1*) emerged as a promising discriminator of exhausted CD8⁺ T-cells in melanoma patients, and as a potential target when blocked together with TIM3 (*HAVCR2*) using a mouse melanoma model.

[0581] Previous studies have shown that the number of infiltrating CD8⁺ T-cells detected in patient biopsies is significantly enriched in responders. However, some inconsistencies were found between these studies: the first showed that the number of infiltrating CD8⁺ T-cells detected before treatment can predict clinical outcome⁵, whereas the second showed that only early on-treatment, but not baseline quantification is significantly associated with patient response⁶. Despite potential spatial and temporal heterogeneity in biopsy-based studies of cancer, Applicants identified robust predictive markers in baseline and on-treatment samples, reflecting quality metrics of T-cell states (TCF7⁺CD8⁺), rather than the quantity of T-cells (CD8⁺). Indeed, the number of CD8⁺ T-cells was comparable across responders and non-responders within the cohort, consistent with the fact that the T-cell number is not a reliable predictor in pretreatment biopsies⁶. Several other studies found that

the spatial distribution of the T-cells was more important in predicting survival⁴⁵⁻⁴⁷. Hence, further studies are needed to explore the relationship between the spatial distribution of T-cells and the cellular states Applicants identified using highly multiplexed tissue imaging systems.

[0582] One of the key markers expressed in clusters associated with response, which was found in an unbiased manner as a result of data analysis and discriminated responders from non-responders, was the transcription factor *TCF7*. Although, these results are in line with previous studies showing that *TCF7* is required for reinvigorating CD8⁺ T-cells in response to anti-PDI or anti-PDL1 therapies to resolve chronic LCMV infection in mice^{29,30}, its association with clinical outcome in patients treated with checkpoint immunotherapy has not been previously demonstrated. More specifically, it has been shown that *TCF7* is essential for the expansion of CXCR5⁺TFM3⁻ CD8⁺ T-cells (but not TFM3⁺ cells) that are important for the control of the virus³⁰. Indeed, Applicants also found that TEVI3⁻ cells are *TCF7*⁺ and are associated with response; however Applicants did not find CXCR5 expression associated with *TCF7*⁺ cells, suggesting that this chemokine receptor may not be a critical marker for cells associated with response in the melanoma context. Additionally, a recent study suggested that decreased open chromatin regions at *TCF7* sites are associated with reduced activation of non-programmable, dysfunctional PD1^{hi} T-cells⁴⁸.

[0583] Furthermore, the data revealed significant heterogeneity of exhausted CD8⁺ T-cell states, with 2 of the 3 clusters showing stronger association with non-responding lesions. While most studies have focused on PDI, TIM3, LAG3 and CTLA-4 to mark exhausted cells, Applicants identified CD39 as an additional marker for human CD8⁺ T-cell exhaustion in melanoma, and found it to be enriched in non-responsive lesions. While not previously observed in melanoma or tested functionally, this result is consistent with recent findings, demonstrating that CD39 is a marker for terminally exhausted CD8⁺ T-cells in HIV and HCV infected patients³². The results in mice support the targeting of CD39 together with TIM-3 for boosting anti-tumor immunity. Additionally, Applicants found that in all samples, regardless of whether they were collected at baseline or during treatment, there was a heterogeneous mixture of all the identified CD8⁺ T-cell states, but that their proportions were associated with clinical outcome. Further studies will be needed to test whether other immunotherapeutic combinations or targets (e.g KTR2DL4 and CD38), which Applicants found to be enriched in clusters associated with lack of response, could be used to enhance immunity and overcome resistance.

[0584] A critical question that still remains unanswered is how the 6 CD8⁺ T-cell states Applicants identified relate to each other. In line with previously published studies, Applicants propose that cells can transition between memory and exhausted states, and bolster this hypothesis through finding divergent memory and exhaustion phenotypes in clonal T cell populations (determined based on identical TCR sequences). Surprisingly Applicants found that CD8_5 T cells, which were predominantly found in post-therapy tumor samples, hardly share TCRs with the baseline sample, suggesting that members of the CD8_5 cluster are generated outside of the tumor and subsequently migrate to the tumor. This is consistent with observations from a recent study ⁴⁹ demonstrating that lymphoid-organ derived T-cells are required for anti-PD1 potency.

[0585] Despite the identification of key components associated with response, the study has technical limitations and open biological questions that necessitate future studies to address them. First, although Applicants used an optimized version of the full length Smart-seq2 protocol, which has the highest sensitivity and lowest dropout probability compared to other methods ⁵⁰, the resulting transcriptomes need to be interpreted with caution because lack of detection of a transcript in a single cell does not guarantee its absence. Applicants address this dropout challenge by relying on signatures composed of many (not single) genes in order to increase confidence in cluster definition. Second, while the approach of scRNA-seq generates relatively unbiased genome-scale transcriptome data, which is then used to identify novel immune cell states and molecular determinants of response, future studies will need to validate these signatures at the protein level (as Applicants did for TCF7) using highly multiplexed tissue imaging or mass cytometry in melanoma as well as other types of cancer. Third, although Applicants identified T cell states associated with responses, Applicants do not know which cells kill the tumors. Fourth, what are the factors that control the proportions of the identified CD8⁺ cell states in patient samples? Is the responder-associated memory phenotype maintained post treatment? Can cells be reprogrammed from an exhausted to a memory phenotype? While the approach in this study is useful in identifying new components associated with response and useful in generating hypotheses, future mechanistic studies will be required to demonstrate these developmental process. Finally, the study focused on melanoma patients treated with checkpoint inhibitors, but Applicants need to address whether these predictive markers of response are relevant to other types of malignancies or therapies.

[0586] This study addresses the long-standing question of which immune cell states are important for the activity of checkpoint therapy in humans, and provide a powerful tool both to the medical and research communities in several ways: first, the data and analysis enables the prediction of clinical outcome in patients treated with anti-PD1 and may impact clinical trial design and execution, as well as application of this therapy as standard of care in thousands of patients across the world. Second, the identification of new targets, and the subsequent validation of two specific ones in the study, may lead to new trials focused on these targets and other combinations of targets associated with success or failure of therapy in the study (e.g. KIR2DL4 and CD38). Third, the finding that specific memory-like signatures are associated with response —together with recent studies showing that CAR T cell activity is enhanced by generating more memory-like cells using the IL-7 or IL-15 pathways, leading to better outcome in preclinical models^{26,51}—suggests that manipulation of T cells prior to adoptive cell therapies by increasing the ratio of CD8_G to CD8_B (especially in patients with a low ratio) would strongly boost clinical responses when combined with checkpoint therapies.

[0587] Since an increasing number of patients are being treated with checkpoint inhibitors as a standard of care, it has become essential to fully understand the determinants of response. Utilizing the data presented in this study, one can potentially help to optimally select patients for therapy, identify new therapeutic strategies and thus increase the chance of durable responses.

Example 19 - Methods

[0588] Patient samples. Metastatic melanoma patients treated with checkpoint blockade therapy at Massachusetts General Hospital (Boston, MA) and University of Texas MD Anderson Cancer Center (Houston, TX) provided written informed consent for the collection of tissue and blood samples for research and genomic profiling, as approved by the Dana-Farber/Harvard Cancer Center Institutional Review Board (DF/HCC Protocol 11-181) and UT MD Anderson Cancer Center (IRB LABOO-063 and 2012-0846). Matched tumor and normal blood samples were obtained from 23 patients at baseline and/or after checkpoint treatment.

[0589] Sample dissociation. Fresh isolated tumor samples were collected immediately after surgery and were dissociated within 1 hour using the human tumor dissociation kit (Miltenyi Biotec; 130-095-929) with the following modifications. Tissue was minced into small pieces using a scalpel and put into a 1.5ml eppendorf tube containing 100μl of enzyme

H, 50 μ l of enzyme R, 12.5 μ l of enzyme A (all provided in the kit), and 837.5 μ l of RPMI, followed by a 20 minute incubation in a therm omixer (Eppendorf; F1.5) at 37C, 600 rpm. After incubation, debris were removed by filtering through a 70 μ m cell strainer, followed by mincing of the remaining tissue left on the strainer with a plunger in order to increase cell yield. Dissociated cells were subsequently washed with cold IX PBS containing 1.5% heat inactivated FCS, spun down at 1300 rpm, 4°C for 5 minutes, resuspended, and counted for yield and viability with trypan blue using a Countess automated cell counter (Invitrogen).

[0590] Flow cytometry and cell sorting. For both flow cytometry and cell sorting, Human TrueStain FcX (Biolegend, 422302) was used for blocking Fc receptors before labeling cells. To discriminate live from dead cells Applicants used Zombie violet Dye (Biolegend, 77477) for 15 min at 4°C, followed by surface labelling of cells for 30 min at 4°C, using standard protocols. The antibodies used for cell surface labelling were PE anti-human CD45 (Biolegend, 304008), APC anti-human CD3 (Biolegend, 300412), FITC anti-human HLA-A,B,C (Biolegend, 311426), APC/Cy7 anti-human CD235a (Biolegend, 349116), PE/Cy5 anti-human CD3 (Biolegend, 300309), BV421 anti-human PD1 (Biolegend, 329919), PE/Cy7 anti-human TEVI3 (Biolegend, 345013), APC/Cy7 anti-human CD39 (Biolegend, 328226), AF700 anti-human CD4 (Biolegend, 317425), BV650 anti-human CD8 (biolegend, 301041). The antibodies used for intracellular staining were FITC anti-human IFNy (Miltenyi Biotec, 130-097-936), PE anti-human IL2 (Miltenyi Biotec, 130-099-391), APC anti-human TNF (Miltenyi Biotec, 130-099-197). Sorting of cells was performed on a BD Fusion instrument using the following antibody panel: Zombie dye, CD45, CD235a and HLA-A,B,C. CD45 $^{+}$ cells from dissociated samples were sorted into 96-well plates (Eppendorf, 951020401) containing 10 μ l of lysis buffer (TCL buffer, Qiagen 1031576, supplemented with 1% β -mercaptoethanol), sealed, vortexed, spun down at 2500 rpm for 30 seconds, immediately placed on dry ice, and then stored at -80°C until processing with the Smart-Seq2 protocol. For flow cytometry Applicants used the Beckman Coulter CytoFLEX instrument and analyzed the data with FlowJo v10.0.8rl software.

[0591] Single cell RNA sequencing procedure. Libraries from single cell lysates were generated with the Smart-Seq2 protocol ⁵² with some modifications in the reverse transcription step as recently described ²¹. 96-well plates containing cell lysates were thawed on ice, spun down at 1500 rpm for 30 seconds, and mixed with Agencourt RNAClean XP SPRI beads (Beckman Coulter) for RNA purification. Purified RNA was resuspended in 4 μ l of Mix- 1 , denatured at 72°C for 3 min and placed immediately on ice for 1 min before 7 μ l of

Mix-2 was added. Reverse transcription was carried out at 50°C for 90 min, followed by 5 min incubation at 85°C. 14 μ l of Mix-3 was added in each well and the whole-transcriptome amplification step was performed at 98°C for 3 min, followed by 21 cycles at (98°C for 15 sec, 67°C for 20 sec and 72°C for 6 min), and final extension at 72°C for 5min. cDNA was then purified with Agencourt AMPureXP SPRI beads (Beckman Coulter) as described ²¹, to remove all primer dimers residues. Quality control steps were performed on samples before library construction and included the following steps: (1) concentration measurements, using the Qubit dsDNA high sensitivity assay kit on the Synergy HI Hybrid Microplate Reader (BioTek); (2) cDNA size distribution using the High-Sensitivity Bioanalyzer Kit. Libraries were generated using the Nextera XT Library Prep kit (Illumina) with custom indexing adapters ²¹ in a 384-well PCR plate, followed by a cleanup step to remove residual primer dimers. Combined libraries from 384 cells were then sequenced on a NextSeq 500 sequencer (Illumina), using paired-end 38-base reads.

[0592] Immunofluorescence assay and analysis. Multiplex staining was performed on 4 μ m formalin-fixed paraffin-embedded sections using the Opal multiplex IHC system (PerkinElmer; NEL800001KT) according to the manufacturer's instructions. Briefly, slides were baked for 1 hour at 65C followed by deparaffinization with xylene and a graded series of ethanol dilutions (100%, 95% and 70%), fixation with 10% neutral buffered formalin for 30 minutes, microwave antigen retrieval using the AR9 buffer (PerkinElmer; AR900250ML), and blocking. Primary antibodies used for staining were: CD8a (Biolegend; C8/144B; 372902; 1:100) detected with OPAL520 (1:100; Cy2); TCF7 (Cell Signaling; #2203; 1:100) detected with OPAL690 (1:100; Cy5.5). Counterstain was done using DAPI (1:1000) and subsequently mounted using Vectashield (Vectra; H-1000) fluorescence media. Slides were imaged using the Olympus 1X83 confocal microscope by scanning 10 random fields on each sample at 40X magnification, and analyzed with CellProfiler 2.2.0 ³¹ to detect the total number of nuclei, CD8⁺, TCF7⁺, and CD8⁺TCF7⁺ cells. Due to cellular heterogeneity between different slides/patients, in each sample the percentage of CD8⁺TCF7⁺ or CD8⁺TCF7⁺ was calculated out of the total nuclei detected. For the analysis, a new pipeline was made for detection of cells positive for CD8 and TCF7 (**pipeline**).

[0593] Immunohistochemistry. Procedures were done on the automated Ventana Discovery Ultra staining system, using 4 μ m formalin-fixed paraffin-embedded sections. Sections were deparaffinized in xylene and graded alcohols, followed by antigen retrieval (EDTA), blocking with Discovery inhibitor (Ventana; 760-4840), incubation with primary

antibodies for 16 minutes, washing and incubation with a secondary antibody conjugated with horseradish peroxidase (HRP). Sections were developed with discovery purple chromogen kit (Ventana; 760-229) and were then counterstained with hematoxylin. Primary antibodies used were: B2M (Abeam; ab27588; 1:1000); anti melanoma triple cocktail (Ventana; 790-4677; 1:100) containing antibodies against melanosome (HMB45), Mart-1/melan A (A103), tyrosinase (T31 1). The melanoma triple cocktail was used to separate tumor from normal cells enabling detection of B2M in the cancerous cell fraction.

[0594] Intracellular cytokine detection. For intracellular cytokine analysis of human CD8⁺ T-cells, 5x10⁵ cells from dissociated samples (n=12) were cultured in the presence of soluble LEAF purified anti-CD3 (Biolegend, 317303, 2µg/ml), anti-CD28 (Biolegend, 302913, ^g/ml) and GolgiPlug (BD, 555029) for 6 hours at 37°C. Intracellular cytokine labelling was performed following surface staining, fixation and permeabilization using the BD Cytofix/Cytoperm Plus kit (BD, 555028) according to the manufacturer's instructions.

[0595] Single cell RNA-seq data generation and processing. FASTQ files were aligned to the NCBI Human Reference Genome Build GRCh37 (hg19) using STAR⁵³. Expression levels were quantified as Transcripts Per Million (TPM) and were computed by the RSEM tool⁵⁴. For each cell Applicants used three quality control (QC) measures. Applicants excluded: (1) cells with a zero expression of both CD45 and CD3E; (2) cells expressing less than 1000 genes; (3) cells with an average expression of housekeeping genes, $\log_2(\text{TPM}+l) < 2.5$. For downstream analysis Applicants used the set of genes with expression levels $\log_2(\text{TPM}+l) \geq 4.5$ in at least 10 cells or genes with a particularly high expression level ($\log_2(\text{TPM}+l) > 12$) in one or more cells.

[0596] A supervised classification of single cells to cell types. To classify each single cell that passed QC to a pre-defined cell type, Applicants performed a supervised analysis based on a list of known marker genes (**table 3**). This was done by defining a set of genes per cell type which must or must not be expressed. On average, this approach led to the unambiguous classification of 80% of the cells. The remaining cells were then annotated using a manual review process. Following this step Applicants validated that no cell had an ambiguous classification (e.g., a T-cell and a B-cell).

[0597] Unsupervised clustering of immune cells. To cluster all cells that passed QC Applicants applied the k-means algorithm with a correlation distance metric, testing $k = 3, \dots, 15$. The algorithm was applied using all genes with variance >6 , yielding -4000

genes. This value was selected based on the relation between the variance and the number of cells expressing each gene (**fig 49**). To determine the optimal number of clusters, Applicants first computed the Pearson correlation matrix \mathbf{R} and the distance matrix \mathbf{D} as $(1 - \mathbf{R})$. Applicants then computed the sum of pair-wise distances between all cells in different clusters $Dis_b = \sum_{l=1}^k (\sum iec_{i,j} i_{ci} D(i,j))$ and the total distance $Dis_t = \sum i_j D(i,j)$. The ratio between these two measures $V = Dis_b/Dis_t$ was used to estimate the variance explained by a given solution (**fig 50**). Specifically, in the extreme case where all cells are clustered together or the case where each cell is a single cluster, this ratio would be 0 and 1, respectively. Applicants then selected the optimal number of clusters, k , as the number of clusters for which there was no extreme increase in beyond it. As a few such similar solutions exist, Applicants used prior biological knowledge on distinct cell types (B-cells, myeloids and regulatory T-cells), and selected the solution in which these groups were separated into different clusters ($k = 11$). In addition, Applicants excluded solutions in which the number of gene markers significantly increased in a given cluster is smaller than 20 genes. To determine the robustness of this clustering solution, Applicants performed 100 iterations in which Applicants randomly removed 10% of the cells, and re-ran the k-means algorithm and checked the stability of the clustering solution. Applicants quantified the agreement of a given solution with the original one as the number of pairs of cells that were either clustered together, or not clustered together, in both solutions, divided by the total number pairs shared between the runs. This process yielded a robustness measure of 0.94 for the selected .

[0598] To examine if there is a significant difference between responders and non-responders for a given cluster i , Applicants computed the fraction of cells in each lesion assigned to cluster i , and applied the Wilcoxon rank-sum test to the corresponding values of responders and non-responder lesions. P-values were corrected using the Benjamini-Hochberg False Discovery Rate (FDR) procedure and were considered significant if the FDR q-value 0.1.

[0599] Unsupervised clustering of CD8 T-cells. To identify different CD8 T-cell clusters Applicants first extracted all single-cells classified as CD8 in the supervised analysis. Applicants performed a similar clustering analysis as described above (**fig 51**). Applicants identified $k = 6$ as the optimal number of clusters since: (1) no significant increase in V was observed *for K >= 7*; (2) All splits up to $k=6$ followed a hierarchical pattern in which whenever Applicants increased k a single cluster split into two sub-clusters (**fig 52**). This

pattern was not seen for $K \geq 7$. Performing a robustness analysis as defined above, Applicants found that this solution yields a robustness value of 0.9. Computation of significant difference between responders and non-responders for a given cluster was done as explained above.

[0600] **Differential expression analysis.** In all cases, differential expression analysis was applied to all genes that had an average expression level $\log_2(\text{TPM}+1) > 2$ in either tested groups, G_1 and G_2 . Then, for each gene i, Applicants count the number of cells in G_1 and G_2 that express it with an expression level $\log_2(\text{TPM}+1) > 2$ or 2. Applicants then apply Fisher's Exact test for the corresponding 2x2 table. To identify significant differences Applicants considered genes with a Bonferroni-corrected q-value 0.05 and $\log_2(\text{fold-change}) > 0.5$.

[0601] **Trajectory analysis of CD8 T-cells.** To analyze the trajectory of CD8 T-cells based on single-cell RNA-seq expression data, Applicants used Monocle v. 2.5.4⁵⁵. As input to Monocle's Reversed Graph Embedding algorithm, Applicants selected a set of 426 genes that was the union of the top 100 differentially expressed genes ordered by ascending q-value (as described above) for each of the six CD8 T-cell clusters (or all such genes for two clusters that had fewer than 100 significant genes).

[0602] **T-cell Receptor (TCR) reconstruction.** Applicants applied the MixCr tool for reconstructing TCRs from all identified T-cells⁴⁴. Applicants defined *persistent* TCRs as TCRs having an identical CDR3 sequence in both chains and were detected in baseline (pre-therapy) and post-therapy samples from the same patient. *Enriched* TCRs were defined as TCRs having an identical CDR3 sequence in both chains and detected in the same patient at a single time point, or in two parallel time points (e.g., multiple biopsies collected at the same time point). Lastly, *common* TCRs were defined as those having an identical CDR3 sequence in both chains and detected in different patients.

[0603] **Mice and tumor transplant.** Female C57BL/6 mice, age of 8-9 weeks were purchased from Jackson Laboratory and were housed at Massachusetts General Hospital under SPF conditions. All experiments followed protocols approved by the Massachusetts General Hospital Institutional Animal Care and use Committee (IACUC). B16-F10 was generously provided by Mikael Pittet. B16-F10 cells (0.5×10^6) were intradermally injected into the right flank using a 30g needle and tumors were measured every 4 days in two dimensions using a digital caliper. Tumor volume (mm^3) was calculated using the following formula $V = (L * W^2)/2$ (V =volume, L =tumor length, tumor width). All treatments started on day 4 post transplantation after 100% of tumors were visible. *Invivo* plus rat IgG2a isotype

control (BioXCell; 2A3; BE0089) 10(^g/dose (for TIM3 experiments) and 20(^g/dose (for PD1 experiments), was intraperitoneally (i.p.) injected to the control (untreated) group every 3 days. *Invivo* plus anti-mouse TEVI3 (BioXCell; RMT3-23; BE01 15) 10(^g/dose was i.p. injected every 3 days. *Invivo* plus anti-mouse PD1 (BioXCell; 29f.1A12; BE0273) 200 μg/dose was i.p. injected every 3 days. POM-1 (polyoxometalate-1) 5mg/kg/day (ChemCruz; sc-203205), a CD39 inhibitor, was i.p. injected on a daily basis.

[0604] ATAC-seq tagmentation. Methods for tagmentation are as previously reported⁵⁶. Briefly, 5,000-10,000 cells were cell sorted into RPMI containing 10% FBS, 1% Pen/Strep, 1% L-Glutamine, and 1% HEPES. The cells were then centrifuged at 500xg at 4°C for 10 minutes, the supernatant aspirated, and resuspended in tagmentation mixture (25 u1 tagmentation buffer (Illumina, FC-121-1031), 2.5 u1 TBE (Illumina, FC-121-1031), 0.5 u1 1% digitonin (Promega, G9441), and 22 u1 H2O). The cells were then incubated at 37°C in a therm omixer, mixing at 300 RPM for 30 min. Following tagmentation, the sample was immediately purified via minElute PCR cleanup column (QIAGEN, 28006), and eluted in 10 u1. The tagmented DNA was then PCR'ed using Nextera indexing primers with sequencing adapters for 5 cycles in a 50 u1 reaction. 5 u1 of the reaction was then used for qPCR to determine the remaining number of PCR cycles required (as determined by the cycle number of each sample when it reaches 1/3 the fluorescence threshold), followed by PCR of each individual sample according to this cycle number. The samples were purified using 1.5X Agencourt AMPure XP beads (A63880), followed by two 70% EtOH washes, and elution of DNA in 15 u1 buffer EB (QIAGEN, 19086). Each sample was quantified by Qubit, and measured for fragment lengths on a Tape Station. The samples were pooled and sequenced on an Illumina Nextseq 500 using 75 bp PE reads to a sequencing depth of 30 million reads per sample.

[0605] ATAC-seq analysis. Sequencing reads for each sample were aligned to hg19 using Bowtie 2.2.1⁵⁷ with a max insert size of 2000 bp. SAM files were converted to BAM files and sorted using Samtools 1.3⁵⁸. Duplicate (as defined by broadinstitute.github.io/picard) and mitochondrial reads were removed, and peaks were called, initially by making tag directories according to chromosome and then by finding peaks (areas with more sequencing reads than expected by chance) for each sample, using the "DNase" peak finding style ('makeTagDirectory -format sam' and 'findPeaks -style dnase', Homer version 4.9)⁵⁹. Overlapping peaks were then merged. The number of Tn5 transposition events (5' ends of reads) lying within each peak were quantified for each

sample, yielding a matrix of peaks by samples containing ATAC read counts. EdgeR 3.14.0 was used to call CD39⁺TIM3⁺ (DP)/CD39TIM3⁻ (DN)-specific peaks, first by grouping the samples by cell type (DP and DN) and pairing the samples from each patient, and then using EdgeR⁶⁰ to estimate the tagwise dispersion using generalized linear models (estimateGLMTagwiseDisp function). Applicants then performed a likelihood ratio test to identify differential accessibility between paired samples from each patient (glmFit, glmLRT). Applicants obtained the top differential peaks (topTags), sorting peaks by their FDR q-value. Differential peaks between DP and DN were called significant if their FDR q-value was 0.01.

[0606] Motif Analysis. To identify TF motifs that distinguish DP- and DN-specific peaks from non-specific (background) peaks, each peak was scanned with the human motifs from the CIS-BP database⁶¹, using the GOMER approach⁶², yielding a binding score for each peak for each TF motif. The minimum hypergeometric (minHG) test was then used to gauge how well motif scores enrich DP- or DN-specific peaks (FDR q < 0.01) compared to background peaks, considering the top N (1 up to 3000) highest scoring peaks. Here, background peaks included those whose ATAC DP-vs-DN FDR was over 0.1 (i.e. not significantly DP- or DN-specific) and had an average counts per million (CPM) greater than the minimum CPM of DP/DN-specific peaks (i.e. enough reads that a difference could have been detected). MinHG P-values were corrected by Benjamini-Hochberg FDR, counting each minHG test as independent (resulting in more conservative FDR q-values).

[0607] Whole exome sequencing (WES). WES of DNA from tumor and matched normal blood samples was done as previously described¹⁵. Briefly, 250-500ng of extracted DNA, using Qiagen AllPrep DNA/RNA Mini Kit (cat# 80204), was used as input for library preparation. Sample were barcoded using unique 8 base molecular barcodes followed by a library enrichment process, and all libraries above 40ng¹ were considered acceptable for solution-phase hybrid selection and sequencing. Libraries preparation was carried out using the SureSelect Target Enrichment System Sequencing Platform Library Prep v2 (Agilent Technologies, G3360-90000), according to manufacturer's specifications, followed by quantification and normalization using PicoGreen to ensure equal concentration. Libraries were then quantified using qPCR (KAPA Biosystems, KK4832), denatured with 0.2M NaOH and diluted to 20pM using hybridization buffer (Illumina). Cluster amplification was performed according to the manufacturer's protocol (Illumina), HiSeq 2500 v4 cluster chemistry and flowcells, as well as Illumina's Multiplexing Sequencing Primer Kit. Libraries

were sequenced using the HiSeq 2500 v4 Sequencing-by-Synthesis method (paired end 76bp reads) followed by analysis with RTA v.1.12.4.2. The minimum depth of coverage was 150X and 80X for tumor and normal samples respectively. All procedures were done at the Genomics Platform of the Broad Institute of Harvard and MIT.

[0608] Survival analysis based on IFC data. Applicants used the TCF7⁺CD8ATCF7⁻ CD8⁺ ratio to split samples into two groups (ratio>1 and <1). A standard Kaplan-Meier survival analysis was then used to determine the association of these groups with survival rate. In case two or more samples for the same patient exist, Applicants selected the baseline sample for this analysis.

[0609] Mutation calling pipeline. WES BAM files were aligned to the NCBI Human Reference Genome Build GRCh37 (hg19) and were checked for contamination by DNA originating from a different individual using ContEst ⁶³. Somatic single nucleotide variations (sSNVs) were then detected using MuTect ⁶⁴. Following this standard procedure, Applicants filtered sSNVs by: (1) removing potential DNA oxidation artifacts ⁶⁵; (2) realigning identified sSNVs with NovoAlign (www.novocraft.com) and performing an additional iteration of MuTect with the newly aligned BAM files; (3) removing technology- and site-specific artifacts using a panel of ~7000 TCGA normal samples (PoN filtering). Finally, sSNVs were annotated using Oncotator ⁶⁶.

[0610] Tables

Table 1

#	Patient ID	Gender (F/M)	Age	Therapy	Clinical response (RECIST; R=CR, PR; NR=SD, PD)	Baseline Biopsy (days from baseline; site; lesion response)	Post I biopsy (days from baseline; site; lesion response)	Post II biopsy (days from baseline; site; lesion response)	WES	Overall survival (days)	Status (Alive=0; Dead=1)	Mutations/indels in known melanoma drivers	Mutations/indels in antigen presentation and/or interferon-gamma pathways
1	P1	M	49	CTLA4 (baseline); PD1 (post I and II)	Resistance	0; right chest; regression	48; anterior neck; regression	437; anterior neck; progression	Y	822	0	ND	ND
2	P2	M	75	PD1	NR	0; small bowel; progression	35; left axilla; progression	NA	Y	347	1	ND	ND
3	P3	F	83	PD1	NR	0; right anterior lower leg;	63; right distal anterior thigh;	161; right anterior knee; progression	Y	521	1	NRAS,TPT	ERAP1, HLA-CRFX5, B2M, IRF6

						progression	progression						
4	P 4	M	2 9	CTLA 4+PD 1	R	(-2); left shoulder; progression prior to therapy	35; left shoulder; regression	NA	Y	539	0	TPTE	ND
5	P 5	M	3 3	PDI	NR (patient had mix response)	NA	199; Post_P#5- right iliac soft tissue mass; progression. Post_P#5_2- right buttock; regression	NA	Y	369	1	TP53	ND
6	P 6	F	6 6	CTLA 4 (baseline); PDI (post I)	NR	(-70); left upper back; progression	270; right colectomy mass in cecum; progression	NA	Y	777	0	NRAS,TPT E	ND
7	P 7	M	7 4	CTLA 4+PD 1	R	0; left forehead; regression	22; left forehead; regression	NA	Y	339	0	TPTE	IFNGR1JPP2, ADAR
8	P 8	M	4 9	CTLA 4+PD 1	R	(-12); left axillary lymph node; regression	62; left axillary lymph node; regression	NA	N	388	0	NA	NA
9	P 1 0	F	6 0	PDI	NR	NA	760; small bowel mass; progression	NA	Y	119 7	0	TP53	IFNGR1,PSMB9, ,TBX21
10	P 1 1	F	6 8	PDI	NR	NA	118; right inguinal lymph node; progression	NA	Y	300	1	NRAS, TP53	ND
11	P 1 2	M	6 8	PDI	NR	(-16); small bowel resection; progression	77; left anterior shoulder; progression	NA	Y	101	1	NRAS	TPP2
12	P 1 3	M	4 8	CTLA 4+PD 1	NR (patient had mix response)	NA	297; porta hepatis; progression	NA	Y	507	0	MAP2K1, TP53, TPTE	ND
13	P 1 4	M	7 0	PDI	NR	NA	462; left axilla lesion; progression	NA	Y	588	1	CTNNB1, NRAS, TPTE	PSMB9
14	P 1 5	M	7 2	PDI	NR	0; right back subcutaneous; progression	73; left lower back; progression	NA	Y	163	1	TPTE	JAK1, STAT1, CD45- cells lack HLA-I expression
15	P 1 6	M	6 2	PDI	NR	NA	67; right abdomen soft tissue; progression	NA	N	476	1	NA	NA

16	P 1 7	F	6 8	PD1	R	NA	61; right axillary lymph node; regression	NA	N	361	0	NA	NA
17	P 1 8	M	7 9	PD1	NR (patient had mix response)	NA	237; right inguinal lymph node; progression	NA	Y	746	0	ND	ND
18	P 1 9	M	5 3	PD1	R	NA	21; left axillary lymph node; regression	NA	N	570	0	NA	NA
19	P 2 0	F	6 4	PD1	NR (patient had mix response)	0; right inguinoiliac lymphadenectomy; progression	199; right pelvic mass; progression	NA	Y	413	0	ND	ND
20	P 2 1	F	7 5	PD1	R	NA	867; jejunum; regression	NA	Y	134 0	0	ND	ND
21	P 2 2	M	5 6	PD1	NR	NA	31; right supraclavicular mass; progression	NA	N	64	1	NA	NA
22	P 2 3	M	6 2	PD1	NR	NA	403; left frontal craniotomy; progression	622; left neck mass; progression	Y	674	0	TP53	IL3RA, IRF6
23	P 2 4	M	7 3	PD1	R	0; left lower back; regression	NA	NA	N	54	0	NA	NA
24	P 2 5	M	7 4	PD1	NR	0; adrenal gland; progression	NA	NA	Y	676	0	ND	CD45- cells lack HLA-I expression
25	P 2 6	M	7 2	CTLA 4+PD 1	R	(-56); axillary lymph node, regression	NA	NA	N	517	0	NA	NA
26	P 2 7	F	6 2	PD1	NR	(-35); upper abdomen ; progression	NA	NA	N	73	0	NA	NA
27	P 2 8	F	6 7	CTLA 4+PD 1	Resistance	0; right groin; regression	41; right groin; progression	89; right groin; progression	Y	61	0	ND	ND
28	P 2 9	M	7 9	PD1	R	(-67); left axillary lymph node; regression	NA	NA	N	417	0	NA	NA
29	P 3 0	M	6 4	PD1	NR	573; left laparoscopic adrenalectomy; progression	NA	NA	N	580	0	NA	NA

30	P 3 1	M	5 2	PD1	NR	(-7); right axilla; progression	NA	NA	N	126	0	NA	NA
31	P 3 3	F	6 5	PD1	R	(-66); left axillary lymph node; regression	NA	NA	N	130	0	NA	NA
32	P 3 5	M	7 0	PD1	R	(-31); right iliac lymph node; regression	NA	NA	Y	511	0	ND	ND

F- female; M- male; NR- nonresponder; R- responder; NA- not available; ND- not detected; DOD- dead of disease; AWD- alive with disease

Table 2A

Gene Name	P-value	G1- B cells		Mean expression non-G1	adjusted P-value = 2.4c-5	GenCN amc	G2- plasma cells		Mean expression non-G2	adjusted P-value = 2.4c-5
		Mean expression G1	Mean expression non-G1				P-value	Mean expression G2		
IGHD	<le-300	5.377108613	0.024126888			SDC1	<le-300	4.233860833	0.008624746	
PAX5	<le-300	4.830784038	0.023214384			IGLV6-57	9.81E-130	2.402558175	0.026153709	
FCRL1	<le-300	5.758715472	0.033840633			IGHV3-OR16-9	3.35E-147	2.229910869	0.02923494	
CR2	<le-300	3.177964545	0.026060567			TNFRSF17	4.73E-257	3.814521814	0.060427652	
VPREB3	<le-300	3.779631599	0.031216078			IGLV3-1	6.62E-229	4.116293177	0.067925619	
FCER2	<le-300	5.175642662	0.04801409			HID1	1.13E-207	2.530148775	0.041918971	
CD19	<le-300	6.879495185	0.067404363			IGHG4	3.21E-239	7.435463173	0.14692745	
EBF1	<le-300	2.578367556	0.025833821			IGHV3-48	1.50E-135	2.640490419	0.060235217	
CD22	<le-300	6.81743677	0.068541106			IGHA2	2.18E-131	4.015662376	0.093785671	
BANK1	<le-300	6.739130158	0.070328664			IGHV3-7	5.17E-127	3.148975371	0.07603257	
CLEC17A	<le-300	3.053371072	0.038423373			IGKV3-OR2-268	5.87E-151	2.660244692	0.069676661	
FCRLA	<le-300	3.62133246	0.056407837			IGHV3-11	6.43E-108	2.138997825	0.057035625	
FCRL2	<le-300	2.350303618	0.036669806			IGHG2	1.87E-225	8.704980223	0.253948458	
MS4AI	<le-300	9.428714865	0.153577919			IGHA1	2.97E-249	7.081368071	0.212315823	
BLK	<le-300	3.434999629	0.090733356			IGHG3	6.04E-223	8.936777049	0.280586437	
RALGPS2	<le-300	3.757215267	0.104227862			DERL3	<le-300	7.472148401	0.24163785	
TCL1A	<le-300	4.653788476	0.147762744			IGLC1	<le-300	7.390641029	0.242357044	
TLR10	<le-300	2.033374459	0.070305566			IGKV4-1	2.60E-105	2.766940507	0.091570966	

FAM 129C	<le- 300	3.3258764 84	0.13062055 5		IGKV3 D-15	4.23E-97	2.6442219 4	0.08987916	
CNR2	<le- 300	2.2307334 39	0.08769665 5		IGLC7	8.72E-116	2.7149164 12	0.09271453 2	
ARH GAP2	<le- 4	3.9866383 22	0.16394866 6		IGLL5	3.08E-262	5.7985527 99	0.20536312 1	
KIAA 0125	9.02 E- 271	2.1033251 1	0.08667368 8		IGHV3- 21	2.09E-117	2.7039222 07	0.09651321 1	
HLA- DOB	<le- 300	3.8311540 47	0.16554764 9		IGHV3- 11	1.40E-80	2.7711741 04	0.10253285	
IGHM	<le- 300	9.3272050 93	0.40745498 7		IGHV1- 69	1.36E-88	2.2326666 27	0.08526387	
KIAA 0226	<le- L 300	4.8298782 93	0.21132716 9		IGJ	6.11700000 000000e- 321	11.105462 5	0.47109533 8	
CD79	<le- A 300	8.3395337 91	0.37017964 7		IGHG1	2.80E-181	11.944645 57	0.50683376 5	
BCL1	<le- 1A 300	7.4105008 24	0.38361486 6		IGHV3- 23	8.56E-108	3.5764142 72	0.15943693 9	
STAP	<le- 1 300	3.6246375 62	0.19870336 5		TXND5	1.92909797 995300e- 312	5.7598913 81	0.26051721 3	
IGLC3	8.54 E- 270	3.3730505 02	0.18979311 8		PYCR1	8.08E-123	2.0684614 38	0.09843747 9	
PKIG	5.82 E- 295	2.7683946 43	0.15679259		FCRL5	4.59306305 897400e- 312	5.0366142 5	0.24157025	
FCRL 5	1.17 E- 285	2.3237129 41	0.13594682 1		SPAG4	3.46E-165	3.6150117 52	0.20271791 3	
AFF3	<le- 300	3.1703638 03	0.20072994 3		IGKV1 D-39	1.94E-62	2.2990930 53	0.13406608 3	
COBL LI	4.92 E- 254	2.1471729 35	0.13739352 1		IGHV3- 20	2.66E-79	2.8044501 55	0.16530640 4	
SPIB	<le- 300	3.8307691 52	0.25308751 4		IGLC2	1.01E-104	5.1124481 2	0.33468206 3	
SWA P70	<le- 300	4.0124140 59	0.28577176 7		TRAM2	1.38E-156	2.1565034 84	0.15028415 7	
IGLL5	9.41 E- 205	2.0018349 23	0.14416444 1		CPNE5	1.27E-199	2.9167284 94	0.20362128 7	
ADA M28	<le- 300	5.9514160 93	0.44283933 1		MZB1	1.21E-302	9.5794608 42	0.71062497 3	
BLNK	<le- 300	4.2832777 5	0.34011246 9		PNOC	8.98E-103	2.0434333 7	0.15669701 3	
IGLC2	2.61 E- 171	2.6218525 91	0.20859588 9		GAB1	2.78E-127	2.3821253 52	0.18761370 3	
IGKC	<le- 300	8.1930256 68	0.77094020 6		IGHV3- 30	1.77E-62	2.1871708 65	0.17941979 9	
IGHG 3	<le- 300	2.5036609 02	0.24051935 7		IGLC3	3.42E-89	4.7205773 62	0.39308038 8	
WDF Y4	<le- 300	4.1436026 65	0.40502326 3		P2RX1	1.03E-145	2.7366232 27	0.22969453 5	
MEF2 C	<le- 300	4.6828284 75	0.46396047 3		COBLL 1	6.99E-133	3.0150997 92	0.26541357 8	
IGHG 2	<le- 300	2.2823146 94	0.22875917 6		IGKC	1.10E-152	13.299584 29	1.20744076 3	
CCR6	2.39 E- 258	2.4969530 86	0.25601685 8		HIST1H 2BG	1.52E-74	2.0597600 08	0.20012590 2	
IGLC1	5.30 E- 148	2.0376411 02	0.21324463 6		SPATS2	9.27E-117	2.4238597 73	0.25377366	

SMI M14	<le- 300	4.4621507 24	0.48399052		CHPF	6.71E-113	2.1172678 81	0.23937570 5	
POU2 AF1	<le- 300	3.3936003 9	0.39071229 5		P0U2A FI	1.59E-250	4.8877913 44	0.57822593 7	
CD79 B	<le- 300	7.4388940 85	0.85846768 5		PRDX4	3.92E-231	7.4269151 9	0.88751611 8	
CD40	<le- 300	4.1118914 01	0.47520585 6		SLC17A 9	2.59E-160	3.8785040 64	0.49242135 1	
HVC N1	<le- 300	4.3279409 92	0.50900210 5		FKBP11	7.61E-229	8.1904327 5	1.20152174 3	
TCF4	<le- 300	4.1921032 72	0.51113149 9		MANE A	2.20E-108	2.4504403 04	0.36045405 6	
BTK	5.19 E- 251	3.3263092 41	0.41213627 2		EAF2	3.97E-118	3.5457098 94	0.53754626 3	
RASG RP3	1.78 E- 215	2.5120607 72	0.31231054 1		TRIB1	1.88E-86	2.5988827 97	0.41054515 1	
CXCR 5	<le- 300	4.0616372 92	0.52828268 8		CLIC4	7.16E-112	2.5113124 79	0.39781662	
IGHG 1	<le- 300	3.3995342 55	0.45827980 1		CD79A	1.94E-231	6.0211621 52	0.98771127 6	
PDLI M1	3.20 E- 181	2.4710901 4	0.33851478 8		GSTM2	4.02E-72	2.4217418 55	0.42956167	
PHAC TR1	3.69 E- 186	2.3958530 64	0.35486534 2		PDK1	4.75E-153	4.0749106 42	0.72848350 4	
NCF1	<le- 300	5.7400938 48	0.87282239 7		XBP1	3.60E-191	7.4017733 39	1.32811729	
FGD2	<le- 300	5.5083362 92	0.84910130 4		CRELD 2	1.16E-140	4.5239181 3	0.81958857 9	
SIGLE C14	1.44 E- 214	2.3357328 16	0.36998714 2		ANKRD 28	3.29E-135	4.4179062 49	0.81418153 7	
HLA- DOA	2.66 E- 288	3.8922205 22	0.62143877 9		BLNK	4.26E-128	3.4462109 92	0.63974623 8	
MICA L3	1.17 E- 232	2.5276118 32	0.40864441 6		HIST1H 2BC	1.11E-51	2.1770801 38	0.40594921 2	
RABE P2	7.54 E- 268	2.9819509 51	0.48550724 3		SELM	2.26E-125	3.9591166 83	0.74975693	
LY86	1.38 E- 164	2.7455667 33	0.45130304 5		MCEE	5.97E-74	2.4963666 48	0.50443314 5	
IRF8	<le- 300	8.2253571 04	1.35248868 7		MANF	6.46E-96	3.2275295 68	0.65488149	
EAF2	3.46 E- 136	2.4511374 36	0.41171819 3		ITM2C	1.38E-183	6.3132285 8	1.32298693 1	
CD72	7.59 E- 238	4.0631204 1	0.68967018 2		RRBP1	3.02E-126	3.5329881 87	0.74580555	
SYK	2.89 E- 234	3.3182829 8	0.58709507 2		RAB30	7.00E-146	5.6214664 13	1.20587415 3	
MGA T5	8.23 E- 167	2.0470160 6	0.37114365 5		MEI1	1.10E-120	4.9375096 34	1.06227396 8	
CD83	<le- 300	7.0683684 38	1.28259194 5		CASP1 0	1.65E-93	2.7707266 58	0.59676504 9	
PLCG 2	1.68 E- 241	3.4078868 51	0.66163942 8		CLPTM 1L	2.55E-91	2.8803341 85	0.62190792 5	
ALOX 5	7.96 E- 86	3.1353779 3	0.61349966 3		GMPPB	3.08E-80	2.8998356 97	0.63516364 6	

	240								
DOK3	7.02 E-109	2.0283968 62	0.39839755 5		FKBP2	2.68E-176	6.8001727 81	1.50761265 5	
CIITA	<le-300	6.0498043 8	1.20936299 6		SLC38A 5	2.20E-65	2.4902881 97	0.55858077	
CDCA7L	1.11 E-120	2.3678953 82	0.48998398 1		PPAPD C1B	1.74E-145	5.6849268 1	1.27835742 1	
PIK3C2B	2.30 E-170	2.5277381 3	0.56069691 6		SDF2L1	6.09E-154	5.6746439 55	1.27872055 2	
LAT2	3.01 E-206	4.1941277 29	0.93733647 1		ST6GA LNAC4	4.64E-111	3.6721054 1	0.83025034 6	
TPD52	1.87 E-186	3.5031240 66	0.82100815 8		TXNDC11	7.00E-110	3.9557783 44	0.90105499 7	
P2RX5	3.54 E-134	2.7402950 41	0.65728211 9		FNDC3B	7.02E-109	3.1007955 96	0.70734375 3	
AKAP2	5.13 E-239	3.4734620 21	0.83809341 1		Cllorf24	5.47E-87	2.8488585 32	0.65603053 9	
CYBB	1.25 E-268	3.0424786 3	0.73699245		SIL1	3.34E-106	3.6529351 99	0.84542770 4	
SNX29	2.02 E-175	3.5072329 08	0.85951663 4		BSCL2	4.43E-73	3.1966938 24	0.74502181 2	
RAB30	3.89 E-210	4.0336645 35	1.01932256 4		TPD52	1.73E-130	4.2420551 5	0.99985585 7	
STX7	1.64 E-193	3.5256183 77	0.89798035 8		FBXO18	6.84E-47	2.0686278 34	0.49933066 5	
POU2F2	8.69 E-172	2.9196701 22	0.74518104 5		SEC24D	4.01E-59	2.0158296 51	0.51016637 1	
FCCR2B	3.63 E-89	2.1139355 14	0.54248685 2		MAGEDI	1.05E-84	3.0415323 73	0.78686818 5	
HLA-DMB286	3.98 E-286	5.9664905 31	1.54648711 3		PLCG2	1.90E-101	3.3110496 05	0.86104640 3	
LY9	<le-300	6.5857928 11	1.75540362 5		HSPA13	1.67E-79	2.5741560 37	0.67114269	
GPR18	7.19 E-94	2.3319386 5	0.62606994 3		HIST1H2BD	9.55E-47	2.2845815 51	0.60317058 3	
HLA-DQB2	<le-300	4.2743911 03	1.19916080 4		SYVN1	5.03E-138	5.2101655 5	1.37834919 2	
ORAI2	8.21 E-115	2.5670928 06	0.72927749 3		SEC11C	3.25E-149	8.0929174 61	2.17075745 6	
CHD7	1.20 E-143	2.5204324 37	0.72548600 5		CPEB4	4.40E-72	2.3359406 54	0.62910679 8	
CYB561A3	3.67 E-149	4.3889844 01	1.27069167 1		SEC24A	1.82E-74	2.1928282 01	0.59495932 4	
IFT57	4.23 E-78	2.2359310 78	0.65173541 2		UBE2J1	2.81E-110	3.9398115 69	1.08444808 6	
HLA-DQA2	<le-300	4.5270101 85	1.33478294 7		EIF2AK4	9.96E-63	2.2038700 35	0.61099433 1	
CTSH	1.00 E-148	4.1447422 88	1.22477577 1		TMEM214	4.99E-69	2.3959600 83	0.67059762 7	
FCHS	3.28	2.5872657	0.77041480		TCF4	4.60E-70	2.8509049	0.80152203	

D2	E-110	55	8				64		
PIK3API	1.49 E-96	2.6084593 62	0.77856491 9		SEL1L	4.43E-117	4.0849177 84	1.15366096 9	
PLEKHF2	3.19 E-75	2.1382494 98	0.63926057 2		WIP1	1.29E-46	2.2869580 47	0.66278086 5	

Table 2B

G3- Monocytes/Macrophages				G4- Dendritic cells					
Gene Name	P-value	Mean expression G3	Mean expression non-G3	Gene Name	P-value	Mean expression G4	Mean expression non-G4	adjusted P-value = 1.4e-5	
MARCO	<1e-300	4.4946082 21	0.006001435	PTCR A	5.81E-271	4.3175811 26	0.006904934	adjusted P-value = 1.6e-5	
FPR3	<1e-300	3.5119140 39	0.006271509	CLEC4 C	<1e-300	5.3111988 87	0.009184315		
CLEC5A	<1e-300	3.2750983 3	0.007399723	KRT5	6.93E-200	3.0499963 64	0.006357704		
AQP9	<1e-300	2.8488569 1	0.006991484	LAMP 5	4.99172460 000000e-316	4.3400202 64	0.017556374		
CLEC10A	<1e-300	2.6332055 08	0.007192837	PLEKHD1	2.31E-139	2.0235391 58	0.009645317		
HNM T	<1e-300	3.6781714 29	0.010075893	EPHB 1	7.32E-291	4.3662541 54	0.022027954		
OLR1	<1e-300	4.3752717 71	0.012256769	PLVAP	4.48E-162	2.5317113 3	0.013197905		
CD30OE	<1e-300	3.5735240 67	0.010515607	LILRA 4	<1e-300	9.9222770 08	0.071632434		
ARHG_EF10L	<1e-300	2.4876774 25	0.007498458	SLC12A3	4.03E-213	3.2869122 61	0.02510332		
HK3	<1e-300	4.6847067 29	0.014382697	SCAM P5	6.06E-249	3.4243873 56	0.028139895		
ANPEP	<1e-300	4.4883317 73	0.013850118	SCN9A	3.88E-167	2.0611172 64	0.018780406		
LGALS2	<1e-300	2.7282798 83	0.009296675	SMIM5	9.00E-168	3.1014429 5	0.031183255		
FCN1	<1e-300	5.9782588 81	0.021182951	DNASE1L3	7.43E-302	5.7287990 87	0.058258115		
GPR84	<1e-300	2.9235444 51	0.010617089	PPM1J	1.37E-128	2.4065832 45	0.026936668		
CCL2	<1e-300	3.1933968 57	0.011675144	PTGDS	1.70E-190	5.9834253 64	0.074921091		
CLEC4E	<1e-300	2.6811803 18	0.010350464	MAP1A	1.99E-277	3.5729558 54	0.045249564		
CSTA	<1e-300	3.2500921 54	0.012863282	PTPRS	<1e-300	7.0610468 6	0.093646469		
TREM1	<1e-300	5.5851147 96	0.022911938	TPM2	3.80E-212	4.5253423 99	0.068376039		
FCGR	<1e	3.3762330	0.014487789	PACSI	2.19E-231	3.3481285	0.056410238		

IB	- 300	92			N1		74		
TME M176 B	<le - 300	4.3234431 09	0.019148981		IL3RA	<le-300	7.8430376 23	0.164531576	
C19or f59	<le - 300	2.1367613 61	0.009551562		TNFR SF21	2.69E-272	4.9147146 9	0.108152266	
OSCA R	<le - 300	2.7721739 46	0.013255969		SERPI NF1	<le-300	10.173735 96	0.229645401	
CYP2S 1	<le - 300	2.3044915 72	0.011100612		PLD4	<le-300	9.4336148 52	0.214274098	
STAB 1	<le - 300	4.0994885 88	0.020052129		Clorf 186	<le-300	5.0013238 06	0.1207404	
VCAN	<le - 300	4.5961653 61	0.022515064		EGLN 3	7.11E-284	6.2967444 88	0.156199475	
FPR1	<le - 300	5.1645491 95	0.026620686		SMPD 3	4.28E-296	6.8078525 94	0.177311825	
SERPI NA1	<le - 300	8.3727086 28	0.043244148		TLR9	7.19E-246	4.4901623 4	0.121049661	
TLR2	<le - 300	4.6254615 25	0.023946353		PFKFB 2	3.90E-242	4.8249719 34	0.153731269	
VSIG4	<le - 300	4.4465904 87	0.023094787		AC02 3590. 1	1.88E-129	3.0395205 05	0.096914264	
TME M176 A	<le - 300	3.0264785 1	0.015882793		SUSD 1	5.71E-152	4.0040703 08	0.131104383	
VMO 1	<le - 300	2.0354873 96	0.010703715		NOTC H4	3.28E-155	3.4725321 72	0.118969313	
MAFB	<le - 300	2.9928616 75	0.015811575		P2RY1 4	1.35E-203	4.4071141 62	0.1535015	
FCGR 1A	<le - 300	4.8519994 26	0.026156116		PALD 1	5.89E-126	2.6923977 99	0.095958683	
SLC37 A2	<le - 300	2.9143086 16	0.015812681		TSPA N13	6.59E-301	7.6960552 4	0.283339689	
CD14	<le - 300	8.0021263 2	0.043956193		TLR7	3.18E-164	3.3808985 45	0.126456752	
TLR4	<le - 300	2.3741139 3	0.013300338		IGJ	7.08537600 000000e- 316	11.545195 18	0.473094778	
CD30 OLF	<le - 300	2.5172761 73	0.014931206		P2RY6	4.04E-174	4.3254757 94	0.181862467	
CXCL 3	<le - 300	3.0294443 75	0.018142863		MYBL 2	4.90E-168	3.9908586 01	0.16860412	
CD33	<le - 300	3.7931208 8	0.02326843		GAS6	1.64E-173	4.1618408 58	0.184720295	
GPN MB	<le - 300	4.1596039 64	0.026249315		CBFA 2T3	5.41E-152	2.7873381 58	0.123981343	
FOLR 2	<le - 300	2.0139894 41	0.012770956		DERL 3	1.09E-238	6.1121380 88	0.273064677	

LILRA 3	<le - 300	2.5074171 95	0.016065897		ZFAT	9.03E-243	6.3454445 88	0.29792319	
LPCA T2	<le - 300	2.9686494 23	0.019073429		FAM2 13A	2.86E-129	3.3969341 72	0.16353134	
TREM 2	<le - 300	2.4552597 75	0.016066822		GAB1	1.49E-134	3.4534979 27	0.170253515	
PLXD C2	<le - 300	5.4368663 99	0.035654355		GAPT	1.23E-145	4.3396492 59	0.215757525	
CD16 3	<le - 300	5.5308949 72	0.037030431		SPIB	7.71E-297	8.5019642 16	0.428911059	
RIN2	<le - 300	3.6373415 57	0.02440608		NREP	4.93E-123	3.3251600 2	0.168844447	
NFA M1	<le - 300	2.8748527 44	0.020133036		ST3G AL4	6.64E-112	3.1598160 53	0.169957579	
CD30 OC	<le - 300	3.2144954 59	0.023083907		CD36	4.11E-138	4.3738733 27	0.255742007	
IL1B	<le - 300	5.2962177 01	0.038055675		NRP1	7.44E-205	5.5219420 83	0.323117957	
MSR1	<le - 300	3.7368474 75	0.026936993		CHAF 1A	1.37E-167	4.0702542 34	0.238618802	
LILRA 6	<le - 300	5.1758856 68	0.038924775		RASD 1	1.27E-122	3.7795056 97	0.221965192	
ALDH 3B1	<le - 300	2.6350708 01	0.02038212		PHEX	1.39E-110	2.4798880 3	0.149720997	
CXCL 2	<le - 300	3.9778533 47	0.031323706		PPP1 R14B	6.60E-152	4.3138905 59	0.266111523	
C5AR 1	<le - 300	4.2058530 03	0.034953849		CSF2R B	6.36E-222	5.9752871 71	0.373497351	
ADAP 2	<le - 300	3.1712736 05	0.027014433		NEK8	4.00E-131	2.9816248 91	0.186443087	
IL8	<le - 300	5.0120101 15	0.042849971		APP	1.83E-209	5.8915837 87	0.369494378	
C15orf 48	<le - 300	5.1781134 29	0.044304113		SOX4	5.16E-180	5.5504400 52	0.362248494	
MRC1 LI	<le - 300	2.8297811 52	0.024428465		FAM1 29C	4.25E-203	5.0460045 04	0.332085215	
BST1	<le - 300	2.4047426 09	0.020785466		ENPP 2	5.26E-94	2.9438129 2	0.194393078	
PLAU	<le - 300	3.0400962 05	0.026456826		P2RX 1	8.11E-111	3.2485971 13	0.222765687	
SIGLE C9	<le - 300	3.8603448 07	0.03384819		LILRB 4	1.49E-266	7.7782579 07	0.533816599	
MS4A 4A	<le - 300	3.8179327 51	0.033895953		MPEG 1	6.52E-247	7.8778999 88	0.555668797	
SIRPA	<le - 300	3.1918564 25	0.028409212		ACO1 0441.	6.42E-120	4.1366180 47	0.301243358	

	300				1				
RNAS EI	<le - 300	2.7463772 01	0.024449745		ADC	6.29E-72	2.4105865 52	0.175729336	
CPVL	<le - 300	6.0127783 12	0.055310537		SRC	3.66E-110	3.2654938 69	0.240164554	
PLBD 1	<le - 300	3.9598696 83	0.036493191		TMIG D2	1.29E-74	2.1987156 46	0.164006857	
LILRA 2	<le - 300	2.8416304 17	0.026715789		TCL1A	4.00E-130	6.0102163 57	0.451253519	
SLC11 Al	<le - 300	5.3149622 94	0.050314006		TCF4	4.86E-264	8.9319796 74	0.693230615	
PYGL	<le - 300	2.4689004 05	0.023422496		SLC15 A4	1.81E-219	6.1538278 06	0.483410876	
HCK	<le - 300	6.7803785 85	0.064583888		SEMA 7A	4.55E-127	3.6873092 11	0.291190115	
LRP1	<le - 300	4.6304714 18	0.044479138		THBD	1.47E-103	3.5108020 43	0.284711606	
C1QC	<le - 300	4.1790296 93	0.040417513		TEX2	6.20E-97	2.8059010 92	0.228630174	
LYZ	<le - 300	10.467341 62	0.10234471		CMKL RI	3.84E-67	2.1761236 02	0.183074451	
LRRC 25	<le - 300	4.8712661 78	0.048319185		RNAS E6	2.13E-210	7.8186070 5	0.665833	
ADM	<le - 300	3.0033969 35	0.030070209		ABHD 15	1.49E-83	2.4298811 82	0.207279178	
C1QA	<le - 300	4.7144827 08	0.047416804		LGMN	1.55E-166	5.8889632 37	0.508285977	
APOB EC3A	<le - 300	2.5643538 86	0.027152185		GNG7	1.56E-116	2.9334683 53	0.259987053	
ZNF3 85A	<le - 300	3.7984419 61	0.041277352		TTYH 2	1.70E-70	2.0035256 61	0.1786112	
TGM2	<le - 300	2.7962499 49	0.031313461		BCL11 A	1.32E-264	9.4106769 83	0.858977097	
TNFAI P2	<le - 300	5.4589619 46	0.061582086		GRAS P	4.07E-119	3.3791020 98	0.310016663	
CSF3R	<le - 300	4.9947132 67	0.05686716		SERPI NG1	3.19E-95	3.1233230 76	0.289184775	
SLCO 2B1	<le - 300	2.9873971 71	0.034015469		TRAF 4	6.79E-170	4.1845265 26	0.392893634	
LILRB 2	<le - 300	5.5957398 66	0.064663485		MGLL	2.58E-63	2.0758400 1	0.196630971	
SIGLE C7	<le - 300	2.7731938 53	0.032173181		CTNS	2.97E-97	3.5312551 22	0.343834585	
TNS1	<le - 300	2.8172360 45	0.032767759		AMIG 03	1.45E-98	3.0839135 02	0.301714405	
SIOO	<le	6.8772470	0.08156152		PMEP	2.00E-82	2.5864085	0.254602597	

A9	- 300	71			AI		61		
RAB20	<le - 300	4.0781917 31	0.048534589		CSF2RA	1.47E-136	4.0209824 98	0.399443425	
EPB41L3	<le - 300	2.6992298 24	0.032284735		SULF2	1.02E-138	4.5072736	0.452889575	
C1QB	<le - 300	4.6062838 3	0.055422362		VEGFB	6.87E-78	2.8007675 18	0.281769742	
PTAFR	<le - 300	4.4501100 65	0.055337388		CLIC3	2.24E-207	8.1950721 95	0.845830106	
CD93	<le - 300	2.1319824 01	0.026659193		TXND5	3.00E-85	3.0461155 35	0.314856668	
PRAM1	<le - 300	2.1704571 04	0.027443247		COBL	5.85E-82	2.6456055 34	0.274687913	
ST3GAL6	<le - 300	2.4407832 37	0.030918923		TGFBI	1.25E-241	8.5005083 06	0.885183081	
S100A8	<le - 300	4.9731729 07	0.063309664		MGST2	2.59E-97	3.9239166 2	0.409905018	
IL1RN	<le - 300	5.0750386 88	0.064728296		UNC93B1	2.91E-61	2.0502737 65	0.214828122	
SLC8A1	<le - 300	3.0474639 65	0.039628064		BLNK	5.13E-156	5.7030553 01	0.601474387	
FBP1	<le - 300	5.8314141 28	0.077908026		MAP2K6	5.06E-65	3.0653799 9	0.329634877	
LILRA1	<le - 300	2.6012098 61	0.034799015		WDFY4	5.11E-178	5.9252299 47	0.644931587	
ANKRD22	<le - 300	2.3435528 43	0.031797091		TNFSF13	1.05E-124	4.7210034 69	0.51561044	

Table 2C

G5- Lymphocytes				G6- Exhausted CD8 T cells					
Gene Name	P-value	Mean expression G5	Mean expression non-G5		Gene Name	P-value	Mean expression G6	Mean expression non-G6	
LMNA	3.33 E-178	4.4832574 61	1.61215679	adjusted P-value = 2.5e-5	FASLG	5.63E-200	2.9509723 65	0.744562663	adjusted P-value = 2.4e-5
ELL2	3.35 E-130	2.8527702 09	1.054356773		VCAM1	1.53E-160	2.7515680 05	0.87739373	
NR4A3	8.45 E-167	3.5085654 31	1.336583126		CCL3	1.01E-265	5.3647035 48	1.712536222	
RALGAPA1	6.58 E-104	2.5944767 01	1.030588483		LAG3	9.39E-239	3.6872254 31	1.222011504	
IL7R	1.75 E-214	5.5459078 5	2.245179471		CXCR6	1.10E-251	4.9969235 01	1.659284933	
FAM177A1	6.62 E-154	4.9461948 04	2.099983146		IFNG	1.75E-264	5.0267715 81	1.710214715	
RNF1	2.33	2.3299363	0.995233018		KLRC4	1.10E-179	2.9780760	1.026292822	

25	E-82	69					91		
PIK3R1	1.74 E-99	3.6799295 88	1.573737097		PDCD1	<le-300	5.9431912 37	2.065076325	
TIPARP	4.97 E-63	2.1591307 56	0.97265346		KLRD1	1.49E-214	4.7996519 08	1.732473246	
RGCC	2.65 E-71	3.3363927 34	1.528185149		HAVC R2	2.64E-287	5.8692595 95	2.141327751	
FOSL2	1.20 E-185	4.7908417 16	2.246716335		CD8B	3.39E-299	6.0294166 41	2.230363101	
MPZL3	9.57 E-59	2.6757125 37	1.280435677		SIRPG	5.70E-308	5.9784707 16	2.23501884	
SLC7A5	1.27 E-67	2.8553289 1	1.375475703		SNAP47	4.05E-160	3.8850853 8	1.491724953	
AIM1	1.20 E-63	2.9419118 59	1.451222283		DTHD1	3.87E-139	2.5467491 08	0.984506671	
TSPYL2	9.89 E-110	4.9269395 53	2.440610963		PRF1	<le-300	8.3647015 74	3.238149632	
KDM6B	4.00 E-100	2.9706642 79	1.480739369		GZMH	4.09E-246	5.9527826 25	2.305309885	
CREM	6.19 E-169	7.4753081 79	3.77645027		F2R	3.92E-142	2.9050976 28	1.126664628	
KIAA1683	4.51 E-41	2.2122063 53	1.126839204		CD38	4.38E-228	5.0181312 8	1.983841205	
DCTN6	2.60 E-46	2.6643594 23	1.382766102		GZMK	6.92E-301	6.7569904 04	2.67261115	
MYADM	2.11 E-143	6.2150737 13	3.276258653		CXCL13	4.42E-136	3.6482092 99	1.452309961	
GABARAPL1	7.59 E-61	3.5667257 77	1.907228181		CCR5	4.09 E-144	3.6931719 52	1.475745868	
REL	1.97 E-84	4.2527146 59	2.2751205		CCL4L2	9.22E-254	4.3489088 93	1.746026052	
TCF7	1.41 E-77	2.9767793	1.60343374		KLRC4 - KLRK1	6.46E-227	3.6506047 91	1.476008806	
PERI	1.09 E-120	4.6767376 22	2.521043554		MY07A	4.79E-81	2.0528285 01	0.836785249	
TUBA4A	4.22 E-108	6.8549537 36	3.717830757		JAKMIP1	9.60E-95	2.3097883 01	0.943808472	
PRMT10	2.18 E-29	2.2363756 13	1.216909552		CD8A	<le-300	8.4269526 97	3.465174357	
JMJD6	3.79 E-48	3.6432959 75	1.998480473		NKG7	<le-300	11.395756 29	4.754483909	
PLK3	6.53 E-27	2.2476905 17	1.237673831		GZMA	<le-300	8.8258618 63	3.682454312	
TSC22D2	4.49 E-56	3.0853759 12	1.703252796		CHST12	1.44E-137	3.7317506 49	1.568500397	
ANXA1	6.20 E-102	7.0311357 99	3.886933672		CCL4	<le-300	8.3968792 61	3.549577564	
ZC3H12A	6.48 E-47	3.2099781 66	1.77598105		TOX	2.10E-190	3.5531606 65	1.508607328	
VPS37B	5.37 E-72	3.1698481 17	1.756348442		GZMB	2.30E-207	6.3291433 52	2.698950167	
OAT	9.98 E-25	2.3532383 37	1.313506979		CCL4L1	4.29323244 682430e-310	5.9314730 57	2.549172007	
S1PR1	1.19 E-32	2.0941256 61	1.169873052		GIMA P6	1.25E-139	3.4024694 61	1.470285917	
CCR7	5.86 E-35	2.5340046 03	1.428024657		CTSW	1.03E-271	6.5450727 77	2.829444772	

CSRN PI	1.40 E-53	3.5171784 16	2.013970694		RAB37	2.33E-80	2.3101133 28	1.011989153	
YPEL5	6.75 E-139	7.8436773 5	4.507122902		TRGC2	7.48E-127	4.0584680 18	1.795837039	
STAT 4	5.83 E-105	5.8978002 97	3.397232288		CD27	4.90E-252	6.5606467 54	2.908896232	
NR4A 2	1.93 E-144	7.9895424 35	4.687070745		GPR56	2.52E-98	2.7247135 26	1.2097145	
SYTL3	1.70 E-77	3.6940725 75	2.168625854		KLRK1	<le-300	8.8727567 23	3.959454854	
TC2N	1.79 E-42	3.1855513 27	1.891364802		ABCA2	1.45E-78	2.3526470 33	1.057820435	
DENN D4A	1.54 E-30	2.5383291 45	1.509037124		TIGIT	4.55E-252	6.2474470 8	2.814440919	
HEXI M1	5.04 E-29	2.4175776 75	1.439410801		RGS3	9.29E-56	2.1062507 17	0.950067873	
IFRD1	2.89 E-58	5.6423788 9	3.36326887		OASL	2.02E-133	4.1586867 7	1.886711198	
SERT AD1	6.77 E-29	2.9952575 36	1.796025464		DDX6 0	1.33E-71	2.3405282 57	1.067558341	
CDKN 1A	1.61 E-39	3.3335814 62	2.009148775		GPR17 4	2.16E-107	3.2106098 94	1.468120263	
USP3	1.85 E-63	4.5509763 24	2.757655349		SLAM F7	1.18E-186	4.8982056 92	2.254239908	
ZFP36 L2	5.72 E-114	6.9629370 85	4.221571711		IKZF3	1.32E-222	5.5381765 17	2.54936791	
SIK1	3.24 E-38	2.3421081 78	1.423268667		PVRIG	5.60E-135	4.3931138 42	2.032352139	
ZNF3 31	3.31 E-115	7.4139979 87	4.508218766		SIT1	2.46E-133	4.4151420 02	2.073420107	
AREG	3.06 E-18	2.0313270 77	1.236962164		UBAS H3A	6.64E-74	2.3445691 55	1.106470693	
PFKFB	9.91 3	3.2996466 E-39	21	2.012609218	S100P BP	1.45E-96	2.8751556 48	1.360138834	
CDK1 7	1.17 E-25	2.3082832 41	1.411191671		TNFRS F9	9.34E-86	2.8150343 99	1.332115915	
SLC38 A2	2.14 E-64	5.2532354 66	3.226172013		ZBP1	6.45E-71	2.3848972 31	1.137656242	
TME M2	1.28 E-74	5.5135837 96	3.387405094		THEMI S	8.28E-93	2.7104687 61	1.296225702	
SKIL	2.04 E-55	4.2817540 02	2.657730029		GPR17 1	1.32E-99	3.3343836 09	1.598490188	
NEU1	2.41 E-18	2.6480532 86	1.653251836		SLAM F6	1.32E-77	2.6711716 9	1.28672022	
HSPH 1	5.58 E-32	4.0884574 58	2.556352483		FCRL3	4.72E-90	3.3990303 91	1.681186377	
SELK	2.51 E-53	5.6585629 28	3.548092381		TRGC1	2.01E-79	2.0865904 09	1.035235899	
CRTA M	1.19 E-19	2.5021677 97	1.569776846		ADOR A2A	1.11E-54	2.1996978 71	1.092217814	
RUNX 3	1.27 E-40	3.2940670 63	2.071626058		GIMA P4	4.02E-182	6.1697307 17	3.069270893	
RORA	7.47 E-28	2.4699324 44	1.558178799		GBP5	1.03E-213	5.9173846 7	2.949760732	
PTGE R4	7.14 E-39	4.1532374	2.640138825		CCL5	<le-300	11.822072 05	5.912713485	
CD55	8.13 E-48	5.0211737 92	3.198932122		BCAS4	1.09E-50	2.1508810 21	1.079645464	
RANB P2	1.48 E-63	4.2898198 67	2.738383746		ITGAE	1.41E-97	4.0055436 43	2.013638465	
JUND	4.43 E-96	3.5944134 74	2.29973852		FUT8	1.76E-64	2.3257367 23	1.169220628	
GZM	1.08	2.4436539	1.564558264		SLFN1	1.24E-105	3.0655010	1.54217004	

M	E-20	4			2L		62		
TUBB	2.91	4.7290502			MCTP	2	2.3760611		
4B	E-38	16	3.033912363			5.08E-72	96	1.202772368	
POIR	3.40	3.5481420			TRAFD	1	2.9770641		
3E	E-28	07	2.292755371			2.66E-63	02	1.507174063	
SCML	3.02	2.6541162			ITGB7	4.22E-53	2.5789484		
4	E-29	39	1.720547954			22	1.312803444		
CNOT	6.42	3.9249018			RAB27	A	4.3269805		
6L	E-50	73	2.548661221			5.11E-125	03	2.226815906	
RGPD	5.54	2.4064476			GIMA	P5	4.7102969		
6	E-27	77	1.564306238			1.13E-120	03	2.436961134	
CHD1	3.17	4.0711205			INPP4	B	4.5783291		
E-41	2	2.653545764				1.04E-146	96	2.375307719	
DUSP	2.87	8.7564122			PYH1N	1	4.9201338		
2	E-112	5	5.738855078			6.81E-148	87	2.554327371	
TNFAI	1.90	10.826684			GIMA	P7	5.5920004		
P3	E-143	31	7.147865185			1.71E-153	59	2.909624761	
RGPD	3.67	5.2943787			C5orf5	6	3.2496926		
5	E-48	89	3.507206484			1.80E-73	25	1.69486526	
PDE4	9.74	5.0508445			LYST		6.5426074		
B	E-39	07	3.358452739			2.20E-240	46	3.416273912	
IDII	5.31	3.8826586			CST7	<le-300	8.9736041		
E-24	42	2.586954705				37	4.695878907		
CCNH	2.81	4.6078479			APOB	EC3G	7.3387360		
E-23	25	3.070532419				6.76E-230	2	3.846833696	
FAM4	8.53	4.9789809			CXCR3	2.43E-99	4.1797921		
6C	E-61	87	3.332095051			99	2.199446151		
ATXN	1.17	2.5908792			CD84	3.21E-108	4.2215505		
7	E-30	6	1.741425098			44	2.223035064		
FYN	9.57	7.2658541			CLSTN	3	2.0502569		
E-117	76	4.888805828				4.45E-60	79	1.081603794	
ATP1	1.18	4.8720507			ABI3	1.47E-63	3.0288603		
B3	E-40	81	3.285414683			86	1.598221831		
NFE2	5.11	3.7012983			IL2RB	1.74E-224	6.2547584		
L2	E-21	89	2.498795494			04	3.301967305		
IVNS1	1.90	4.6762437			RARRE	S3	7.2571218		
ABP	E-28	09	3.183118599			8.68E-193	26	3.867006375	
POLR	4.12	5.3186457			APOB	EC3D	3.3504240		
2A	E-47	97	3.62575721			8.50E-121	48	1.798179903	
CAMK	2.20	2.3282003			SLFN5	1.14E-125	3.9087131		
4	E-19	69	1.589207478			59	2.098640434		
CHM	8.02	2.8690279			HAPLN3	1.57E-34	2.0269549		
P1B	E-18	66	1.958603621			15	1.091698363		
NR4A	2.73	3.8452825			PAM	1.25E-77	2.9765576		
1	E-19	37	2.656379795			81	1.607617497		
GSPT	1.30	2.4693307			PCED1	B	3.1088282		
1	E-12	42	1.708454721			3.69E-86	84	1.679076643	
SLC2A	4.58	7.8657934			ITM2A	3.98E-153	6.1609274		
3	E-63	95	5.4474217			76	3.332344533		
IQGA	1.05	2.4613802			GBP1	2.78E-76	3.6776148		
P2	E-15	66	1.709898404			84	2.002535044		
GPR6	1.75	2.3277246			GOLI	M4	2.1588695		
5	E-08	67	1.624386821			1.18E-57	7	1.176344532	
HBP1	6.96	2.6681814			SH2D1	A	4.8816047		
E-08	06	1.865144355				4.72E-127	51	2.670995027	
PAF1	3.43	2.6057951			MPHO	SPH9	2.5389079		
E-II	32	1.822622722				5.36E-46	74	1.402397418	
AMD	1.34	3.6013408			GIMA	P2	2.3660093		
1	E-17	8	2.518994593			1.96E-35	31	1.312623963	
SORL	5.21	2.1109703			TTN	4.68E-69	2.2642140		
1	E-13	13	1.479529912			31	1.256504449		
KLRK1	8.05	6.2437143			IFI44L	3.63E-37	2.3519123		
E-54	1	4.382216785			DENN	D2D	5.9230809		
GZMK	3.71	4.3404489				6.62E-132	79	3.313248223	

AKIRI N1	6.70 E-12	2.7053080 28	1.90930518		GYG1	1.15E-29	2.2053505 84	1.237052039	
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Table 2D

G7- Regulatory Tcells				G8- Cytotoxicity (Lymphocytes)					
Gene Name	P-value	Mean expression G7	Mean expression non-G7		Gene Name	P. value	Mean expression G8	Mean expression non-G8	
FOXP3	<le-300	2.9084137 27	0.090556475	Adjusted P-value = 2.4e-5	FGFBP2	1.00 E-235	2.1722153 04	0.173170381	adjusted P-value = 2.6e-5
CCR8	2.700000000 000000e-322	2.1881641 06	0.075687229		FCRL6	1.00 E-116	2.2316089 38	0.590102161	
TNFRSF4	<le-300	4.5184001 42	0.477269932		TGFBR3	6.11 E-151	2.0398588 98	0.553300244	
ICAI	1.79E-242	2.3655144 85	0.259492653		GNLY	1.75 E-191	4.8512506 65	1.372456654	
FBLN7	4.85E-210	2.0808570 66	0.259247645		SPON2	5.07 E-69	2.1381768 42	0.746447667	
RTKN2	6.92E-238	2.1282877 92	0.272269648		SAMD3	1.59 E-198	4.3753273 62	1.55410411	
IL2RA	3.03E-227	2.7816289 19	0.385409782		TRDC	7.32 E-67	2.0939496 76	0.751546817	
TNFRSF18	<le-300	5.7161355 23	0.96786451		KLRG1	1.38 E-150	3.9808797 33	1.474660126	
MAGEH1	3.53E-159	2.6642863 29	0.531885504		GZMH	6.95 E-173	5.6871855 53	2.360734181	
MAF	<le-300	3.9734528 44	0.968129857		TRGC1	2.65 E-97	2.3241629 58	1.003067036	
ETV7	1.94E-114	2.0284881 35	0.511507871		A2M	2.25 E-111	2.3927909 02	1.047033015	
CD4	<le-300	6.5343907 19	1.678805026		FCGR3A	1.47 E-48	2.0998591 68	0.93181407	
TBC1D4	4.78E-223	3.6002661 05	0.956829353		GZMM	2.05 E-82	3.1939197 68	1.449569907	
IKZF2	8.60E-133	2.1604360 96	0.585303833		AOAH	1.07 E-148	4.9506080 16	2.349056852	
DUSP16	1.83E-103	2.2566122 97	0.664144261		GZMA	1.01 E-257	7.9535536 9	3.836901532	
ICOS	2.18E-270	5.3008590 72	1.572669758		HOPX	4.28 E-51	2.6218969 2	1.276417898	
ZC3H12D	2.26E-192	3.4497790 47	1.093100253		KLRB1	4.28 E-38	2.1119916 64	1.040188137	
STAM	3.50E-126	2.8529699 28	0.904163289		NKG7	<le-300	10.012419 4	4.993297224	
HS3ST3B1	1.99E-116	2.5191118 22	0.799545175		GRAP2	7.15 E-52	2.3736282 19	1.209239579	
CTLA4	2.58E-306	6.0954591 1	1.962910377		PXN	4.30 E-52	2.5123005 25	1.282253401	
TIAM1	2.80E-116	2.3450606 93	0.779900258		KLRD1	8.89 E-79	3.7307681 27	1.908670512	
TNFRSF25	1.25E-156	3.5464261 16	1.183587732		PTPN4	3.07 E-45	2.3109873 45	1.219806765	
GK	9.70E-136	3.0055006 3	1.011438666		TRGC2	2.82 E-74	3.5584181 63	1.881606394	
BTLA	7.44E-82	2.2287275 56	0.763674526		CTSW	4.15 E-31	5.5964711 31	2.989823745	

					126			
BATF	7.65E-238	5.5037579 11	1.90427697	S1PR1	1.53 E-42	2.1631445 37	1.159294979	
COR		3.4146587			1.08			
OIB	7.06E-123	02	1.250470601	PRF1	E- 142	6.3362025 69	3.569730675	
SDC4	1.89E-82	2.2607945 44	0.829654367	CCL5	<le- 300	10.780699 07	6.096162864	
CD28	8.86E-200	4.5050039 5	1.658919166	TC2N	2.27 E-54	3.2739789 48	1.877812076	
THAD		2.7593358		C12orf	5.50	2.3557915		
A	1.95E-94	86	1.022705012	75	E-27	56	1.376957789	
PHTF		3.7095480		PLAC8	6.48 E-41	2.9472812 72	1.723352085	
2	3.08E-137	69	1.400635409	ITM2C	4.45 E-27	2.2064813 28	1.295325762	
TME		5.7339646		CCL4	1.89	6.5404257		
M173	2.94E-209	6	2.183616303	CCL4L	E-97	91	3.853663502	
KLRB		2.6114620		2.5764040				
1	2.92E-84	59	1.011766586	2	E-73	37	1.923970079	
MICA		2.1712700		TSEN5	7.10	2.9227893		
L2	1.83E-66	8	0.848952074	4	E-29	62	1.735034013	
SLAM		2.8641614		GZMB	1.27	4.9263317		
Fl	4.59E-86	42	1.132013256	E-69	19	2.928598206		
SPOC		7.3804938						
K2	<le-300	92	2.971304765	KLRK1	4.41 E- 140	7.0792678 39	4.254156938	
PBX4		2.0627111						
	1.39E-65	25	0.841335307	CST7	1.27 E- 142	7.8977928 01	4.878022605	
PHAC		2.5941003		CCL4L	5.63			
TR2	9.50E-114	34	1.063992414	1	E-70	4.4551144	2.789091846	
MBO		3.3577118		C20orf	3.45	2.5280536		
ATI	6.49 E-104	23	1.432443676	112	E-31	72	1.588078455	
FAS		3.2410823		MYO1	2.09	4.1495897		
	5.43E-90	65	1.384655322	F	E-44	38	2.633861547	
RORA		3.4256895		GIMA	5.03	4.7743723		
	3.74E-114	33	1.470520079	P7	E-51	23	3.045760988	
PELII		3.5335599		NLRP3	9.30	2.0464433		
	6.50E-100	77	1.524060798	E-25	27	1.315260556		
TNIK		2.2697023		S0RL1	7.21	2.2613018		
	3.34E-75	32	0.983370734	E-26	01	1.456489584		
LTB		5.0627007		ZAP70	9.08	6.0943854		
	4.24E-138	04	2.20604619	E-91	7	3.928736743		
GEM		2.0211641		C5orf5	1.82	2.7517180		
	4.56E-42	59	0.882068113	6	E-23	8	1.777460478	
TIGIT		6.5124357		SYNE1	3.87	4.1511302		
	7.46E-212	29	2.896471619	E-52	92	2.682600875		
DNP		4.0375236		PYHIN	1.21	4.1402258		
HI	2.19E-88	86	1.796331637	1	E-46	25	2.683405094	
		2.0199412		SCML	1.40	2.6453228		
UXS1	1.95E-45	13	0.904382548	4	E-44	65	1.721895658	
PBXIP		5.5660359		SLFN1	4.14	2.5059065		
1	4.17E-189	5	2.594328128	2L	E-30	24	1.634082262	
NCF4		2.2185155		GPR56	2.80	2.0120719		
	7.94E-46	09	1.040444721	E-17	98	1.325049617		
HTAT		2.0845470		CD8A	1.68	5.8197329		
IP2	4.81E-37	89	0.979359507	E-69	55	3.884787277		
CD5		4.2639545		STOM	1.81	4.2445547		
	3.64E-116	83	2.025795414	E-40	74	2.836417195		
ARID		6.1005792		PIM1	6.06	3.6643899	2.45178595	
5B	1.55E-214	62	2.907675605					
TRAF		2.1708050						
3	4.82E-47	54	1.051843069	KLRC4	3.18	2.4987754		
RAB1		3.1904023		-	E-35	16	1.66131693	
1FIP1	1.64E-75	51	1.561024115	KLRK1				
RHBD		4.2990189						
D2	2.73E-86	6	2.148754716					
LY75		2.47E-53	2.2916954					
			1.146266509					

		35				E-26	71		
SUSD 3	1.39E-42	2.3875103 18	1.197390982		SYTL1	3.57 E-18	2.2686837 15	1.52264347	
P2RY 10	2.41E-54	2.8782431 63	1.465873443		BIN2	2.89 E-28	4.3528364 41	2.939740624	
CNST	1.06E-49	2.2665537 02	1.161711281		MGAT 4A	7.87 E-19	2.2552044 47	1.528885156	
DUSP 4	1.09E-170	5.6578306 36	2.900523194		PATL2	1.17 E-09	2.1228387 93	1.441287402	
IL6ST	7.78E-84	3.8667124 42	1.98752116		SLC9A 3R1	1.12 E-32	4.2347234 5	2.884820027	
LIMS 1	1.15E-66	3.3573149 2	1.737804209		TNF	8.77 E-II	2.0431275 7	1.392053653	
TP53I NPI	2.08E-66	2.8724630 91	1.503327014		GLPR 2	3.52 E-19	2.7921785 89	1.912894809	
MSI2	2.54E-57	2.6908571 01	1.423990667		TBCD	4.13 E-18	3.1545789 83	2.165678834	
ZC3H 7A	2.93E-68	3.5947056 83	1.908123109		TPST2	6.75 E-14	2.6201149 52	1.802195474	
SIRP G	1.06E-92	4.6958376 11	2.512396711		THEMI S	8.20 E-17	2.0313112 02	1.406022387	
NR3C 1	1.35E-55	4.0869234 76	2.195774422		GZMK	1.11 E-34	4.4009421 68	3.050188245	
HNR NPPLL	2.22E-102	4.4283221 16	2.403323959		ANXA 1	6.75 E-63	5.8603125 23	4.066378128	
CARD 16	1.59E-43	3.5717153 15	1.947131057		SLFN5	2.72 E-26	3.1854581 87	2.216792859	
OTU D5	4.05E-28	2.0181702 52	1.115322696		GIMA P5	6.39 E-28	3.7277182 99	2.596727721	
PHLD A1	9.62E-80	3.0608492 44	1.693115558		STAT4	1.21 E-40	5.0141155 74	3.532668889	
CD82	1.44E-78	4.9682357 1	2.757717194		RASAL 3	3.30 E-21	2.2700476 77	1.601342177	
GOLG A8B	1.39E-58	3.1153146 77	1.732030277						
EPSTI 1	1.40E-65	3.5544964 23	1.993749084						
UGP2	5.96E-43	3.4327849 06	1.940519106						
KIAA 0319 L	1.16E-35	2.6531823 68	1.512545427						
TLK1	9.92E-99	4.7885064 53	2.734997166						
SYT1 1	9.75E-30	2.0580062 9	1.193397514						
TRAF 1	1.30E-38	2.8201441 04	1.637534766						
CNIH 1	1.24E-33	2.9791535 1	1.731344333						
ARNT L	8.59E-27	2.0547142 99	1.19965166						
PIK3I PI	2.09E-82	5.4202786 43	3.165901718						
PIM2	8.08E-117	7.3141169 09	4.310127865						
NABP 1	4.51E-51	4.2093022 01	2.483672766						
LAT	1.85E-79	5.3986283 99	3.19395051						
PCED 1 B	7.85E-45	2.9466145 8	1.745834401						
GOLG A8A	8.62E-42	2.2627747 27	1.346343169						
ITM2 A	4.94E-82	5.8193082 01	3.46689161						
IFNA R2	3.36E-29	2.5657000 43	1.535927592						

BTG3	6.37E-35	3.2082184 46	1.924165026						
GATA 3	4.11E-33	2.1584706 98	1.304861133						
DDH DI	6.85E-33	2.5726039 76	1.555313671						
CD24 7	5.64E-80	5.1637785 36	3.122026701						
SKAP 1	5.90E-71	4.8244731 17	2.918432343						
TULP 4	1.66E-31	2.1705300 54	1.319962353						
TRIM 59	2.00E-33	2.6674135 82	1.628028112						
GRSF 1	1.65E-26	2.857782	1.74443896						
PMAI PI	2.15E-35	3.6155053 76	2.207346783						
CD2	1.17E-183	8.6777275 72	5.331415144						
NDFI PI	7.69E-49	3.2576471 15	2.005866319						

Table 2E

G9- Exhausted/HS CD8 T cells				G10- Memory T cells					
GeneName	P-value	Mean expression G9	Mean expression non-G9		Gene Name	P-value	Mean expression G10	Mean expression non-G10	
VCAM 1	<1e-300	4.5086744 8	0.751053745	adjusted P-value = 2.5e-5	LEF1	5.45 E-211	2.94444894 8	0.579553106	adjusted P-value = 2.6e-5
KIR2DL 4	9.85 E-114	2.0886682 3	0.441388636		TCF7	<1e-300	5.15937531 7	1.373967472	
TNFRS F9	1.61 E-258	4.5369895 31	1.194621837		SERIN C5	1.71 E-96	2.16870596 4	0.679370498	
GEM	1.93 E-106	2.7923067 02	0.801348743		IL7R	<1e-300	6.77351122 9	2.184382166	
CXCL1 3	3.69 E-209	4.8533439 18	1.400869995		CCR7	8.82 E-149	3.86012851 4	1.295935281	
NAB1	4.21 E-244	4.5897093 21	1.33732745		TNFR SF25	6.66 E-122	3.39672114 5	1.196499513	
DFNB3 1	6.82 E-154	2.7030782 02	0.833537198		S1PR1	1.30 E-100	2.85176368 1	1.102302782	
CADM 1	3.67 E-87	2.3982720 64	0.746128607		PASK	9.70 E-61	2.06658203 2	0.81085611	
CRTA M	4.53 E-176	4.4411835 1	1.382798846		FLT3L G	9.78 E-81	2.65233014 5	1.071446837	
GPR56	1.03 E-162	3.6286863 29	1.166018599		CAMK 4	8.70 E-102	3.47591791 1	1.468996844	
CTLA4	1.58 E-239	5.8504188 68	2.014356959		SORL 1	2.09 E-106	3.15297662 1	1.369325162	
MYO7 A	1.57 E-94	2.3791614 07	0.846889327		DHRS 3	5.84 E-43	2.05941662 5	0.985173246	
DUSP4	<1e-300	7.4397301 71	2.714721226		TME M63A	3.90 E-39	2.09396385	1.011937545	
HAVCR 2	6.71 E-3	6.0431135	2.265831154		MGA T4A	3.97 E-62	2.98639176 4	1.459200768	

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TNFSF 9	6.17 E-97	2.6082542 9.6	0.984891442		LTB	4.87 E-98	4.55425031 3	2.261647026	
METR NL	8.21 E-71	2.5979422 0.7	0.991089694		RCAN 3	1.45 E-41	2.04886648 5	1.032986603	
DTHD1	3.04 E-110	2.6612400 2	1.031970441		ABLI M1	5.04 E-59	3.39735833 4	1.765303513	
CXCR6	6.08 E-123	4.5895815 9.6	1.834458258		PLAC 8	1.05 E-77	3.28999900 3	1.714545204	
CCDC6 4	1.82 E-97	2.7829619 8.7	1.134034007		DGKA	3.29 E-115	6.07316654 6	3.188214222	
PHLDA 1	3.41 E-155	3.9245383 28	1.603236536		TC2N	5.40 E-74	3.54392655 1	1.882542777	
PDE3B	9.09 E-95	2.6252089 43	1.075625687		SELL	7.14 E-71	4.33455900 3	2.33094736	
GZMB	9.87 E-196	6.7657533 35	2.789941845		KLRB1	4.46 E-27	2.00685008 3	1.081968203	
LAG3	4.95 E-107	3.2572123 74	1.366009638		C20or fl12	1.02 E-58	2.90572337 1	1.56733606	
SLC7A 5	2.71 E-95	3.2628231 67	1.380835101		TESPA 1	6.96 E-54	2.95543883 8	1.599015236	
KLRC4	1.74 E-82	2.6730914 74	1.136286936		CCDC 109 B	1.51 E-28	2.25524559 9	1.229294002	
PDCD1	5.07 E-161	5.3276882 32	2.284706391		GIMA P5	4.85 E-81	4.58146216 6	2.52300272	
NELL2	1.60 E-90	3.1661749 48	1.388173452		OXNA D1	5.15 E-54	3.51018896 8	1.942033	
SNAP4 7	8.05 E-79	3.6521344 97	1.610645941		FAM1 02A	5.89 E-53	3.08675121 2	1.726293832	
ENTPD 1	3.31 E-87	3.6914248 23	1.642028119		SATB 1	1.34 E-34	2.40485877 8	1.394529769	
CD8A	2.18 E-247	8.2345908 9.5	3.678834602		NOSIP	2.09 E-24	2.80203827 2	1.626433851	
TTN	4.66 E-103	2.7452726 88	1.241043601		FAM6 5B	1.67 E-62	3.88711977 2	2.26804134	
PRF1	4.91 E-257	7.7103445 62	3.510458727		ICAM 2	2.92 E-27	2.18270200 3	1.284960731	
CD8B	1.38 E-140	5.3728182 59	2.451585597		ATM	1.39 E-37	3.27833228 3	1.964122784	
MCTP2	3.72 E-61	2.6597350 58	1.21604996		SCML 4	2.13 E-38	2.81405540 6	1.726222746	
TOX	2.58 E-103	3.4700798 34	1.597080102		CD5	1.27 E-41	3.44049213 4	2.121272729	
AHI1	1.84 E-71	3.3306424 86	1.537543074		PIK3I PI	3.86 E-55	5.07133406 8	3.203392026	
GZMH	6.34 E-125	5.4177403 56	2.506915595		FOXP 1	3.30 E-51	4.07345850 5	2.575606277	
SYTL3	8.59 E-127	4.5531870 43	2.124468616		EPB4 1	7.62 E-25	2.44318226 1	1.55896682	
GOLIM 4	4.27 E-58	2.4872746 0.3	1.177182963		CD28	1.79 E-32	2.89722081 5	1.848799225	
PAM	3.00 E-72	3.3874657 66	1.614064805		GOLG A8A	4.09 E-24	2.12875824 6	1.360626747	
KLRC4-	1.16	3.3260711	1.596832114		GIMA	4.27	4.79852873	3.089485076	

KLRK1	E-86	01			P7	E-42	6		
LYST	2.04 E- 225	7.0356632 6	3.481392078		CHMP 7	5.22 E-17	2.44473006 6	1.600860764	
SLA2	1.09 E-87	3.7675833 48	1.868466871		NELL2	1.15 E-16	2.25627984 5	1.484964873	
NKG7	2.74 E- 283	10.330181 18	5.131904651		DENN D2D	2.69 E-51	5.19676944 8	3.482663105	
RAB27 A	5.95 E-96	4.5752992 69	2.279940422		GOLG A8B	8.87 E-18	2.62515418	1.788746503	
ASXL2	5.43 E-57	2.5929348 51	1.309028158		GIMA P2	5.90 E-09	2.01572164 7	1.387980767	
HNRRN PLL	1.24 E- 118	4.7168145 2	2.382302874		TME M123	3.74 E-17	3.70834674 4	2.577253683	
ITPRIP	6.46 E-37	2.1708418 3	1.096513915		GPR1 83	1.15 E-24	3.72459834 9	2.59332694	
TGIF1	3.21 E-54	3.4684281 25	1.767563879		TTC39 C	1.03 E-20	3.18872930 8	2.221132449	
BANP	1.85 E-38	2.5288651 95	1.291785586		KIAAO 922	7.98 E-12	2.19047557 1	1.529233079	
CREM	4.86 E- 145	7.6031547 17	3.890628938		RAPG EF6	1.49 E-15	2.75122537 5	1.924237613	
PON2	8.34 E-30	2.3309910 9	1.201677945		AAK1	1.98 E-27	3.90153266 7	2.757927038	
CCL4L1	9.15 E- 129	5.2824120 68	2.753423964						
CCL4L2	5.19 E-88	3.6636294 45	1.924232711						
PDE4D	5.60 E-97	4.1134502 86	2.173825861						
CCL4	1.95 E- 138	7.2643259 96	3.865196352						
VPS37 B	4.16 E-83	3.3504431 76	1.785074504						
ATXN1	1.40 E- 111	5.1021377 4	2.731878905						
CTSW	3.37 E- 105	5.7210895 38	3.066381						
KLRK1	6.50 E- 187	7.9299330 59	4.256157611						
GABAR APL1	8.57 E-61	3.6380692 33	1.956872151						
CCL5	<1e- 300	11.487184 24	6.179148138						
F2R	2.04 E-40	2.3394388 4	1.259450691						
JMJD6	4.98 E-47	3.7878260 21	2.039332494						
KLRD1	1.87 E-56	3.6594218 22	1.980115484						
BTG3	4.44 E-49	3.4986691 11	1.898669583						
DCTN6	3.63 E-36	2.6359978 09	1.430548667						
SAMS N1	8.49 E- 105	6.0545345 74	3.289197393						
TIGIT	6.76 E- 103	5.5532594 72	3.025760097						

PARDM 1	2.59 E- 182	6.7357567 15	3.672503705						
ZBTB1	2.15 E-43	3.1763025 31	1.735354528						
CBLB	1.93 E- 171	7.2627623 79	3.980358924						
DNAJA 4	1.58 E-25	2.0541604 95	1.128412534						
CST7	3.88 E- 200	8.8627709 69	4.873858559						
STAT5 B	3.00 E-38	2.5010117 28	1.393403362						
CD27	2.14 E-99	5.6504160 42	3.15312123						
CHST1 2	3.04 E-41	3.0890163 89	1.724890392						
FUT8	2.63 E-30	2.1933378 69	1.228929587						
TP53IN PI	3.39 E-46	2.7263175 68	1.52772226						
TMEM 2	1.94 E- 113	6.0482769 39	3.400850472						
GSPT1	1.05 E-39	2.9793238 47	1.67721026						
GATA3	2.46 E-37	2.2862794 29	1.295298574						
PMAIP 1	5.81 E-45	3.8563160 71	2.188180593						
HSPH1	6.26 E-49	4.5013981 37	2.562912897						
PFKFB 3	5.94 E-58	3.5602642 02	2.027882148						
CN0T6 L	4.58 E-88	4.4273056 74	2.539677675						
IFNG	7.99 E-43	3.4821455 24	2.013260286						
GZMK	5.47 E-67	5.1956545 48	3.00724257						
ATHL1	1.23 E-42	2.8224168 69	1.633708809						
TSPYL2	1.37 E-56	4.4555010 87	2.580429436						
SH2D2 A	1.35 E- 104	5.2101123 69	3.023296269						
FCRL3	8.86 E-38	3.0725312 15	1.784567474						
IPCEF1	8.03 E-35	2.6652383 82	1.54872764						
LRMP	1.47 E-45	3.1838864 78	1.853455577						
TRAT1	7.67 E-40	3.4047511 84	1.984115363						
OASL	6.35 E-39	3.5079387 29	2.048212867						
STAT5 A	6.91 E-26	2.2583584 33	1.330095834						
IRF4	6.00 E-35	2.8651492 34	1.69212468						
ETS1	1.64 E-74	4.4114021 93	2.613554596						
PTPN2 2	5.49 E-75	4.7823150 91	2.833610963						

Table 2F

Gil- Lymphocytes exhausted/cell-cycle			
GeneName	P-value	Mean expression Gil	Mean expression non-Gil
SPC25	<le-300	2.55158841	0.044683954
CDC45	<le-300	3.655492107	0.067760256
KIF15	<le-300	2.174404427	0.045021031
DLCAP5	<le-300	2.74204077	0.060438937
HIST1H3G	<le-300	2.048930929	0.048216741
KIF18B	<le-300	2.299645768	0.054267922
RRM2	<le-300	5.979473049	0.144311828
UBE2C	<le-300	4.605312013	0.11221674
HJURP	<le-300	2.440545198	0.059740348
ESCO2	<le-300	2.281039379	0.056143688
SPC24	<le-300	3.790205449	0.099149221
BIRC5	<le-300	4.160162746	0.110489205
CDC6	<le-300	2.129610899	0.061460209
CDCA8	<le-300	2.917928579	0.085707394
AURKB	<le-300	3.938514949	0.118029222
ZWINT	<le-300	4.880944943	0.146815449
CDCA2	3.00000000000000e-323	2.154636598	0.064819609
GTS E1	<le-300	2.237362094	0.067415946
DTL	<le-300	3.01936579	0.091448243
RAD51	<le-300	3.006106426	0.091861703
CDCA3	<le-300	2.843509088	0.089148695
MELK	<le-300	3.045934257	0.096305647
CKAP2L	<le-300	2.484127304	0.087002675
ANLN	<le-300	2.32543532	0.082123655
ASF1B	<le-300	4.341703889	0.156818226
TYMS	<le-300	6.623959258	0.242937564
NCAPG	<le-300	2.760840243	0.102509553
TK1	<le-300	5.275512434	0.196568648
PKMYT1	<le-300	4.173006819	0.155507194
KIFC1	<le-300	2.986890319	0.112381109
KIAA0101	<le-300	6.132531375	0.231188565
CCNB2	<le-300	3.346553414	0.127119045
DEPDIC1B	2.35E-295	2.120095818	0.081054515
CDC20	<le-300	2.99029734	0.11496007
TROAP	<le-300	2.730634101	0.108622291
CLSPN	<le-300	2.412512241	0.097904245
ASPM	<le-300	2.524410973	0.102914917
GINS2	<le-300	2.591389266	0.105848356
KIF23	<le-300	2.899077376	0.120677081
KIF2C	<le-300	2.919485764	0.124529204
RAD51AP1	<le-300	2.831802232	0.12281219
NUF2	<le-300	2.855727508	0.125977392
SHCBP1	4.53593553773400e-312	2.325559651	0.103508687
TOP2A	<le-300	4.491338648	0.202597836
CDK1	<le-300	4.802007428	0.2176185
MKI67	<le-300	4.119201621	0.187968014
MLF1IP	<le-300	3.974550094	0.185022883
PLK1	3.37E-284	2.527243378	0.121149365
DHFR	<le-300	3.408690236	0.163807152
KIF11	<le-300	3.028640082	0.15488364
CENPW	<le-300	3.230577206	0.16790494
TPX2	<le-300	3.581022453	0.188783365
CASC5	<le-300	3.000396744	0.163541724
CDKN3	<le-300	3.713849904	0.211251442
CCNA2	<le-300	4.048129904	0.235156885
BUB1B	<le-300	2.778128153	0.163793368
MCM2	<le-300	4.087032817	0.247259574
UBE2T	<le-300	4.557859797	0.280427572
BRCA1	6.91E-280	2.22729081	0.137544941
MCM4	<le-300	4.751741111	0.313942117

GGH	5.68E-276	2.771416642	0.184573495	
TCF19	<le-300	3.548525036	0.238036736	
BUB1	<le-300	3.051032141	0.208116055	
HMGB3	8.20E-265	2.387864303	0.167993506	
ECT2	2.03E-245	2.001276411	0.141510846	
FEN1	<le-300	4.493377252	0.318417899	
WDR34	<le-300	3.08337534	0.221409972	
NCAPG2	<le-300	3.252214597	0.234183609	
CCNB1	2.26E-241	2.726825158	0.198010971	
ORC6	2.16E-269	2.345457084	0.172056348	
CHEK1	5.54E-300	2.730370116	0.201519298	
SGOL1	1.48E-238	2.263546757	0.169551266	
CENPH	3.54E-266	2.439082667	0.183665985	
CENPF	<le-300	3.371903982	0.254122052	
MAD2L1	<le-300	4.377747502	0.333811558	
SPAG5	6.22E-282	2.864969673	0.222602813	
NCAPH	<le-300	3.187949506	0.253944902	
CCNF	1.34170272600000e-315	2.479216797	0.197966267	
CENPE	1.50E-280	2.332072352	0.186533873	
RFC3	2.62E-245	2.14291466	0.171840946	
FANCI	<le-300	4.024540198	0.324976352	
CENPM	<le-300	4.983236941	0.435256782	
CDCA7	<le-300	4.084697463	0.359021698	
TIMELESS	3.68E-304	2.602007914	0.232190616	
FBXO5	7.81E-263	2.533158157	0.229785725	
PRC1	3.87E-284	3.176132843	0.291974824	
RNASEH2A	<le-300	3.454471068	0.333041016	
SMC2	<le-300	3.925122486	0.380064933	
STMN1	<le-300	8.387592123	0.818322649	
AURKA	1.86E-208	2.246869717	0.220381455	
RACGAP1	8.14E-261	2.852109531	0.289040451	
HIST1H2AM	4.23E-223	2.14309683	0.218864764	
APOBEC3B	1.83E-204	2.520044219	0.259552616	
BRCA2	1.81E-235	2.324802474	0.241846519	
ATAD5	8.59426392276280e-310	2.356822748	0.246544856	
CENPN	2.52E-269	3.366971033	0.352640995	
HIRIP3	5.74E-307	3.07915335	0.323115763	
CKS1B	<le-300	5.284506046	0.574726378	
NDC80	2.18175000000000e-318	3.410466555	0.383395396	

Table 3

T cells	CD4 T cell	CD8 T cells	Regulatory T cells	Regulatory CD4 T cells	Regulatory CD8 T cells
CD3E	CD4	CD8A;CD8B	FOXP3	FOXP3	FOXP3
CD2	CD3E	(-)NCR1	CD3E	CD3E	CD3E
(-)NCR1	(-)NCR1	(-)NCAM1	CTLA4;IL2RA A	CD4	CD8A;CD8B
(-)NCAM1	(-)NCAM1	(-)FOXP3	CD4	CTLA4;IL2RA	CTLA4;IL2RA
(-)FOXP3	(-)FOXP3		CD8A	(-)CD8A	(-)CD4
			CD8B	(-)CD8B	
Regulatory CD4\CD8 T cells	NKT cells	NK cells	B cells	Activated T cells	Exhausted T cells
FOXP3	CD3E	FCGR3A	CD19;MS4A1	CD3E	CD3E
CD3E	NCR1;NCAM1	NCR1;NCAM1	(-)CD3E	CD2	CD2
CD4	(-)FOXP3	(-)CD3E	(-)FOXP3	CD28	PDCD1;CTLA4;BTLA;KIR3DL1;LAG3;HAVCR2;ADORA2A;HAVC

					R1
CD8A;CD8B	(-)FOXP3		IL2RA;CD69;ICOS;TNFRSF4;TNFRSF9;CD27		
CTLA4;IL2RA			IL2;TNF;IFNG	CD8A;CD8B;CD4	
			CD8A;CD8B;CD4	(-)NCR1	
			(-)NCR1	(-)NCAM1	
			(-)NCAM1		
Memory T cells	Memory CD4 T cells	Memory CD8 T cells	Memory CD4\CD8 T cells	Macrophage immature	Macrophage mature
CD3E	CD3E	CD3E	CD3E	CD163	CD163
SELL	SELL	SELL	SELL	ITGAM	ITGAM
CCR7	CCR7	CCR7	CCR7	CD4	CD4
CD28	CD28	CD28	CD28	(-)CD3E	HLA-DRA
(-)FOXP3	CD4	CD8A;CD8B	CD4	(-)HLA-DRA	(-)CD3E
(-)CD4	(-)FOXP3	(-)FOXP3	CD8A;CD8B		
(-)CD8A	(-)CD8A	(-)CD4	(-)FOXP3		
(-)CD8B	(-)CD8B				
Monocyte immature	Monocyte mature	cDCs dendritic cells	pDCs	Myeloid cells general immature	Myeloid cells general mature
CD14	CD14	MHCII	IL3RA	CD33	CD33
FCGR1A	FCGR1A	CD4	CLE4C	(-)CD3E	MHCII
(-)HLA-DRA	HLA-DRA	ITGAX;THBD	NRP1	(-)MHCII	(-)CD3E
	(-)CD3E	(-)CD3E	LILRA4		
			MHCII		
			(-)CD3E		

Immune cell classification based on known markers. Essential markers are bolded; non-bolded refers to markers where only one in each cell is essential; (-) marker should be absent;

Table 4

Responder

GI- B cells				G10- Memory T cells			
Gene Name	P-value	% exp in R	% exp in NR	Gene Name	P-value	% exp in R	% exp in NR
IGHD	8.44E-72	0.11095 8904	0.03503 4347	LEF1	1.13E-35	0.14500 9785	0.07899 9019
PAX5	4.56E-85	0.11819 9609	0.03424 9264	TCF7	8.03E-50	0.33287 6712	0.22021 5898
FCRL1	1.86E-91	0.12446 184	0.03542 6889	CCR7	8.92E-148	0.29706 4579	0.12198 2336
FCER2	1.14E-58	0.10391 3894	0.03640 8243	S1PR1	1.20E-34	0.22367 9061	0.14317 9588
CD19	4.54E-107	0.15009 7847	0.04435 7213	LTB	4.66E-41	0.35479 4521	0.24985 2797

CD22	1.17E-92	0.14794 5205	0.04867 5172		PLAC8	3.41E-37	0.28101 7613	0.18930 3238
BANK1	1.97E-95	0.15440 3131	0.05142 2964					
MS4A1	1.66E-140	0.19354 2074	0.05750 736					
BLK	1.04E-59	0.10195 6947	0.03473 9941					
RALGPS2	8.21E-66	0.11213 3072	0.03827 2816					
FAM 129C	7.47E-43	0.10195 6947	0.04308 1452					

Non-responder

G6- Exhausted CD8 T cells				G7- Regulatory T cells			
Gene Name	P-value	% exp in R	% exp in NR	Gene Name	P-value	% exp in R	% exp in NR
FASLG	1.64E-41	0.07397 2603	0.14720 314	TNFRSF4	1.21E-15	0.08630 137	0.12924 4357
VCAM 1	3.43E-80	0.06692 7593	0.17330 7164	TNFRSF18	9.19E-26	0.13463 7965	0.20245 3386
CCL3	5.62E-158	0.11174 1683	0.29774 2885	MAF	2.31E-31	0.17632 0939	0.25927 3798
LAG3	2.58E-59	0.14109 589	0.25250 2453	ETV7	2.05E-56	0.04266 1448	0.11658 4887
CXCR6	2.69E-64	0.15068 4932	0.26977 4289	CD4	6.77E-51	0.20782 7789	0.32188 42
IFNG	1.26E-66	0.16086 1057	0.28459 2738	CTLA4	7.61E-52	0.21311 1546	0.32914 6222
PDCD1	7.40E-87	0.20058 7084	0.35279 686				
KLRD1	8.31E-43	0.17045 0098	0.26830 2257				
HAVCR2	5.12E-168	0.17514 6771	0.38891 0697				
SIRPG	1.65E-40	0.24559 6869	0.35053 9745				
SNAP47	9.99E-83	0.14500 9785	0.28105 9863				
DTHD1	8.10E-25	0.13933 4638	0.20657 5074				
PRF1	1.05E-135	0.29256 3601	0.50058 8813				
GZMH	2.44E-103	0.17964 775	0.34298 3317				
F2R	1.94E-31	0.13933 4638	0.21629 0481				
CD38	7.97E-172	0.16046 9667	0.37301 2758				
CXCL13	5.60E-28	0.13522 5049	0.20647 6938				
G8- Cytotoxicity (Lymphocytes)				G9- Exhausted/HS CD8 T cells			
Gene Name	P-	% exp in	% exp in	Gene Name	P-	% exp in	% exp in

	value	R	NR			value	R	NR
FCRL6	3.00E-23	0.063209393	0.111776251	TNFRSF9		9.64E-39	0.143052838	0.230716389
SPON2	1.26E-18	0.099412916	0.149656526	GEM		3.04E-24	0.084735812	0.14033366
KLRG1	9.23E-25	0.155381605	0.225024534	NAB1		7.05E-43	0.17260274	0.270951914
TRGC1	3.26E-32	0.134050881	0.211187439	DFNB31		5.84E-28	0.132876712	0.203729146
A2M	2.03E-27	0.143835616	0.215799804	CADM 1		2.66E-58	0.063405088	0.149067713
FCGR3A	2.92E-106	0.048923679	0.165358194	CRTAM		1.97E-28	0.157142857	0.232777233
GZMA	5.83E-97	0.314285714	0.490088322	GPR56		4.55E-50	0.129158513	0.22747792
HOPX	1.22E-23	0.121722114	0.184003925	MY07A		8.82E-51	0.0962818	0.186555447
NKG7	7.00E-120	0.373581213	0.572423945	DUSP4		1.33E-78	0.334637965	0.493228656
PXN	1.24E-27	0.183170254	0.261236506	METRNL		5.92E-60	0.093542074	0.192345437
				PHLDA1		4.81E-50	0.242465753	0.359960746

Table 5

GeneName	p-value	mean_exp_in R	mean_exp_in NR	%in R	%in NR	log2(R/NR)	GeneName	p-value	mean_exp_in R	mean_exp_in NR	%in R	%in NR	log2(R/NR)
CCL3	5.62E-158	0.93958905	2.80390774	0.111741683	0.297742885	-1.5773	LAMTOR1	1.18E-51	1.471970188	2.265803825	0.182778865	0.293915604	-0.6223
LGALS1	1.62E-171	1.434364197	3.469757489	0.166731898	0.380863592	-1.2744	UQCRCFS1	3.66E-73	1.956621799	3.0106469006	0.244227832	0.389008693	-0.6217
CD38	7.97E-172	1.224144242	2.896771762	0.160469667	0.373012758	-1.2427	NDUFB4	1.04E-65	1.821027274	2.801058765	0.218199609	0.351226693	-0.6212
EPST11	1.35E-164	1.169193654	2.669176018	0.165949119	0.374877331	-1.1909	CAPZA2	1.06E-68	2.025194051	3.114300151	0.249315068	0.389892051	-0.6208
WARS	7.87E-151	1.506308103	3.324907134	0.190998043	0.395878312	-1.1423	BRK1	1.76E-105	2.767872656	4.2536961472	0.319569011	0.503631011	-0.6199
PLEK	8.00E-104	0.98374674	2.139448522	0.126027397	0.275466143	-1.1209	ADRM1	2.18E-73	1.978008457	3.0377110227	0.24344402	0.38842002	-0.6189
HAVCR2	5.12E-168	1.466442103	3.177078253	0.17514653	0.388910771	-1.1154	NDUFB2	7.93E-63	1.979068666	3.037540019	0.232681018	0.364474975	-0.6181
LGALS3	1.82E-103	1.151931789	2.473107353	0.139921722	0.293326791	-1.1023	ETFA	2.81E-49	1.548497983	2.376481302	0.19549999	0.30578999	-0.6180
FABP5	8.12E-116	1.227485851	2.634565658	0.160078278	0.329538763	-1.1019	VDAC3	2.29E-54	1.561910504	2.3962644276	0.178669301	0.292247301	-0.6175
MT2A	5.36E-180	1.857507939	3.984219391	0.216634051	0.448086359	-1.1009	NUDT5	2.48E-42	1.347260422	2.064886871	0.169863014	0.267026497	-0.6160
GBP1	1.20E-133	1.272533906	2.683489122	0.165166341	0.350539745	-1.0764	IFITM3	1.02E-53	1.844810093	2.826167546	0.331115829	0.460745829	-0.6154
PLSCR1	1.58E-103	1.084594548	2.284260645	0.141878669	0.295878312	-1.0746	BANF1	5.74E-64	1.870524604	2.861604859	0.238747554	0.372620216	-0.6134
CCR5	1.30E-113	1.032817583	2.140534911	0.131506849	0.290873405	-1.0514	ZNHIT1	4.43E-57	1.472004662	2.251268529	0.198630137	0.318842002	-0.6130
GSTO1	2.42E-110	1.299902209	2.667379622	0.157925636	0.322178606	-1.0370	CAPG	3.71E-29	1.341358738	2.051292507	0.150880626	0.226594701	-0.6128
ANXA5	6.77E-203	2.167251976	4.429830147	0.258317025	0.511678116	-1.0314	NHP2	4.49E-48	1.495319661	2.286599938	0.176125245	0.281648675	-0.6128
GLUL	7.02E-165	1.022070976	2.080857014	0.125244618	0.238469087	-1.0257	LASP1	1.11E-110	2.355484491	3.59954771	0.331506849	0.521000981	-0.6118
PYCARD	6.66E-113	1.270979866	2.579921887	0.154207436	0.319725221	-1.0214	TOMM5	2.02E-42	1.407280385	2.150542248	0.174559687	0.272620216	-0.6118
TYMP	1.16E-93	1.061001216	2.150678099	0.153816047	0.30235525	-1.0194	MVP	3.84E-89	2.345602791	3.583600734	0.301565558	0.468792934	-0.6115
IFI6	6.30E-205	2.087718034	4.226932063	0.298238748	0.556722277	-1.0177	CTSW	1.29E-74	2.430435004	3.712773088	0.26555773	0.414425908	-0.6113
VAMP5	1.49E-94	1.118835892	2.256616205	0.132485323	0.27595682	-1.0122	AURKAIP1	8.70E-55	1.644344099	2.509162353	0.202544031	0.32060844	-0.6097
OASL	7.77E-109	1.325351664	2.641854175	0.161056398	0.32492651	-0.9952	RARRES3	4.54E-103	3.161469824	4.818914617	0.344227006	0.527379784	-0.6081
GZMB	2.85E-111	1.920523317	3.756160821	0.191976517	0.364965653	-0.9678	PSMB10	2.47E-117	3.228693957	4.920731633	0.382778865	0.579587831	-0.6079
TXN	9.43E-126	1.442051697	2.819578423	0.198825832	0.385475957	-0.9674	TMEM173	7.92E-68	1.947127742	2.964367654	0.231311155	0.368596663	-0.6064
SQRDL	6.00E-107	1.286360337	2.514026773	0.160078278	0.321982336	-0.9667	SLX1A	1.27E-67	1.815061258	2.763275323	0.252446184	0.392149166	-0.6064
RHOC	6.20E-197	1.197562723	2.337664704	0.14579209650	0.29774209650		APOBEC3	2.10E-310	3.1829344484	4.84437430391976	0.5921490592149	0.6060	

	100	23	96	564	885		G	121	44	11	517	166			
AP2S1	6.73E-108	1.3598651	2.6489837	0.165949	0.330225	711	-0.9620	GIMAP4	1.75E-92	2.5357111	3.8592703	0.282387	0.451226		
GZMH	2.44E-103	1.7200628	3.3109680	0.179647	0.342983	75	317	-0.9448	EIF4E	3.03E-57	1.5414772	2.3457221	0.224853	0.349067	
CCL4L2	1.76E-125	1.3004095	2.5014725	0.190606	0.375368	654	008	-0.9438	CTLA4	7.61E-52	1.8095938	2.7522660	0.213111	0.329146	
SNAP47	9.99E-83	1.1315289	2.1684310	0.145009	0.281059	785	863	-0.9384	NDUFS8	1.61E-57	1.7917094	2.7250487	0.213307	0.336211	
LAP3	3.15E-113	1.5817031	2.9999184	0.206262	0.383807	25	231	655	CYB5B	2.74E-48	1.3256188	2.0148820	0.165166	0.269087	
ATP6V1B	6.58E-278	1.1007832	2.0846786	0.144814	0.276251	95	09	227	-0.9213	PIK3R5	1.45E-60	1.5200465	2.3098091	0.214677	0.341413
CCL4L1	1.11E-157	1.8964317	3.5704857	0.240900	0.460058	82	6	196	HEXB	1.02E-40	1.4908470	2.2652883	0.190998	0.289401	
LAMP2	1.34E-88	1.2215841	2.2690528	0.173776	0.322374	91	13	908	STXBP2	2.86E-67	1.8057928	2.7437675	0.241487	0.379489	
PSMA4	1.58E-114	1.8074211	3.3292677	0.216438	0.396859	58	26	356	-0.8813	PSMD8	6.12E-93	2.5702503	3.9049762	0.310176	0.481844
SERPINB1	1.65E-121	1.7026278	3.1317016	0.218003	0.404710	75	17	914	SEC61B	1.03E-78	2.4161883	3.6703986	0.275146	0.429440	
HIGD1A	1.04E-72	1.1287488	2.0745524	0.143639	0.269676	94	56	922	RGS10	4.15E-64	1.8468360	2.8051734	0.226614	0.359077	
UBE2F	1.72E-75	1.2189528	2.2393471	0.150880	0.281452	43	22	626	PHB	2.15E-43	1.4114308	2.1420913	0.172015	0.270951	
TALDO1	8.74E-108	1.7123209	3.1325602	0.204696	0.377134	34	82	673	ATP5C1	2.39E-80	2.6231652	3.9794711	0.304892	0.463493	
CD63	2.60E-115	2.0112927	3.6773479	0.227788	0.410794	97	79	65	ARF5	1.37E-88	2.4032085	3.6429113	0.286692	0.452109	
CLTA	8.43E-98	1.5341084	2.8042214	0.184148	0.343572	13	71	728	SUMO3	1.36E-58	1.4003285	2.1219504	0.194520	0.315799	
S100A11	2.39E-176	2.8010202	5.1149024	0.317025	0.557311	04	44	089	PRDX6	2.09E-73	2.2118503	3.3503239	0.268688	0.416584	
PHPT1	1.48E-75	1.1263335	2.0535281	0.141291	0.269479	31	46	585	RNH1	3.54E-55	1.8536901	2.8060357	0.226418	0.348380	
GBP4	9.39E-96	1.2304515	2.2338023	0.173972	0.329244	52	82	603	-0.8603	ATP5F1	8.18E-70	2.4246553	3.6690777	0.270645	0.414818
PRDX3	6.15E-96	1.5475218	2.8002446	0.192759	0.352109	06	19	295	UQCRC1	3.69E-66	2.2292268	3.3732486	0.250293	0.388125	
PSMB2	1.00E-101	1.3131596	2.3758162	0.190410	0.354661	04	86	959	SARNP	4.90E-50	1.5169815	2.2953160	0.191976	0.302649	
BST2	8.95E-134	2.0184439	3.6447752	0.240117	0.440529	8	99	417	PLIN2	2.05E-50	1.7609813	2.6643484	0.205870	0.319038	
GBP5	4.82E-157	2.1971032	3.9613693	0.298825	0.523945	27	97	832	PIN1	1.17E-52	1.5872533	2.4007546	0.198434	0.313346	
CTSC	2.10E-135	2.2139049	3.9830427	0.264187	0.469087	1	51	867	SDHC	5.29E-45	1.3319602	2.0143703	0.167318	0.267517	
NDUFB3	9.09E-88	1.4603191	2.6255505	0.187866	0.338567	21	75	928	POLR2G	2.00E-52	1.4359498	2.1702641	0.176320	0.276349	
NPC2	9.68E-52	1.3074755	2.3361826	0.155772	0.261923	75	3	994	SF3B14	1.21E-43	1.4359498	2.1761329	0.205088	0.315309	
GALM	9.43E-107	1.6239229	2.8998633	0.217025	0.390775	17	44	27	-0.8365	CAPRIN1	2.58E-56	1.5188820	2.2948595	0.204892	0.325220
GLIPR2	1.11E-79	1.3348547	2.3727618	0.174951	0.315112	7	82	931	-0.8299	COX7B	4.10E-48	1.8807370	2.8370972	0.237769	0.368302
CCL4	5.54E-142	2.7820563	4.9434126	0.273972	0.485181	46	77	603	UQCRC10	3.43E-61	1.8807370	2.8370972	0.237769	0.368302	
PRF1	1.05E-135	2.5574755	4.5406419	0.292563	0.500588	32	73	601	FBXO7	5.26E-66	1.8755818	2.8287125	0.233855	0.369479	
IFNG	1.26E-66	1.4115251	2.5059962	0.160861	0.284592	11	96	057	NDUFB6	1.27E-45	1.3426607	2.0248572	0.187866	0.292247	
IFI30	5.39E-37	1.3651475	2.4235968	0.163405	0.252404	2	14	551	S100A4	1.41E-139	4.0381042	6.0861035	0.422701	0.641020	
CHST12	4.22E-74	2.1216820	2.1631991	0.159295	0.290382	77	499	728	-0.8267	PRELID1	2.55E-93	2.7300748	4.1132891	0.316829	0.489303
ISG15	4.17E-139	2.5647149	4.5272951	0.282778	0.492639	71	15	865	TRPV2	7.31E-43	1.3448934	2.0257974	0.164774	0.261727	
MYD88	1.94E-85	1.4383883	2.5384026	0.175342	0.321197	03	32	466	SF3B5	3.37E-48	1.5833588	2.3830412	0.189041	0.296957	
IDH2	2.70E-110	1.8587392	3.2789539	0.233659	0.413150	38	63	491	MYO1F	1.62E-87	2.1236714	3.1961730	0.276712	0.440137	
MTHFD2	2.44E-103	1.7763969	3.1300099	0.220352	0.391560	3	01	25	-0.8172	SCAMP2	1.09E-73	2.0897825	3.1447163	0.278473	0.427674
CHMP2A	3.80E-89	1.5638800	2.7554489	0.186888	0.338763	81	58	454	RNF7	1.33E-56	1.8726135	2.8147306	0.226418	0.350049	
NDUFQ9	1.51E-72	1.1724580	2.0651109	0.150684	0.278312	56	12	932	SHKBP1	1.53E-67	1.9950486	2.9935604	0.242661	0.381157	
CHMP5	3.92E-72	1.1593523	2.0407322	0.143052	0.268302	77	89	257	-0.8158	CXCL13	5.60E-28	1.3553941	2.0367558	0.135225	0.206476
CALM3	2.20E-141	2.3129989	4.0674707	0.350684	0.566437	93	25	932	RAB1B	4.25E-94	2.2541074	3.3865585	0.325636	0.499411	
ANXA2	5.54E-149	2.7876194	4.9013876	0.316634	0.536800	38	87	051	HM13	1.55E-73	2.2465151	3.3649010	0.293542	0.443866	
PPT1	9.83E-74	1.3811669	2.4272364	0.169667	0.302649	82	1	319	-0.8134	ARPC5	2.05E-103	3.1088386	4.6457101	0.376516	0.560942
GTF3C6	1.12E-67	1.1660123	2.0487441	0.141095	0.261334	18	47	89	VTI1B	4.71E-38	1.4175552	2.1213906	0.170645	0.262315	
NDUFAB	2.01E-62	1.1838248	2.0792735	0.144031	0.259470	12	03	311	S100A6	1.63E-119	3.6675534	5.4840222	0.444227	0.641707	
CXCR6	2.69E-64	1.3979260	2.4534013	0.150684	0.269774	45	23	932	ARPC5	2.05E-103	3.1088386	4.6457101	0.376516	0.560942	
RNF181	5.41E-71	1.2927979	2.2671343	0.152054	0.278410	59	94	795	FDPS	2.19E-37	1.3557385	2.0250780	0.160078	0.249067	
LGALS9	5.23E-76	1.3762552	2.4122425	0.183365	0.321491	46	52	658	MINOS1	3.77E-52	1.8607885	2.7777520	0.222700	0.340431	
COX5A	6.53E-112	2.2281467	3.8999338	0.248140	0.431207	8	19	9	RAB10	5.59E-63	1.8240348	2.7220311	0.239921	0.372914	
OAS2	1.57E-65	1.2790768	2.2373470	0.174951	0.313935	18	47	89	NEDD8	8.71E-87	2.2624559	3.3751935	0.266144	0.404121	

	78	85	45	076	231			65	11	97	814	688	
PDCD1	7.40E-87	1.72553088	3.0060584	0.200587	0.352796		BATF	7.23E-49	1.71925502	2.5645622	0.209784	0.321687	
SNRPC	2.07E-65	1.26293582	2.1979570	0.157338	0.278999	019	PHB2	5.57E-57	1.9102453	2.8484840	0.234833	0.360058	-0.5769
BHLHE40	4.33E-125	1.8866285	3.2800998	0.244031	0.437782	1	ERH	2.86E-51	1.5436088	2.3015961	0.203326	0.317173	-0.5764
TWF2	7.99E-102	1.7479537	3.0363854	0.214090	0.382826	2	NCOA4	4.84E-56	1.6329471	2.4300187	0.212524	0.333660	-0.5735
SLAMF7	3.21E-96	1.6922208	2.9535336	0.223091	0.388125	977	PDIA4	2.94E-49	1.4276364	2.1237107	0.196673	0.307065	-0.5730
TXN2	1.36E-61	1.1862706	2.0563994	0.144227	0.258881	29	PSMB9	2.69E-128	3.9344412	5.8518098	0.435812	0.640628	
CARD16	2.47E-75	1.4331246	2.4827208	0.168688	0.303140	83	C11orf48	1.88E-43	1.4600641	2.1708607	0.175146	0.274681	-0.5722
ANAPC1	8.37E-1	1.5610818	2.7015337	0.180430	0.322865	54	TMEM50	1.39E-73	2.4670195	3.6674399	0.306066	0.457507	
MRPL51	1.83E-68	1.2108621	2.0914171	0.150880	0.274288	97	TIGIT	5.12E-64	2.4380679	3.6208723	0.281604	0.420215	
LIMS1	7.33E-76	1.2867094	2.2174213	0.178669	0.315799	83	NDUFA1	5.56E-1	2.3750169	3.5243912	0.284540	0.431305	-0.5694
NDUFA1	2.11E-60	1.2634146	2.1749976	0.148923	0.263395	58	NELFE	4.36E-44	1.4206416	2.1077404	0.171819	0.271638	
RANBP1	3.77E-56	1.2594019	2.1646465	0.162818	0.275466	12	COX6C	3.71E-75	2.5865262	3.8356489	0.291583	0.443572	
GBP2	6.09E-162	2.6030231	4.4652743	0.319569	0.549656	81	SLA2	2.46E-45	1.5474489	2.2943931	0.211741	0.319234	
PSMC1	4.42E-81	1.4587605	2.4986960	0.188454	0.332777	63	PSMB8	4.58E-123	3.9042989	5.7870190	0.434442	0.635132	-0.5678
ACTR1A	3.34E-77	1.3190861	2.2578798	0.176712	0.314720	61	NDUFS7	1.09E-59	2.0996545	3.1119762	0.258904	0.390186	
CD2BP2	3.00E-96	1.5850530	2.7081273	0.210176	0.373110	94	RER1	3.06E-68	2.1575013	3.1968937	0.279256	0.422473	
VDAC1	1.37E-92	1.8477282	3.1565251	0.231506	0.394308	39	RAB8A	6.50E-68	2.0130797	2.9820130	0.273385	0.415701	
EMC7	6.48E-79	1.4665601	2.5052475	0.175146	0.314524	89	COX5B	6.50E-80	2.76749234	3.9609171	0.322896	0.482531	-0.5669
MX1	8.91E-61	1.3882219	2.3710763	0.180821	0.302257	41	MRPL20	3.48E-41	1.4234813	2.1084268	0.179647	0.276938	
GPS1	3.97E-67	1.2507436	2.1345483	0.153424	0.276054	34	COX5B	3.22E-80	2.6749234	3.9609171	0.322896	0.482531	-0.5663
ATP5J2	3.41E-77	1.6149456	2.7545093	0.191193	0.331992	72	SEC13	1.97E-49	1.6619368	2.4602369	0.207632	0.319921	-0.5659
USMG5	2.84E-71	1.6083060	2.7352702	0.182778	0.315897	53	FKBP1A	2.84E-102	3.2271460	4.7761153	0.405675	0.589205	
SHFM1	3.31E-105	1.7859719	3.0363156	0.235420	0.410598	53	PRDM1	2.60E-131	3.0461454	4.5070656	0.418590	0.626398	
ATP5I	1.29E-81	1.7218526	2.9238076	0.199804	0.346712	91	RAB1A	2.49E-51	1.6686619	2.4718058	0.213111	0.337095	
FAM96A	2.22E-63	1.3242874	2.2445134	0.159686	0.279784	31	RHOG	3.38E-59	2.0530466	3.0367467	0.242074	0.370853	
CASP1	7.11E-64	1.3083735	2.2149259	0.157729	0.277919	95	CYB5R3	1.63E-47	1.4462247	2.1386632	0.201174	0.310107	
PARP9	1.11E-107	1.7952268	3.0378186	0.238356	0.416192	66	AIP	1.01E-60	2.1240364	3.1345732	0.253620	0.385475	-0.5615
NOP10	2.84E-75	1.6392939	2.7729252	0.190215	0.328949	1	ABRACL	1.72E-49	1.7443349	2.5733800	0.209197	0.321687	
GNG5	5.67E-119	2.3905559	4.0344560	0.294911	0.489205	49	PSMB7	1.21E-54	1.9809738	2.9219619	0.245009	0.368596	
CYC1	8.29E-69	1.4559315	2.4568809	0.175733	0.304906	37	COX6B1	4.58E-72	2.5185887	3.7145663	0.298043	0.447203	
RAB11A	6.28E-65	1.3327128	2.2471991	0.162818	0.285181	81	PSMD7	1.24E-49	1.6970174	2.5021294	0.220352	0.334641	
PGAM1	1.84E-160	3.0718762	5.1712800	0.357338	0.587536	83	PPA1	6.56E-47	1.8533577	2.7314713	0.217221	0.327576	
ENTPD1	6.80E-69	1.3047179	2.1955525	0.180821	0.311089	2	PCMT1	2.80E-45	1.6819016	2.4764371	0.207827	0.314818	
PDI6	1.21E-106	2.0003300	3.3630284	0.254011	0.433071	5	SURF4	2.48E-86	2.5285099	3.7229681	0.326614	0.492836	
PSMC3	8.49E-69	1.4509485	2.4340033	0.182191	0.312561	58	ENY2	1.25E-38	1.4596677	2.1489067	0.173972	0.266928	
TMBIM1	2.67E-65	1.2512575	2.0977976	0.172015	0.296663	96	TCEB2	1.30E-90	2.7771682	4.0870322	0.336986	0.507948	
UBE2L6	2.82E-152	2.8781886	4.8231607	0.336007	0.559666	43	MAP2K3	8.12E-58	1.7939505	2.6398409	0.237377	0.363886	
PSMA6	3.17E-113	2.5093797	4.1979085	0.2890401	0.477821	15	AL35335	5.26E-42	1.4922521	2.1958708	0.186497	0.291167	
EIF6	7.35E-64	1.3495745	2.2519253	0.163600	0.284985	31	AKIRIN2	3.13E-54	1.5244375	2.2421765	0.208414	0.326692	
DCTN3	2.49E-62	1.3515248	2.2551567	0.156751	0.274975	97	MAPRE1	1.87E-55	1.8264825	2.6859560	0.249315	0.374386	
SEC11A	7.16E-84	1.8120636	3.0211505	0.2232827	0.376349	27	SRGP1	8.08E-45	1.4112337	2.0746972	0.187084	0.290284	
CSTB	3.85E-86	1.5776704	2.6300131	0.234637	0.391658	56	DUSP4	1.33E-78	2.4693540	3.6299274	0.334637	0.493228	
ETFB	1.71E-63	1.2279117	2.0467178	0.152054	0.270559	17	ATG3	9.38E-41	1.5967873	2.3467935	0.210567	0.311776	
DBI	2.28E-87	1.9966341	3.3264481	0.232876	0.390775	43	SRGP2	7.74E-61	1.5976863	2.3445844	0.253816	0.385868	
GRN	4.21E-45	1.5140072	2.5208054	0.175342	0.277036	22	LRPAP1	1.67E-50	1.8356992	2.6927072	0.247162	0.352306	
ELOVL1	9.44E-65	1.3481176	2.2408958	0.168297	0.291658	19	NEFLCD	2.43E-52	1.6369278	2.4002364	0.201174	0.316094	
UBE2L3	1.26E-101	1.8116644	3.0104821	0.238551	0.410991	55	ATP6V0D	3.45E-50	1.7087560	2.5033939	0.225244	0.340824	
PSMB3	6.98E-122	2.5373744	4.2163049	0.288258	0.484396	28	C14orf16	4.22E-6	2.1925180	3.2110773	0.261252	0.393228	
NDUFB7	1.06E-1	1.4673648	2.4289968	0.176125	0.299901	-0.7271	SNRPB2	7.94E-7	1.7795934	2.6060840	0.235420	0.364082	-0.5503

	63	58	27	245	865			60	27	75	744	434	
DOK2	3.48E-102	2.075998091	3.434995984	0.250097847	0.424533857	-0.7265	CHMP4A	1.17E-54	2.02630114	2.967111219	0.246183953	0.369872424	-0.5502
SEC61G	1.15E-66	1.538491233	2.544376225	0.187279843	0.316388616	-0.7258	SFT2D1	2.94E-47	1.697558219	2.484717431	0.21702544	0.327772326	-0.5496
IGFLR1	7.15E-86	1.927914089	3.187751194	0.226614481	0.382139352	-0.7255	CASP4	4.68E-71	2.66907205	3.906028223	0.321722295	0.471442114	-0.5494
ATP5H	9.49E-72	1.716160763	2.826487301	0.205283757	0.342983317	-0.7198	NME1-NME2	7.28E-69	2.595755028	3.798697123	0.292759295	0.437880275	-0.5494
COPZ1	3.01E-85	1.855234152	3.051188527	0.218590998	0.37232581	-0.7178	FAM96B	8.90E-59	2.1325260187	3.124325994	0.252250489	0.381648675	-0.5491
ATP6V1F	3.87E-70	1.792661754	2.947324786	0.210176125	0.346908734	-0.7173	FDFT1	1.94E-53	1.751753245	2.5616793746	0.216829746	0.335328754	-0.5483
BNIP3L	4.41E-56	1.253003626	2.05842955	0.164774951	0.277723258	-0.7162	SLC25A3	9.11E-43	1.488180264	2.175633856	0.180039139	0.279489696	-0.5479
NUTF2	5.21E-68	1.418983233	2.320652089	0.178864971	0.307850834	-0.7097	LMAN2	6.19E-79	2.661937145	3.890814745	0.326418787	0.484887144	-0.5476
AKR1A1	8.17E-49	1.294003494	2.113973959	0.15146771	0.253287537	-0.7081	MDH1	1.49E-56	2.159071577	3.155077466	0.248923679	0.375368008	-0.5473
MDH2	2.62E-89	1.881179422	3.062693403	0.24090049	0.401864573	-0.7032	RHBDD2	4.59E-48	1.83720283	2.684039216	0.234050881	0.347988224	-0.5469
VAMP8	2.31E-93	2.227780803	3.622727611	0.258904159	0.426202159	-0.7015	ARPC5L	1.85E-40	1.410309582	2.058636406	0.195694716	0.294406281	-0.5457
ROMO1	1.27E-52	1.261924135	2.051872493	0.152641879	0.259175662	-0.7013	TBCA	6.19E-39	1.424502166	2.079241489	0.177886497	0.271933268	-0.5456
CXCR3	4.85E-69	1.735693895	2.818283904	0.209393346	0.344749755	-0.6993	EBC	1.03E-35	1.455331933	2.12162066	0.171232877	0.259764475	-0.5438
SAMHD1	2.91E-72	1.626140311	2.639789163	0.230528376	0.37252208	-0.6990	SEC14L1	7.87E-45	1.414991413	2.061812909	0.191976517	0.295976447	-0.5431
NUCB1	3.94E-113	2.007036656	3.256525129	0.270254403	0.457016683	-0.6983	EIF2S2	1.74E-57	1.575000588	2.294852147	0.253424658	0.381452404	-0.5430
ACTN4	4.27E-112	2.003257236	3.249339968	0.281017613	0.468007851	-0.6978	CST7	5.56E-104	4.008575455	5.8396813303	0.418395605	0.603336605	-0.5428
ZYX	4.38E-89	1.593372605	2.583501132	0.240313112	0.400981354	-0.6972	STARD7	2.56E-66	1.818963736	2.6485082045	0.272407605	0.412757605	-0.5421
FLOT1	6.28E-54	1.290240197	2.091419648	0.167514677	0.278606477	-0.6968	SOD2	3.98E-28	1.601534898	2.331465365	0.205870841	0.287536801	-0.5418
BLOC1S1	2.55E-66	1.611287393	2.609882868	0.193933368	0.323748613	-0.6958	SPN	3.25E-48	1.664731979	2.4230900628	0.232289786	0.346221786	-0.5416
STAT1	4.08E-175	3.219916712	5.213467124	0.417221135	0.656624141	-0.6952	FAM32A	1.12E-43	1.526685075	2.221696059	0.185518591	0.287046124	-0.5413
VIMP	6.60E-60	1.370165426	2.215374882	0.170058802	0.28842002	-0.6932	SEC11C	6.61E-42	1.738577937	2.52889762	0.203718819	0.3055932	-0.5406
PAM	1.67E-57	1.282912354	2.073551854	0.17162464	0.287536801	-0.6927	TNFRSF1B	8.77E-101	2.773295247	4.032776097	0.362426614	0.544160942	-0.5402
NUDT21	1.95E-66	1.534835476	2.480580337	0.191389432	0.320902846	-0.6926	POLR2E	1.30E-62	2.210020053	3.213015133	0.28590998	0.423258096	-0.5399
MYO1G	2.54E-73	1.624594799	2.621995609	0.233072407	0.376643768	-0.6906	NDUFA1	6.43E-3	3.080454378	4.478465372	0.357338552	0.525515211	-0.5399
C17orf49	2.06E-88	3.465120214	2.04598884	0.245988258	0.406771344	-0.6905	OSTC	2.09E-41	1.636289987	2.378072241	0.201174771	0.301962709	-0.5394
GTF2A2	8.51E-46	1.291329708	2.083248968	0.147358619	0.244651619	-0.6900	UFC1	3.40E-53	1.900381413	2.761257513	0.225048924	0.344357213	-0.5390
HIST2H2AA4	3.42E-65	1.62102739	2.615123423	0.19275923	0.321197295	-0.6900	C18orf32	3.69E-41	1.470983154	2.136809024	0.175146771	0.271736997	-0.5387
C19orf10	2.19E-65	1.55602868	2.51021178	0.20058785	0.330520084	-0.6899	SRP19	1.37E-33	1.381728576	2.006885213	0.174363992	0.260353288	-0.5385
ABI3	3.02E-51	1.273931631	2.052517581	0.17260274	0.281354269	-0.6881	C14orf2	1.86E-49	1.849955851	2.686458394	0.222113503	0.336310108	-0.5382
TRAPPC5	2.57E-50	1.248130735	2.010460435	0.151076321	0.254563297	-0.6878	UQCR11	1.00E-77	2.450119097	3.55671787	0.319765166	0.476545633	-0.5377
PSMC4	4.15E-53	1.266475368	2.036536064	0.156360078	0.264180569	-0.6853	PDCD6	1.40E-40	1.752876187	2.544398287	0.217416544	0.319234544	-0.5376
NDUFC2	8.86E-78	1.934442192	3.109172729	0.226614481	0.373895976	-0.6846	AP2M1	5.67E-70	1.478464646	2.344949646	0.28590998	0.431697743	-0.5366
HN1	3.36E-68	1.742967474	2.801062863	0.203913894	0.337487733	-0.6844	PPP1CA	2.61E-110	3.542087159	5.137175971	0.414285714	0.604906771	-0.5364
SNRPD3	4.95E-66	1.551930033	2.490946015	0.207436399	0.339254171	-0.6826	SRP19	1.28E-33	2.076934676	3.010873662	0.260665362	0.389597645	-0.5357
CMC1	2.20E-42	1.246352417	2.000456268	0.146379648	0.239352306	-0.6826	SSR3	3.23E-60	2.38146269	3.449707614	0.330332681	0.468007851	-0.5346
RAB27A	3.63E-64	1.752236143	2.811528835	0.235616438	0.369381747	-0.6822	UNC13D	2.12E-43	1.399377817	2.027070952	0.197260274	0.300196271	-0.5346
NDUFA6	9.95E-83	1.912148225	3.064326815	0.236594912	0.390382728	-0.6804	FERMT3	4.68E-65	2.416923975	3.497774375	0.292367906	0.433071639	-0.5333
POMP	1.22E-105	2.183841496	3.499591321	0.335029854	0.520215898	-0.6803	ARHGAP1	1.35E-51	1.438764431	2.082036838	0.238943569	0.364180569	-0.5332
PFKP	1.25E-65	1.462573605	2.342213701	0.198825832	0.32875368	-0.6794	EIF3I	5.12E-41	2.031993349	2.940346899	0.247553816	0.371933268	-0.5331
ATP5G3	1.46E-102	2.557809892	4.094370323	0.292759539	0.472129539	-0.6787	CECR1	7.73E-46	1.522110632	2.200790822	0.198043053	0.304317959	-0.5319
TMEM17	3.61E-55	1.490689438	2.38415221	0.17906084	0.293719665	-0.6775	MRPS6	2.15E-49	1.438764449	3.058838249	0.258317025	0.376643768	-0.5299
PSMD9	2.52E-49	1.309469049	2.093070449	0.15890411	0.262806673	-0.6766	DNPH1	2.35E-37	1.554209872	2.244003976	0.18003976	0.272227674	-0.5299
IRF7	1.14E-56	1.52817564	2.441759251	0.20489251	0.325515368	-0.6761	DCXR	5.17E-47	1.963483091	2.834656476	0.233463515	0.346025515	-0.5298
CNIH1	1.07E-51	1.322861705	2.106205977	0.161448141	0.268694799	-0.6710	PSMF1	7.85E-63	2.414674743	3.485419989	0.2962818	0.434739941	-0.5295
DYNLRB	5.77E-61	1.696766307	2.701256507	0.207632421	0.341609421	-0.6708	SNRPG	1.13E-43	1.896120791	2.734222101	0.234833659	0.343081452	-0.5281
APOL2	9.23E-106	1.921882275	3.056362968	0.292759295	0.475171737	-0.6693	CNDP2	5.21E-46	1.741016257	2.509372113	0.216242661	0.32531894	-0.5274
TKT	2.09E-58	1.569007046	2.494917602	0.197847358	0.319430815	-0.6691	ANXA11	6.36E-65	2.142825583	3.087794383	0.284735812	0.424631992	-0.5271
DCTN2	1.05E-54	1.435568143	2.280425351	0.184148728	0.299116781	-0.6677	SLMO2	1.45E-65	2.008723259	2.894532786	0.282778865	0.423258096	-0.5271
GSDMD	2.47E-107	1.702681147	2.704585920	0.20136932	0.3280666676	-0.6676	C160rf13	3.60E-46	1.485867027	2.141105909	0.1818000273895	0.27389505271	-0.5271

	62	97	35	863	732			37	71	03	391	976					
STOM	7.50E-99	2.1706993	3.4448285	0.270841	0.444651	-0.6663	CAPN2	7.67E-64	2.0944503	3.0178355	0.284148	0.422767					
CTSD	4.88E-137	3.1683157	5.0273997	0.378473	0.591265	947	-0.6661	BSG	1.31E-87	2.5218764	3.6333937	0.359686	0.528655	-0.5268			
KDELR2	6.57E-69	1.5339651	2.4302939	0.210371	0.345731	109	-0.6639	LAMTOR	4.68E-5	1.8600217	2.6788069	0.210371	0.315996	-0.5263			
ATP5J	4.43E-62	1.6797033	2.6604600	0.204305	0.331207	066	-0.6635	SIVA1	1.56E-35	1.6735393	2.4096606	0.202935	0.295682	-0.5259			
RPS27L	2.94E-55	1.4364182	2.2748803	0.182778	0.298233	865	562	-0.6633	TRAPP1	2.21E-70	1.9844350	2.8572448	0.295303	0.442296	-0.5259		
PSME2	1.92E-179	4.1025635	6.4935525	0.459099	0.698429	804	833	-0.6625	TMCO1	1.06E-47	1.8964796	2.7297653	0.227984	0.340726	-0.5255		
DRAPI	1.28E-100	1.8083730	2.8598344	0.263992	0.438665			PSMD13	2.76E-54	2.1457914	3.0878781	0.261252	0.386162	-0.5251			
NDUFB1	5.21E-52	1.4860807	2.3501134	0.182778	0.294308	865	145	-0.6612	PSMB1	1.02E-79	3.1967223	4.6001454	0.374755	0.536113	-0.5251		
DECRI	3.67E-54	1.5173121	2.3963560	0.188258	0.303336	93	317		RSU1	7.79E-58	1.4232862	2.0479159	0.186497	0.280274			
GSTP1	1.93E-93	2.6739736	4.2227557	0.297651	0.468891	96	663	07	-0.6592	NDUFA1	1.04E-58	2.2697082	3.2656196	0.290019	0.423061	-0.5248	
TMED9	3.37E-56	1.4427785	2.2774706	0.182191	0.298626	781	104	-0.6586	TUBB	1.75E-56	2.7386052	3.9384522	0.331506	0.464671	-0.5242		
MGAT1	3.10E-72	1.8789267	2.9633386	0.248140	0.392443	36	572	-0.6573	DCTN1	5.26E-43	1.4230973	2.0463528	0.188649	0.289793	-0.5240		
HSPB1	2.41E-75	1.8861179	2.9715088	0.240313	0.386947	83	112	988	-0.6558	SH3GLB1	7.29E-46	1.6126741	2.3181320	0.215459	0.324239	-0.5235	
COX8A	8.81E-101	2.8036112	4.4141092	0.318590	0.498331	91	998	698	-0.6548	BCAP31	2.84E-71	2.5215066	3.6233229	0.306849	0.455740	-0.5230	
ZEB2	3.94E-81	1.8846597	2.9661365	0.249510	0.403434	12	763	74	-0.6543	RTFDC1	7.09E-54	1.9670145	2.8259179	0.241682	0.363886	-0.5227	
ILK	4.10E-60	1.5217182	2.3927813	0.191585	0.314131	89	94	127	-0.6530	UFD1L	5.86E-37	1.6126741	2.2210332	0.206066	0.301373	-0.5226	
PSMB6	6.77E-91	2.4075606	3.7823465	0.283757	0.451226	48	339	693	-0.6517	GPI	1.03E-94	3.4913159	5.0144169	0.419178	0.595682	-0.5223	
HK1	1.33E-60	1.3709654	2.1533188	0.186301	0.308537	51	38	37	-0.6514	DNAJB11	1.90E-61	2.1354793	3.0651450	0.277495	0.412659	-0.5214	
CD58	1.37E-60	1.5712473	2.4671449	0.194520	0.318056	32	79	548	-0.6509	SNX17	1.17E-59	2.1330577	3.0603740	0.254794	0.385574	-0.5208	
TMX1	8.52E-76	1.5538714	2.4396573	0.216438	0.360157	94	335	017	-0.6508	SH2D2A	1.81E-70	2.5212377	3.6156951	0.363405	0.514425	-0.5201	
GZMA	5.83E-97	3.1239287	4.9037393	0.314285	0.490088	47	12	714	322	-0.6505	C1orf43	1.97E-52	2.0095103	2.8799724	0.250880	0.372423	-0.5192
SRI	3.66E-75	2.0308568	3.1869986	0.232289	0.377625	36	87	628	123	-0.6501	BUD31	3.75E-51	2.1480785	3.0784947	0.249706	0.369381	-0.5192
PSMG2	8.91E-53	1.4516389	2.2768840	0.177103	0.288616	16	41	718	29	-0.6494	PSTPIP1	9.64E-64	2.4586589	3.5230037	0.285714	0.424435	-0.5189
ARL8B	5.95E-62	1.5601222	2.4466130	0.204305	0.331010	87	81	284	795	-0.6491	CTSA	2.54E-46	1.8086910	2.5914730	0.216438	0.325907	-0.5188
NKG7	7.00E-120	4.0438809	6.3387197	0.373581	0.572423	04	47	213	945	-0.6485	TPST2	8.97E-38	1.4524817	2.0808857	0.181996	0.275073	-0.5187
GPX1	8.45E-53	1.8996909	2.9759259	0.234246	0.354268	52	78	575	891	-0.6476	MPV17	1.43E-33	1.4070455	2.0153614	0.164774	0.249165	-0.5184
ACP5	3.77E-49	1.4915764	2.3344076	0.177690	0.284887	14	808	144	-0.6462	APMAP	3.57E-60	2.1280594	3.0450313	0.263405	0.395682	-0.5169	
CHP1	7.06E-113	3.7429531	0.330528	0.521884		77	376	2	-0.6460	CMC2	2.65E-43	1.8109942	2.5912941	0.230136	0.337291	-0.5169	
GPR171	2.57E-45	1.3232806	2.0705291	0.155968	0.254465	11	79	689	162	-0.6459	UQCRRQ	6.75E-65	2.6189367	3.7463753	0.306066	0.447693	-0.5165
ATP6V0B	1.39E-68	2.0618341	3.2260163	0.238747	0.377821	34	79	554	394	-0.6458	TBCB	1.49E-60	2.3373997	3.3299020	0.264774	0.397644	-0.5106
KLRD1	8.31E-43	2.3876838	0.170450	0.268302		15	098	257	-0.6452	C9orf16	1.57E-53	2.1147846	3.0109286	0.266927	0.391462	-0.5097	
H2AFY	2.44E-71	1.5115433	2.3639380	0.224853	0.365161	86	229	923	-0.6452	PARK7	4.28E-93	2.6189367	3.7463753	0.306066	0.447693	-0.5079	
PPM1G	4.40E-71	1.6953811	2.6485192	0.217221	0.356035	9	41	135	329	-0.6436	ATP5EP2	9.45E-66	1.5180788	2.1581656	0.306653	0.449362	-0.5076
PRDX5	5.89E-87	2.3062335	3.6020746	0.272994	0.435426	65	92	129	889	-0.6433	SHISA5	2.68E-84	3.0792085	4.3766346	0.375538	0.541609	-0.5073
PSMA5	2.63E-88	2.4822086	3.8764148	0.305675	0.474243	7	87	147	945	-0.6431	SMC4	1.21E-33	1.5201545	2.1605025	0.188454	0.276447	-0.5071
FBXW5	1.51E-54	1.2841732	2.0053932	0.173189	0.286064	07	32	824	769	-0.6430	TAP1	5.12E-120	3.8947457	5.5355041	0.423874	0.598724	-0.5071
ATP6AP1	1.94E-54	1.5731799	2.4547351	0.200391	0.317664	59	69	389	377	-0.6419	SCAND1	3.08E-49	1.9144377	2.7205675	0.242661	0.359077	-0.5070
CD4	6.77E-51	1.6721698	2.6081315	0.207827	0.321884	38	86	789	2	-0.6413	SIRPG	1.65E-40	2.0629931	2.9312577	0.245596	0.350539	-0.5068
SNRPD1	3.30E-53	1.4822622	2.3105511	0.186301	0.299803	53	65	37	729	-0.6404	HDLBP	1.59E-44	1.7415053	2.4738995	0.244618	0.355053	-0.5065
XAF1	9.54E-78	1.9772618	3.0790094	0.287671	0.442100	3	5	233	098	-0.6390	EMC4	9.20E-42	1.8886722	2.6829203	0.229745	0.334739	-0.5064
LY6E	1.02E-148	3.0208881	4.7002432	0.402739	0.624141	18	22	726	315	-0.6378	FIS1	1.12E-38	1.6458136	2.3375461	0.198043	0.294602	-0.5062
DYNLT1	5.75E-58	1.7844067	2.7759842	0.204305	0.326398	61	61	29	43	-0.6376	TPI1	1.02E-112	4.6748847	6.6393883	0.508414	0.696172	-0.5061
AK2	2.00E-63	1.5655499	2.4352786	0.203718	0.331992	88	28	149	-0.6374	GOLGA7	3.63E-41	1.5319385	2.1756038	0.191780	0.290873	-0.5061	
PSMA2	1.94E-104	2.5547466	3.9731633	0.328571	0.512266	33	44	429	928	-0.6371	POLR2J	1.24E-31	1.5581931	2.2121944	0.203718	0.290677	-0.5056
YIPF3	1.27E-49	1.3182282	2.0483335	0.158512	0.262708	7	55	72	538	-0.6359	EIF2S1	3.71E-33	1.4221180	2.0188628	0.186301	0.273307	-0.5055
S100A10	9.45E-92	3.0323873	4.7106878	0.331115	0.502944	25	54	46	063	-0.6355	UBA3	8.86E-37	1.5041879	2.1344845	0.195499	0.289106	-0.5049
SCP2	3.67E-69	1.9286084	2.9926364	0.228767	0.367222	68	11	123	767	-0.6339	P4HB	8.31E-73	1.5319385	2.3375461	0.198043	0.294602	-0.5047
MRPS34	3.99E-54	1.5018058	2.3300215	0.180430	0.294111	67	21	528	874	-0.6336	UQCRH	1.94E-54	2.5064059	3.5547126	0.286692	0.414131	-0.5041
PSMD4	3.86E-	2.0419639	3.1679981	0.236399	0.379097	-0.6336					CSNK2B	8.75E-	2.7788759	3.9395846	0.327592	0.472816	-0.5035

	72	77	22	217	154			67	86	04	955	487	
CDC123	1.47E-42	1.3084439	2.0295503	0.164579	0.261138	-0.6333	SZRD1	2.12E-49	1.4585345	2.0677229	0.220352	0.334249	
BTG3	4.41E-58	1.5204100	2.3582179	0.198434	0.319725	-0.6332	NDUFA3	7.18E-46	2.0387607	2.8888620	0.251076	0.363984	-0.5028
TMEM25	2.01E-8	1.8066072	2.7997560	0.216634	0.350343	-0.6320	ATP50	6.26E-52	2.3496685	3.3292900	0.275538	0.398822	
TSPO	1.19E-91	2.7269172	4.2245322	0.306457	0.476545	-0.6315	DERL2	7.82E-31	1.4551077	2.0608343	0.176908	0.259175	-0.5021
SDHB	6.97E-48	1.5487784	2.3989809	0.186888	0.294013	-0.6313	COPS6	8.06E-48	2.0114845	2.8477113	0.229158	0.342198	
TCEB1	1.80E-53	1.3485601	2.0885951	0.177299	0.289597	-0.6311	COPE	5.72E-100	3.3600614	4.7568472	0.421917	0.603238	
WDR83O	6.81E-69	2.0709966	3.2063396	0.245988	0.386359		SNX6	1.75E-50	2.0293512	2.8726787	0.260273	0.380274	
HCST	6.09E-128	3.2165322	4.9702309	0.353620	0.558684	-0.6278	FLII	8.24E-65	2.5581658	3.6207383	0.329158	0.472129	
NAA10	9.16E-48	1.3375178	2.0652254	0.160665	0.263002	-0.6267	ERGIC3	1.33E-51	2.1250593	3.0064792	0.251272	0.371736	
CTSB	1.29E-75	3.0319066	4.6810889	0.355577	0.511972	-0.6266	PLAC8	3.41E-37	2.3564279	1.6493225	0.281017	0.189303	0.5147
YARS	5.53E-56	1.5077823	2.3261791	0.197651	0.316388	-0.6255	LTB	4.66E-41	3.1017332	2.1301584	0.354794	0.249852	
GLRX	7.33E-47	1.3921477	2.1472628	0.173972	0.277625	-0.6252	LY9	5.25E-35	2.8133853	1.8223044	0.333268	0.238272	
RBCK1	2.05E-73	1.9950751	3.0763109	0.253816	0.400098	-0.6248	SELL	9.52E-60	3.3683809	2.1304791	0.382583	0.253581	
RBX1	8.52E-72	1.8037309	2.7792821	0.252641	0.397055	-0.6237	TCF7	8.03E-50	2.2825178	1.4218820	0.332876	0.220215	
							IGKC	1.96E-44	2.0769379	1.1057896	0.211741	0.123650	
							CCR7	8.92E-148	2.6707545	1.0403105	0.297064	0.121982	0.6828
											579	336	1.3602

Table 6

GeneName	P-value	Table 6A		Table 6B			
		Mean expression G1	Mean expression G2	GeneName	P-value	Mean expression G2	Mean expression G1
CD38	2.45E-297	4.503877948	1.067546812	IL7R	4.45E-217	4.032099183	0.82470727
CCL3	1.08E-189	4.490273548	1.162498	GPR183	6.53E-64	2.140831891	0.640823245
STMN1	1.02E-82	2.306672782	0.627967181	LMNA	4.70E-72	2.280851687	0.746486735
MYO7A	7.55E-130	2.150132553	0.587955869	NR4A3	5.45E-80	2.171854112	0.71446374
GOLM4	1.39E-193	2.474678345	0.724612237	TCF7	1.22E-99	2.67922891	0.945088234
VCAM1	9.48E-152	3.278485922	0.967664744	MGAT4A	2.63E-40	2.074649553	0.972061001
WARS	1.67E-151	3.549322801	1.056419085	CD55	2.02E-62	3.611008837	1.731567098
HAVCR2	6.33E-277	5.240416049	1.5619818086	AIM1	3.33E-31	2.221582511	1.204797635
LGALS9	2.21E-92	2.00800184	0.600009745	PER1	3.81E-46	3.370857406	1.90500073
ID3	5.82E-87	2.012380498	0.605569109	FOSL2	1.90E-50	3.389389476	1.995926462
PRDX3	9.43E-156	3.321987666	1.007631697	EGR1	1.99E-19	2.316122377	1.399420975
MCM5	3.21E-101	2.526148252	0.776346958	TSPYLY2	6.35E-28	3.842494104	2.327893495
LSM2	4.50E-120	2.664535289	0.823047531	YPEL5	2.28E-41	5.890086333	3.80231451
MTHFD1	3.37E-97	2.230218039	0.690497842	CSRN1	6.49E-15	2.359107803	1.542059683
FASLG	1.88E-117	2.741295656	0.866662195	REL	5.86E-20	2.71949729	1.780867484
SNAP47	1.65E-174	3.769141235	1.224212618	SKIL	2.61E-22	3.266384049	2.155358757
IFI35	1.13E-91	2.43525005	0.805054574	PIK3R1	1.90E-15	2.952741142	1.959612009
SKA2	1.45E-115	2.515678821	0.858582012	FOXP1	3.94E-40	2.845200237	1.888462587
NDUFB3	8.58E-120	3.068411059	1.059675286	RGCC	1.17E-09	2.18586806	1.463110207
FABP5	1.35E-141	3.902150107	1.354881465	PFKFB3	1.89E-12	2.51054407	1.694388984
IFI27L2	3.19E-96	2.43103298	0.853275802	MYADM	1.01E-22	4.463153142	3.02595635
PTTG1	3.55E-83	2.427179679	0.854702167	ZFP36L2	2.53E-54	6.249733989	4.243453221
ENTPD1	6.50E-121	2.869960348	1.0117944	USP36	8.56E-19	3.423887672	2.34467909
EPSTI1	6.66E-116	2.925729029	1.035336741	TC2N	3.05E-18	3.14866233	2.158806238
PDCD1	6.84E-240	5.448490436	1.93012534	FAM177A1	1.53E-12	3.136884147	2.165526181
TRAF1	1.33E-101	2.669610001	0.947092587	BTG2	1.52E-23	4.610064959	3.185196999
SIRPG	1.10E-217	5.174793521	1.87397397	TSC22D2	5.99E-11	2.133916347	1.47500329
RGS3	7.93E-73	2.125645365	0.775453366	FAM65B	2.02E-11	2.291796896	1.584523044
UBE2F	3.45E-95	2.883976138	1.0539014	STAT4	2.55E-28	5.177656351	3.582523427
SNRPD1	1.71E-99	2.8909447	1.059663948	RGPD5	2.42E-18	4.3650224	3.037824875
FIBP	1.93E-83	2.429236471	0.892221704	NEU1	2.95E-08	2.147711506	1.497547611
CLTA	2.60E-105	3.039002863	1.122896472	IFRD1	1.90E-14	4.199147598	2.932395951
CXCL13	3.64E-120	3.951890716	1.460527346	PDE4B	1.51E-16	3.806892143	2.664211096
NMI	1.29E-82	2.176688391	0.805571318	NR4A1	3.97E-09	2.611477311	1.842902699
DNPH1	3.84E-79	2.381720722	0.883250405				
PCNA	1.06E-61	2.182262811	0.810186297				
ACP5	1.17E-98	2.885465491	1.073573821				
MRPL28	1.51E-71	2.024609025	0.754615011				
FARSA	2.49E-73	2.001442588	0.747150763				
COX5A	5.98E-157	4.676954848	1.761865508				
MRPL51	7.34E-85	2.457427972	0.925772862				
SNRPE	2.44E-75	2.404227446	0.907001261				
RANBP1	2.33E-84	2.670265445	1.016696907				
NOP10	2.34E-97	2.939269738	1.121937984				
PYCARD	1.41E-95	2.752462272	1.051076801				
GTF3C6	2.43E-79	2.330310059	0.898953967				
CCR5	9.54E-127	3.32856227	1.28734614				
GSTO1	2.89E-88	2.653561322	1.027965346				
OAS3	1.17E-87	2.267550807	0.879138328				
IGFLR1	1.44E-118	3.636915037	1.41015425				

HLA-DMA	2.9SE-144	4.115547348	1.607931363				
STRA13	1.95E-63	2.095400445	0.820913992				
HSD17B10	6.03E-69	2.108135203	0.828597124				
VAMP5	1.36E-S7	2.727461347	1.081471049				
NDUFAB1	1.62E-72	2.286416107	0.907988058				
BATF	1.20E-106	3.284547348	1.305467896				
NDUFS2	2.46E-65	2.191532071	0.873496229				
C17orf49	S.32E-134	4.085892692	1.629745209				
GNG5	1.61E-151	4.397466903	1.757469193				
PSMB2	3.17E-117	2.884465746	1.155182139				
PDI46	1.40E-148	3.943893622	1.579997884				
COMMD3	6.05E-60	2.035170674	0.8192485				
CD63	4.08E-144	4.568304134	1.847290214				
PSMA4	3.50E-118	3.326465017	1.551917638				
SAE1	2.67E-71	2.324560064	0.943978493				
ATP5J	1.44E-104	3.193882543	1.29728302				
MEA1	1.79E-73	2.330314867	0.949658623				
EXOSC9	3.63E-69	2.020491198	0.824607562				
ARPCSL	1.04E-111	3.068312322	1.253247292				
BL0C1S1	5.50E-102	3.225259843	1.318190273				
HELLS	4.55E-76	2.130695664	0.871697169				
CXCR6	5.36E-126	4.305209437	1.774354247				
BCAS4	6.50E-65	2.098722766	0.865111332				
ETFB	6.75E-S1	2.418481829	0.997801189				
TXN2	1.99E-66	2.306400284	0.953598266				
PTPN6	3.30E-156	5.015528117	2.077075152				
SIT1	1.00E-144	4.332124739	1.796590741				
FKBP1A	1.29E-227	5.929887981	2.462211689				
COPZ1	1.12E-107	3.528959222	1.466159688				
HLA-DRA	1.57E-195	6.430869882	2.672266924				
CDC123	S.06E-66	2.391351443	0.996238928				
AP2S1	3.62E-S2	2.615042085	1.089662717				
FUTS	7.70E-SS	2.38753102	0.995229203				
BST2	3.80E-133	4.205209714	1.757935193				
ATP6V1E1	7.69E-66	2.184741648	0.916464286				
CD2BP2	2.25E-131	3.693163626	1.549681009				
HLA-DQA1	6.24E-104	3.237470161	1.360566264				
ZCRB1	1.20E-61	2.010211694	0.845742982				
MX1	1.00E-75	2.837779179	1.194997598				
TNFRSF9	9.65E-106	3.467676923	1.462547382				
SQRDL	6.22E-79	2.622852717	1.106783424				
SERPINB1	2.57E-110	3.515777369	1.483727648				
PHPT1	1.4SE-65	2.194049577	0.926881074				
CALM3	3.56E-192	5.170473018	2.185344461				
TOX	9.95E-155	3.545285595	1.49868579				
SNRPC	1.47E-70	2.474584608	1.046413984				
MRPS34	4.95E-S4	2.768976948	1.175826428				
NUTF2	2.74E-S2	2.608754415	1.108160937				
NDUFS6	2.85E-72	2.035602108	0.864947239				
PSMB3	1.75E-155	4.904107876	2.08491137				
CHMP2A	4.84E-S1	2.857076253	1.222009155				
SLC25A11	6.27E-61	2.027829326	0.867613358				
SHFM1	9.64E-130	3.777245159	1.616846165				
TMEM179B	2.42E-76	2.767358685	1.18524941				
EIF6	2.20E-72	2.502067278	1.074616725				
ANXA5	5.84E-162	5.223830407	2.244806053				
JAKMIP1	1.03E-71	2.312789337	0.994466875				
TALDO1	9.37E-S9	3.134857922	1.349123107				
GLRX3	6.4SE-57	2.089535894	0.90076918				
ANAPC11	1.04E-S0	2.8896458	1.252200712				
DUT	4.99E-53	2.104622655	0.913261117				
PDCD5	9.09E-55	2.041601583	0.885963761				
ATP5G3	1.69E-143	4.749757651	2.062994434				
CHMP5	1.42E-65	2.186115189	0.950427238				
TWF2	1.86E-107	3.526082815	1.536184109				
IDH2	4.64E-133	4.501434308	1.965274937				
MPG	5.23E-65	2.276372945	0.994200645				
SNRPF	7.80E-SS	2.269196926	0.991701642				
NDUFC1	2.30E-53	2.074849616	0.907011779				
GBPI	2.07E-117	3.524455833	1.542528045				
DCTN3	1.76E-65	2.617143149	1.145880627				
ERH	2.0SE-S0	2.64205175	1.161491201				
NDUFA12	7.16E-61	2.312758316	1.017291863				
LIMS1	1.13E-64	2.066400504	0.909789851				
BANF1	5.56E-92	3.26276779	1.436691138				
NDUFC2	S.04E-97	3.456390807	1.527036232				
PSMC3	1.79E-73	2.746119429	1.213266782				
PON2	2.47E-62	2.403166306	1.0623325344				
PRDX5	1.39E-125	4.231392267	1.870991009				
TMX1	1.34E-101	2.956400489	1.312311214				
STOML2	5.02E-61	2.169463241	0.963097654				
RPS6KA1	5.43E-62	2.231976177	0.991943879				
PAM	1.41E-102	3.194179878	1.420230794				
ATP5J2	3.01E-77	2.948363375	1.311670832				
GIMAP6	2.57E-98	2.930366838	1.304224929				
NDUFB7	7.55E-70	2.601941528	1.158242834				
DBI	2.97E-96	3.575932852	1.592327842				

IFI6	3.3SE-154	5.201445339	2.318825526				
TSTA3	5.7SE-63	2.319464423	1.036532055				
SSNA1	7.93E-62	2.042647073	0.913112139				
ADOR2A	2.25E-55	2.0S016922S	0.930560714				
FDPS	2.83E-62	2.425943892	1.086244465				
CYCI	1.72E-66	2.539964355	1.137807765				
PSMD4	3.20E-104	3.665547931	1.646002485				
FAM96A	1.89E-56	2.081417771	0.934343428				
0AS2	5.39E-90	2.575625307	1.292503506				
ERCC1	1.64E-49	2.0S5642591	0.938003773				
PDHB	2.18E-52	2.055225667	0.926591496				
CD27	3.60E-194	6.136699677	2.773576115				
SNRPA	6.25E-61	2.067275057	0.935086166				
UBE2L3	9.31E-115	3.592S59999	1.625412091				
MDH1	9.43E-99	3.721279971	1.683801365				
SDHC	2.76E-63	2.347811248	1.064694838				
PSMG2	2.76E-64	2.555665014	1.159473162				
Clorf48	2.63E-76	2.890040778	1.313040137				
PSMA2	2.43E-15S	4.802864375	2.188108226				
C7orf73	2.09E-62	2.160459497	0.984637314				
MRPS16	9.40E-56	2.076926967	0.948382985				
MCM7	2.41E-42	2.11796775	0.969278006				
SNX20	2.36E-59	2.087926298	0.957095188				
AK2	1.15E-75	2.611969942	1.197612803				
RBBP7	1.21E-69	2.818309884	1.293003788				
TIGIT	3.77E-190	5.930720988	2.725798008				
TMPO	3.11E-79	2.779105016	1.27759614				
CTSB	1.01E-83	3.306011196	1.520579095				
PARP1	1.79E-S7	2.902379776	1.33623695				
USB1	4.00E-56	2.178707226	1.003328766				
MRPS7	1.90E-51	2.0636019	0.951959238				
NHP2	2.37E-60	2.464790149	1.137484098				
ATPS1	2.14E-S1	3.169807005	1.463809999				
PSMC1	6.43E-77	2.849473341	1.317477763				
VDAC1	6.12E-S6	3.404397887	1.574958304				
CARD16	3.28E-65	2.589653803	1.198332561				
RNF1S1	2.3SE-55	2.235233402	1.035620008				
PGAM1	1.06E-178	6.143413578	2.855010843				
NTSC	6.96E-49	2.010909629	0.935299453				
IRF2	9.60E-101	3.388234685	1.577120507				
NUDT22	1.82E-54	2.244019782	1.045018606				
NDUFA9	5.79E-62	2.3334169	1.08808713				
SRI	1.91E-100	4.000315311	1.870272516				
GBP4	S.65E-92	2.833516253	1.325233526				
NDUFSS	2.74E-75	3.092642125	1.446840419				
PSMC2	7.23E-49	2.012346972	0.941902901				
FPGS	3.10E-62	2.264011843	1.060107164				
PLSCR1	4.91E-51	2.23546722	1.048506785				
P0LR2G	2.80E-73	3.048974111	1.430789702				
COXSA	6.54E-123	4.967137562	2.331202931				
SLX1B	5.75E-73	2.245393336	1.054066173				
TRAPPC1	2.17E-124	3.492707062	1.643504305				
ABI3	7.75E-79	3.099603274	1.461146971				
CBX5	7.46E-S9	2.590646216	1.221380459				
PSMD14	2.33E-56	2.112233684	0.999656109				
UBE2L6	1.55E-175	5.968070327	2.832430432				
IFNG	1.S1E-123	4.707056597	2.234490186				
DECRI	4.66E-62	2.629425264	1.248765457				
ITGB1BP1	3.53E-56	2.406344071	1.143163002				
AKR1B1	4.64E-62	2.540626941	1.209149866				
PSMA5	S.29E-125	4.746837028	2.25955883				
NUDT21	2.99E-S0	3.06028903	1.457121678				
NDUFAF3	S.SSE-55	2.383919436	1.137322319				
MTD1	3.07E-47	2.009722381	0.960963898				
NUDT5	LSSE-56	2.434893144	1.165636277				
SNRPD3	6.21E-74	2.92881676	1.403249077				
TMEM25S	3.10E-72	2.970486925	1.424616179				
UQCRC1	7.64E-S2	3.645888901	1.749594365				
TRIM59	S.92E-S2	2.643379535	1.268587986				
DTX3L	1.69E-79	2.33543546	1.121027282				
PHB	2.03E-54	2.272531231	1.092024482				
VOPPI	6.19E-77	2.780981344	1.336514767				
GTF2A2	6.39E-48	2.25475435	1.085682726				
PSMB10	1.16E-171	6.030269925	2.906188687				
WDRS30S	3.57E-S6	3.557054354	1.715109338				
NDUFB10	1.43E-59	2.688975493	1.296723088				
YARS	4.21E-92	3.297927977	1.591534117				
AIP	1.21E-102	4.122714607	1.990604407				
CHST12	5.59E-99	3.595802492	1.736806178				
DCTN2	1.59E-60	2.576256487	1.244453561				
ATP5F1	5.24E-S4	3.962567401	1.921573174				
SMC3	5.54E-72	2.682357487	1.302035445				
CISC	2.24E-115	4.560963578	2.216349374				
SAMD9L	3.25E-66	2.495152105	1.213261926				
PEF1	2.74E-44	2.106208751	1.024666417				
H2AFY	4.36E-67	2.366480968	1.151785138				
C9orf16	2.77E-97	3.649773462	1.776991219				

SEC11A	2.43E-74	3.183121169	1.550593035				
SF3B14	2.53E-56	2.470585546	1.203633519				
SNX17	8.57E-81	3.340407842	1.630944085				
SLX1A	9.65E-94	3.397692612	1.659810557				
CNP	3.35E-51	2.010494426	0.98281337				
PIN1	9.51E-75	3.030442S03	1.48319666				
CNIH1	6.20E-48	2.222542426	1.090375464				
AL353354.2	9.95E-63	2.61S432506	1.286402608				
TOMM5	3.00E-55	2.536152718	1.246669896				
RAB27A	2.13E-123	4.687421809	2.304364209				
PSMDS	9.26E-117	4.575753315	2.256456493				
NDUFS7	1.21E-86	3.590142368	1.771365198				
GSDMD	1.89E-75	3.363352126	1.660918068				
SEC61G	5.37E-55	2.522814855	1.246782876				
ETFA	2.63E-56	2.651933137	1.311401693				
AP0BEC3D	5.34E-130	3.380245529	1.671679024				
GSTP1	5.11E-113	4.912488909	2.430082491				
FDF1	5.14E-62	2.704718169	1.338918193				
LAMTOR1	3.10E-49	2.16882921	1.075600567				
LGALS1	5.85E-64	3.357009584	1.664933266				
LAG3	7.60E-111	3.541064229	1.757280767				
ACTRIA	3.40E-65	2.510683291	1.245955297				
DYNLRB1	1.92E-65	2.957742724	1.468856794				
PPP1CC	3.64E-52	2.203796011	1.094519379				
GYG1	5.40E-45	2.230119979	1.108318616				
BRK1	2.25E-116	4.649300583	2.312956199				
PHB2	1.97E-71	3.202588722	1.594157768				
SFT2D1	3.01E-51	2.34543133	1.167512921				
PLERKH1	9.84E-52	2.354150689	1.174696649				
PUF60	4.55E-55	3.561505558	1.777203021				
FCRL3	1.64E-73	3.174160935	1.586070711				
SARNP	5.01E-61	2.731949001	1.365444301				
HK1	1.84E-62	2.457208967	1.228174703				
CTLA4	2.91E-99	3.690690102	1.845201044				
MDH2	1.86E-SS	3.481165243	1.741079456				
ATP5H	1.41E-62	2.92556102	1.463512768				
BAK1	1.44E-57	2.406565266	1.20447788				
PCMT1	1.83E-66	2.945615435	1.474281001				
TRIM69	2.32E-49	2.077449634	1.040347476				
GORASP2	1.20E-48	2.164740148	1.085770361				
PPM1M	1.05E-59	2.236488966	1.122173655				
RABSA	1.66E-82	3.273781635	1.643043985				
AURKAIP1	5.64E-65	2.88947544	1.450645103				
ATP5C1	1.41E-106	4.552287027	2.285753391				
TXN	2.64E-69	2.769173937	1.392219502				
SNRNP40	3.25E-47	2.257709257	1.135424061				
M6PR	1.07E-103	4.274687856	2.150113046				
C12orf75	3.35E-60	2.676137792	1.346789383				
RAB11A	4.43E-51	2.302870226	1.160980005				
TMCO1	5.19E-70	3.2016094	1.615421544				
NME1-NME2	1.16E-84	3.973214185	2.005844343				
OSTC	3.86E-51	2.420274514	1.222245854				
PARP9	1.54E-91	3.484009902	1.761121297				
ELOVL1	4.25E-50	2.330492905	1.179464783				
NEDDS	1.07E-85	3.862897554	1.956859328				
MT2A	6.11E-105	4.731740516	2.397041493				
TKT	2.12E-52	2.34031621	1.185718352				
CDK2AP2	2.99E-106	4.37439044	2.217013994				
HLA-DQB1	6.95E-81	3.631009793	1.8425553206				
TXLNA	4.84E-76	2.517656577	1.277762337				
PPP1R7	2.80E-41	2.08959601	1.060776221				
PPM1G	3.17E-74	3.137917906	1.593139835				
GBP5	2.61E-152	5.386152272	2.735263811				
ARPCS	7.91E-140	5.353269289	2.718813537				
SDHB	1.15E-45	2.398175813	1.219644736				
EIF2S1	7.82E-45	2.351574855	1.196180759				
KDELR2	6.33E-62	2.459379931	1.251223349				
NDUFA2	2.96E-47	2.074992277	1.056426235				
FIS1	2.87E-55	2.583194526	1.315724451				
HIGD1A	1.00E-40	2.019928924	1.030208517				
TAF9	1.62E-48	2.322383485	1.184774779				
CHMP4A	4.27E-77	3.474750511	1.77335484				
PFKP	3.56E-66	2.753096572	1.405205921				
YIPF3	6.39E-47	2.248718147	1.148418285				
CSK	2.52E-81	3.250034273	1.660326862				
COPS6	1.99E-65	3.276262524	1.674127159				
NELFCD	1.10E-71	3.08944576	1.579246323				
TMED9	1.32E-44	2.196556187	1.12350714				
PDAP1	3.32E-75	2.481359623	1.269843284				
CXCR3	5.75E-90	3.985402999	2.039885776				
SIVA1	1.54E-48	2.687595565	1.375766449				
TMEM140	4.32E-50	2.069594409	1.062932531				
THYN1	1.05E-42	2.105211154	1.081406696				
RBX1	3.36E-76	3.036093563	1.562362913				
C14orf2	1.12E-61	2.912725644	1.499001063				
TEX264	1.45E-41	2.101028148	1.081949894				
C14orf166	4.93E-78	3.620612979	1.864921756				

EZH2	1.13E-57	2.403595203	1.239474049				
CLNS1A	1.57E-55	3.025513649	1.560188965				
UOCR10	3.47E-71	3.075630459	1.587072528				
PSMD9	3.70E-47	2.3512S4S54	1.213670213				
EIF4E	S.17E-66	2.775116394	1.43287626				
TUBB	4.49E-76	4.440002507	2.294311683				
RPA2	7.46E-47	2.296714526	1.187201726				
ATP50	5.10E-71	3.559S77S7	1.841606686				
PSMA6	2.01E-91	4.545432082	2.35375124				
HLA-DRB5	3.29E-172	5.3202965S1	2.756910928				
EID1	2.82E-133	5.106215408	2.647522052				
HMGN3	1.15E-53	2.782395503	1.442863787				
LBR	5.99E-73	3.041556205	1.577781934				
GZMB	1.7SE-115	6.601718045	3.42740232				
ROM01	5.21E-42	2.177555494	1.132261022				
MPV17	2.40E-40	2.173491046	1.132459063				
HSPB11	1.01E-44	2.009504626	1.047387698				
PSMD13	4.20E-67	3.529735619	1.842882774				
C11orf31	2.S1E-63	3.060043S36	1.598063589				
BOLA2B	1.17E-45	2.106214295	1.099994061				
LASPI	6.79E-133	4.465323537	2.332109448				
PPP2R1A	1.46E-55	3.785512027	1.97977335				
DCAF7	4.35E-60	2.33S42S225	1.223321312				
MDM2	4.05E-75	3.1845S2499	1.667095595				
DGUOK	7.95E-56	2.808834118	1.471314642				
SF3B5	1.37E-52	2.630323364	1.377975865				
S100PBP	3.52E-60	2.455692833	1.286831295				
COX6B1	1.S2E-90	4.154064004	2.177319719				
GALM	5.53E-SS	3.780473588	1.981554833				
POMP	1.31E-106	3.792006612	1.988048302				
LAMTOR5	7.17E-54	2.805109982	1.470782799				
CYBSB	3.67E-49	2.222962355	1.166862154				
USMG5	1.27E-49	2.818179477	1.481020226				
PMF1	2.53E-54	2.979208475	1.567302781				
UBE2N	2.77E-71	3.458312581	1.819652579				
TSG101	6.93E-35	2.008441993	1.056868043				
COX6C	2.47E-90	4.297502135	2.261527732				
MMADHC	5.25E-54	2.546849333	1.340626269				
PDCD6	1.39E-56	2.867232793	1.509515224				
PRMT1	7.16E-51	2.292687821	1.207942454				
LAMP2	1.32E-43	2.060477022	1.085928068				
PPA1	1.00E-55	2.837052137	1.495534417				
RPS27L	2.25E-45	2.229881524	1.175627681				
CASP3	4.24E-45	2.186665748	1.153377383				
ABRACL	4.19E-61	2.984389399	1.574402165				
MRPL20	6.59E-46	2.360837079	1.245620918				
SCP2	1.21E-66	3.275205948	1.728686685				
PSMB9	5.99E-195	7.561703783	3.992218943				
IKZF3	1.93E-141	5.1333572	2.717179773				
GPAA1	4.28E-44	2.145220765	1.13554062				
PSMB7	2.11E-60	3.085829834	1.635268266				
NDUFB9	3.00E-53	2.86716967	1.519616379				
DCAF11	4.32E-44	2.09743389	1.111780514				
VAMPS	S.17E-71	3.617767006	1.918122959				
SRSF4	5.57E-63	2.855488067	1.515999785				
SDHD	5.47E-55	2.573395818	1.368524673				
CAPRIN1	1.42E-57	2.578084513	1.372114847				
PSMC4	1.52E-43	2.18285663	1.16256485				
TRAF5	2.43E-93	3.831282066	2.040560405				
DRAPI	2.69E-98	3.548162957	1.891345203				
SMARCE1	7.62E-61	2.922024113	1.560821455				
ATPSA1	4.80E-118	5.270852224	2.816853507				
SRP9	5.99E-93	4.658812631	2.490284429				
TSTD1	4.01E-49	2.745580493	1.468068825				
GPS1	9.25E-44	2.323324562	1.242993963				
LAP3	7.20E-51	2.803015323	1.500994577				
PSME2	1.96E-193	7.712640895	4.131300065				
FAM195B	1.40E-49	2.331982837	1.249248514				
GBP2	1.42E-129	5.627221555	3.015042873				
CCTS	2.17E-74	3.71843876	1.992518788				
PSMB8	5.15E-170	7.132616946	3.82284933				
RAB1B	3.30E-110	4.215409902	2.259559985				
MPC2	2.79E-43	2.161625503	1.159002392				
NDUFS3	S.34E-37	2.142760678	1.149303577				
DAXX	2.90E-46	2.244036601	1.204997514				
ITGAE	1.43E-77	3.917097291	2.104574161				
NAAS3	1.39E-50	2.377261287	1.279778449				
ARF5	1.S5E-S7	4.060602326	2.186438939				
OSBPL3	5.S1E-63	2.73529629	1.474000296				
TIMM17B	5.43E-43	2.079702541	1.12088566				
MUSS1	S.91E-52	2.134593232	1.150493376				
TBCA	3.4SE-41	2.124444985	1.145265082				
ANP32A	5.13E-71	2.136382557	1.151790944				
CAPZA2	1.S7E-61	3.152541287	1.700959916				
CLPP	3.3SE-39	2.245357913	1.212130221				
PPP1CA	2.73E-146	6.212183494	3.35851502				
NDUFB6	9.53E-42	2.125216074	1.15197839				

DENR	1.29E-4S	2.143S0554	1.162452736				
NDUFA11	6.66E-76	3.815650312	2.070588162				
PSMB6	2.46E-79	4.124762054	2.239163153				
P0LR2J	4.27E-44	2.406944153	1.306660817				
CSNK2B	S.94E-91	4.43712145	2.409240456				
PDCD10	4.11E-3S	2.169152245	1.178244394				
COPS3	6.67E-35	2.044398957	1.110864996				
CASP1	9.07E-41	2.17195558	1.180502204				
RER1	3.75E-76	3.593428378	1.95333784				
ATXN10	3.21E-42	2.148757117	1.169066474				
HNRNPf	3.SSE-123	5.428744397	2.953899961				
SASH3	2.69E-112	4.801901906	2.613223269				
HNRNPd	6.81E-106	3.831133687	2.085635183				
RBCK1	4.42E-S0	3.890691947	2.118157521				
ADRM1	1.70E-66	3.369979655	1.834832786				
GPR174	7.12E-62	2.909857314	1.584737538				
SZRD1	5.51E-60	2.387023453	1.300070432				
UQCRCFS1	2.63E-71	3.398175193	1.85184326				
NDUVF2	6.02E-76	4.018898441	2.19071813				
PREDU1	9.33E-94	4.54782629	2.479670957				
SRGAP2	2.3SE-64	2.393103187	1.305643597				
PRF1	S.71E-221	8.148002222	4.447049496				
ZNHIT1	3.S7E-4S	2.358081672	1.287827441				
DNAJCS	1.05E-6S	3.507921737	1.916118334				
ECH1	5.91E-95	4.513875901	2.465864659				
AP1M1	3.92E-51	2.606686949	1.424541654				
STSSIA4	5.44E-52	2.13008172	1.164971994				
ATP6V1F	3.74E-46	2.778310201	1.520163309				
DBNL	1.26E-51	3.119955701	1.707484627				
SDHAF2	2.55E-42	2.231651492	1.221549855				
SNX5	3.71E-63	3.272448498	1.791443438				
RHOC	1.52E-43	2.351991862	1.287709659				
TCEB1	S.92E-44	2.176761792	1.191861997				
PCED1B	1.30E-67	2.944884906	1.613053079				
DCP2	4.12E-54	2.574367761	1.411556472				
POLR2E	2.34E-66	3.389657353	1.859132157				
PPT1	2.1SE-40	2.226713439	1.221724453				
CDS2	1.S7E-5S	3.550286691	1.949856093				
SUMO3	2.22E-4S	2.192537316	1.204675279				
PDIA4	1.37E-45	2.060217384	1.13206285				
OASL	6.62E-77	4.080162676	2.242370091				
SF3B3	5.14E-53	2.718364208	1.494574908				
GARS	5.03E-43	2.095421762	1.152341989				
HLA-DPA1	9.63E-171	7.690356053	4.231368311				
UQCRRH	5.60E-65	3.748982903	2.063606297				
SMC4	4.74E-49	2.877003225	1.584007396				
CCT5	3.05E-50	3.07573076	1.693789585				
TSPO	9.94E-S7	4.503418544	2.480531605				
GDI2	5.63E-91	4.137894871	2.279348624				
AAMP	7.66E-41	2.476207502	1.364373891				
UBASH3A	3.96E-41	2.172861948	1.198199292				
HDAC1	1.77E-73	3.679902245	2.030395951				
GPR56	4.7SE-55	2.845762606	1.570335058				
COX6A1	9.74E-S5	4.597021905	2.538102143				
CKLF	S.31E-70	3.533151041	1.950775788				
SNX1	1.S0E-59	2.947913118	1.628494353				
TINF2	3.49E-3S	2.47000819	1.365517115				
MPHOSPH9	1.62E-47	2.462069474	1.361302101				
HNRNPR	S.30E-S1	3.944593996	2.181355015				
ATPIF1	9.94E-64	3.827276198	2.117266119				
DPF2	1.50E-45	2.283166496	1.264710228				
CAPN1	1.S1E-49	2.676883079	1.483514192				
ANP32B	1.77E-73	3.75028518	2.080260257				
NELFE	2.43E-40	2.304716716	1.279213279				
RTFDC1	2.7SE-56	3.009168631	1.672110798				
SEC11C	3.55E-40	2.49896405	1.389247286				
S100A11	2.27E-92	4.795138112	2.6666238822				
C16orf13	2.92E-43	2.413956514	1.342681762				
SUPT16H	2.51E-52	2.486538637	1.383065963				
RFC1	S.03E-47	2.081093915	1.157564642				
AATF	S.09E-43	2.24825377	1.250722686				
STT3A	9.31E-59	2.831860143	1.575388056				
SYNGR2	1.92E-77	4.083210069	2.272278003				
MFF	6.65E-41	2.265772763	1.261193134				
UBE2V2	S.14E-3S	2.058332586	1.146110328				
SF3A3	5.66E-37	2.130428547	1.186285181				
MCTP2	3.35E-49	2.348089564	1.3081431				
PARK7	4.34E-131	6.514285459	3.629195583				
CYB5R3	5.56E-40	2.149492284	1.1977339754				
SCAMP2	5.22E-73	3.533358638	1.969288978				
ZNF706	S.40E-65	3.758731285	2.094961179				
LYPLA2	4.77E-3S	2.111783157	1.17720598				
NCOA4	1.11E-46	2.42508573	1.352964533				
PA2G4	1.7SE-65	3.542495188	1.977145652				
NDUFB4	1.05E-52	2.869072844	1.60149626				
COX5B	2.60E-S2	4.197187824	2.347363182				
PTPN7	7.32E-100	4.962296328	2.775731868				

SEC13	3.27E-40	2.3S747S6S1	1.335942068				
EMC7	7.94E-47	2.6S300402S	1.501941678				
ILK	6.62E-44	2.434167629	1.362800687				
DAD1	S.74E-69	3.736519S99	2.091949725				
TMBIM4	7.46E-S0	3.63S3 19372	2.037367883				
SRP19	9.00E-40	2.27542S772	1.274995319				
ITM2A	3.24E-117	6.150S54246	3.448522261				
C19orf53	4.47E-62	3.439266377	1.9285571				
XRCSCS	3.62E-SS	4.3207SS579	2.42472928				
EIF3I	9.57E-54	3.119987352	1.750940226				
VDAC3	1.94E-44	2.625250731	1.474648028				
UBE2K	7.02E-3S	2.257519674	1.268418919				
MRS21	3.15E-46	2.32412113	1.306502385				
GIMAP4	1.94E-104	5.335535476	3.003143476				
MEAP6	7.14E-52	2.66745427	1.502330936				
PET100	1.02E-42	2.511283089	1.414738891				
TCEB2	1.QSE-95	4.602014608	2.593134304				
NA10	6.03E-37	2.273235998	1.281421157				
NDUFB11	3.32E-57	2.853365581	1.608439899				
SNRPB	3.41E-96	5.128555101	2.891284185				
C21orf33	3.50E-50	2.512882177	1.416695681				
SF3B4	1.60E-4S	2.225339306	1.254652104				
LSM7	2.19E-43	2.597846536	1.464910635				
CCT3	7.14E-62	3.702446152	2.088709284				
C12orf57	3.S3E-60	3.323240918	1.874858495				
DYNLL1	5.S1E-60	3.684041564	2.07877425				
ESYT1	4.54E-60	3.285822163	1.856171524				
F2R	1.65E-52	2.625227856	1.483165607				
FIP1L1	4.06E-43	2.372173615	1.340613928				
CASP2	2.95E-69	3.030701507	1.713160684				
STAT2	1.61E-59	2.8142147	1.591185386				
PVRIG	1.23E-65	3.861993979	2.183660575				
SNW1	1.90E-40	2.240520696	1.266900748				
PSMB1	1.73E-S6	5.074311692	2.870398857				
LY6E	7.30E-165	6.009189042	3.400342445				
KIF22	5.72E-32	2.041998279	1.155711967				
ISG15	1.S1E-90	5.278851896	2.98841662				
FERMT3	2.77E-70	3.763681847	2.131397907				
CDK6	5.S5E-51	2.443856705	1.384757455				
ZC3H7A	2.93E-52	2.456371941	1.393177961				
C19orf24	7.11E-3S	2.038582649	1.156930025				
PSMD11	5.S1E-62	3.193231368	1.812524189				
ACTR3	2.62E-133	6.151550845	3.491770598				
ARHGAP1	3.02E-55	2.283923702	1.296543845				
NDUFB2	9.SSE-47	2.955264647	1.683076084				
SMIM7	2.36E-32	2.045495605	1.166469824				
VTH1B	5.3SE-32	2.271012962	1.296326986				
CCT7	3.73E-65	3.781517822	2.159130382				
COX7A2	3.52E-75	4.547667793	2.599484015				
TUFM	1.06E-59	3.423716784	1.960362498				
EIF2S2	S.96E-54	2.413538809	1.383047818				
UBE2A	2.16E-43	2.493342543	1.429235196				
LSM4	4.05E-40	2.447377251	1.40315026				
PKM	1.33E-153	6.179521227	3.54300505				
PFKL	3.57E-47	2.406279403	1.38002417				
HADHA	2.29E-49	2.810865774	1.612627924				
MYO1G	1.65E-59	3.240016542	1.859070572				
SPCS2	7.25E-65	3.811066908	2.189851049				
HLA-DRB1	3.77E-165	6.967266382	4.00442777				
CNOTS	1.50E-40	2.432739323	1.398761456				
EIF3CL	1.23E-55	2.748844976	1.580784317				
GRSF1	S.36E-37	2.143522011	1.233334784				
CHFR	1.69E-37	2.114229595	1.216750772				
DDOST	9.62E-90	4.673164159	2.69003846				
SCAMP3	1.09E-33	2.173346259	1.251186304				
ACTR2	4.15E-68	3.191222066	1.837780259				
SLBP	4.37E-33	2.034977143	1.172823601				
RAB10	1.59E-50	2.696983817	1.554789953				
PRDX6	1.03E-64	3.885077785	2.241772484				
NDUFA13	3.66E-90	4.998517507	2.885087091				
SNRPG	2.61E-45	2.976214081	1.718308873				
ACLY	1.15E-44	2.379660011	1.374353644				
NDUFA1	1.65E-55	3.37702517	1.950682295				
ACPI	2.45E-47	2.81906422	1.628461461				
ZBP1	1.12E-33	2.006786173	1.159547377				
ATP6V0E2	1.44E-34	2.168896362	1.253228804				
MED4	4.61E-41	2.454344233	1.418229547				
SEC61B	1.54E-60	3.6693737	2.122504368				
CNDP2	4.03E-42	2.433574726	1.408888708				
MTHFD2	2.25E-52	3.216472819	1.864006829				
DERL2	7.65E-35	2.317280583	1.342915				
CLTC	5.54E-4S	2.286040953	1.325431599				
APOBEC3G	4.39E-169	7.387496204	4.285227047				
HNRNPH2	2.12E-35	2.105987544	1.221991416				
ATF6B	2.04E-51	2.730617224	1.58502401				
SHKBP1	1.40E-55	3.246521987	1.88515449				
CBX3	1.41E-S6	4.370196565	2.5392877				

STATI	3.35E-119	5.871721173	3.414712047				
AP2M1	7.2SE-62	3.3642249S5	1.958107331				
PSMD7	2.90E-39	2.5S4577S46	1.505225089				
CNPY2	1.42E-34	2.22220602	1.294765954				
PSMF1	4.73E-62	3.5S6697471	2.247402371				
RPA1	7.21E-40	2.028592351	1.182269083				
DEK	4.13E-65	3.3S5775009	2.237733473				
GMFG	3.75E-77	4.617705133	2.694975168				
ARPP19	4.12E-54	3.13275547	1.829847012				
CMTM6	3.83E-57	2.719332241	1.588656201				
HN1	9.22E-51	3.1642303SS	1.849404684				
P0LR1D	4.80E-46	2.837670349	1.659097961				
CCDC12	1.68E-39	2.703333781	1.580761946				
NUCB1	5.75E-68	3.349321427	1.959011308				
POLD4	2.03E-64	3.4130S5521	1.998190932				
ARPC1B	1.78E-160	7.440338777	4.356042726				
CNPY3	2.19E-45	2.906730404	1.702201148				
WDR1	5.44E-123	5.301253851	3.104574596				
DLD	S.96E-3S	2.173121961	1.273371189				
NCKAP1L	1.S3E-41	2.177225621	1.275859341				
SPN	2.05E-59	3.265SSSS22	1.917193626				
UBA3	2.40E-42	2.346926931	1.37756974				
OTUB1	5.06E-72	4.001172429	2.348997366				
RALY	1.34E-51	3.235S47541	1.902042773				
C19orf10	5.77E-39	2.194410859	1.28894004				
CTSS	1.07E-4S	3.23S021065	1.902534072				
PIH1D1	7.1SE-33	2.156620901	1.268662345				
MYDSS	1.11E-35	2.296118284	1.350893587				
ARHGAP30	S.37E-73	3.643988384	2.144212036				
SP140	1.95E-61	3.581496238	2.107700389				
TOMM6	9.35E-40	2.636942561	1.551895264				
ATP5D	2.80E-77	3.97069663	2.337834529				
MINOS1	1.34E-36	2.619683698	1.542628797				
FAM96B	3.52E-56	3.495232888	2.060010521				
HOPX	S.26E-33	2.671478308	1.575465985				
SNRNP27	1.60E-3S	2.27848107	1.344322105				
CASP4	3.S1E-70	4.500271672	2.655700938				
MAPRE1	2.48E-46	2.855612801	1.685446275				
NDUFBS	2.12E-S1	4.562793927	2.69319591				
VCP	2.63E-S3	4.176202737	2.465209746				
ENSA	S.65E-S1	3.347095606	1.975961694				
UQCRO	3.13E-65	3.971405319	2.34596961				
PPCS	4.30E-36	2.263033129	1.336883791				
DNMT1	3.30E-51	3.355560977	1.982540994				
TMEM109	1.25E-45	2.922630843	1.727400943				
RBBP4	5.39E-61	3.884808608	2.296584282				
UFC1	4.09E-47	2.929065197	1.731861768				
CTNNBL1	9.03E-34	2.011805059	1.190096386				
PSMA1	1.57E-S0	4.470406933	2.646200971				
RAD21	2.47E-40	2.188661996	1.295760943				
KARS	1.5SE-37	2.482437262	1.472273377				
GTF3C1	1.1SE-27	2.053035546	1.217688343				
SPCS1	9.S5E-70	4.342427969	2.576198999				
LMAN2	1.90E-74	4.233762231	2.512699961				
CHMP1A	1.07E-36	2.167820355	1.287451502				
SNX14	2.76E-39	2.307111802	1.371304965				
NUBP2	1.40E-31	2.060139802	1.225689618				
ACAA1	4.SSE-30	2.092522101	1.245199394				
NDUFA4	5.69E-75	3.639458342	2.166682567				
CIAO1	S.35E-3S	2.405011278	1.432469594				
PSMD2	1.42E-33	2.334094941	1.392275458				
DBB1	4.95E-42	2.532118824	1.510505688				
ATP6V0B	6.96E-39	2.80574037	1.674421148				
ILF2	3.70E-51	3.376083165	2.015288469				
ITGB7	6.39E-36	2.472216783	1.476023317				
UBE2V1	4.45E-SS	4.775893402	2.852785225				
XAF1	2.49E-63	3.621032676	2.163568099				
NUCB2	5.31E-41	3.045367444	1.820277194				
ATP6V0D1	1.21E-33	2.443517425	1.454761148				
BABAM1	1.11E-35	2.489980121	1.4885406				
SLAMF7	1.S2E-S4	4.403054002	2.633365188				
RGS10	1.64E-44	2.760205778	1.65087194				
SH3BGRL	2.43E-63	3.673615864	2.198524241				
TMEM230	5.66E-44	2.948120907	1.764685206				
SFXN1	1.13E-61	3.425027863	2.050331362				
GOLGA7	1.14E-41	2.4748934	1.481793613				
TMEM14B	3.30E-39	2.669337892	1.598221489				
OCIAD1	1.6SE-3S	2.550827587	1.527459075				
IAH1	2.56E-47	3.310667233	1.982882161				
Clorf43	4.04E-44	2.913590992	1.746072105				
USP39	6.46E-34	2.253191519	1.351529262				
CHTOP	1.1SE-39	2.455200876	1.472895285				
SNRPD2	1.29E-61	4.556391844	2.733660493				
CAPI	6.20E-159	6.587729015	3.953535771				
CKAP2	1.34E-32	2.019820487	1.212316639				
HCFC1	3.40E-52	2.405949753	1.445431568				
COXA2L	5.09E-51	3.165310074	1.903124683				

SLC25A39	4.30E-31	2.1S69746S3	1.316076859				
IRF9	4.84E-S2	4.85290S536	2.920824885				
UCP2	2.15E-93	6.566217146	3.952308259				
MGAT1	6.60E-46	2.76656S464	1.665299426				
CTSD	7.99E-115	6.002393763	3.613129473				
SSBP1	1.59E-37	2.8742402	1.730879067				
UFD1L	2.12E-33	2.45043032S	1.475774991				
CNPPD1	7.27E-30	2.003207449	1.207019064				
NUP50	4.57E-4S	2.427163276	1.462662319				
TMEM219	1.01E-33	2.3623323	1.424213697				
UNC13D	4.86E-41	2.400S66965	1.448024258				
RNASEK-C17orf49	4.1SE-74	2.6266132S1	1.584930988				
FLU	5.16E-62	3.9554545S33	2.388711356				
KLRD1	2.50E-62	4.7S52324S7	2.88846001				
NMT1	S.6SE-44	2.55701375	1.544151148				
SUMO2	1.32E-135	6.334S40357	4.127950433				
CMTM3	4.20E-44	2.03741SS71	1.231124403				
GHTM	1.SSE-65	3.564410453	2.335491023				
ANX6	2.S1E-12S	6.137607S03	3.714349198				
pKN1	9.57E-33	2.091753964	1.266131927				
MIS1SBP1	1.14E-39	2.266019504	1.372095957				
RPS19BP1	2.21E-30	2.045430756	1.238694653				
TOR1AIP1	4.60E-31	2.118965183	1.285089538				
UOCR11	6.03E-70	3.81499423	2.313899577				
RNPS1	1.04E-51	3.621261758	2.196520824				
PPP1R1S	4.59E-41	2.407582254	1.46043692				
ATRAID	3.97E-33	2.582840298	1.566956202				
SNRPB2	1.37E-44	2.76625871	1.678255108				
HADHB	1.41E-45	2.864129737	1.738105589				
EIF3C	5.87E-70	4.306295836	2.613902117				
TBCB	2.00E-54	3.688196621	2.240515824				
SH3GLB1	1.14E-37	2.53906017	1.543070541				
HIGD2A	4.75E-5S	3.980579233	2.419574317				
IRF7	7.49E-32	2.380521094	1.447232527				
PITPNB	1.34E-37	2.325670154	1.415011998				
TP1I	1.79E-121	7.395881465	4.500070553				
CEP57	1.50E-34	2.212695351	1.346684502				
PHF11	9.09E-49	3.158822197	1.922889936				
TRPV2	S.SOE-32	2.367709838	1.441911108				
SRSF10	4.47E-44	2.980511358	1.815932343				
OSTF1	2.00E-42	2.999144375	1.827395893				
MTG1	1.22E-31	2.218643439	1.3521046				
CPSF3L	1.63E-31	2.211174717	1.347725009				
PIGT	2.SOE-34	2.513227558	1.532672741				
ARPC4	1.30E-157	6.851827127	4.181712088				
HMOX2	4.77E-41	3.022930956	1.844946179				
C19orf66	1.40E-47	2.993548301	1.827579941				
MVP	1.73E-60	3.722136708	2.272412176				
SSR3	1.45E-44	3.40424105	2.079401191				
FBX07	3.01E-47	3.198461449	1.956190438				
EMC4	1.17E-35	2.835750302	1.735406425				
C17orf62	1.71E-S3	5.33172607	3.263614179				
PSMD6	4.03E-3S	2.830631129	1.733510045				
RAN	1.37E-9S	6.544539291	4.009440789				
NT5C3A	1.35E-27	2.140812368	1.311837323				
CLIC1	7.42E-17S	8.433449003	5.169662289				
ERGIC3	1.60E-41	2.995469656	1.837187244				
PYURF	5.47E-34	2.348458197	1.440953676				
VPS26A	2.11E-30	2.078535177	1.275365333				
GIMAP2	4.S2E-24	2.035579516	1.249501889				
VPS29	1.05E-44	3.142644748	1.929865286				
NDUFA3	3.10E-44	3.176811748	1.951182522				
ARL6IP4	5.99E-55	3.819418005	2.346764209				
COPE	4.S2E-99	5.259363554	3.231909649				
PRKAR1A	1.22E-76	4.416608671	2.714563501				
ANP32E	4.47E-6S	4.261755181	2.622919456				
FAS	1.63E-30	2.02784923	1.248420175				
TIA1	1.04E-44	3.042713453	1.873605809				
POLR2K	1.01E-36	2.458093031	1.51421495				
HSPB1	6.13E-4S	2.906386041	1.790537005				
IFI16	5.40E-119	6.172864049	3.804689139				
CALCCOC02	5.2SE-46	3.250633353	2.004822383				
C5orf56	S.27E-42	2.961541242	1.826940818				
BUB3	2.SSE-77	5.1357333	3.169195142				
STXBp2	5.59E-43	2.690955946	1.661080838				
AP3S1	5.75E-35	2.455741841	1.516783649				
TPM4	3.79E-70	4.197012241	2.593640032				
GPI	1.02E-90	5.719836631	3.534864332				
ANXA11	3.41E-52	3.361449258	2.078475622				
SNX6	9.10E-41	2.969658784	1.83647297				
RSU1	4.77E-29	2.181984201	1.349562929				
COX7B	5.S4E-32	2.110043765	1.305363144				
NDUFA10	5.3SE-36	2.822888697	1.746415241				
HM13	3.35E-42	3.185304677	1.970775858				
ACSL5	3.69E-31	2.131074781	1.318955199				
MAP2K3	2.09E-36	2.818078082	1.744193579				
GABARAP	1.06E-107	6.463268119	4.002597503				

RAD23A	3.28E-52	2.939828452	1.820626087				
FBXW5	1.12E-31	2.166893027	1.342509072				
ATP5L	4.72E-85	5.902632358	3.660315242				
NDUFV1	1.13E-46	3.654319147	2.266213741				
RNF7	3.21E-35	2.966729911	1.841285164				
CPSF6	5.24E-28	2.188809842	1.358854684				
FAM32A	6.74E-27	2.26521548	1.406355453				
RWD2D1	1.75E-33	2.328616423	1.445913612				
TLN1	2.13E-55	2.83477322	1.760609391				
REEP5	7.21E-56	4.012890333	2.493302187				
NAB1	2.51E-43	2.583117048	1.605329927				
SUB1	5.51E-119	6.926868887	4.305358205				
RNF167	2.14E-59	3.251788854	2.021327615				
ACTN4	2.54E-65	4.017527807	2.497511726				
CD164	3.44E-84	4.798223656	2.984003276				
RBPI	1.08E-72	4.599572129	2.860650726				
SYNCRIP	1.43E-29	2.075675416	1.291195927				
SF3B2	8.25E-74	3.749571466	2.333567052				
MAP4	3.98E-61	3.519160811	2.190424566				
HLA-DPB1	5.57E-130	7.298193735	4.54313944				
DNAJB11	3.95E-52	3.215496625	2.003386312				
EIF4G1	3.68E-46	3.023582729	1.884145293				
ATP5EP2	5.57E-59	2.336498104	1.456041078				
BCAP31	7.14E-57	3.681222571	2.294156976				
SPATA13	3.34E-58	3.308985653	2.062711146				
NOL7	1.59E-30	2.079211994	1.296612709				
TMEM173	5.44E-34	2.610443626	1.628024411				
C1QBP	4.96E-33	2.536382203	1.582148766				
ZBTB38	3.52E-36	2.27467782	1.419366096				
RCSD1	2.10E-30	2.100879039	1.310998204				
RASSF1	1.82E-41	3.140334137	1.96011208				
TROVE2	1.64E-57	3.282108424	2.049797753				
OS9	1.70E-41	2.880583433	1.799945872				
HPS1	1.50E-37	2.564393391	1.602542015				
DCXR	5.94E-35	2.961817246	1.851052392				
APOL2	3.23E-74	3.629181003	2.268673089				
MAT2B	8.08E-87	4.937590513	3.086767073				
C4orf3	1.61E-61	3.01178837	1.88408595				
PSTPIP1	3.60E-73	4.936433608	3.097994208				
NUCKS1	9.06E-67	2.647890735	1.66186903				
FRG1	7.94E-33	2.277768457	1.43174302				
NSD1	3.06E-38	2.088236806	1.313409035				
APOBEC3C	5.37E-186	7.962933634	5.011603653				
NEDD9	1.02E-42	2.908674059	1.831089133				
NRD1	1.96E-50	3.205726873	2.01817484				
ATP6AP1	1.35E-26	2.189337028	1.378495297				
ZYX	2.36E-44	2.874590561	1.811519884				
ARHGEF6	1.55E-37	2.143868022	1.351594141				
NDUFA6	3.29E-45	3.223481384	2.032653591				
SHISA5	6.88E-79	5.16156127	3.255939187				
TAP1	5.20E-120	6.827121183	4.3072061				
USP10	4.16E-32	2.594303424	1.637529873				
VDAC2	1.80E-40	3.340362467	2.108772919				
BSG	3.75E-73	3.917750256	2.473335439				
SLAMF6	4.50E-33	2.312527898	1.46025311				
GIMAP7	1.04E-73	4.837423871	3.056014				
GZMA	3.88E-133	8.404353185	5.310526823				
PARVG	4.32E-59	3.786798932	2.393050277				
ENY2	2.49E-23	2.079785881	1.314895961				
SUMO1	1.48E-51	3.888958758	2.458807328				
CTS4	3.31E-33	2.567656219	1.624903178				
YWHAE	3.14E-82	4.46749523	2.829183738				
GABARAPL2	1.49E-42	3.272339636	2.073958931				
TM9SF2	3.12E-42	2.842103705	1.801523074				
TMEM9B	1.07E-26	2.101387846	1.332514489				
RHOG	1.89E-33	2.783782498	1.768194821				
ATPSG2	2.86E-113	7.216807229	4.585891172				
HMGN1	1.44E-90	5.907073802	3.756389242				
CCT4	3.99E-43	3.391043905	2.157415929				
DGKZ	5.02E-34	2.105760861	1.339720442				
MOB3A	2.80E-40	2.685889951	1.709771621				
YY1AP1	1.60E-29	2.052913444	1.307074888				
SCAND1	2.84E-41	2.970307459	1.891248721				
SURF4	1.44E-72	4.488437276	2.858364656				
KRTCAP2	2.30E-50	3.937107647	2.508411369				
DGCR6L	7.08E-24	2.081946672	1.326516078				
CCL4L2	2.16E-64	3.938525142	2.509655941				
RNASEK	2.03E-94	6.224449345	3.966543979				
PDHA1	7.17E-23	2.151780493	1.373902705				
EIF3M	4.27E-44	3.974408381	2.539108444				
SEL1L3	1.98E-30	2.018099472	1.289543953				
HMGN4	1.31E-39	3.042008177	1.944082372				
C18orf32	2.47E-26	2.166044511	1.384511589				
GZMH	1.30E-73	5.894512086	3.777397432				
DARS	1.15E-36	3.084375538	1.97732192				
U2AF1L4	1.43E-29	2.17184753	1.39265974				
AC040977.1	1.29E-47	2.916354617	1.87128586				

RAB11B	5.73E-39	2.050342686	1.31608312				
IDH3B	2.35E-40	2.954707051	1.898308248				
STIP1	2.79E-44	3.537545486	2.274889059				
BECN1	8.05E-37	2.722915881	1.754051395				
P4HB	8.44E-56	4.165336865	2.683632485				
LSP1	3.35E-159	7.616866902	4.912004868				
GPR171	1.11E-38	3.012734056	1.943265783				
ADAR	3.26E-55	3.151379602	2.034872014				
TMEM50A	4.96E-48	4.089997255	2.642051936				
DNAJC7	1.57E-34	2.740896263	1.771205422				
UBE2I	8.71E-55	3.93333298	2.542167011				
APEH	1.66E-26	2.475388449	1.600109311				
TRAF3IP3	1.19E-67	4.851180082	3.137389949				
TYK2	1.22E-25	2.394766099	1.549470291				
ATP5E	1.22E-109	3.975723569	2.57244789				
DCTN1	4.03E-29	2.18443937	1.413688251				
PSMC5	1.04E-54	4.193865191	2.714172324				
EIF4EBP2	1.79E-41	2.068074222	1.33852577				
SELT	7.76E-49	4.143695783	2.682014846				
ATP6VOE1	5.68E-58	4.56706923	2.956505851				
HMGGB2	1.22E-44	4.506775593	2.919106762				
MTF2	9.03E-27	2.197695309	1.420938046				
TRMT112	4.34E-60	4.489644923	2.909558402				
UXT	8.23E-28	2.6923466	1.745444661				
KXD1	1.54E-30	2.345004629	1.520294147				
PAK2	1.75E-38	2.210017912	1.432838882				
RARRES3	1.62E-94	6.596902368	4.277118092				
AP2B1	2.87E-45	3.091383683	2.006271352				
IL2RB	1.08E-113	5.921323797	3.843423808				
PSMC6	6.33E-33	2.726625769	1.770379729				
MOB1A	1.33E-78	4.783929954	3.107595821				
VIMP	7.05E-27	2.126371165	1.381467199				
ARPC3	1.47E-124	7.484204265	4.86977031				
ARCN1	5.77E-28	2.220026945	1.444717564				
ADD1	2.77E-28	2.103970307	1.369235942				
MAP4K1	8.59E-82	5.155918314	3.357699393				
VPS28	5.98E-49	4.09828844	2.668959842				
ZNF106	4.46E-30	2.101794045	1.36882088				
DR1	5.20E-67	3.668343403	2.390324384				
UBL5	2.19E-52	4.147431556	2.70273483				
SH2D1A	4.70E-66	4.779902653	3.117910972				
TCEA1	3.94E-51	3.467395717	2.261883953				
EXOSC10	2.12E-31	2.432550976	1.586969488				
LRMP	2.25E-39	2.499096302	1.630516899				
CTSW	4.34E-105	6.84297274	4.466128898				
CD84	9.09E-45	3.469082844	2.26450223				
MIF	9.58E-80	6.014896083	3.927408813				
SLFN5	1.70E-54	3.398082577	2.219132774				
QARS	9.65E-26	2.452104416	1.601861754				
CHCHD2	2.90E-94	6.538412547	4.273878212				
IP6K2	1.84E-26	2.484193419	1.624111357				
CHP1	4.51E-61	3.739156984	2.444679058				
ADSS	1.54E-26	2.178959942	1.425127795				
MED28	4.90E-29	2.067390347	1.352321366				
FAM192A	9.47E-33	2.568577547	1.682271284				
CANX	4.09E-58	3.870752171	2.536022704				
HBS1L	4.44E-23	2.179472352	1.42795995				
TLK1	3.55E-56	3.260350119	2.136328049				
MRPS6	1.64E-35	3.200933281	2.098333354				
MARS	5.52E-28	2.763540175	1.812189436				
ZC3H15	1.97E-29	2.5166636389	1.651347011				
MFSD10	1.13E-34	2.918352126	1.916295808				
FBXW2	1.21E-28	2.085014048	1.369322522				
ANAPCS	4.23E-42	3.366507941	2.211121676				
LAMTOR4	1.30E-32	3.05638239	2.007499692				
ARL6IP1	3.79E-71	5.300427623	3.482998051				
DNAJC1	8.01E-25	2.003317792	1.316599617				
TOMM22	3.94E-24	2.27015687	1.492370255				
PHLDA1	1.07E-45	2.69859196	1.775240081				
RHBDD2	5.80E-31	2.777370902	1.827336336				
NFATC3	7.51E-34	2.606514375	1.715450148				
H CST	1.89E-90	6.511946895	4.285861261				
EBP	1.70E-25	2.55007453	1.678394885				
DYNLT1	5.85E-28	2.872671838	1.891727315				
CCT6A	2.54E-38	3.321361789	2.187737041				
UBE2G1	2.56E-26	2.197962624	1.44790689				
METTL17	4.44E-26	2.563268413	1.688628376				
PEBP1	3.54E-38	3.324489184	2.190503711				
RASSF5	5.73E-48	3.619722393	2.385040754				
LDHB	3.28E-106	7.217618466	4.755923988				
CYCS	1.11E-49	4.056734588	2.673620261				
ARRDC1	3.13E-22	2.187529473	1.44210077				
TMED2	4.52E-47	3.759359558	2.478331276				
SERBP1	2.42E-52	4.079834454	2.690620905				
PPP6R1	2.24E-27	2.075315769	1.368888607				
SLC25A5	7.59E-81	6.146292619	4.05672534				
ASCC2	2.70E-26	2.2935164	1.514241414				

XRCC6	2.24E-66	5.332S6631	3.521552017				
HINT1	2.00E-69	5.444269191	3.595154415				
COMMID6	1.80E-47	3.942989244	2.603894656				
GTF2I	1.03E-39	2.605337448	1.720800727				
ARFGAP2	1.11E-26	2.162S78566	1.428586221				
RPN2	1.63E-50	4.076933247	2.693455312				
CACVBP	2.40E-40	3.568917103	2.360132739				
FAM49B	2.80E-54	4.50407231	2.978784542				
LYST	2.88E-111	6.035206783	3.993901492				
CCT2	4.17E-27	2.457412094	1.627710862				
GIMAP5	5.74E-43	3.971161226	2.630381797				
ABCA2	1.06E-23	2.03954305S	1.351010325				
ARL6IP5	2.79E-S0	5.55165428	3.677661258				
PJA2	5.00E-31	2.135106401	1.416392377				
PAG1	9.42E-44	2.771094813	1.838611606				
ICAM3	2.50E-55	4.184529093	2.77808883				
RABGGTB	2.94E-24	2.190477192	1.455249049				
CBX6	1.35E-65	3.207576836	2.131129713				
ANAPC16	1.60E-60	4.44125641	2.951423548				
UBE2D2	1.54E-49	3.332037896	2.214660952				
CMC2	1.34E-32	3.045057414	2.024752334				
CYTH4	6.93E-29	2.419694933	1.609750572				
DHPS	1.64E-21	2.141145494	1.424754178				
ARLSB	3.82E-27	2.315513892	1.541026135				
SRP54	S.06E-25	2.172518355	1.446446899				
HNRNPA3	2.02E-51	4.177375906	2.782387515				
WDR33	1.44E-29	2.355311056	1.569538437				
PSD4	2.22E-37	2.687355534	1.791678741				
ARMCS	1.33E-2S	2.176526092	1.451279875				
PRRC2A	2.37E-36	2.172182912	1.450127423				
DERL1	1.07E-30	2.65210845	1.770523144				
U2SURP	S.87E-3S	3.084151355	2.059096389				
CERS5	9.81E-2S	2.570971162	1.716920578				
NUDC	2.88E-44	3.484075924	2.327423089				
PRDX2	1.75E-35	3.43953608	2.298228498				
AHII	6.76E-31	2.398367212	1.603880689				
SSR1	4.06E-43	3.517697239	2.353620607				
POLR2L	1.06E-32	2.491882379	1.667384234				
INPP4B	2.00E-71	4.37672435	2.9288058				
ASXL2	3.17E-32	2.108317351	1.411605009				
ATG4B	5.16E-25	2.225661294	1.492125773				
LSM12	2.66E-42	3.000791735	2.011861684				
PPP6C	1.94E-25	2.328711003	1.562259411				
RNF4	9.65E-49	3.772541129	2.532500365				
EFCAB14	6.51E-36	2.717792101	1.826863823				
EWSR1	1.02E-61	5.27069016	3.543415803				
ATP6AP2	3.67E-33	2.87956881	1.938545201				
EIF3H	7.42E-66	5.23594245	3.525377653				
TCIRG1	2.95E-47	4.148180031	2.793223022				
CMCI	3.18E-25	2.600570046	1.752536789				
SEPW1	7.17E-3S	3.616324667	2.437144725				
BTG3	5.87E-33	2.661145175	1.793626458				
HSPA4	S.41E-27	2.044235635	1.378377771				
BUD31	1.89E-30	3.232431906	2.180395611				
MLF2	6.07E-37	3.252881601	2.194581786				
BRDS	1.89E-23	2.094298243	1.413056636				
ARHGEF3	3.03E-30	2.707102202	1.82704047				
ZFR	5.21E-29	2.405365423	1.623894417				
RNHI	2.16E-24	2.394140938	1.616911589				
RQCD1	2.16E-65	4.384180221	2.960975759				
RHOA	6.46E-133	7.902922231	5.338622161				
IRF3	1.57E-25	2.452275937	1.656606717				
ERAP1	1.11E-29	2.384571644	1.611020213				
EDF1	3.39E-76	5.942447836	4.016529304				
CCNDBP1	S.39E-51	4.382605668	2.962971368				
ATG3	1.21E-20	2.021986514	1.367075446				
C7orf55-LUC7L2	9.40E-37	2.3808909	1.609954217				
ST6GALNAC6	4.05E-28	2.719470888	1.839496906				
OST4	4.22E-67	5.471192314	3.700928585				
TPR	1.13E-3S	3.246980918	2.197245647				
CCL4L1	3.04E-S2	5.49689949	3.723660885				
RALGDS	6.97E-33	3.030525765	2.053538761				
LARP4B	6.35E-3S	2.326104909	1.577917765				
CNTRL	6.04E-29	2.35429884	1.597295692				
TESPA1	1.97E-25	2.168661239	1.472198248				
RBM17	2.30E-33	3.037559551	2.062735027				
PARP14	1.15E-4S	3.051280657	2.073373049				
IRF4	1.26E-26	2.303676426	1.565454138				
OGDH	1.69E-2S	2.241572448	1.523431079				
DOK2	5.3SE-3S	3.74488755	2.545605284				
JTB	2.32E-21	2.104395367	1.430642404				
CNOT7	1.06E-31	2.801609526	1.904721688				
SRP14	2.15E-103	7.49178249	5.093426717				
ELMO1	1.01E-37	3.216027022	2.186654238				
SDHA	2.92E-33	3.468793636	2.359065802				
CAPZB	1.29E-97	6.388918429	4.345279637				
EIF4H	5.60E-55	4.6389405	3.157794688				

METTL23	1.50E-19	2.124483219	1.446921131				
PRDX1	1.44E-44	4.396664763	2.995606468				
ISCU	2.06E-44	3.938433077	2.683637768				
ERP29	9.57E-40	3.419556374	2.331961517				
TMED10	9.34E-41	3.511693538	2.395543973				
MRFAP1L1	4.11E-23	2.229358073	1.520923709				
TOX4	5.77E-30	2.797710276	1.909218279				
PCBP1	6.62E-58	4.102823361	2.800690533				
DENNND2D	5.62E-57	5.158701613	3.522687205				
SARS	4.60E-31	2.986622641	2.039576497				
RPN1	3.79E-55	4.36650348	2.983135575				
PSMA3	8.73E-37	4.340402057	2.965569046				
NECAP2	3.29E-28	2.409938585	1.646810367				
GLIPR2	1.15E-20	2.331104148	1.592954483				
ETNK1	2.37E-S4	4.798208956	3.278870917				
YWHAQ	9.21E-42	4.104026719	2.804935287				
SPCS3	1.55E-34	3.127057817	2.13767614				
BROX	2.77E-43	2.996718284	2.051504294				
MRPL10	1.51E-20	2.354203605	1.613160182				
GIT2	3.73E-30	2.698452322	1.849510866				
COX7C	1.32E-55	5.3881874146	3.690008942				
PSMA7	1.17E-96	7.078722592	4.855421638				
SOD1	1.35E-72	6.411638789	4.399025983				
USP4	1.65E-24	2.307293925	1.58314858				
GPS2	1.21E-26	2.906658924	1.994953297				
NHP2L1	7.82E-43	3.977571773	2.733500307				
HDLBP	1.43E-26	2.457331413	1.689720212				
CCL4	7.70E-S7	7.808356716	5.372671392				
RAPGEF1	3.42E-27	2.063916233	1.420260587				
LCP2	1.00E-S6	5.663713418	3.901307532				
MYL12B	7.68E-99	7.213916034	4.970445418				
PRR13	1.24E-62	5.322610825	3.667346792				
SS1SL2	4.25E-19	2.106912803	1.452105718				
TNFRSF1B	2.25E-53	4.644268949	3.201693272				
PTBP1	1.45E-45	3.794388964	2.616270243				
CCND2	1.44E-51	4.061882522	2.800977741				
RNF114	3.71E-26	2.173974866	1.501754604				
PPIB	1.91E-S2	6.548210729	4.523737308				
PRPF40A	1.S4E-26	2.648378951	1.831100989				
Clorf58	3.73E-62	5.222474652	3.611557859				
APOL6	6.90E-35	2.198346714	1.520484108				
PNRC2	2.01E-47	4.010840361	2.774926298				
PSMB4	5.20E-4S	4.66978486	3.232451809				
ANKRD10	4.35E-99	6.007181504	4.160622794				
ATP5B	4.73E-65	6.115050004	4.235792502				
DDX39A	4.84E-30	3.712509471	2.572770358				
SLFN12L	2.12E-39	2.927231643	2.028795903				
SSR4	6.53E-49	4.995265015	3.462286143				
PCIF1	4.10E-22	2.301368176	1.595419291				
NFAT5	1.31E-25	2.204989861	1.528663709				
GPRIN3	2.50E-40	2.567966118	1.780423811				
LRBA	1.57E-31	2.339541687	1.622592678				
RAP1GDS1	1.45E-29	2.557433928	1.774580173				
TCERG1	3.09E-32	2.785889889	1.933391533				
DEGS1	4.72E-20	2.016088116	1.399270935				
HMGN2	2.02E-71	7.681448766	5.331525844				
RAC2	7.60E-141	8.860802821	6.150823213				
BIN1	2.66E-29	2.83383341	1.97085633				
CASC4	9.33E-24	2.159554092	1.501983373				
TARDBP	1.41E-32	2.739400892	1.90547687				
TANK	2.S6E-44	4.063031988	2.827364459				
RAB7L1	3.07E-24	2.459221023	1.71154895				
EXOC7	4.89E-22	2.105264834	1.465731362				
SSU72	2.09E-18	2.027775189	1.412453934				
SEC24C	5.05E-28	2.414842903	1.682107238				
ANXA2	3.71E-40	4.612027355	3.215640818				
FYTTDI	3.22E-22	2.049003575	1.428906094				
NDUFA5	1.S4E-25	2.468249327	1.722233131				
TPST2	2.41E-23	2.472095316	1.725695558				
H2AFV	1.13E-16	2.238773457	1.56333869				
RABAC1	2.SSE-40	4.222925453	2.949687442				
CRKL	1.06E-24	2.120279667	1.481085685				
PIM1	1.57E-29	3.223902327	2.252243029				
SH3KBP1	2.17E-59	4.755111806	3.32353741				
PTBP3	1.51E-31	2.530875076	1.768986375				
UBXN1	2.34E-47	4.586560378	3.207664016				
GRB2	5.S4E-48	3.973754698	2.779366956				
U2AF1	5.42E-48	4.509899275	3.15466215				
PPIE	2.77E-26	2.616046102	1.830196288				
ZNF410	2.91E-18	2.218616014	1.552580564				
NONO	1.36E-73	5.933940446	4.153338329				
DNAJA2	2.06E-26	2.432867867	1.703382509				
KIF2A	3.43E-24	2.134265978	1.494728594				
NKG7	1.05E-169	11.35967924	7.957885544				
NPEPPS	S.93E-47	3.173894128	2.22416716				
VAPA	1.70E-33	3.681594647	2.58107836				
ADIPOR1	1.65E-22	2.367477939	1.662704735				

DAP3	5.05E-25	2.657307061	1.866391882					
LRPAP1	1.04E-20	2.290634382	1.608865841					
LCK	3.46E-101	7.533473442	5.292448282					
SYNRG	2.86E-27	2.450990742	1.722655895					
ANXA7	4.56E-26	3.011478691	2.117222328					
NAPA	8.82E-36	3.548349886	2.494764913					
ERP44	3.07E-27	2.191002041	1.54054131					
LAT	1.42E-43	4.725957504	3.324566062					
S100A4	1.04E-62	6.339108393	4.461573784					
STARD7	1.18E-39	2.890297078	2.034417983					
UBAP2L	6.25E-25	2.621606628	1.846663437					
CLK3	2.07E-22	2.558200789	1.802038652					
PCNP	1.74E-31	3.078049292	2.168410705					
PRDM1	2.33E-84	5.477404345	3.860395533					
C2orf68	4.24E-41	2.77814339	1.958678758					
HP1BP3	1.16E-37	3.260553477	2.300180925					
USP22	2.33E-29	2.161033427	1.526466989					
SS18	6.42E-21	2.168153993	1.532124355					
PYHIN1	4.65E-50	4.441494759	3.139252438					
COTL1	2.43E-91	7.728695226	5.463550006					

Table 7

Significant in Non-responder			
Gene Name	P-value	% exp. in R	% exp. in NR
CD38	1.97E-112	0.1522694	0.427109974
EPSTI1	4.28E-56	0.141532455	0.321553127
GOLIM4	1.38E-49	0.162030259	0.335038363
WARS	2.44E-49	0.155197657	0.3257382
PDCD1	8.07E-47	0.292337726	0.48058591
CCL3	1.45E-45	0.187408492	0.357823762
SNAP47	2.24E-43	0.19228892 1	0.358986282
VCAM1	8.75E-43	0.145436798	0.299697745
SKA2	3.86E-38	0.133235725	0.273889793
HAVCR2	2.66E-37	0.273792094	0.438502674
LGALS9	8.18E-32	0.096632504	0.211578703
PRDX3	1.15E-31	0.169350903	0.303882818
FASLG	2.46E-29	0.133235725	0.253429435
ENTPD1	3.76E-29	0.167886774	0.29597768
FABP5	2.54E-28	0.221571498	0.356893746
SIRPG	3.49E-28	0.310395315	0.454545455
LSM2	2.20E-27	0.133235725	0.24854685
NDUFB3	5.28E-27	0.167886774	0.290165078
TRAFD1	2.07E-25	0.153733529	0.268774704
UBE2F	4.24E-25	0.152757443	0.266914671
NMI	4.08E-24	0.123474866	0.227853987
IFI35	1.58E-22	0.128355295	0.229946524
CLTA	3.50E-22	0.170326989	0.279702395
MTHFD1	5.40E-21	0.12249878	0.21832132 1
MY07A	1.36E-20	0.132259639	0.229249012
IFI27L2	1.46E-16	0.138604197	0.224831435
MCM5	6.19E-16	0.142020498	0.227156475
STMN1	2.12E-15	0.110297706	0.186933271
ID3	1.31E-14	0.108833577	0.1825 15694

RGS3	1.65E-14	0.131283553	0.209486166
SN RPD1	1.66E-14	0.179599805	0.2662 17159
PTTG1	2.02E-14	0.134699854	0.21320623 1
FIBP	1.72E-13	0.142996584	0.219948849

Significant in Responder			
Gene Name	P-value	% exp in R	% exp in NR
IL7R	9.60E-48	0.41727672	0.236921646
TCF7	3.02E-20	0.354807223	0.24226924
GPR183	1.54E-16	0.21522694	0.132527319
MGAT4A	1.28E-12	0.263543192	0.184840735

Table 8A - Patient

F- female; M- male; NR- nonresponder; R- responder; NA- not available; DOD- dead of disease; AWD- alive with disease

#	Patient ID	Gender (F/M)	Age	Therapy	Clinical response (RECIST; R=CR, PR, NR=S D, PD)	Baseline Biopsy (days from baseline; site; lesion response)	Post I biopsy (days from baseline; site; lesion response)	Post II biopsy (days from baseline; site; lesion response)	Overall survival (days)	Status (Alive=0; Dead=1)
1	P36	F	80	PD1	R	NA	42; right upper arm; regression	NA	823	0
2	P37	M	67	PD1	NR		42; left posterior; progression	NA	144	1
3	P38	F	62	PD1	R	(-1); adreanal gland (left biopsy); regression	NA	NA	1496	0
4	P39	M	71	PD1	Resistance	0; upper back lesion; regression	21; upper back lesion; regression	207; adrenalectomy; progression	490	0
5	P40	M	81	PD1	NR	(-55); lung wedge biopsy (left lobe); progression	NA	NA	175	1
6	P41	M	86	PD1	R	NA	341; left lower leg; regression	NA	642	0
7	P42	M	77	PD1	R	(-30); Skin left flank; regression	NA	NA	597	1
8	P43	F	45	PD1	R	(-86); lymph node right axillary; regression	NA	NA	383	0
9	P44	M	52	PD1	NR	NA	9; small bowel resection; progression	NA	558	0
10	P45	F	77	PD1	NR	(-192); left iliac lymph node; progression	30; left retroperitoneal lymph node; progression	290; brian right temporal; progression	371	1
11	P46	M	81	PD1	NR (patient)	(-22); brain lesion; regression	97; right chest wall; progression	NA	558	1

					had mix respo nse)					
1 2	P47	M	8 5	PD1	R	(-112); left forehead; regression	NA	NA	642	0
1 3	P49	M	7 0	PD1	NR	(-15); liver; progression	141; left parotid gland mass; progression	NA	345	1
1 4	P50	M	6 4	PD1	NR	(-132); proximal jejunum; progression	NA	NA	328	0
1 5	P51	M	7 2	PD1	NR	0; right neck (skin); progression	142; right neck (skin); progression	NA	241	1
1 6	P52	F	6 7	PD1	Resist ance	(-195); left leg (skin); regression	342; left arm; progression	NA	194 3	0
1 7	P53	M	6 1	PD1	Resist ance	NA	113; left groin; progression	NA	492	1
1 8	P54	M	8 2	PD1	Resist ance	0; lower left abdomen; regression	209; left groin; progression	NA	420	1
1 9	P55	M	7 5	PD1	NR (patie nt had mix respo nse)	0; left neck; progression	NA	NA	504	0
2 0	P56	M	6 6	PD1	R	0; left upper arm; regression	NA	NA	658	0
2 1	P57	F	5 6	PD1	R	0; left upper arm; regression	NA	NA	612	0
2 2	P58	M	7 8	PD1	R	(-2); left chest; regression	NA	NA	605	0
2 3	P59	F	6 5	PD1	R	(-66); left axillary lymph node; regression	NA	NA	413	0
2 4	P60	M	7 6	PD1	R	(-1); right lateral calf (leg); regression	NA	NA	438	0
2 5	P61	F	6 7	PD1+C TLA4	NR		23; lung; progression	NA	27	1
2 6	P62	M	6 0	PD1	R	NA	126; skin back; regression	NA	189 5	0
2 7	P63	M	7 2	PD1	NR	(-105); gastric tumor; progression	NA	NA	954	1
2 8	P64	M	6 3	PD1	NR	NA	39; left frontal brain; progression	NA	129 5	0
2 9	P65	F	4 0	PD1	R	(-17); right temple inferior lower; regression	146; right cheek (skin); regression	NA	113 8	0
3 0	P66	M	7 5	PD1	NR	(-174); axillary lymph node; progression	NA	NA	963	0
3 1	P67	F	7 1	PD1	NR	(-20); lung wedge lower left lobe; progression	NA	NA	929	0
3	P68	M	6	PD1	NR	NA	244; right lower	NA	756	1

2			3			lobe- lung biopsy; progression			
3	P69	M	65	PD1	R	(-21); left upper arm; regression	NA	NA	406 0

Samples from the scRNAseq cohort

#	Patient ID	Gender (F/M)	Age	Therapy	Clinical response (RECIST; R=CR, PR, NR=S D, PD)	Baseline Biopsy (days from baseline; site; lesion response)	Post I biopsy (days from baseline; site; lesion response)	Post II biopsy (days from baseline; site; lesion response)	Overall survival (days)	Status (Alive=0; Dead=1)
34	P1	M	49	CTLA4 (baseline); PD1 (post I and II)	Resistance	NA	48; anterior neck; regression	437; anterior neck; progression	822	0
35	P2	M	75	PD1	NR	0; small bowel; progression	35; left axilla; progression	NA	347	1
36	P4	M	29	CTLA4 +PD1	R	(-2); left shoulder; progression prior to therapy	35; left shoulder; regression	NA	539	0
37	P24	M	73	PD1	R	0; left lower back; regression	NA	NA	54	0

Table 8B - Lesion

Patient	Sample name	Response status; R-responder, NR-non-responders	CD8% of nuclei	CD8+TCF7+/CD8+TCF7- ratio
P36	Post_P36	R	23.02	2.103
P37	Post_P37	NR	6.06	0.849
P38	Pre_P38	R	5.65	1.36
P39	Pre_P39	R	6.65	1.93
P39	Post_P39	R	32.4	2.07
P39	Post_P39_2	NR	8.34	0.77
P40	Pre_P40	NR	5.35	0.926
P41	Post_P41	R	12.7	4.17
P42	Pre_P42	R	12.67	0.81
P43	Pre_P43	R	17.96	1.33
P44	Post_P44	NR	19.65	0.852
P45	Pre_45	NR	18.62	0.882
P45	Post_P45	NR	16.67	1.049
P45	Post_P45_2	NR	3.32	0.655
P46	Pre_P46	R	3.94	1.718
P46	Post_P46	NR	3.23	0.946
P47	Pre_P47	R	23.51	1.599
P49	Pre_P49	NR	3.988	0.792
P49	Post_P49	NR	8.85	1.29
P50	Pre_P50	NR	12.2	0.533
P51	Pre_P51	NR	14.62	0.413
P51	Post_P51	NR	18.72	0.174
P52	Pre_P52	R	16.74	2.73
P52	Post_P52	NR	33.6	0.534
P53	Post_P53	NR	8.7	1.12

P54	Pre_P54	R	4.919	0.57
P54	Post_P54	NR	12.5	0.471
P55	Pre_P55	NR	27.7	0.36
P56	Pre_P56	R	36.52	1.189
P57	Pre_P57	R	20.45	1.2
P58	Pre_P58	R	3.877	0.86
P59	Pre_P59	R	22.2	1.202
P60	Pre_P60	R	66.36	0.961
P61	Post_P61	NR	29.73	0.243
P62	Post_P62	R	9.242	1.272
P63	Pre_P63	NR	16.68	1.22
P64	Post_P64	NR	4.3	0.238
P65	Pre_P65	R	2.2	1.657
P65	Post_P65	R	11.12	1.885
P66	Pre_P66	NR	12.88	0.27
P67	Pre_P67	NR	8.357	0.82
P68	Post_P68	NR	7.723	0.634
P69	Pre_P69	R	1.53	3.416
Samples from the scRNAseq cohort				
PI	Post_PI	R	50.7	1.01
PI	Post_PI_2	NR	17.57	0.576
P2	Pre_P2	NR	3.8	0.395
P2	Post_P2	NR	12.04	0.858
P4	Pre_P4	NR	25.39	0.209
P4	Post_P4	R	9.48	3.44
P24	Pre_P24	R	7.366	1.457

Table 9

CD8_1					CD8_2				
GeneName	P-value	Mean expression G1	Mean expression non-G1		GeneName	P-value	Mean expression G2	Mean expression non-G2	
SPC25	7.29E-183	2.152793671	0.022709593	adjusted P-value = 1.4e-5	GEM	3.01E-97	3.212921547	0.626392714	adjusted P-value = 2.5e-5
CDCAS5	9.61E-249	3.283968204	0.048568291		LAYN	1.71E-57	2.341179483	0.605117134	
ESCO2	2.05E-173	2.107874319	0.031717053		VCAM1	9.49E-153	5.745571447	1.494735054	
CDC45	2.77E-199	2.662546645	0.041064042		RDH10	1.81E-55	2.085194324	0.574960204	
ZWINT	<1E-300	4.54154968	0.078683491		FAM3C	4.62E-68	2.699856838	0.758692255	
SHCBP1	5.45E-176	2.166191529	0.038651757		KIR2DL	1.25E-45	2.743079022	0.78306608	
DLGAP5	1.19E-173	2.297602153	0.044316935		TNFRSF18	1.80E-47	2.222603508	0.666522377	
RAD51	1.01E-197	2.792017792	0.056664368		MTSS1	3.59E-51	2.379706089	0.729873791	
KIF18B	5.15E-197	2.197946499	0.044829274		CADM1	5.38E-46	2.394829138	0.744721504	
RRM2	<1E-300	5.545835323	0.113463575		ENTPD1	7.16E-101	4.438595811	1.500307988	
BIRC5	2.61E-284	3.649241861	x		ETV1	1.38E-41	2.042430288	0.712259261	
TK1	<1E-300	5.010059873	0.107424617		AFAP1L2	2.42E-40	2.174279724	0.762835224	
HJURP	1.34E-173	2.210552183	0.048501849		TNFRSF9	3.53E-113	5.512980442	1.93475996	
UBE2C	2.82E-231	3.899002096	0.085658551		NAB1	1.73E-99	4.684753101	1.663550573	
CCNB2	5.21E-194	2.890822394	0.065006324		PEL1	5.89E-58	3.121591718	1.127848712	
CENPW	1.08E-193	2.850658243	0.068347806		DFNB31	1.97E-75	3.091277624	1.143209508	
GIN52	3.56E-212	2.566792107	0.061865207		CTLA4	2.28E-106	5.903760001	2.228307516	
RAD51A	9.10E-206	2.754092108	0.066994063		HSPB1	2.48E-81	5.018019213	1.901655837	

DTL	2.77E-246	3.010444717	0.075483325		FKBP4	2.07E-32	2.357262179	0.900943487	
SPC24	9.51E-257	3.284411816	0.086262498		NAMPT	3.70E-55	2.828322525	1.082805125	
CDCA3	1.26E-148	2.310214642	0.062926119		MY07A	2.70E-55	2.851829292	1.094796871	
PKMYT1	2.46E-274	3.912074882	0.111843438		CXCL13	3.20E-76	5.537703257	2.196116878	
MELK	1.37E-206	2.827927308	0.083703387		GOUM	1.70E-71	3.257832778	1.292870894	
ANLN	9.63E-163	2.167852433	0.064238576		PHLDA	5.37E-105	4.61451026	1.840925822	
CDCA8	3.22E-188	2.516171112	0.076333804		DNAJA	7.45E-35	2.466346883	0.987327141	
KIAA01	<1E-01	300	6.014545588	0.184748739	TGIF1	5.04E-52	3.707163014	1.49388894	
GGH	3.61E-165	2.628317969	0.082894389		HAVCR	1.70E-125	6.721208897	2.782150341	
AURKB	1.19E-202	3.417845343	0.109220569		APLP2	8.51E-37	2.829096569	1.193574792	
ASF1B	7.64E-275	3.989642884	0.131899853		GPR56	3.81E-67	4.305002658	1.84604347	
CDC20	9.13E-155	2.571097633	0.090072626		BPGM	3.55E-24	2.112090785	0.912324729	
NCAPG	1.48E-186	2.466952224	0.086576421		SEC14L	6.30E-45	3.165973951	1.368216333	
DHFR	2.55E-243	3.380729828	0.119371977		TNIP3	1.08E-28	2.29389018	1.02796927	
KIFC1	9.14E-172	2.507275778	0.090625638		METRN	9.31E-31	2.530023686	1.139591291	
TYMS	<1E-300	6.372852514	0.234303573		HSPH1	5.06E-59	5.204278335	2.347337913	
CKAP2L	4.81E-154	2.219315268	0.085362763		KLRC2	1.86E-16	2.029681849	0.941238889	
CLSPN	2.56E-223	2.431790608	0.095589012		PMAIP	4.13E-48	4.357067179	2.021451401	
MLF1IP	5.73E-257	3.889404448	0.159321142		DUSP4	2.68E-131	7.476213847	3.48564483	
TROAP	4.14E-159	2.472509996	0.102008367		IGFLR1	3.22E-50	4.574899349	2.14231225	
KIF2C	7.79E-181	2.510278759	0.1044348		HSPA1A	5.69E-59	6.348691619	2.974468662	
WDR34	1.19E-195	3.019929166	0.126847555		ZFAND	2.19E-20	2.186631309	1.027281707	
CDK1	1.80E-244	4.306984329	0.183943699		NDFIP2	1.27E-21	2.2106574	1.050155852	
KIF23	9.39E-172	2.617700489	0.1120404		PAM	6.49E-47	4.145597678	1.971693122	
PLK1	2.33E-120	2.080904536	0.089427249		TP53IN	5.31E-38	2.693198774	1.296099613	
TOP2A	1.43E-254	3.95379396	0.176154169		AHI1	6.99E-39	3.595532505	1.730724301	
NUF2	8.11E-150	2.292506517	0.108500218		UBE2F	4.76E-34	3.487548578	1.681697896	
HMGB3	2.22E-129	2.007443518	0.095277255		HSPA4	9.10E-29	3.032981266	1.486959447	
ASPM	5.98E-192	2.24647022	0.107948753		ICOS	1.30E-31	3.618907106	1.775485924	
MCM2	1.89E-266	4.24019511	0.206950214		CHORD	2.26E-43	4.649213815	2.289473394	
ORC6	1.54E-134	2.019910216	0.099102929		TRPS1	5.81E-26	2.212680953	1.094915301	
CASC5	5.68E-191	2.758978817	0.136299446		TBC1D4	9.10E-24	2.108456723	1.04372663	
CENPH	1.89E-152	2.446495791	0.123996713		RALA	2.93E-25	2.579740721	1.286294041	
FEN1	1.26E-255	4.146844098	0.210752282		CD82	5.25E-46	4.774476431	2.390377878	
BRCA1	4.31E-149	2.194349072	0.11261379		SEMA4	3.03E-18	2.121371796	1.063602252	
MCM4	5.26E-298	4.748202624	0.248402619		PON2	3.54E-24	2.966853817	1.503367546	
TIMELE	1.17E-175	2.504879494	0.131966386		ACP5	7.77E-29	3.375622269	1.711869243	
SS	7.00E-281	3.779424913	0.215975467		CCDC6	1.08E-36	3.054795949	1.549817138	
MKI67	1.16E-168	3.42287434	0.19826645		BHLHE	1.33E-40	4.938329191	2.513266968	
CDKN3	4.05E-113	2.356293552	0.14094421		NAMPT	4.65E-42	4.558315	2.3391288	
APOBEC	1.25E-112	2.286673065	0.14388862		AHSA1	3.43E-40	4.846807326	2.496974426	
TPX2	6.61E-161	3.249133415	0.205462527		BANP	1.49E-	2.471967382	1.28050943	

	197					24			
NCAPG	3.85E-2	2.933777691	0.187046063		RHBDD	4.79E-2			
	212					33	3.890107086	2.028687397	
KIF11	6.72E-186	2.706508395	0.173099323		CREM	2.62E-71	7.56604644	3.946265062	
TCF19	1.24E-205	3.19100032	0.212179404		SLC7A5	4.60E-35	3.268624015	1.705861583	
	2.92E-220	4.231471082	0.291429352		CACYBP	6.31E-38	5.00305574	2.613466019	
UBE2T	1.11E-132	2.566249104	0.178360671		NUSAP1	2.82E-20	2.072815795	1.092495033	
SPAG5	5.99E-146	2.351130485	0.171149721		STIP1	1.99E-39	4.832561502	2.571147298	
	4.68E-179	3.743000261	0.280210068		LRMP	2.71E-33	3.424957531	1.828855879	
CCNA2	2.51E-143	2.660515065	0.199743709		PDE3B	6.25E-33	2.710816144	1.451470982	
BUB1B	2.16E-147	2.688171153	0.207169333		RGS2	1.06E-73	8.050777843	4.323433952	
	1.33E-157	2.793982614	0.216484658		CCDC141	1.64E-22	2.035200121	1.096683788	
CHEK1	1.35E-220	3.866443915	0.324877844		SNAP47	7.17E-31	4.052440361	2.183869746	
	1.85E-215	4.506968995	0.380463346		DEDD2	1.76E-27	3.450491584	1.861237221	
RNASEH2A	3.04E-175	3.344405757	0.285050354		BTG3	7.47E-32	3.662598245	1.979769384	
	1.27E-169	3.013656013	0.2597295		ITPRIP	8.91E-19	2.207231209	1.193183727	
HIRIP3	1.82E-195	3.956829854	0.342664735		HSPA1B	5.19E-37	5.352638687	2.904830246	
	3.32E-137	2.207423429	0.194829078		GALNT2	5.41E-22	2.233759079	1.218402379	
CCNF	<1E-300	8.059433725	0.733797002		TNFSF9	5.15E-22	2.70962443	1.478562319	
					RANGA	1.28E-17	2.368495249	1.296540042	
STMN1	2.09E-234	3.743958836	0.341639173		PDCD1	2.71E-54	5.925199432	3.243600815	
	9.89E-243	4.8473316	0.44974339		DDX3Y	6.08E-26	3.145532035	1.722551562	
CKS1B	1.14E-174	3.383508732	0.317117771		ARID5B	7.16E-48	4.680313383	2.564657935	
	1.03E-138	2.80566619	0.26310969		DUSP10	9.99E-28	3.510076713	1.933146697	
NCAPH	6.59E-164	2.381247448	0.226256736		ZBTB1	2.12E-28	3.436305385	1.899592732	
	2.25E-118	2.741020483	0.274952715		SAMSN1	3.73E-58	6.360674706	3.525389463	
PRC1	1.64E-107	2.354298256	0.23995904		IRF4	4.39E-25	3.082040237	1.735357355	
	7.52E-171	3.067207453	0.312810158		CD2BP2	2.22E-32	4.111559791	2.329946702	
CENPF	2.26E-126	3.141653788	0.320472248		SYNGR2	5.84E-38	5.013596264	2.841604396	
	5.48E-208	4.143105633	0.425232965		CDK6	1.25E-22	3.020199495	1.71270042	
CDCA7	6.60E-111	2.294463343	0.237158675		MCTP2	7.30E-21	2.876967415	1.635926964	
	1.22E-106	2.009763256	0.214883208		RAB27	4.01E-45	5.459190278	3.124067888	
CENPE	1.13E-191	3.226942728	0.362578907		HSPD1	8.82E-39	6.077649059	3.480454567	
	4.35E-110	2.317128112	0.260966949		NFAT5	9.99E-28	2.931103048	1.682294193	
WDR76	2.06E-93	2.054712356	0.232381135		BATF	4.37E-23	3.555787596	2.043815825	
	1.49E-151	3.563842214	0.409382908		GZMB	1.30E-56	7.758277758	4.499249256	
POLD1	1.29E-130	2.936785803	0.3384978		NEU1	6.93E-16	2.886639105	1.67483792	
	1.21E-100	2.259551767	0.263080986		SYT11	1.08E-18	2.440568325	1.419244954	
LRR1	1.13E-108	2.46958953	0.287645547		CXCR6	2.58E-28	4.655644289	2.718000218	
	9.20E-144	3.439113101	0.417194751		CNIH1	1.12E-16	2.541655109	1.487216627	
SNRNP2	5.06E-202	4.078080883	0.495378126		FCRL3	1.67E-22	3.65262676	2.137913589	
	1.06E-160	3.833035974	0.474433354		CRTAM	7.97E-24	4.445338565	2.601965542	
NUDT1	2.66E-132	3.145357502	0.400520512						
ACOT7									

CD8_3

CD8_4

GeneName	P-value	Mean expression G3	Mean expression non-G3		GeneName	P-value	Mean expression G4	Mean expression non-G4	
CCL3	8.51E-117	5.015059335	1.887855906	adjusted P-value = 2.6e-5	LMNA	7.56E-209	5.130250252	0.897090823	adjusted P-value = 2.6e-5
EPSTI1	4.29E-74	3.295223458	1.420359201		NR4A3	1.42E-148	3.975385123	1.022291452	
CD38	5.20E-113	4.545668162	2.005928356		GPR18-3	2.24E-90	3.818257547	0.990291243	
FASLG	8.75E-56	2.94645835	1.309504723		CDKN1A	2.22E-101	3.549498599	0.94656863	
IFI44L	1.37E-32	2.11646469	0.964395941		CCR7	2.63E-70	2.960438007	0.803254077	
GIMAP6	2.43E-79	3.400092837	1.577918668		S1PR1	7.18E-60	2.603870566	0.834184323	
TRAFD1	1.91E-50	2.885862016	1.343522517		KDM6B	2.71E-93	3.094151685	1.107145291	
LGALS9	1.11E-34	2.070086127	0.967475419		ELL2	7.77E-64	2.644214961	0.95895337	
CXCR6	4.56E-86	4.846134919	2.272311204		TIPARP	9.18E-46	2.385312133	0.926764373	
RAB37	4.19E-43	2.28535694	1.079728993		SC5D	2.88E-26	2.070300226	0.836568015	
CCR5	9.42E-62	3.552480197	1.769370144		PLK3	4.51E-36	2.416236716	0.979321478	
ZBP1	1.16E-40	2.427047475	1.235683441		CD55	2.46E-88	5.44915903	2.217537782	
SAMD9L	4.66E-45	2.823461812	1.44490878		NR4A1	1.31E-65	4.503772288	1.833479149	
SIRPG	8.13E-91	5.329178021	2.732094169		REL	1.05E-92	4.557582437	1.855660913	
MX1	1.41E-42	3.056292666	1.568430104		PBX4	4.34E-34	2.027854951	0.829354777	
HAVCR2	1.09E-86	5.110139367	2.63274115		TNF	3.29E-32	2.403875852	0.983417335	
ACP5	3.56E-41	2.975743001	1.542519863		IL7R	2.09E-90	4.963852356	2.056482076	
DDX60	9.04E-33	2.158055201	1.118673932		RGCC	4.18E-52	3.641583354	1.513335849	
PDCD1	8.20E-93	5.522853643	2.878952853		FOSL2	8.27E-136	5.364391103	2.244521639	
SH2D3C	5.22E-29	2.006760405	1.056157919		SIK1	5.06E-53	2.40027799	1.007615991	
GPR174	4.10E-50	3.358551212	1.782806586		CSRNP1	2.21E-67	3.840776722	1.628759198	
RPS6KA1	3.08E-30	2.377415894	1.281208847		GPR132	3.72E-30	2.031526995	0.862615998	
GBP5	1.68E-118	5.978542127	3.247065468		GLUL	3.47E-28	2.233099233	0.955295655	
GBP1	1.48E-48	3.697213452	2.02745995		KIAA1683	2.40E-34	2.26575626	0.983533953	
PTPN6	1.37E-68	5.151317565	2.841613699		RALGAPA1	1.31E-46	2.72402864	1.193898593	
S100PB	3.82E-43	2.721960992	1.510583409		PRNP	3.55E-55	3.899739622	1.716398012	
IFI35	9.67E-20	2.326512656	1.298901302		PRMT10	4.41E-28	2.203831344	0.9761392	
OAS3	8.08E-27	2.26072679	1.267258312		SORL1	1.27E-35	2.260898386	1.01695799	
SNAP47	1.68E-44	3.566898004	2.007468349		FAM177A1	9.55E-69	4.913747872	2.265796329	
GIMAP4	8.46E-86	5.999604075	3.399364434		CHMP1B	1.35E-36	3.133082345	1.445099161	
PARP9	4.36E-50	3.752100501	2.138026317		ZC3H12A	3.57E-48	3.561182965	1.642960962	
IFNG	2.98E-64	4.95583907	2.826877465		TSC22D2	2.88E-49	3.320356189	1.546466586	
SIT1	9.26E-52	4.360720044	2.489833852		P2RY8	3.38E-31	2.188617927	1.02961924	
PYCARD	3.34E-27	2.698145533	1.544086008		NEU1	3.84E-33	3.292790536	1.572735173	
RGS3	5.80E-22	2.053497623	1.177694189		TCF7	4.31E-55	3.308469276	1.588174381	
XAF1	5.14E-59	4.120833869	2.379035731		ZNF683	2.11E-24	2.138956815	1.033050095	
OAS2	6.39E-35	2.946307406	1.707312929		MYADAM	1.01E-96	6.675116754	3.250421254	
C5orf56	3.50E-36	3.398169743	1.976674608		ATP2B1	1.23E-26	2.195986914	1.083849042	
GIMAP5	1.37E-52	4.670269131	2.737983339		CREM	4.66E-88	7.694627082	3.839214426	
ABI3	3.58E-34	3.200470446	1.877923624		OAT	1.25E-23	2.672595049	1.339019187	

SNX20	6.74E-23	2.133950473	1.253679389		NFE2L2	1.79E-38	3.919369557	1.968883709	
VAMP5	1.76E-24	2.66021222	1.563994728		DNAJB9	5.60E-24	2.504239718	1.2672753	
IRF2	3.28E-40	3.479010551	2.045661958		SKIL	7.06E-62	4.655206906	2.387487241	
UBASH3A	6.78E-27	2.370123324	1.394154502		DENND4A	1.27E-26	2.594070684	1.331951845	
PARP10	3.51E-22	2.17349071	1.281779284		SERTAD1	2.13E-28	3.212803971	1.651394441	
GIMAP7	6.26E-74	5.557729331	3.2779442		YPEL5	8.59E-102	8.312255233	4.272756772	
GBP4	2.47E-35	2.907061262	1.716208583		BCL6	6.10E-18	2.009622059	1.043811958	
PVRIG	8.46E-45	4.243408789	2.50528476		EGR1	3.08E-28	3.166562297	1.644871081	
CYTH4	9.69E-31	2.833281744	1.678057875		PDE4B	2.21E-52	5.475796637	2.859061378	
DTX3L	9.21E-31	2.40929227	1.430280817		ANXA1	1.08E-75	8.252186176	4.309847652	
RHOC	1.54E-25	2.535523964	1.513748945		SOD2	1.87E-17	2.085073676	1.092164253	
SASH3	2.61E-66	5.164050448	3.083855688		RNF125	1.38E-28	2.565902115	1.351957248	
CCL4L2	7.05E-76	4.503046587	2.692677718		GADD45B	6.41E-24	3.364530673	1.776721079	
IFI6	5.35E-56	5.206051374	3.117531087		SELK	9.61E-57	6.050397655	3.235516633	
BCAS4	4.27E-18	2.046159933	1.22756435		RORA	2.42E-28	2.748664848	1.472234075	
IKZF3	1.73E-70	5.425905348	3.277337602		SELL	6.32E-15	2.192817714	1.182804004	
GIMAP2	2.10E-17	2.26933074	1.379274176		MXD1	3.38E-16	2.033120542	1.110371579	
ADORA2A	1.42E-19	2.063948349	1.255927302		IFRD1	1.88E-47	5.818877601	3.190446363	
ARPCSL	3.07E-29	2.95597189	1.799793078		PIK3R1	2.58E-28	4.007311792	2.201212537	
GYG1	1.64E-16	2.292308191	1.39627513		TUBB4B	3.55E-43	5.045470043	2.789748361	
SLFN5	9.32E-56	3.873564682	2.366470161		HECA	9.74E-18	2.036137426	1.129865662	
CHST12	1.76E-35	3.652602619	2.231508059		MPZL3	1.98E-20	2.797447121	1.5528395	
APOBEC3D	6.61E-56	3.436403521	2.124852498		USP36	2.46E-43	4.655958467	2.591389474	
WARS	3.41E-22	3.099554913	1.918950359		INSIG1	2.08E-16	2.55250692	1.439353343	
UBE2L6	7.28E-62	5.964259648	3.704581811		LTB	3.90E-14	2.089285828	1.198337274	
TMEM140	2.16E-23	2.123334107	1.322115881		NR4A2	5.10E-90	8.670934348	4.978803937	
CSK	1.37E-30	3.318055645	2.076003481		SLC2A3	4.55E-74	8.37521012	4.823150952	
F2R	4.40E-27	2.78115134	1.741256588		PERI	9.92E-49	4.141906157	2.404951911	
CTSS	6.45E-30	3.476249917	2.183171663		S100A10	1.27E-33	5.772765577	3.365144408	
SLAMF7	5.65E-56	4.759575299	2.989782655		AIM1	5.94E-21	2.682598663	1.565058021	
CXCR3	1.24E-33	4.057073582	2.552982396		MGAT4A	4.11E-15	2.392257396	1.395993896	
CD27	5.49E-58	5.978865328	3.767214443		CDC42E	3.37E-13	2.06843029	1.208080475	
PPP1R18	4.38E-35	2.60976111	1.646889148		NDEL1	5.54E-19	3.040275733	1.785412472	
TOX	3.27E-43	3.37345819	2.132048391		IDH1	3.48E-25	4.042451327	2.384296543	
CTSC	6.15E-40	4.545762838	2.873160416		EIF4A3	1.37E-22	3.948449863	2.330234315	
SLAMF6	5.36E-23	2.544432842	1.609106178		BIRC3	9.01E-53	6.903173588	4.08020683	
STAT1	9.53E-67	6.247628281	3.954645325		TSPYLN2	2.84E-30	4.732493188	2.827440707	
FUT8	7.21E-21	2.25709318	1.431233895		DCTN6	8.75E-15	2.536182616	1.520922064	
IDH2	4.98E-38	4.318082043	2.738942801		HSPH1	7.87E-20	4.125500345	2.478239555	
PCED1B	1.94E-30	3.054548165	1.941355334		CDK17	2.15E-16	2.666535913	1.601978123	
BST2	1.07E-31	3.973119153	2.525375499		DDX21	8.00E-15	2.162987449	1.305638568	
PSMB1	9.15E-22	5.964359202	3.79864379		PPP1R1	1.89E-1	2.648701678	1.606445949	

0	59				5B	16			
STAT2	6.52E-26	2.950115892	1.879340578		ZNF331	1.66E-60	7.593874475	4.608366594	
RNASET	9.81E-18	2.305998235	1.472168555		BTG2	2.63E-40	5.882677141	3.575700754	
RBCK1	8.05E-34	4.012686146	2.564165695		AMD1	1.48E-21	3.941359871	2.397415885	
SEL1L3	2.35E-21	2.215650861	1.417050007		SLC7A5	3.33E-20	2.859871351	1.744370906	
C14orf1	1.59E-59	2.24808124	1.438035499		POLR3E	4.71E-22	3.753752835	2.299589379	
HLA-DRA	1.20E-53	6.027667429	3.868627815		JMJD6	5.78E-24	4.169181055	2.554897493	
GZMA	5.05E-109	9.15450386	5.885590032		CHD1	3.93E-29	4.143608426	2.545256433	
CD63	3.56E-29	4.240658328	2.726923538		TAF13	4.69E-10	2.0291608	1.246698643	
DENND	8.25E-2D	5.799247975	3.734443517		VPS37B	2.86E-27	3.178217463	1.954695663	
HLA-DQB1	4.75E-28	3.624285685	2.341720123		GTF2B	4.95E-16	3.257013677	2.004816899	
PRF1	1.76E-111	8.352915718	5.397147064		PAF1	5.69E-16	2.842525353	1.760833823	
CD84	1.10E-33	3.816408078	2.467481691		BCAS2	7.19E-13	2.897021121	1.799772551	
TIGIT	2.54E-52	5.710777486	3.699023791		RGPD6	1.68E-18	2.421350288	1.514084212	
CCL4L1	4.81E-83	6.131891763	3.975624769		TUBA4	3.95E-39	7.524898698	4.709933457	
PLSCR1	7.18E-14	2.165168213	1.404721258		RASA3	1.26E-12	2.006153535	1.259197185	
LAG3	1.85E-36	3.496287471	2.269558169		GPCPD	3.23E-16	3.349453356	2.103388505	
DAXX	1.73E-15	2.274292791	1.482063123		RASGEF	1.62E-28	4.382473959	2.754447436	
PHF11	3.85E-27	3.360321274	2.189790278		DNAJA1	6.40E-61	8.74019538	5.494176996	
CD8_5									
GeneName	P-value	Mean expression G5	Mean expression non-G5		GeneName	P-value	Mean expression G6	Mean expression non-G6	
ELL2	1.31E-22	2.258396304	1.067507406	adjusted P-value = 2.7e-5	PLAC8	1.34E-120	2.721051972	0.566040001	adjusted P-value = 2.7e-5
PFKFB3	1.33E-42	3.870436405	1.867088669		S1PR1	1.02E-90	2.557930913	0.711569251	
DTHD1	9.22E-25	3.016910937	1.591313865		SORL1	1.03E-98	2.652430432	0.815150733	
SMAP2	6.22E-27	3.463112365	1.843131344		SELL	1.27E-59	2.718867008	0.962164936	
FKBP5	9.70E-34	4.64587865	2.499918859		TCF7	1.24E-103	3.604480319	1.376047023	
AIM1	3.33E-22	2.886082577	1.570074619		CCR7	6.60E-44	2.205452096	0.844474512	
TMEM3	7.32E-9A	2.40024324	1.311120526		IL7R	2.15E-106	4.655784091	1.918493067	
NR4A3	7.59E-19	2.437574361	1.338149122		MGAT4A	2.63E-54	2.764843807	1.218269602	
PER1	1.35E-33	4.400570708	2.421147359		FAM65B	1.44E-68	3.448613271	1.547656599	
TSPYL2	8.32E-32	5.136559275	2.827654281		LTB	2.50E-36	2.351713108	1.058707102	
TTN	8.07E-33	3.006134786	1.70228753		FLT3LG	1.14E-23	2.056693579	1.041080521	
TMEM2	3.91E-43	6.796935223	3.983607915		PXN	1.99E-26	2.16526495	1.148703348	
IL6ST	1.46E-13	3.235083521	1.945757496		A2M	6.51E-27	2.121310831	1.167318427	
NAB1	3.92E-16	3.143292963	1.914455934		ATM	2.94E-35	3.170115623	1.8197708	
IQGAP2	4.61E-14	3.047102441	1.905282765		C20orf112	6.25E-23	2.417727967	1.428535109	
SLC7A5	9.88E-11	2.838234759	1.782145797		GPR18	6.76E-3	2.058556979	1.254371682	
IPCEF1	1.26E-11	3.07776164	1.953653511		EPB41	3.94E-16	2.376064591	1.550241763	
DCTN6	1.59E-10	2.457942502	1.563838489		ADD3	1.04E-13	2.343780842	1.535037596	
DUSP4	1.00E-35	5.832137839	3.75990696		GRAP2	1.25E-14	2.670324792	1.768772329	
RANBP2	1.32E-19	4.321354137	2.822142334		KLRG1	4.62E-16	3.474387291	2.30863397	
FAM17	3.84E-	3.807664523	2.509239673		GIMAP	6.58E-	4.430095626	2.951842148	

7A1	11					5	26				
GABAR APL1	2.32E- 14	4.077268197	2.709526568			TC2N	1.04E- 20	3.566506845	2.434887756		
RGPD6	4.36E- 11	2.329022986	1.555702802			TXNIP	3.24E- 37	7.185732762	4.9742525		
CTLA4	1.67E- 09	3.774779483	2.570537393			GIMAP	1.21E- 206	2.132831683	1.484839796		
CREM	5.70E- 23	6.131408184	4.18678062			TNFAIP	1.81E- 811	2.489851327	1.738181279		
ETS1	2.79E- 18	4.60707062	3.157814837			IL16	1.41E- 14	3.41919275	2.398557056		
PNRIC1	5.84E- 09	2.786248611	1.92252723								
ZFP36L	4.30E- 29	7.252180572	5.009213671								
RGPD5	1.69E- 12	5.1040558	3.533326688								
ZNF331	1.18E- 26	6.910385854	4.800673733								
CNOT6L	8.52E- 15	4.421764785	3.084404685								
TGIF1	6.44E- 07	2.428224881	1.699522135								
CXCL13	8.39E- 09	3.576063687	2.511057499								
PDE4D	1.37E- 11	3.884342535	2.727585217								
RNF19A	8.14E- 31	7.769482372	5.488817066								

Table 10

First split		Second split		Third split		Fourth split		Fifth split		
CD8 -B	CB5 MS	N i l l i	CD8 -2 +3	CD8 -6	CD8 -7	CD8 -2	CD8 -3	N i l l i	CD8 -1	CD8 -4
VCAM1	IL7R	SPC25	TXNIP	PLAC8	CXCL13	GEM	GIMAP5	s1PR1	CXCL13	
CCL3	TCF7	CDCA5	TTN	GIMAP6	ELL2	APLP2	AOAH	LTB	HAVCR2	
HAVCR2	GPR183	ESC02		GIMAP2	SLC7A5	SLC7A5	GIMAP7	GPR183	CTLA4	
MY07A	LMNA	CDC6		GIMAP5	NR4A3	TGIF1	SCML4	ZNF683	TOX	
G0LIM4	NR4A3	SHCBP1		GIMAP4	LMNA	RDH10	LY9	SELL	TNFRSF9	
CD38	MGAT4A	CDC45		GIMAP7	DUSP4	FKBP4	GIMAP6	CCR7	DTHD1	
ENTPD1	AIM1	RRM2		GNLY	CREM	HSPH1	TC2N	P2RY8	NAB1	
NDFIP2	CD55	DLGAP5		SELL	CDKN1A	NAMPTL	GIMAP2	LMNA	TTN	
MCM5	FAM65B	ZWINT		DENND2D	KPNA2	TNFRSF18	GBP1	TNF	PDCD1	
FASLG	RORA	RAD51		PTPN6	CCDC64	DCTN6	GIMAP4	CDKN1A	IPCEF1	
ACP5	TC2N	DTL		TRPV2	PHLDA1	MTSS1	SAMD9L	S100A10	ITGAE	
SNAP47	PERI	KIF18B		IL16	CKS2	TNFRSF9	PPP1R18	PRNP	FKBP5	
	FAM102A	TK1		FLT3LG	ATP1B3	PFKFB3	GYG1	S0RL1	SIRPG	
STMN1	FAM177A1	CCNB2		S1PR1	TIPARP	PELI1	SAMD3	FAM65B	FCRL3	
CXCL13	ATM	SPC24		FAM65B	PFKFB3	DNAJA4	STOM	TMEM123	CD84	
WARS	REL	BIRC5		LTB	SAMSN1	RANGAP1	CYTH4	TES	PAM	
CXCR6	F0SL2	UBE2C		TNFAIP8	AHI1	CREM	GZMM	PIM1	CXCR6	
PRDX3	STAT4	CDC20		TRAFF3IP3	DCTN6	FAM3C	GLIPR2	EMP3	ETS1	
LSM2	F0XP1	CENPW		PARP9	FAM177A1	SEC14L1	RAB37	GLIPR2	ATHL1	
IGFLR1	EGR1	CDCA3		A2M	KDM6B	CKS2	TBC1D10C	TPM4	CD27	
MTHFD1	PIK3R1	HJURP		SAMD3	GABARAPL1	DFNB31	MY01F	OASL	PYHIN1	
FABP5	TSPYL2	M ELK		GRAP2	INSIG1	VCAM1	GPR174	GLUL	IL6ST	
		RAD51A		PXN	TNFSF9	RGCC	C5orf56	RORA	SMAP2	
DNPH1		PI								
SIRPG		DHFR		KLRG1	GTF2B	AHI1	GRAP2	NR4A1	LYST	
PTTG1		GINS2		SASH3	TSPYL2	KPNA2	SLFN5	CXCR3	ERAP2	
IFI35		PKMYT1		SSBP1	NAMPT	NR4A1	FAM102A	ANXA1	KLRC4	
CCR5		ANLN		SAMHD1	RALGAPA1	TSPYL2	PATL2	ANXA2	LRMP	
TNFRSF9		CENPH		SORL1	JMJD6	ZFAND2A	PVRIG	GPR132	CCL4L2	
SKA2		ASF1B		ADD3	RGCC	LAYN	AGTRAP	LYAR	PRDM1	
UBE2F		CLSPN		DBNL	MTHFD2	NAB1	ARHGAP25	GADD45B	AHI1	

SAE1		CDCA8		MGAT4A	TSC22D2	RALA	PARP9	HSPA1A	PLCB2
TRAFD1		GGH		GIT2	REL	HSPB1	C14orf159	S100A11	EVL
HLA-DQA1		KIFC1		PRMT2	PLK3	PDE3B	RPS6KA1	TMBIM1	LPIN1
EPST11		TYMS		C19orf66	VPS37B	ITPRIP	PKN1	DNAJB9	PDE4D
CTLA4		FEN1		COX7A2L	SELK	PHLDA1	CSK	MTRNR2L	
GTF3C6		KIF2C		RGS10	HSPH1	ETV1	TBCD	PBX4	TCIRG1
IFI27L2		KIAA0101		DAD1	SLC38A2	HSPD1	HMOX2	TCF7	OXNAD1
HLA-DMA		PLK1		PTPN4	OAT	ZEB2	EPST1	MGAT4A	CBLB
PDIA6		SPAG5		XAF1	RASGEF1B	METRNL	CCND3	SOD2	HNRNPL
CD2BP2		ORC6		PVRIG	YPEL5	CTLA4	KLRG1	CSRNP1	DUSP4
BATF		WDR34		SLC44A2	SERTAD1	FOSL2	TES	BIRC2	NAP1L4
RANBP1		KIF23		PIM1	ZNF331	TNFSF9	SASH3	CDC42EP3	TXNIP
MRPL51		TOP2A		TMBIM4	GSPT1	CADM1	IDH2	CD55	RGS1
0AS3		RFC3		CMC1	HNRNPLL	AHSA1	RP4-583P15.14	MT2A	CCL4L1
CDC123		TIMELESS		TXNIP	CRTAM	ZBTB1	SELPLG	VIM	RAP1GD
PCNA		AURKB		ATM	TGIF1	BPGM	SLAMF7	XCL2	TIGIT
HSD17B10		NUF2		PHB2	EIF4A3	CACYBP	RCSD1	ZC3H12A	PARK7
CLTA		NCAPG		COA1	ICOS	GSPT1	MX1	HSPA1B	CCL4
VAMP5		MLF1IP		SIT1	MPZL3	REL	CD52	STIP1	IKZF3
FKBP1A		BRCA1		HERC3	BTG3	ICOS	SP110	TUBA1C	SH2D1A
NDUFB3		SGOL1		PSD4	HBP1	ENTPD1	ARHGEF3	IL21R	ATXN1
NOP10		CKAP2L		AES	TMEM2	HBP1	SLAMF6	TTC39C	ITK
SHFM1		CDK1		POLD4	NR4A2	TBC1D4	LIME1	EIF4A3	
COX5A		MCM4		C20orf112	NASP	NEU1	CXCR3	LGALS1	
TIGIT		TROAP		C2orf68	NFE2L2	CCDC64	RNF166	PPP3CA	
BLOC1S1		ASPM		GMFG	FAM46C	PMAIP1	OAS2	MOB4	
ANXA5		HMGB3		OXNAD1	Fosl2	AMD1	TPST2	DOK2	
PAM		MCM2		GPSM3	ETF1	RASGEF1B		IFNG	
JAkm IP1		NCAPG2		ESYT1	HSPD1	GTF2B	CD48	CCT2	
HLA-DRA		CDKN3		TBC1D10C	ZEB2	SERTAD1	DENND2D	RILPL2	
SNRPD1		CASC5		IRF9	AMD1	NAMPT	PIM1	C14orf166	
GZMB		TCF19		ERP29	SDCBP	CHORDC1		CAST	
COPZ1		KIF11		TMEM230	MYADM	NFE2L2		YWHAQ	
CD63		HIRIP3		ARHGAP25	TUBB4B	DDX3Y		PPP2R3C	
CARD16		BRCA2		DEF6	PAF1	MORF4L2		CHMP1B	
FIBP		CCNB1		GNG5	FAM129A	BIRC3		RGCC	
ATP5J		APOBEC3B		TTC39C	IFRD1	BTG3		REL	
GSTO1		MKI67		BIN2	BIRC3	PERI		STOM	
TOX		TPX2		MFNG	GTPBP1	CRTAM		KPNA2	
TXN2		CCNF		SRP9	HEXIM1	AFAP1L2		PTP4A1	
C17orf49		NCAPH		PTEN	NEU1	EIF4A3		RAB8B	
ADORA2A		ECT2		SYTL1	SYAP1	RHBDD2		SC5D	
CHST12		ATAD5		PRKACB	ZC3H12A	HSPE1		BCAS2	
TRIM59		STMN1		C5orf56	IL21R	JMJD6		CDK17	
TMPO		UBE2T		MY01F	RGPD6	DEDD2		INSIG1	
PSMB2		FANCI		HMOX2	IDI1	IFRD1		NEU1	
HELLS		PRC1		NUCB2	NR4A1	CXCL13		ABLIM1	
SNRPE		RNASEH2A		NME1-NME2	TUBA4A	B3GNT2		PPP2CA	
NDUFAB1		BUB1B		UBE2L6	RNF19A	IL21R		SDCBP	
SQRDL		CKS1B		VPS29	NR3C1	HSPA4		CHP1	
SERPINB1		CENPM		SNX17	IL6ST	BHLHE40		CACYBP	
MX1		SMC2		ST6GAL1	RGS2	SDCBP		SAMD3	
MEA1		CCNA2		CSK	ZBTB1	ATP1B3		MAP1LC3B2	
GPR56		BUB1		RBL2	RANBP2	CHD1		FOS	
NUTF2		CENPN		PPIE	MAT2A	BANP		SLFN11	
BST2		AURKA		STX16	CHMP1B	ZC3H12		TAGLN2	

FUT8		MAD2L1		PILRB	DNTTIP2	EIF5			DDX21
DUT		CDCA7		LENG8	BCAS2	LRMP			TMEM50A
GNG5		CHTF18		Cllorf31	SKIL	CD55			SLM02
ATP6V1E1		CHEK1		ABRACL	TNFRSF1B	ZNF331			CD5
AP2S1		CENPF		ANP32B	POLR3E	POLR3E			SCP2
CALM3		LIG1		TPST2	CSRNP1	TUBB4B			CD28
MRPS34		KNTC1		ATP5A1	LYST	SLC38A2			RAB7L1
CD27		RPL39L		NFATC3	GOLGB1	DUSP4			ATP2B1
TMEM179B		POLE		ARHGEF3	EIF5	CNIH1			CD52
SRI		POLD1		GLIPR2	PDE4B	TMPO			CAMLG
PSMB3		RFC5		C ^K LF	JUND	STIP1			PLP2
IFI6		MXD3		OSTF1	PERI	RGS2			TOR1AIP2
SIT1		RFC4		SNX6	DNAJA1	GLS			MAP2K3
PON2		RACGAP1		LAMTOR4	TFRC	CCT2			CAMK4

[0611] Pipeline for detection of cells positive for CD8 and TCF7

CellProfiler Pipeline: www.cellprofiler.org

Version :3

DateRevision: 20160418141927

GitHash: 9969f42

ModuleCount :16

HasImagePlaneDetails :False

```
Images :[module_num :1 |svn_version :\ 'Unknown \' |variable_revision_n umber :2
|show_window :False |notes :\x5B\ 'To begin creating your project, use the
Images module to compile a list of files and/or folders that you want
to analyze. You can also specify a set of rules to include only the
desired files in your selected
folders. \'|\batch_state :array (\x5B\x5D,
dtype=uint8) |enabled: True |wants_pause :False]
```

```
Metadata : [module_num: 2 |svn_version :\'Unknown\' |variable_revision_number  
:4 |show_window :False |notes :\x5B\ 'The Metadata module optionally allows  
you to extract information describing your images (i.e, metadata) which  
will be stored along with your measurements. This information can be  
contained in the file name and/or location, or in an external  
file.\'\x5D| batch_state :array (\x5B\x5D).
```

```
[115]: ( roi | batch_size array, (roi[roi.dtype=uint8) |enabled: True |wants_pause :False]
Extract metadata?: No
Metadata data type :Text
Metadata types :{}
Extraction method count: 1
Metadata extraction method :Extract from file/folder names
Metadata source: File name
Regular expression :^ (?P<Plate> .* )_ (?P<Well> \x5BA-P\x5D\x5B0-9\x5D{2} )_s (?P<Site> \x5B0-9\x5D) _w (?P<ChannelNumber> \x5B0-9\x5D)
Regular expression: (?P<Date> \x5B0-9\x5D{4} )_\x5B0-9 \x5D{2 }_\x5B0-9\x5D{2} ) $
Extract metadata from: All images
Select the filtering criteria: and (file does contain "")
```

```

Metadata file location:
Match file and image metadata :|\x5B\x5D
Use case insensitive matching? :No

NamesAndTypes :[module_num: 3 |svn_version: \Unknown\ |variable_revision_number :6|show_window :False|notes :\x5B\ 'The NamesAndTypes module allows you to assign a meaningful name to each image by which other modules will refer to it.\ '\x5D|batch_state :array (\x5B\x5D,
dtype=uint8) |enabled: True |wants_pause :False]
    Assign a name to:All images
    Select the image type: Color image
    Name to assign these images :ColorImage
    Match metadata :|\x5B\x5D
    Image set matching method: Order
    Set intensity range from: Image metadata
    Assignments count: 1
    Single images count: 0
    Maximum intensity :255 .0
    Select the rule criteria: and (file does contain "")
    Name to assign these images :DNA
    Name to assign these objects: Cell
    Select the image type :Grayscale image
    Set intensity range from: Image metadata
    Retain outlines of loaded objects? :No
    Name the outline image :LoadedOutlines
    Maximum intensity :255 .0

Groups :[module_num :4 |svn_version :\Unknown\ |variable_revision_number :2|show_window :False|notes :\x5B\ 'The Groups module optionally allows you to split your list of images into image subsets (groups) which will be processed independently of each other. Examples of groupings include screening batches, microtiter plates, time-lapse movies, etc.\ '\x5D|batch_state :array (\x5B\x5D,
dtype=uint8) |enabled: True |wants_pause :False]
    Do you want to group your images?: No
    grouping metadata count: 1
    Metadata category :None

ColorToGray: [module_num: 5 |svn_version :\Unknown\ |variable_revision_number :3|show_window :False|notes: \x5B\x5D|batch_state :array (\x5B\x5D,
dtype=uint8) |enabled: True |wants_pause :False]
    Select the input image :Color Image
    Conversion method: Split
    Image type :Channels
    Name the output image :OrigGray
    Relative weight of the red channel: 1.0
    Relative weight of the green channel: 1.0
    Relative weight of the blue channel: 1.0
    Convert red to gray?: Yes
    Name the output image :OrigRed
    Convert green to gray?: Yes
    Name the output image :OrigGreen
    Convert blue to gray?: Yes
    Name the output image :OrigBlue
    Convert hue to gray?: Yes
    Name the output image :OrigHue
    Convert saturation to gray?: Yes

```

```
Name the output image :OrigSaturation
Convert value to gray?: Yes
Name the output image :OrigValue
Channel count: 3
Channel number :Red\x3A 1
Relative weight of the channel: 1.0
Image name :TCF7
Channel number :Green\x3A 2
Relative weight of the channel: 1.0
Image name :CD8
Channel number :Blue\x3A 3
Relative weight of the channel: 1.0
Image name: DAPI

EnhanceOr Suppress Features :[module_num: 6 |svn_version :\'Unknown \' |variable_revision_number :5 |show_window :False|notes:\x5B\x5D| batch_state :array (\x5B\x5D, dtype=uint8) |enabled :True |wants_pause :False ]
    Select the input image :DAPI
    Name the output image :FilteredDAPI
    Select the operation :Enhance
    Feature size:20
    Feature type :Speckles
    Range of hole sizes: 1,10
    Smoothing scale: 2.0
    Shear angle :0.0
    Decay: 0.95
    Enhancement method :Tubeness
    Speed and accuracy :Fast / hexagonal

IdentifyPrimaryObjects :[module_num :7 |svn_version :\'Unknown \' |variable_revision_number :10 |show_window :False |notes: \x5B\x5D |batch_state :array (\x5B\x5D, dtype=uint8) |enabled: True |wants_pause :False]
    Select the input image :FilteredDAPI
    Name the primary objects to be identified: Nuclei
    Typical diameter of objects, in pixel units (Min,Max) :15,50
    Discard objects outside the diameter range?:Yes
    Try to merge too small objects with nearby larger objects? :No
    Discard objects touching the border of the image?: Yes
    Method to distinguish clumped objects :Shape
    Method to draw dividing lines between clumped objects :Propagate
    Size of smoothing filter: 10
    Suppress local maxima that are closer than this minimum allowed distance :7.0
    Speed up by using lower-resolution image to find local maxima?: Yes
    Name the outline image :PrimaryOutlines
    Fill holes in identified objects? :After both thresholding and declumping
        Automatically calculate size of smoothing filter for declumping? :Yes
        Automatically calculate minimum allowed distance between local maxima? :Yes
        Retain outlines of the identified objects? :No
        Automatically calculate the threshold using the Otsu method?: Yes
        Enter Laplacian of Gaussian threshold :0.5
        Automatically calculate the size of objects for the Laplacian of Gaussian filter?:Yes
        Enter LoG filter diameter: 5.0
```

```

Handling of objects if excessive number of objects
identified: Continue
Maximum number of objects: 500
Threshold setting version: 2
Threshold strategy :Adaptive
Thresholding method: Otsu
Select the smoothing method for thresholding :Automatic
Threshold smoothing scale: 1.0
Threshold correction factor: 1.0
Lower and upper bounds on threshold: 0.0,1.0
Approximate fraction of image covered by objects ?:0.01
Manual threshold :0.0
Select the measurement to threshold with:None
Select binary image:None
Masking objects :None
Two-class or three-class thresholding? :Two classes
Minimize the weighted variance or the entropy? :Weighted variance
Assign pixels in the middle intensity class to the foreground or
the background? :Foreground
Method to calculate adaptive window size: Image size
Size of adaptive window: 10
Use default parameters ?:Default
Lower outlier fraction :0.05
Upper outlier fraction :0.05
Averaging method:Mean
Variance method :Standard deviation
# of deviations :2.0

RescaleIntensity: [module_num: 8 |svn_version :\ 'Unknown \' |variable_revision_number :2 |show_window :False |notes :\x5B\x5D |batch_state :array (\x5B\x5D,
dtype=uint8) |enabled: True |wants_pause :False]
    Select the input image :CD8
    Name the output image :RescaledCD8
    Rescaling method :Stretch each image to use the full intensity range
    Method to calculate the minimum intensity:Custom
    Method to calculate the maximum intensity:Custom
    Lower intensity limit for the input image :0.0
    Upper intensity limit for the input image :1.0
    Intensity range for the input image :0.0,1.0
    Intensity range for the output image :0.0,1.0
    Method to rescale pixels below the lower limit:Mask pixels
    Custom value for pixels below lower limit: 0.0
    Method to rescale pixels above the upper limit :Mask pixels
    Custom value for pixels above upper limit: 0.0
    Select image to match in maximum intensity :None
    Divisor value: 1.0
    Divisor measurement :None

MeasureObjectIntensity: [module_num :9 |svn_version :\ 'Unknown \' |variable_revision_number :3 |show_window :False|notes:\x5B\x5D| batch_state :array (\x5B\x5D,
dtype=uint8) |enabled :True |wants_pause :False ]
    Hidden :1
    Select an image to measure :RescaledCD8
    Select objects to measure :Nuclei

```

```

FilterObjects: [module_num: 10 |svn_version :\'Unknown \' |variable_revision_
number :7 |show_window :False|notes:\x5B\x5D| batch_state :array (\x5B\x5D,
dtype=uint8) |enabled: True |wants_pause :False]
  Name the output objects :CD8PosNuclei
  Select the object to filter :Nuclei
  Select the filtering mode :Measurements
  Select the filtering method :Limits
  Select the objects that contain the filtered objects:None
  Retain outlines of the identified objects? :No
  Name the outline image :FilteredObj ects
  Rules file location :Elsewhere ... \x7C
  Rules file name :rules .txt
  Class number :1
  Measurement count: 1
  Additional object count: 0
  Assign overlapping child to: Both parents
  Select the measurement to filter
by :Intensity_MeanIntensityEdge_RescaledCD8
  Filter using a minimum measurement value?: Yes
  Minimum value: 0.1
  Filter using a maximum measurement value?: No
  Maximum value: 1.0

IdentifyPrimaryObjects :[module_num :11 |svn_version :\'Unknown \' |variable_
revision_number :10 |show_window :False |notes :\x5B\x5D |batch_state :array (\
\x5B\x5D, dtype=uint8) |enabled :True |wants_pause :False ]
  Select the input image :TCF7
  Name the primary objects to be identified: TCF7Nuclei
  Typical diameter of objects, in pixel units (Min,Max) :10,40
  Discard objects outside the diameter range?:Yes
  Try to merge too small objects with nearby larger objects? :No
  Discard objects touching the border of the image?: Yes
  Method to distinguish clumped objects :Intensity
  Method to draw dividing lines between clumped objects :Intensity
  Size of smoothing filter: 10
  Suppress local maxima that are closer than this minimum allowed
distance :7.0
  Speed up by using lower-resolution image to find local maxima?: Yes
  Name the outline image :PrimaryOutlines
  Fill holes in identified objects? :After both thresholding and
declumping
  Automatically calculate size of smoothing filter for
declumping? :Yes
  Automatically calculate minimum allowed distance between local
maxima? :Yes
  Retain outlines of the identified objects? :No
  Automatically calculate the threshold using the Otsu method?: Yes
  Enter Laplacian of Gaussian threshold :0.5
  Automatically calculate the size of objects for the Laplacian of
Gaussian filter?:Yes
  Enter LoG filter diameter: 5.0
  Handling of objects if excessive number of objects
identified: Continue
  Maximum number of objects: 500
  Threshold setting version: 2
  Threshold strategy :Automatic
  Thresholding method: Otsu

```

```

Select the smoothing method for thresholding :Automatic
Threshold smoothing scale: 1.0
Threshold correction factor: 1.0
Lower and upper bounds on threshold: 0.0,1.0
Approximate fraction of image covered by objects ?:0.01
Manual threshold :0.0
Select the measurement to threshold with:None
Select binary image:None
Masking objects :None
Two-class or three-class thresholding? :Two classes
Minimize the weighted variance or the entropy? :Weighted variance
Assign pixels in the middle intensity class to the foreground or
the background? :Foreground
Method to calculate adaptive window size: Image size
Size of adaptive window: 10
Use default parameters ?:Default
Lower outlier fraction :0.05
Upper outlier fraction :0.05
Averaging method:Mean
Variance method :Standard deviation
# of deviations :2.0

MaskObjects :[module_num: 12 |svn_version :\'Unknown\' |variable_revision_number :2 |show_window :False|notes:\x5B\x5D| batch_state :array (\x5B\x5D,
dtype=uint8) |enabled: True |wants_pause :False]
    Select objects to be masked: CD8PosNuclei
    Name the masked objects :TCF7PosCD8PosNuclei
    Mask using a region defined by other objects or by binary
image? :Objects
    Select the masking object :TCF7Nuclei
    Select the masking image:None
    Handling of objects that are partially masked :Remove depending on
overlap
    Fraction of object that must overlap: 0.3
    Numbering of resulting objects :Renumber
    Retain outlines of the resulting objects?:No
    Name the outline image :MaskedOutlines
    Invert the mask?:No

CalculateMath :[module_num: 13 |svn_version :\'Unknown\' |variable_revision_number :2 |show_window :False|notes:\x5B\x5D| batch_state :array (\x5B\x5D,
dtype=uint8) |enabled: True |wants_pause :False]
    Name the output measurement :Count_CD8Pos_TCFNeg
    Operation: Subtract
    Select the minuend measurement type: Image
    Select the minuend objects: None
    Select the minuend measurement :Count_CD8PosNuclei
    Multiply the above operand by: 1.0
    Raise the power of above operand by: 1.0
    Select the subtrahend measurement type :Image
    Select the subtrahend objects: None
    Select the subtrahend measurement :Count_TCF7PosCD8PosNuclei
    Multiply the above operand by: 1.0
    Raise the power of above operand by: 1.0
    Take log10 of result?:No
    Multiply the result by: 1.0
    Raise the power of result by: 1.0

```

```

Add to the result: 0.0
Constrain the result to a lower bound? :No
Enter the lower bound: 0.0
Constrain the result to an upper bound? :No
Enter the upper bound: 1.0

OverlayOutlines :[module_num: 14 |svn_version :\ 'Unknown\ '|variable_revision_number :3 |show_window :False|notes :\x5B\x5D|batch_state :array (\x5B\x5D, dtype=uint8) |enabled: True |wants_pause :False]
    Display outlines on a blank image? :No
    Select image on which to display outlines :Color Image
    Name the output image :OrigOver lay
    Outline display mode: Color
    Select method to determine brightness of outlines :Max of image
    Width of outlines :1
    Select outlines to display:None
    Select outline color :#F8FF2D
    Load outlines from an image or objects ?:Objects
    Select objects to display :CD8PosNuclei
    Select outlines to display:None
    Select outline color :#F1F7F7
    Load outlines from an image or objects ?:Objects
    Select objects to display :TCF7PosCD8PosNuclei

Save Images :[module_num :15 |svn_version :\ 'Unknown\'|variable_revision_number :11 |show_window :False|notes:\x5B\x5D|batch_state :array (\x5B\x5D, dtype=uint8 ) |enabled :True |wants_pause :True ]
    Select the type of image to save: Image
    Select the image to save :OrigOverlay
    Select the objects to save:None
    Select the module display window to save: None
    Select method for constructing file names: From image filename
    Select image name for file prefix :Color Image
    Enter single file name :OrigBlue
    Number of digits: 4
    Append a suffix to the image file name?: Yes
    Text to append to the image name :_Over lay
    Saved file format: tif
    Output file location :Default Output Folder\x7C
    Image bit depth: 8-bit integer
    Overwrite existing files without warning?: No
    When to save: Every cycle
    Rescale the images? :Yes
    Save as grayscale or color image? :Grayscale
    Select colormap :gray
    Record the file and path information to the saved image?: No
    Create subfolders in the output folder? :No
    Base image folder :Elsewhere ... \x7C
    Saved movie format :avi

Export ToSpreadsheet :[module_num: 16 |svn_version :\ 'Unknown\'|variable_revision_number :11 |show_window :False|notes:\x5B\x5D|batch_state :array(\x5B\x5D, dtype=uint8) |enabled :True |wants_pause :False ]
    Select the column delimiter :Comma (",")
    Add image metadata columns to your object data file?: No
    Limit output to a size that is allowed in Excel? :No
    Select the measurements to export: Yes

```

Calculate the per-image mean values for object measurements ?:No
Calculate the per-image median values for object measurements ?:No
Calculate the per-image standard deviation values for object measurements ?:No
Output file location :Default Output Folder\x7C
Create a GenePattern GCT file?:No
Select source of sample row name :Metadata
Select the image to use as the identifier :None
Select the metadata to use as the identifier :None
Export all measurement types?: No

:Image\x7CCount_TCF7Nuclei, Image\x7CCount_Nuclei, Image\x7CCount_CD8PosNuclei, Image\x7CCount_TCF7PosCD8PosNuclei, Image\x7CMath_Count_CD8Pos_TCF Neg
Representation of Nan/Inf:NaN
Add a prefix to file names? :No
Filename prefix :MyExpt_
Overwrite existing files without warning?: No
Data to export: Image
Combine these object measurements with those of the previous object? :No
File name :DATA.csv
Use the object name for the file name?: Yes

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[0612] Various modifications and variations of the described methods, pharmaceutical compositions, and kits of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific embodiments, it will be understood that it is capable of further modifications and that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the art are intended to be within the scope of the invention. This application is intended to cover any variations, uses, or adaptations of the

invention following, in general, the principles of the invention and including such departures from the present disclosure come within known customary practice within the art to which the invention pertains and may be applied to the essential features herein before set forth.

CLAIMS

What is claimed is:

1. A method of detecting a checkpoint blockade (CPB) therapy responder gene signature comprising, detecting in CD45+ cells obtained from a biological sample the expression of a gene signature comprising one or more genes or polypeptides selected from the group consisting of:

- a) TCF7; or
- b) TCF7, PLAC8, LTB, and CCR7; or
- c) TCF7, LEF1, S1PR1, PLAC8, LTB, CCR7, IGHM, PAX5, FCRL1, FCER2, CD19, CD22, BANK 1, MS4A1, BLK, RALGPS2 and FAM129C; or
- d) TCF7, PLAC8, LTB, LY9, SELL, IGKC and CCR7.

2. A method of detecting a checkpoint blockade (CPB) therapy responder gene signature comprising, detecting in CD8+ T cells obtained from a biological sample the expression of a gene signature comprising one or more genes or polypeptides selected from the group consisting of:

- a) TCF7; or
- b) TCF7 and IL7R; or
- c) TCF7, IL7R, FOSL2, REL, FOXPI, and STAT4; or
- d) TCF7, PLAC8, LTB, and CCR7; or
- e) TCF7, LEF1, S1PR1, PLAC8, LTB, and CCR7; or
- f) TCF7, IL7R, GPR183, and MGAT4A; or
- g) TCF7, IL7R, GPR183, LMNA, NR4A3, CD55, AIM1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N and CSRNP1; or
- h) TCF7, IL7R, GPR183, LMNA, NR4A3, CD55, AIM1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N, CSRNP1, FAM65B, PIK3R1, RGPD6, SKIL, TSC22D2, USP36, FOXPI, EGRI, MYADM, ZFP36L2, FAM102A, RGCC, PDE4B, PFKFB3, FOSB, DCTN6 and BTG2; or
- i) CD8_G genes listed in Table 6.

3. The method according to claim 1 or 2, wherein the CD8 T cells do not express ENTPD1 (CD39) and HAVCR2.

4. A method of detecting a checkpoint blockade (CPB) therapy non-responder

gene signature comprising, detecting in CD45+ cells obtained from a biological sample the expression of a gene signature comprising one or more genes or polypeptides selected from the group consisting of:

- a) ENTPD1 and HAVCR2; or
- b) CCL3, CD38 and HAVCR2; or
- c) CD38, PDCD1, CCL3, SNAP47, VCAM1, HAVCR2, FASLG, ENTPD1, SIRPG, MY07A, FABP5, NDUFB3, UBE2F, CLTA and SNRPD1; or
- d) FASLG, VCAM1, CCL3, LAG3, CXCR6, IFNG, PDCD1, KLRD1, HAVCR2, SIRPG, SNAP47, DTHD1, PRF1, GZMH, F2R, CD38, CXCL13, TNFRSF4, TNFRSF18, MAF, ETV7, CD4, CTLA4, FCRL6, SPON2, KLRG1, TRGC1, A2M, FCGR3A, GZMA, HOPX, NKG7, PXN, TNFRSF9, GEM, NABI, DFNB31, CADM1, CRTAM, GPR56, MY07A, DUSP4, METRNL and PHLDAl; or
- e) LAYN, GEM, VCAM1, RDH10, TNFRSF18, FAM3C, AFAP1L2, KIR2DL4, MTSS1, ETV1, CTLA4, MY07A, ENTPD1, TNFRSF9, CADM1, DFNB31, CXCL13, HAVCR2, GPR56, GOLIM4, NABI, PHLDAl, TGIF1, SEC14L1, IGFLR1, NAMPTL, PAM, HSPB1, TNIP3, BPGM, TP53INP1, TRPS1, UBE2F, NDFIP2, PON2, PELI1, METRNL, SNAP47 and APLP2; or
- f) CCL3, LGALS1, CD38, EPSTI1, WARS, PLEK, HAVCR2, LGALS3, FABP5, MT2A, GBP1, PLSCR1, CCR5, GSTO1, ANXA5, GLUL, PYCARD, TYMP, IFI6, VAMP5, OASL, GZMB, TXN, SQRDL, RHOC, AP2S1, GZMH, CCL4L2, SNAP47, LAP3, ATP6V1B2, CCL4L1, LAMP2, PSMA4, SERPINB1, HIGD1A, UBE2F, TALDO1, CD63, CLTA, S100A11, PHPT1, GBP4, PRDX3, PSMB2, BST2, GBP5, CTSC, NDUFB3, NPC2, GALM, GLIPR2, CCL4, PRF1, IFNG, IFI30, CHST12, ISG15, MYD88, IDH2, MTHFD2, CHMP2A, NDUFA9, CHMP5, CALM3, ANXA2, PPT1, GTF3C6, NDUFAB1, CXCR6, RNF181, LGALS9, COX5A, OAS2, PDCD1, SNRPC, BHLHE40, TWF2, SLAMF7, TXN2, CARD16, ANAPCI1, MRPL51, LIMS1, NDUFA12, RANBP1, GBP2, PSMC1, ACTR1A, CD2BP2, VDAC1, EMC7, MX1, GPS1, ATP5J2, USMG5, SHFM1, ATP51, FAM96A, CASP1, PARP9, NOP10, GNG5, CYC1, RAB11A, PGAM1, ENTPD1, PDIA6, PSMC3, TMBIM1, UBE2L6, PSMA6, EIF6, DCTN3, SEC11A, CSTB, ETFB, DBI, GRN, ELOVL1, UBE2L3, PSMB3, NDUFB7, DOK2, SEC61G, IGFLR1, ATP5H, COPZ1, ATP6V1F, BNIP3L, NUTF2, AKRIAI, MDH2, VAMP8, ROMO1, CXCR3, SAMHD1, NUCB1, ACTN4, ZYX, FLOT1, BLOC1S1, STAT1, VFMP, PAM, NUDT21, MYOIG, C17orf49, GTF2A2, HIST2H2AA4, C19orf10, ABI3, TRAPPC5, PSMC4, NDUFC2, HN1, SNRPD3,

CMC1, RAB27A, NDUFA6, POMP, PFKP, ATP5G3, TMEM179B, PSMD9, IRF7, CNIH1, DYNLRB1, APOL2, TKT, DCTN2, GSDMD, STOM, CTSD, KDELR2, ATP5J, RPS27L, PSME2, DRAPI, NDUFB10, DECR1, GSTP1, TMED9, MGAT1, HSPB1, COX8A, ZEB2, ILK, PSMB6, HK1, CD58, TMX1, GZMA, SRI, PSMG2, ARL8B, NKG7, GPX1, ACP5, CHP1, GPR171, ATP6V0B, KLRD1, H2AFY, PPM1G, PRDX5, PSMA5, FBXW5, ATP6AP1, CD4, SNRPD1, XAF1, LY6E, DYNLT1, AK2, PSMA2, YIPF3, S100A10, SCP2, MRPS34, PSMD4, CDC123, BTG3, TMEM258, TSPO, SDHB, TCEB1, WDR830S, HCST, NAA10, CTSB, YARS, GLRX, RBCK1, RBX1, LAMTOR1, UQCRCFS1, NDUFB4, CAPZA2, BRK1, ADRM1, NDUFB2, ETFA, VDAC3, NUDT5, IFITM3, BANF1, ZNHIT1, CAPG, NHP2, LASP1, TOMM5, MVP, CTSW, AURKAIP1, RARRES3, PSMB10, TMEM173, SLX1A, APOBEC3G, GFMAP4, EIF4E, CTLA4, NDUFS8, CYB5B, PIK3R5, HEXB, STXBP2, PSMD8, SEC61B, RGS10, PUB, ATP5C1, ARF5, SUMO3, PRDX6, RNHI, ATP5F1, UQCRCI, SARNP, PLIN2, PIN1, SDHC, SF3B14, CAPRINI, POLR2G, COX7B, UQCR10, FBX07, NDUFB6, S100A4, PRELID1, TRPV2, SF3B5, MY01F, SCAMP2, RNF7, CXCL13, RAB1B, SHKBP1, PET100, HM13, VTI1B, S100A6, ARPC5, FDPS, MINOS 1, RABIO, NEDD8, BATF, PHB2, ERH, NCOA4, PDIA4, PSMB9, C11orf48, TMEM50A, TIGIT, NDUFA1 1, NELFE, COX6C, SLA2, PSMB8, NDUFS7, RER1, RAB8A, CAPN1, MRPL20, COX5B, SEC13, FKBP1A, PRDM1, RABIA, RHOG, CYB5R3, AIP, ABRACL, PSMB7, COX6B1, PSMD7, PPA1, PCMT1, SURF4, ENY2, TCEB2, MAP2K3, AL353354.2, AKTRIN2, MAPRE1, GRSF1, DUSP4, ATG3, SRGAP2, ATP6V0D1, NELFCD, LRPAPI, C14orf166, SNRPB2, CHMP4A, SFT2D1, CASP4, NME1-NME2, FAM96B, FDFT1, SLC25A39, LMAN2, MDHI, RHBDD2, ARPC5L, TBCA, EBP, SEC14L1, EIF2S2, CST7, STARD7, SOD2, SPN, FAM32A, SEC1 1C, TNFRSF1B, POLR2E, NDUFA13, OSTC, UFC1, C18orf32, SRP19, C14orf2, UQCR1 1, PDCD6, AP2M1, PPP1CA, ATP6AP2, SSR3, UNCI 3D, FERMT3, ARHGAPI, EIF3I, CECR1, MRPS6, DNPH1, DCXR, PSMF1, SNRPG, CNDP2, ANXA1 1, SLM02, C16orf13, CAPN2, BSG, LAMTOR5, SIVA1, TRAPPC1, TMCO1, PSMD13, PSMB1, RSU1, NDUFA1, TUBB, DCTN1, SH3GLB1, BCAP31, RTFDC1, UFDIL, GPI, DNAJB11, SNX17, SH2D2A, Clorf43, BUD31, PSTPIP1, CTSA, TPST2, MPV17, APMAP, CMC2, UQCRQ, TBCB, C9orf16, PARK7, ATP5EP2, SHISA5, SMC4, TAPI, SCAND1, SIRPG, HDLBP, EMC4, FIS1, TPI1, GOLGA7, POLR2J, EIF2S1, UBA3, P4HB, UQCRH, CSNK2B, SZRD1, NDUFA3, ATP50, DERL2, COPS6, COPE, SNX6, FLU and ERGIC3.

5. A method of detecting a checkpoint blockade (CPB) therapy non-responder gene signature comprising, detecting in CD8+ T cells obtained from a biological sample the expression of a gene signature comprising one or more genes or polypeptides selected from the group consisting of:

- a) ENTPD1 and HAVCR2; or
- b) CCL3, CD38 and HAVCR2; or
- c) CD38, CCL3, VCAM1, GOLIM4, HAVCR2, PRDX3, ENTPD1, PTTG1, CCR5, TRAFD1, PDCD1, CXCR6, BATF, PTPN6, LAG3 and CTLA4; or
- d) LAYN, GEM, VCAM1, RDH10, TNFRSF18, FAM3C, AFAP1L2, KIR2DL4, MTSSI, ETV1, CTLA4, MY07A, ENTPD1, TNFRSF9, CADM1, DFNB31, CXCL13, HAVCR2, GPR56, GOLIM4, NAB1, PHLD1, TGIF1, SEC14L1, IGFLR1, NAMPTL, PAM, HSPB1, TNIP3, BPGM, TP53INP1, TRPS1, UBE2F, NDFIP2, PON2, PELI1, METRNL, SNAP47 and APLP2; or
- e) CD38, EPSTI1, GOLFM4, WARS, PDCD1, CCL3, SNAP47, VCAM1, SKA2, HAVCR2, LGALS9, PRDX3, FASLG, ENTPD1, FABP5, SIRPG, LSM2, NDUFB3, TRAFD1, UBE2F, NMI, IFI35, CLTA, MTHFD1, MY07A, IFI27L2, MCM5, STMN1, ID3, RGS3, SNRPD1, PTTG1 and FIBP; or
- f) CD8_B genes listed in Table 6.

6. The method according to any of claims 1 to 5, wherein the biological sample is a tumor sample obtained from a subject in need thereof.

7. The method according to any of claims 1 to 6, wherein the gene signature is detected in tumor infiltrating lymphocytes (TILs).

8. The method according to any of claims 1 to 5, wherein the biological sample comprises *ex vivo* or *in vitro* immune cells, preferably CD8+ T cells.

9. The method according to any of claims 1 to 8, wherein the gene signature is detected by deconvolution of bulk expression data such that gene expression in immune cells is detected.

10. The method according to any of claims 1 to 9, wherein detecting a higher proportion immune cells expressing a responder signature as compared to a non-responder signature indicates sensitivity to checkpoint blockade (CPB) therapy and an increased overall

survival, and wherein detecting a higher proportion immune cells expressing a non-responder signature indicates resistance to checkpoint blockade (CPB) therapy and a decreased overall survival.

11. The method according to any of claims 1 to 9, wherein detecting a higher proportion of TCF7+CD8+ as compared to TCF7-CD8+ T cells indicates sensitivity to checkpoint blockade (CPB) therapy and an increased overall survival, and wherein detecting a higher proportion TCF7-CD8+ as compared to TCF7+CD8+ T cells indicates resistance to checkpoint blockade (CPB) therapy and a decreased overall survival.

12. The method according to claim 11, wherein TCF7+CD8+ and TCF7-CD8+ T cells are detected by immunofluorescence.

13. The method according to any of claims 1 to 12, wherein the checkpoint blockade (CPB) therapy comprises anti-CTLA4, anti-PD-L1, anti-PDL1 therapy or combinations thereof.

14. A method of predicting cancer clinical outcome in a subject in need thereof comprising detecting in a sample obtained from the subject the ratio of immune cells enriched for expression of a gene signature according to any of claims 1 to 3 as compared to immune cells enriched for expression of a gene signature according to claims 4 or 5, wherein a ratio greater than one indicates sensitivity to an immunotherapy and an increased overall survival, and wherein a ratio less than one indicates resistance to an immunotherapy and a decreased overall survival.

15. A method of predicting cancer clinical outcome in a subject in need thereof comprising detecting in a sample obtained from the subject the ratio of TCF7+CD8+ to TCF7-CD8+ T cells, wherein a ratio greater than one indicates sensitivity to an immunotherapy and an increased overall survival and wherein a ratio less than one indicates resistance to an immunotherapy and a decreased overall survival.

16. The method according to claim 15, wherein TCF7+CD8+ and TCF7-CD8+ T cells are detected by immunofluorescence.

17. The method according to any of claims 14 to 16, further comprising detecting mutations associated with loss of antigen presentation in tumor cells obtained from the

subject, wherein detecting a mutation associated with loss of antigen presentation indicates resistance to an immunotherapy and a decreased overall survival.

18. The method according to claim 17, wherein the mutations result in the loss of one or more genes or polypeptides selected from the group consisting of B2M, HLA-A, HLA-B, and HLA-C.

19. The method according to any of claims 26 to 32, wherein predicting cancer clinical outcome is performed before, after or during treatment with a checkpoint blockade (CPB) therapy.

20. A method of enriching for memory/effect T cells comprising sorting for CD8+ T cells lacking expression of ENTPD1 and HAVCR2 and/or lacking expression of CD38.

21. A method of enriching for exhausted CD8+ T cells comprising sorting for CD8+ T cells that express ENTPD1 and HAVCR2 and/or express CD38.

22. The method according to claims 20 or 21, wherein the cells are sorted using antibodies specific to ENTPD1 and HAVCR2 and/or CD38.

23. A population of CD8+ T cells, wherein the population of cells comprises CD8+ T cells that lack expression of ENTPD1 and HAVCR2 and/or CD38.

24. The population according to claim 23, wherein the population of cells is depleted for CD8+ T cells that express ENTPD1 and HAVCR2 and/or CD38.

25. The population according to claim 24, wherein the population of cells is enriched for CD8+ T cells that lack expression of ENTPD1 and HAVCR2 and/or CD38.

26. A population of CD8+ T cells, wherein the population of cells comprises cells having a responder gene signature according to any of claims 1 to 3.

27. The population according to claim 26, wherein the population of cells is depleted for cells having a non-responder gene signature according to claims 4 or 5.

28. The population according to claim 26, wherein the population of cells is enriched for cells having a responder gene signature according to any of claims 1 to 3.

29. The population of CD8+ T cells according to any of claims 23 to 28, wherein the population of cells expresses a chimeric antigen receptor (CAR) or an endogenous T cell receptor (TCR).

30. The population of CD8+ T cells according to any of claims 23 to 29, wherein the population of cells comprises CD8+ T cells obtained from a subject suffering from cancer.

31. The population of CD8+ T cells according to any of claims 23 to 30, wherein the cells are modulated to decrease activity or expression of one or more genes or polypeptides selected from the group consisting of:

- a) ENTPD1 and HAVCR2; or
- b) CCL3, CD38 and HAVCR2; or
- c) CD38, CCL3, VCAM1, GOLIM4, HAVCR2, PRDX3, ENTPD1, PTTG1, CCR5, TRAFD1, PDCD1, CXCR6, BATF, PTPN6, LAG3 and CTLA4; or
- d) CD38, EPSTI1, GOLIM4, WARS, PDCD1, CCL3, SNAP47, VCAM1, SKA2, HAVCR2, LGALS9, PRDX3, FASLG, ENTPD1, FABP5, SIRPG, LSM2, NDUFB3, TRAFD1, UBE2F, NMI, IFI35, CLTA, MTHFD1, MY07A, IFI27L2, MCM5, STMN1, ID3, RGS3, SNRPD1, PTTG1 and FIBP; or
- e) CD8_B genes listed in Table 6.

32. The population of CD8+ T cells according to any of claims 23 to 30, wherein the cells are modulated to increase activity or expression one or more genes or polypeptides selected from the group consisting of:

- a) TCF7; or
- b) TCF7 and IL7R; or
- c) TCF7, IL7R, FOSL2, REL, FOXP1, and STAT4; or
- d) TCF7, PLAC8, LTB, and CCR7; or
- e) TCF7, LEF1, S1PR1, PLAC8, LTB, and CCR7; or
- f) TCF7, IL7R, GPR183, and MGAT4A; or
- g) TCF7, IL7R, GPR183, LMNA, NR4A3, CD55, AIM1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N and CSRNP1; or
- h) TCF7, IL7R, GPR183, LMNA, NR4A3, CD55, AIM1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N, CSRNP1, FAM65B, PIK3R1, RGPD6,

SKIL, TSC22D2, USP36, FOXPI, EGRI, MYADM, ZFP36L2, FAM102A, RGCC, PDE4B, PFKFB3, FOSB, DCTN6 and BTG2; or

- i) CD8_G genes listed in Table 6.

33. The population of CD8+ T cells according to claims 31 to 32, wherein the one or more genes are modulated with a genetic modifying agent.

34. The population of CD8+ T cells according to any of claims 23 to 33, wherein the population of cells comprises activated T cells.

35. The population of CD8+ T cells according to claim 34, wherein the population of cells comprises T cells activated with tumor specific antigens.

36. The population of CD8+ T cells according to claim 35, wherein the tumor specific antigens are subject specific antigens.

37. A pharmaceutical composition comprising the population of cells according to any of claims 23 to 36.

38. A method of treating cancer in a subject in need thereof comprising administering an inhibitor of CD39 and an inhibitor of TIM3 or an inhibitor of CD39 and an inhibitor of PDI.

39. The method of claim 38, wherein the inhibitor of TFM3 comprises anti-TIM3 antibodies or wherein the inhibitor of PD1 comprises anti-PDI antibodies.

40. The method of claim 38, wherein the inhibitor of CD39 comprises POM-1.

41. A method of treating cancer in a subject in need thereof comprising:

a) predicting cancer clinical outcome in the subject according to any of claims 14 to 19; and

b) treating the subject,

wherein responders are treated with an immunotherapy comprising checkpoint blockade (CPB) therapy,

wherein non-responders are treated with:

- i) adoptive cell transfer and optionally checkpoint blockade (CPB) therapy;
- or

- ii) an inhibitor of CD39 and an inhibitor of TIM3; or
- iii) an inhibitor of CD39 and an inhibitor of PD1; or
- iv) an agent capable of targeting, inhibiting or depleting CD8+ TILs having said non-responder signature and optionally checkpoint blockade (CPB) therapy; or
- v) an agent capable of activating, maintaining or increasing CD8+ TILs having said responder signature and optionally checkpoint blockade (CPB) therapy, or

wherein non-responders comprising tumors not capable of presenting antigens are treated with a therapy other than checkpoint blockade (CPB) therapy.

42. The method of treatment according to claim 41, wherein the adoptive cell transfer comprises:

- a) autologous T cells having the responder signature; or
- b) autologous T cells specific against tumor antigens, having the responder signature; or
- c) autologous T cells transduced with T cell receptors targeting tumor antigens, having the responder signature; or
- d) autologous CAR T cells having the responder gene signature; or
- e) allogenic T cells having the responder signature; or
- f) allogenic T cells specific against tumor antigens, having the responder signature; or
- g) allogenic T cells transduced with T cell receptors targeting tumor antigens, having the responder signature; or
- h) allogenic CAR T cells having the responder gene signature.

43. The method according to claim 42, wherein the autologous T cells are obtained from the subject and cells having the non-responder signature are depleted and/or cells having the responder signature are expanded.

44. The method according to claim 42, wherein CAR T cells are enriched for cells having a responder signature or depleted for cells having a non-responder signature.

45. The method of treatment according to claim 41, wherein the agent capable of

targeting, inhibiting or depleting CD8+ TILs having a non-responder signature comprises:

- a) an agent capable of binding to a cell surface or secreted CD8+ T cell non-responder signature gene; or
- b) an agent capable of reducing the expression or activity of the non-responder signature.

46. The method of treatment according to claim 41, wherein the agent capable of activating, maintaining or increasing CD8+ TILs having a responder signature comprises an agent capable of increasing or activating the expression of the responder signature.

47. The method according to any of claims 41 to 46, wherein checkpoint blockade (CPB) therapy comprises anti-CTLA4, anti-PD-L1, anti-PDI therapy or combinations thereof.

48. A method of treating cancer in a subject in need thereof comprising administering an agent capable of increasing the expression or activity of one or more genes or polypeptides selected from the group consisting of TCF7, IL7R, GPR183, LMNA, NR4A3, CD55, AIM1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N, CSRNP1, FAM65B, PIK3R1, RGPD6, SKIL, TSC22D2, USP36, FOXPI, STAT4, PLAC8, LTB LEFI, SIPRI, EGRI, MYADM, ZFP36L2, FAM102A, RGCC, PDE4B, PFKFB3, FOSB, DCTN6 and BTG2 in combination with checkpoint blockade therapy.

49. A method of treating cancer in a subject in need thereof comprising administering an agent capable of reducing the expression or activity of one or more genes or polypeptides selected from the group consisting of CD38, CCL3, VCAMI, GOLFM4, HAVCR2, PRDX3, ENTPDI, PTTGI, CCR5, TRAFDI, PDCDI, CXCR6, BATF, PTPN6, LAG3 and CTLA4 in combination with checkpoint blockade therapy.

50. A method of treating cancer in a subject in need thereof comprising administering CD8+ T cells expressing a gene signature comprising of one or more genes selected from the group consisting of TCF7, IL7R, GPR183, LMNA, NR4A3, CD55, AFM1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N, CSRNP1, FAM65B, PIK3R1, RGPD6, SKIL, TSC22D2, USP36, FOXPI, STAT4, PLAC8, LTB LEFI, SIPRI, EGRI, MYADM, ZFP36L2, FAM102A, RGCC, PDE4B, PFKFB3, FOSB, DCTN6 and BTG2 in combination with checkpoint blockade therapy.

51. The method according to any of claims 41 to 49, wherein said agent comprises a therapeutic antibody, antibody fragment, antibody-like protein scaffold, aptamer, protein, genetic modifying agent or small molecule.

52. A method of monitoring a subject in need thereof undergoing treatment with checkpoint blockade (CPB) therapy, said method comprising detecting in a tumor sample obtained from the subject the expression or activity of a gene signature comprising one or more genes or polypeptides selected from the group consisting of:

- a) ENTPD1 and HAVCR2; or
- b) CCL3, CD38 and HAVCR2; or
- c) CD38, CCL3, VCAM1, GOLIM4, HAVCR2, PRDX3, ENTPD1, PTTG1, CCR5, TRAFD1, PDGCD1, CXCR6, BATF, PTPN6, LAG3 and CTLA4; or
- d) CD38, EPSTI1, GOLEVI4, WARS, PDGCD1, CCL3, SNAP47, VCAM1, SKA2, HAVCR2, LGALS9, PRDX3, FASLG, ENTPD1, FABP5, SIRPG, LSM2, NDUFB3, TRAFD1, UBE2F, NMI, IFI35, CLTA, MTHFD1, MY07A, IFI27L2, MCM5, STMNI, ID3, RGS3, SNRPD1, PTTG1 and FIBP; or
- e) CD8_B genes listed in Table 6,

wherein the treatment is adjusted if the signature is increased in CD8+ TILs after treatment.

53. A method of monitoring a subject in need thereof undergoing treatment with checkpoint blockade (CPB) therapy, said method comprising detecting in a tumor sample obtained from the subject the expression or activity of a gene signature comprising one or more genes or polypeptides selected from the group consisting of:

- a) TCF7; or
- b) TCF7 and IL7R; or
- c) TCF7, IL7R, FOSL2, REL, FOXP1, and STAT4; or
- d) TCF7, PLAC8, LTB, and CCR7; or
- e) TCF7, LEF1, S1PR1, PLAC8, LTB, and CCR7; or
- f) TCF7, IL7R, GPR183, and MGAT4A; or
- g) TCF7, IL7R, GPR183, LMNA, NR4A3, CD55, AIM1, MGAT4A, PERI, FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N and CSRNP1; or
- h) TCF7, IL7R, GPR183, LMNA, NR4A3, CD55, AIM1, MGAT4A, PERI,

FOSL2, TSPYL2, REL, FAM177A1, YPEL5, TC2N, CSRNP1, FAM65B, PIK3R1, RGPD6, SKIL, TSC22D2, USP36, FOXP1, EGRI, MYADM, ZFP36L2, FAM102A, RGCC, PDE4B, PFKFB3, FOSB, DCTN6 and BTG2; or

- i) CD8_G genes listed in Table 6,

wherein the treatment is adjusted if the signature is decreased in CD8+ TILs after treatment.

54. A method of manufacturing cells for use in adoptive cell transfer comprising:

- a) obtaining CD8+ T cells; and

- b) depleting cells having a non-responder signature as defined in claims 4 or 5 or selecting for cells having a responder signature as defined in any of claims 1 to 3.

55. The method according to claim 54, further comprising expanding cells having a responder signature.

56. The method according to claims 54 or 55, further comprising activating the cells.

57. The method according to any of claims 54 to 56, further comprising expressing a chimeric antigen receptor (CAR) or an endogenous T cell receptor (TCR) in the cells.

58. A kit comprising reagents to detect at least one gene or polypeptide according to a gene signature as defined in claims 1 or 5.

59. The kit according to claim 58, wherein the kit comprises at least one antibody, antibody fragment, or aptamer.

60. The kit according to claim 58, wherein the kit comprises primers and/or probes or fluorescently bar-coded oligonucleotide probes for hybridization to RNA.

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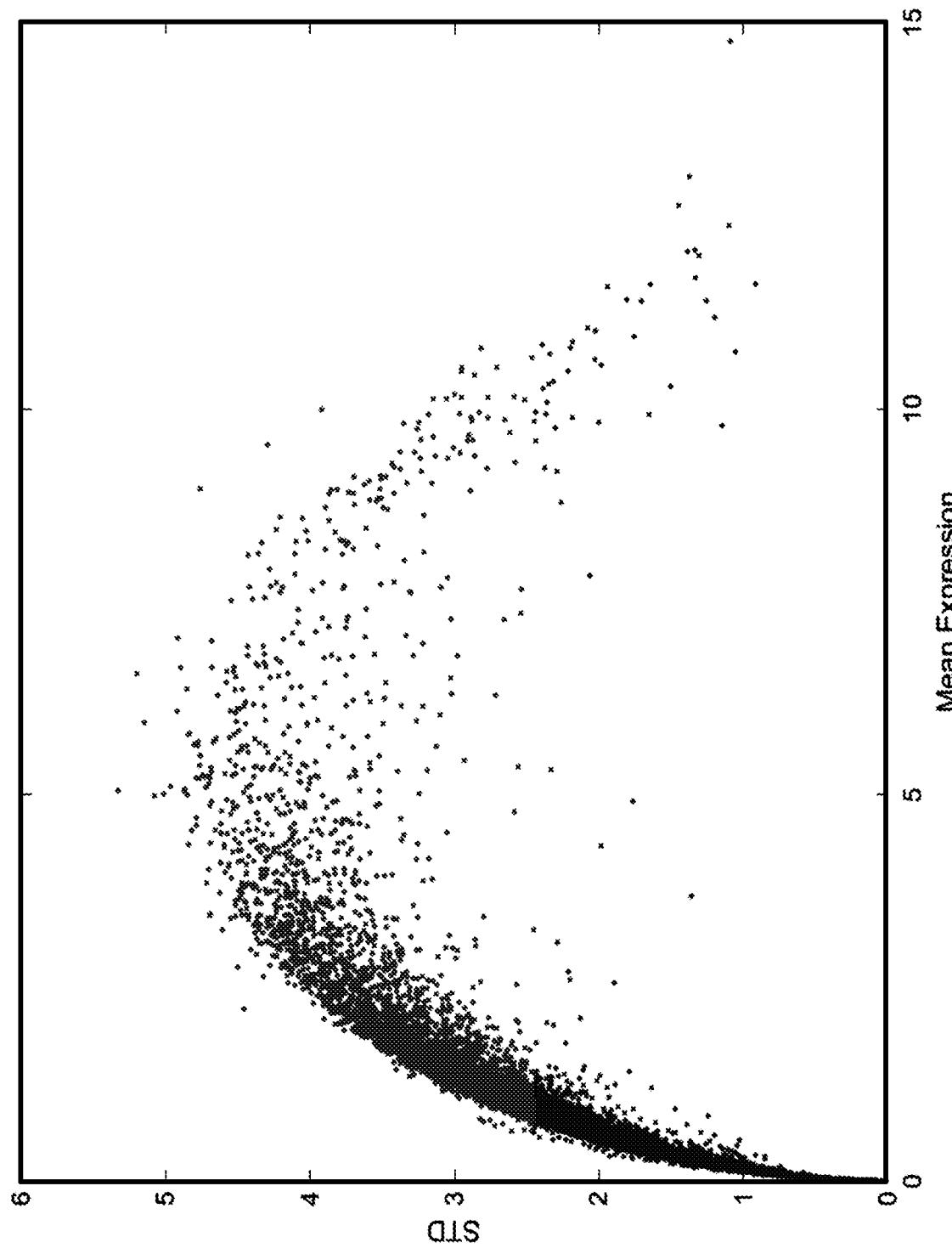


FIG. 1

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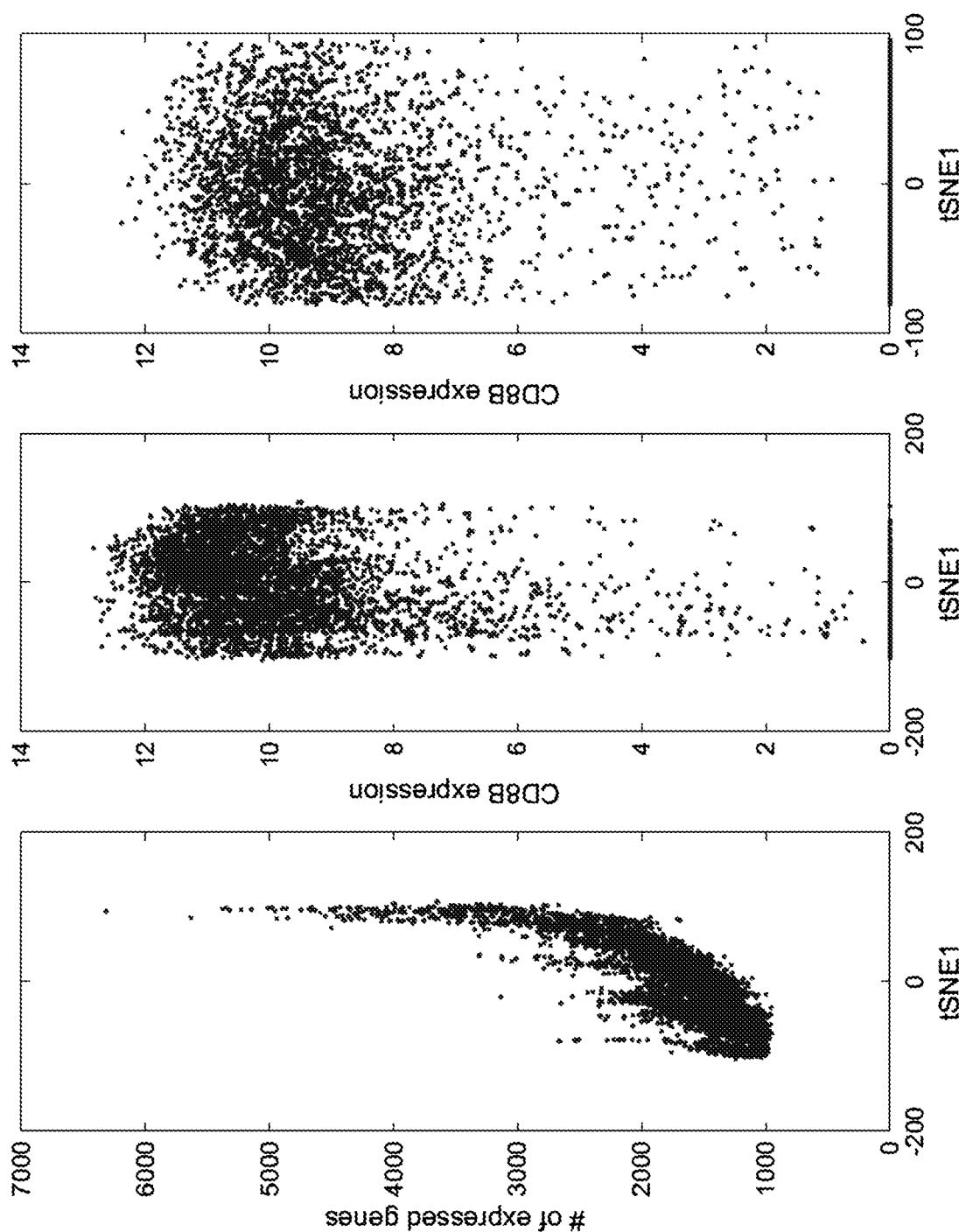


FIG. 2

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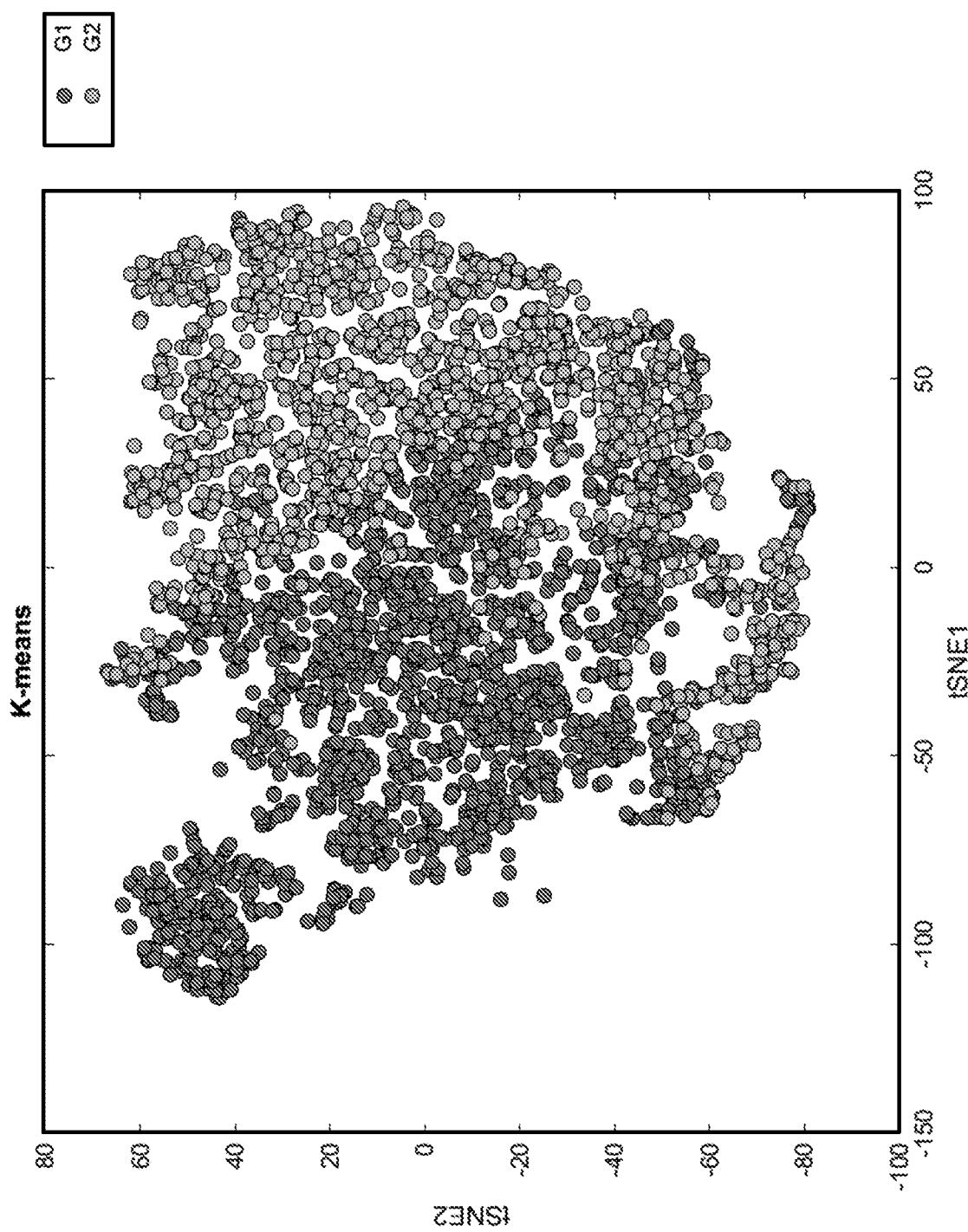


FIG. 3

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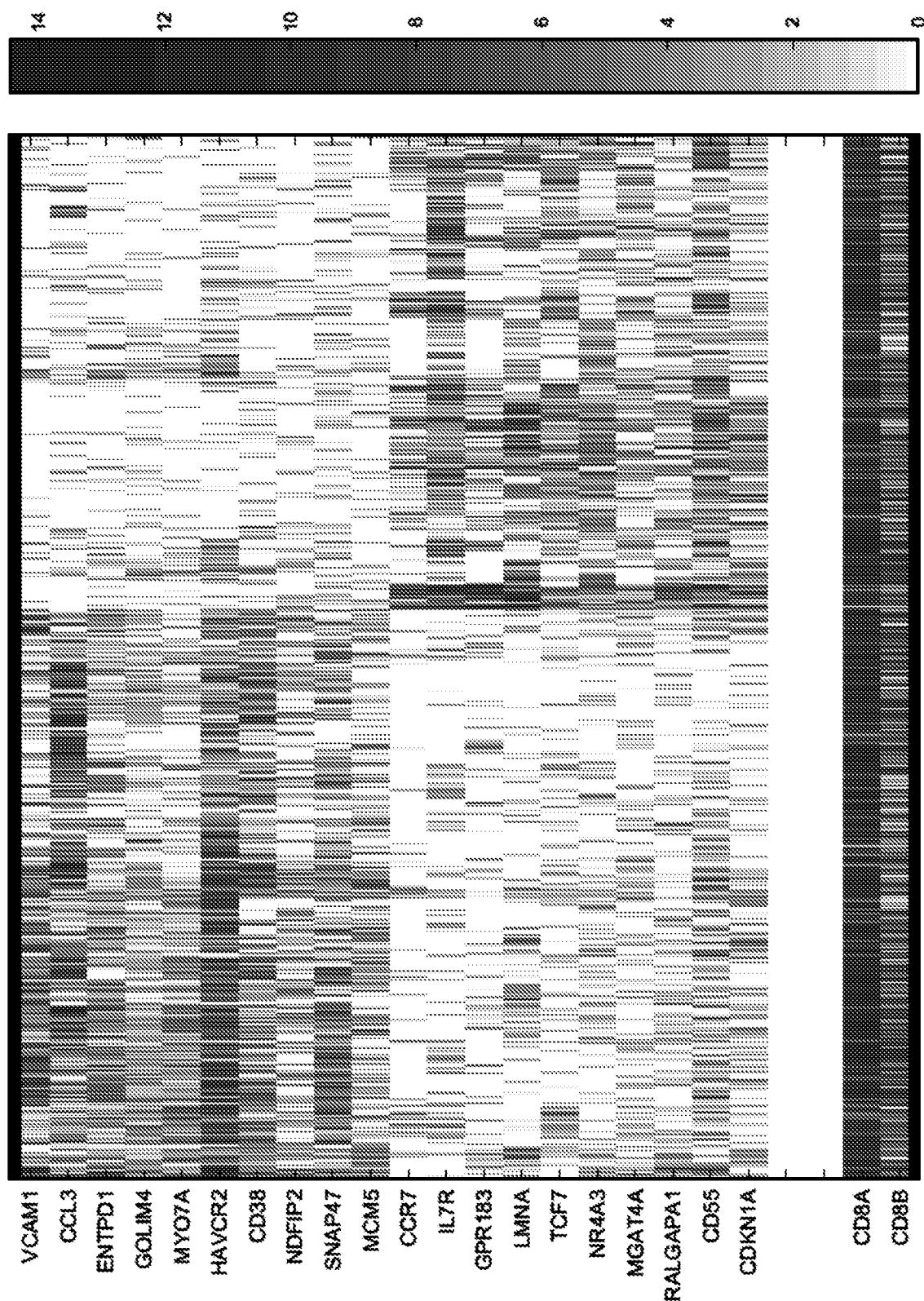


FIG. 4

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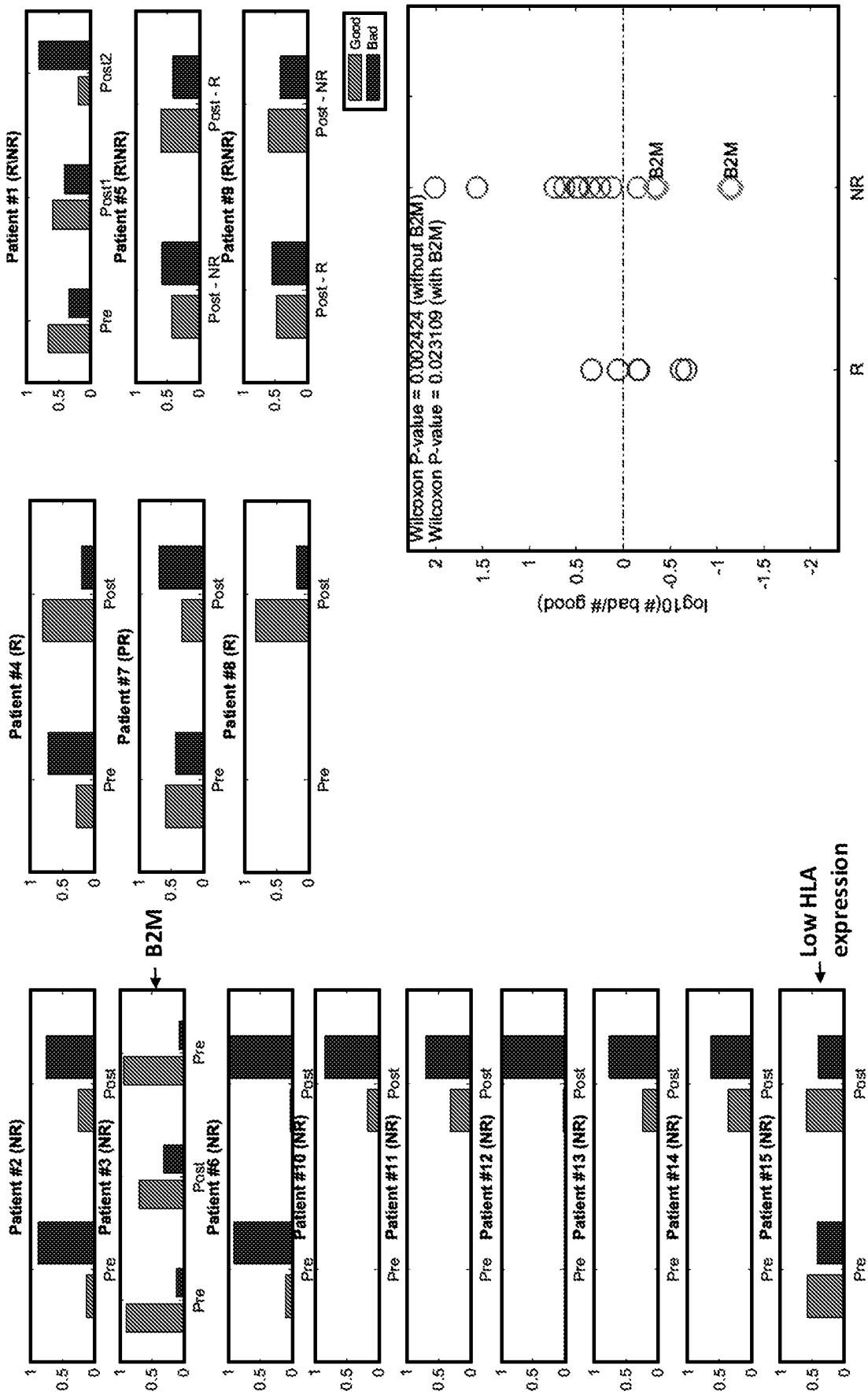


FIG. 5

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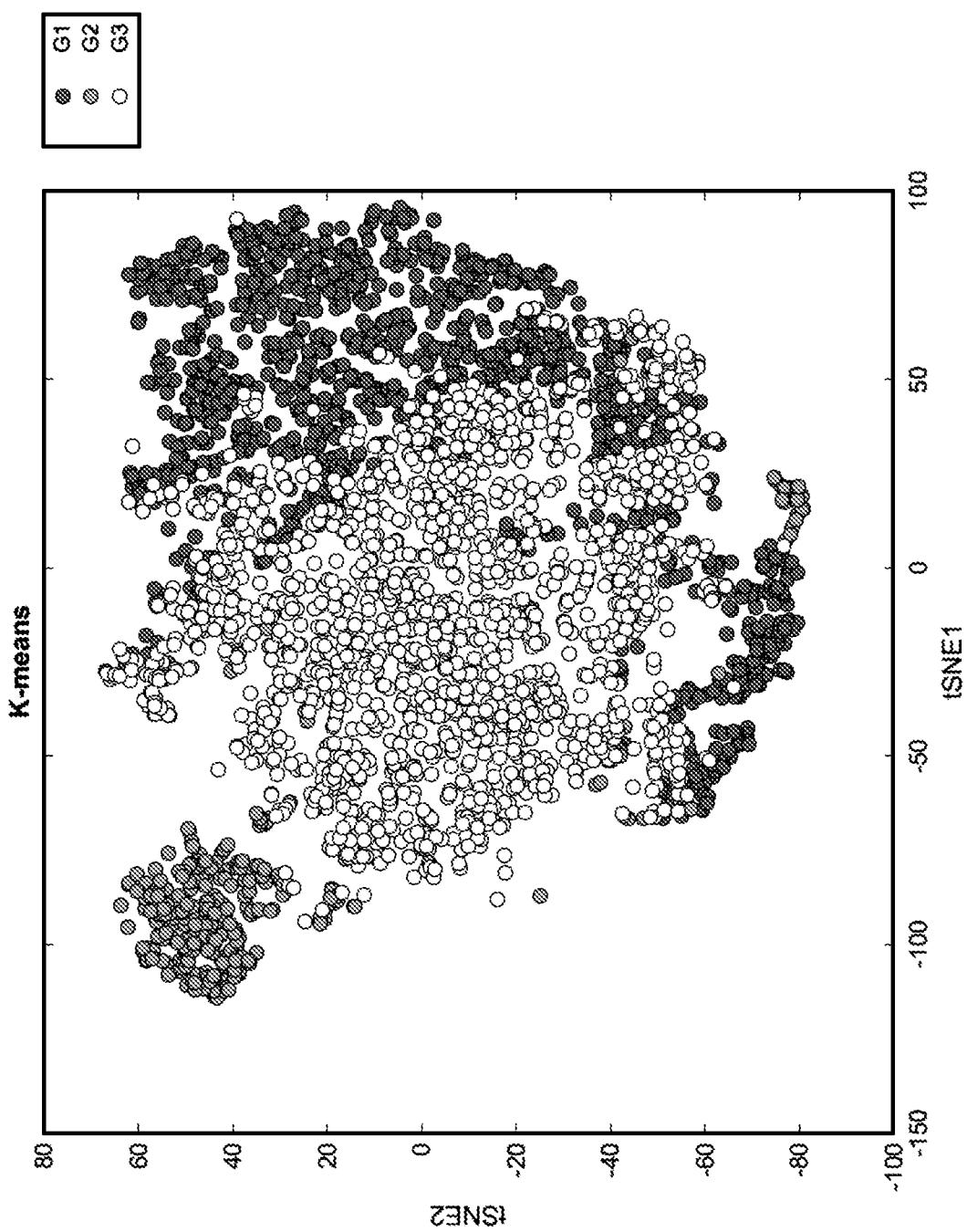


FIG. 6

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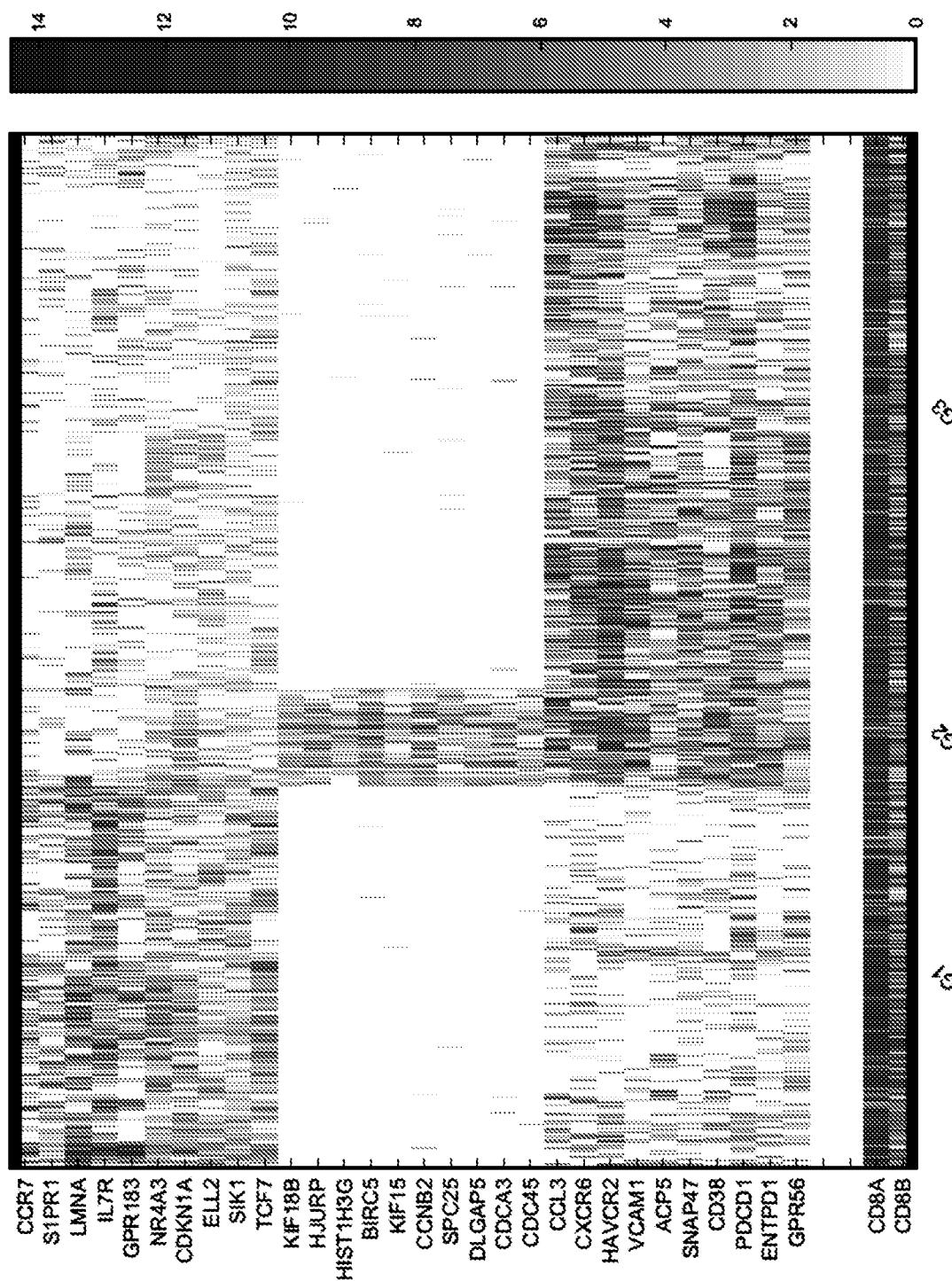


FIG. 7

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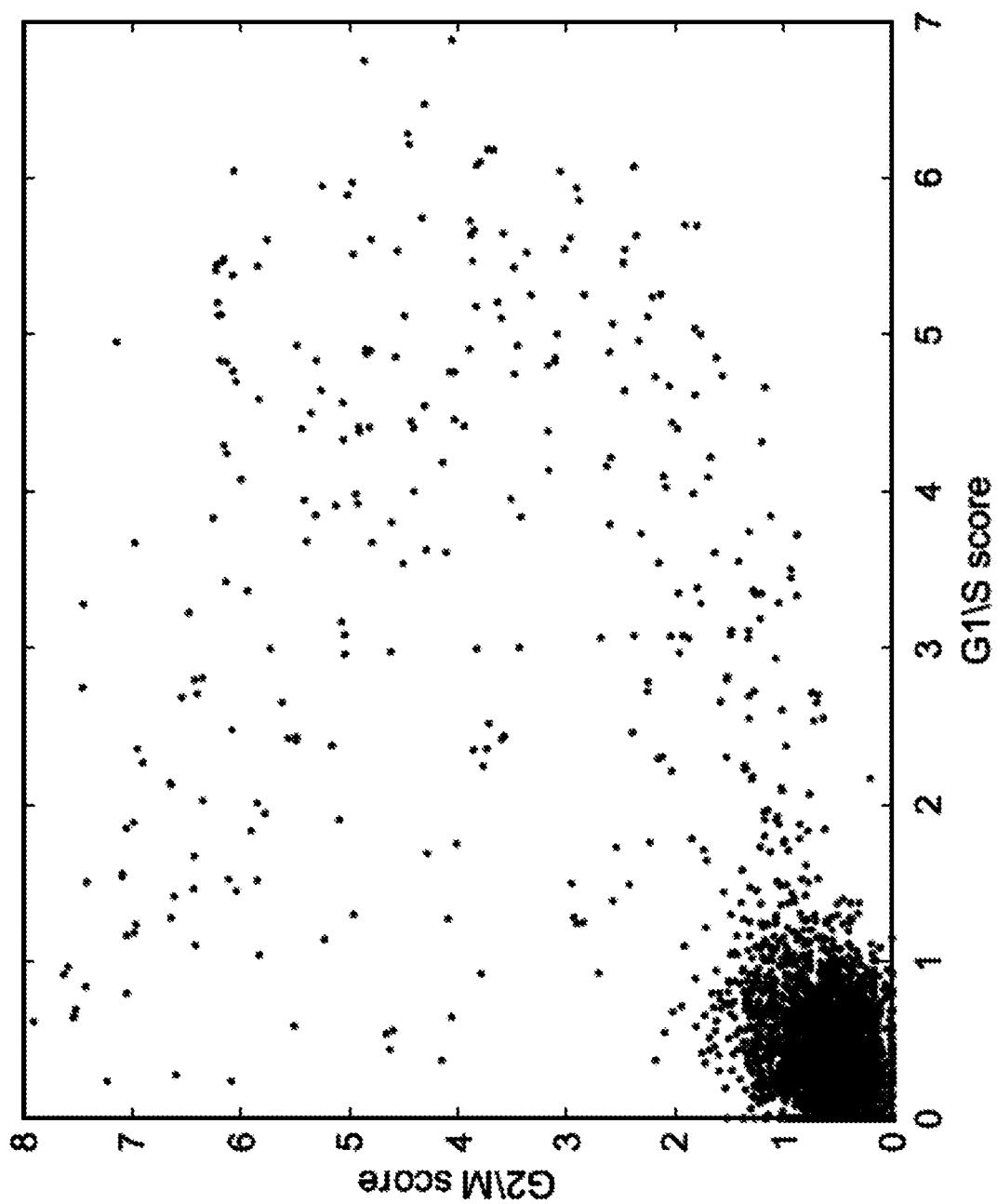


FIG. 8

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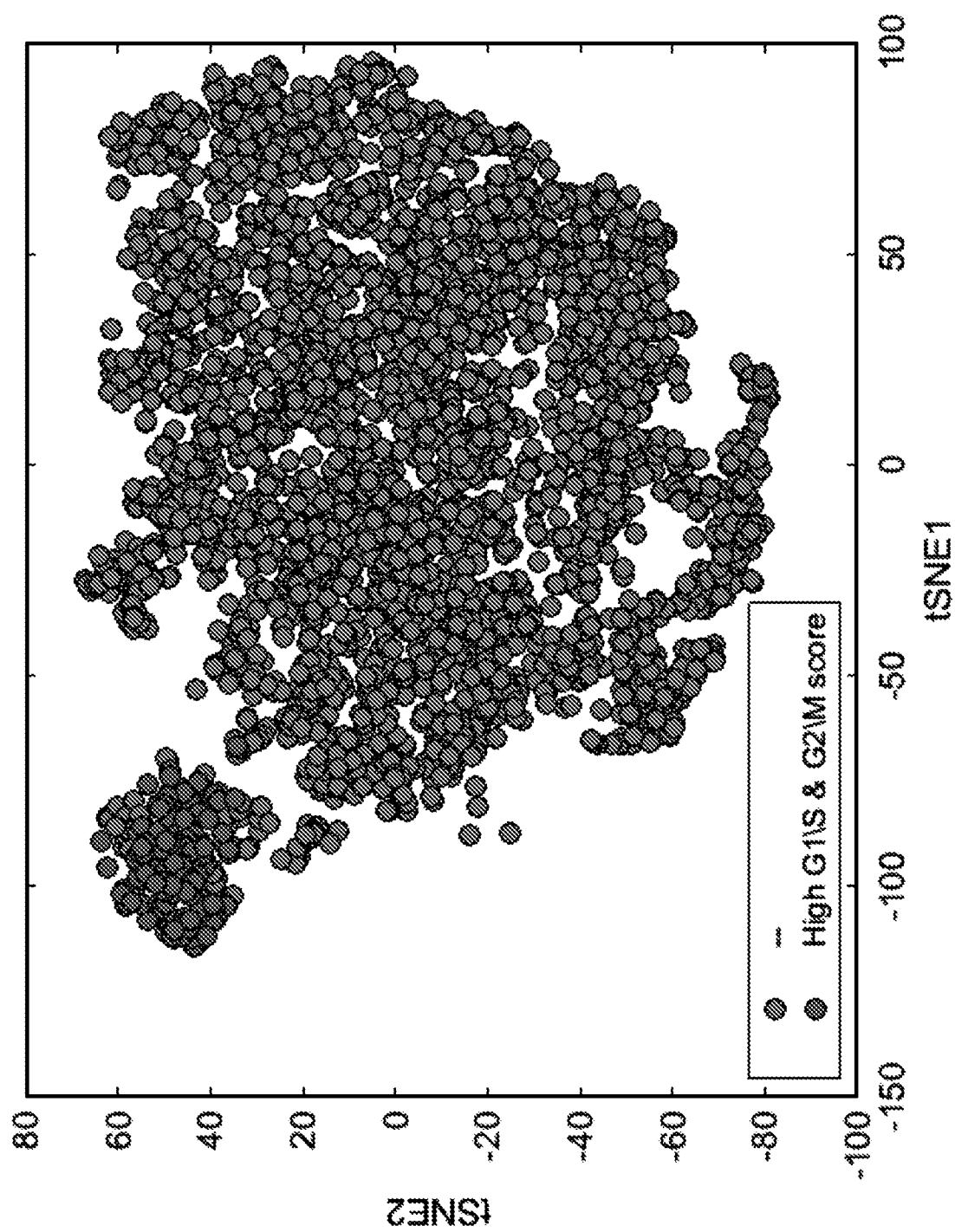


FIG. 9

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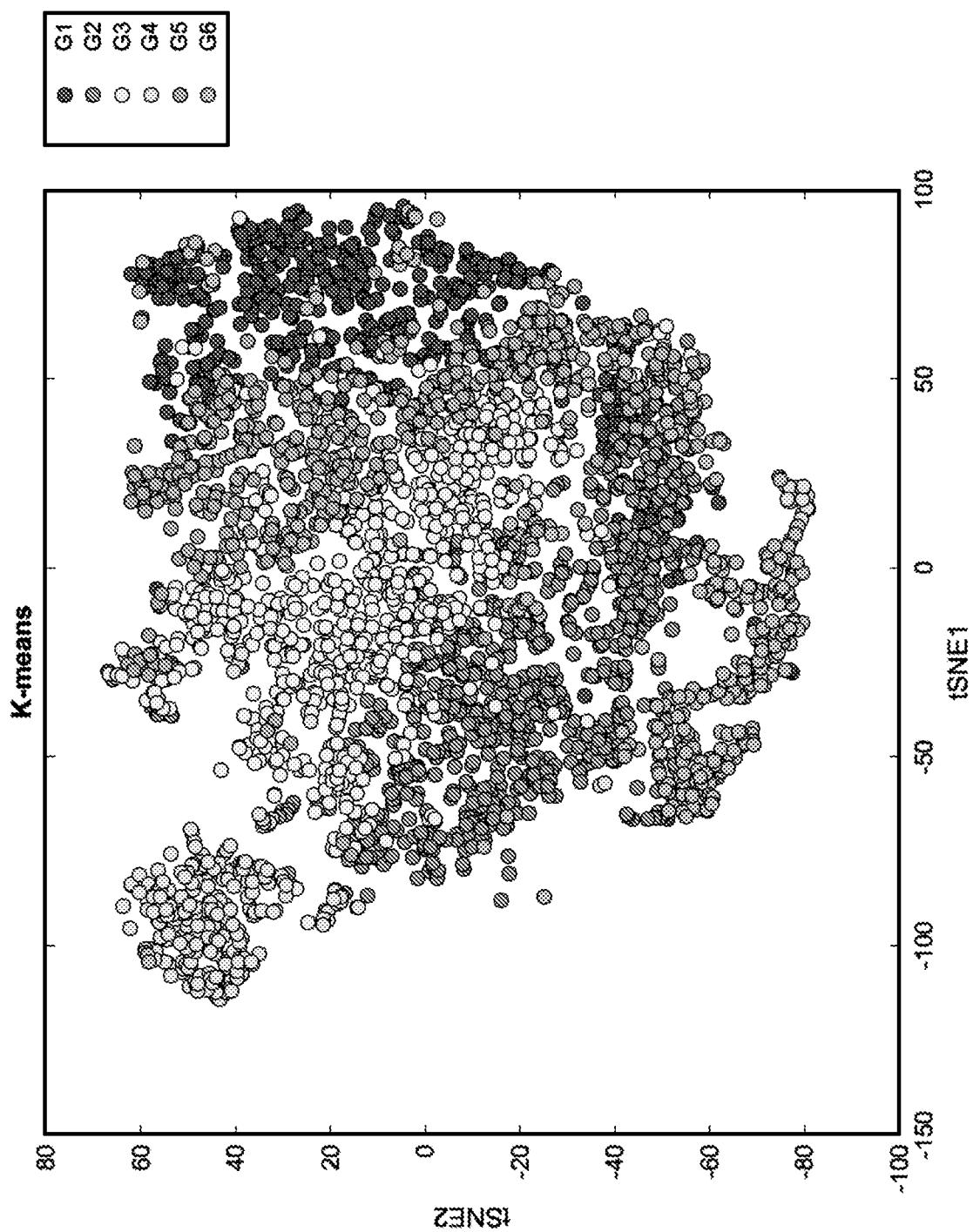


FIG. 10

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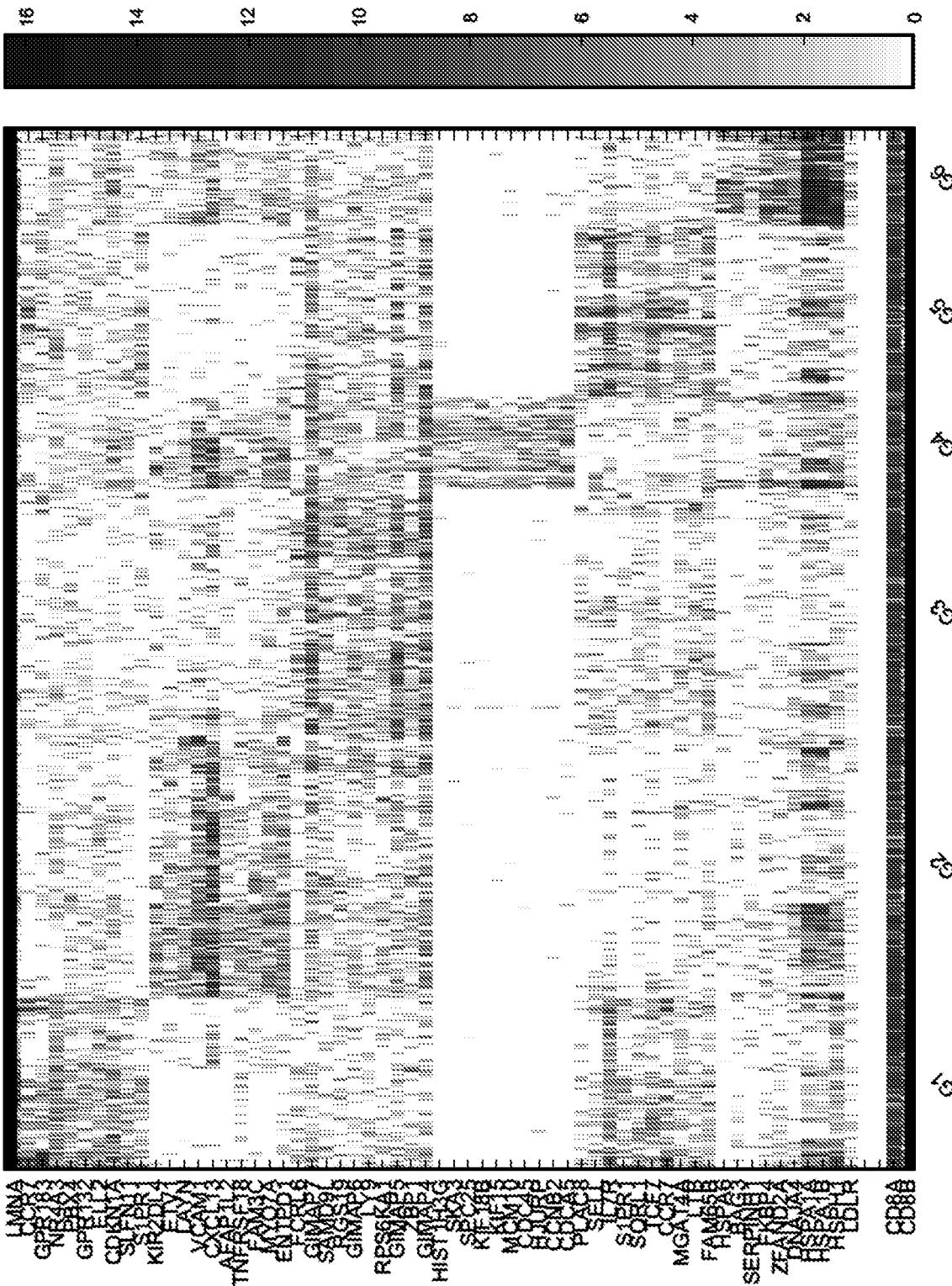


FIG. 11

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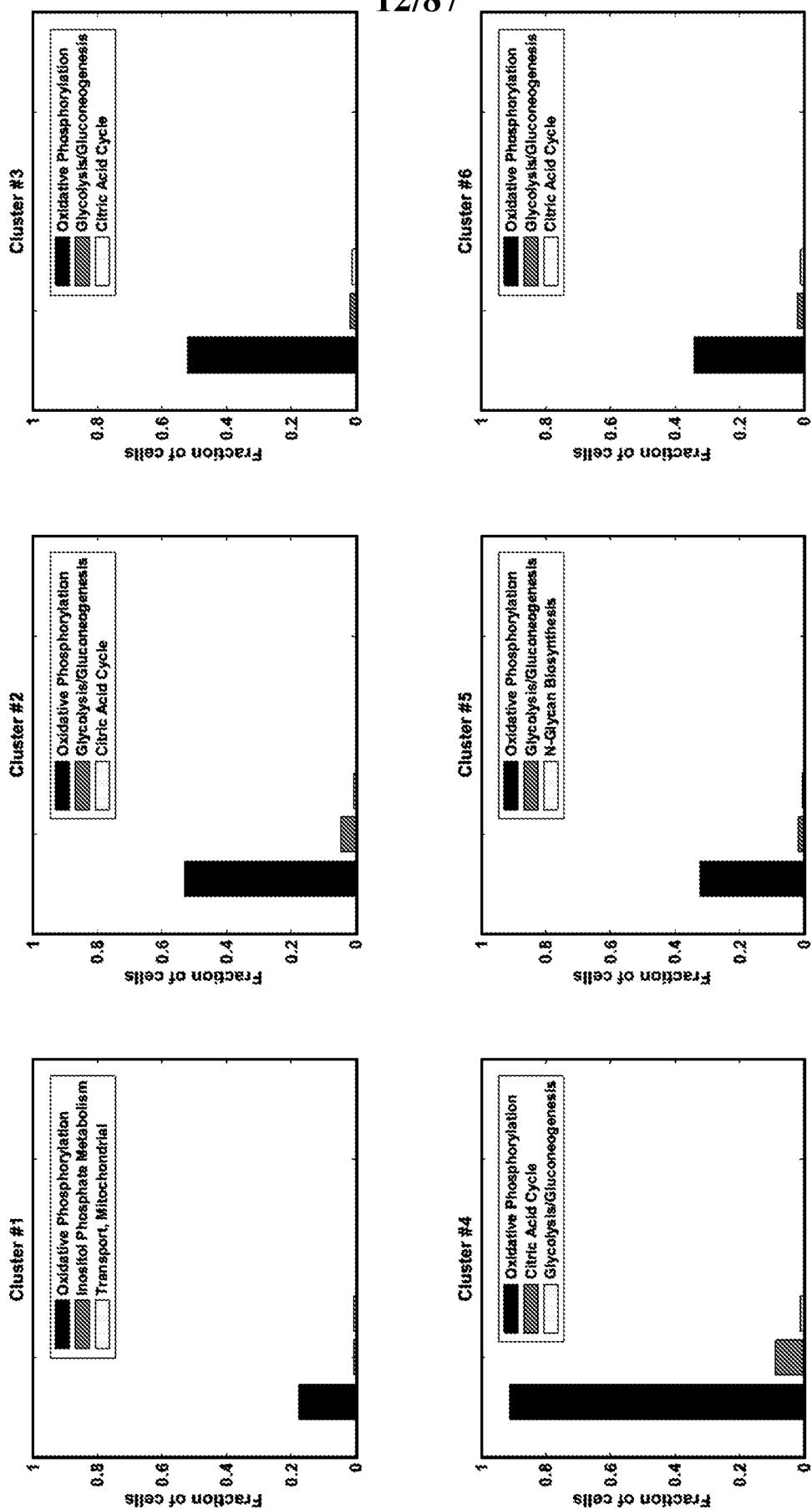


FIG. 12

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Metabolites associated with the transporter genes

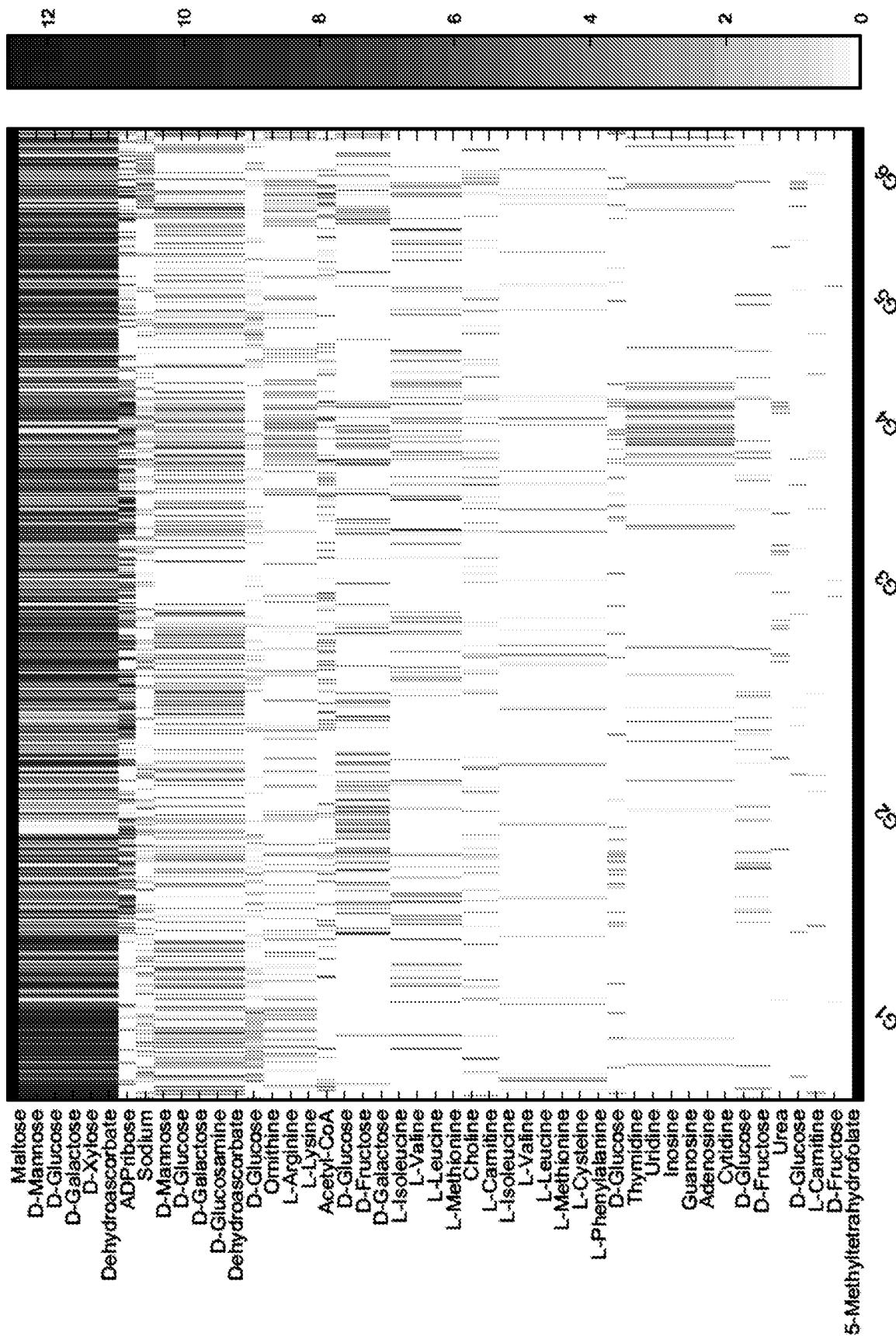


FIG. 13

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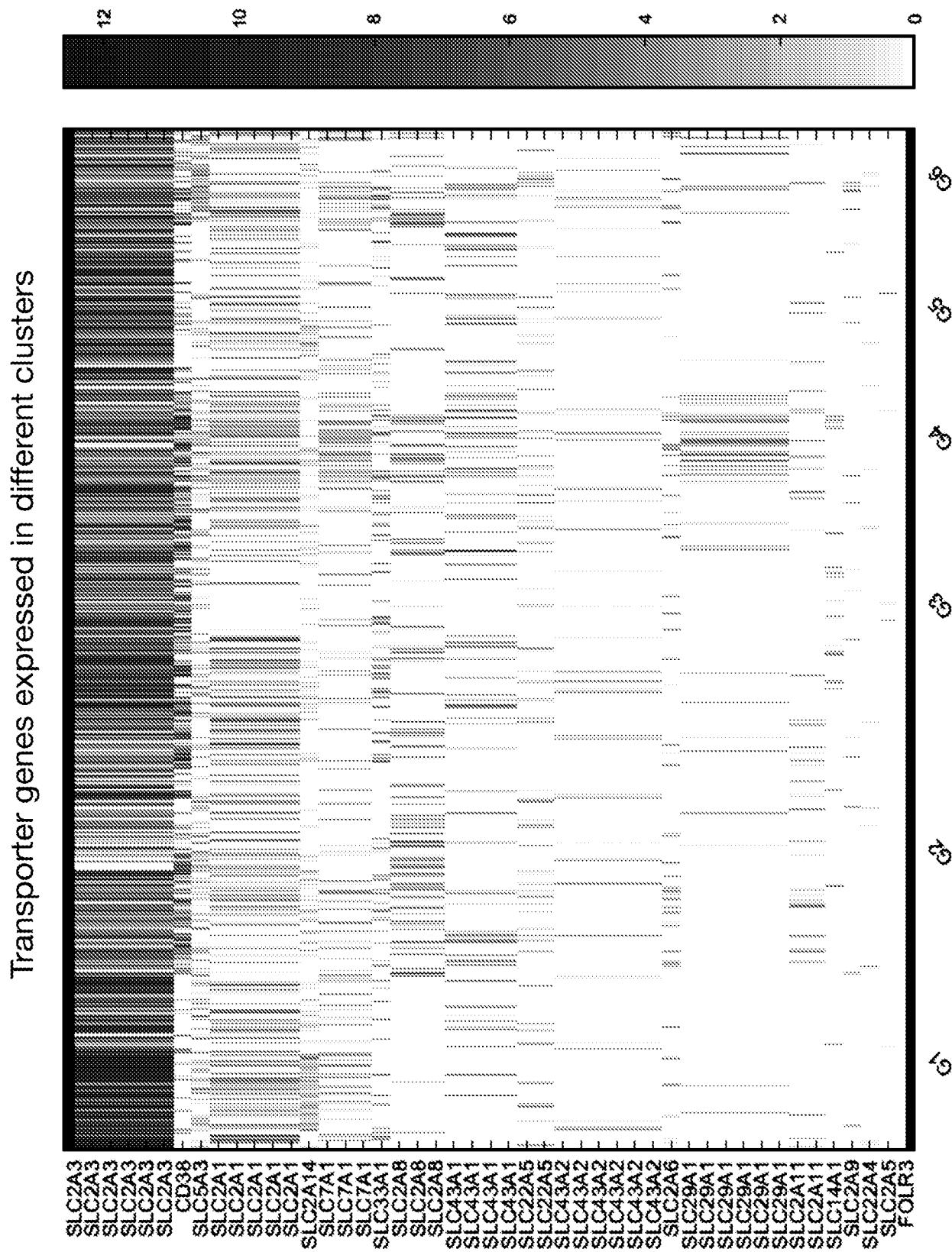
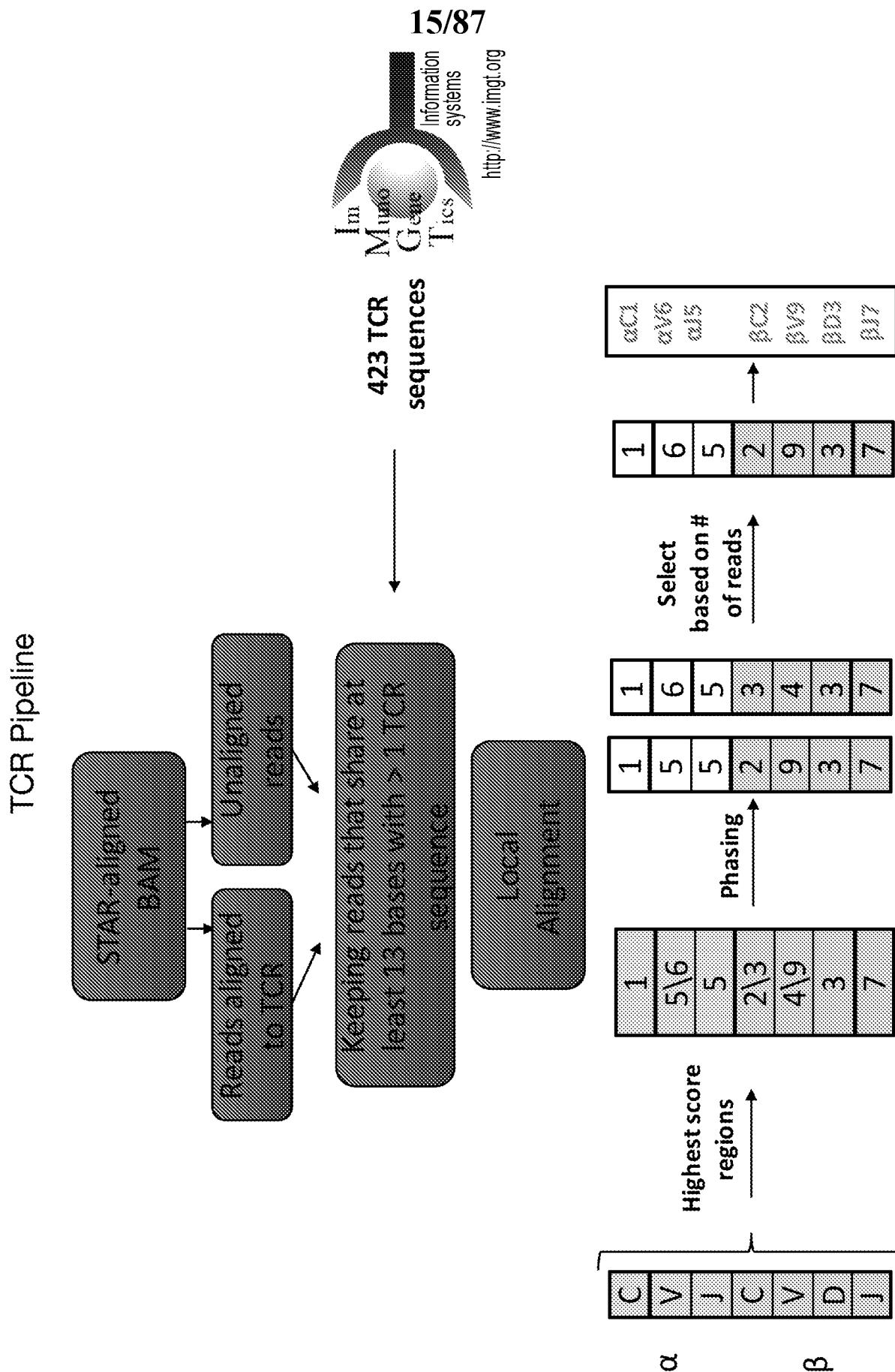


FIG. 14

**FIG. 15**

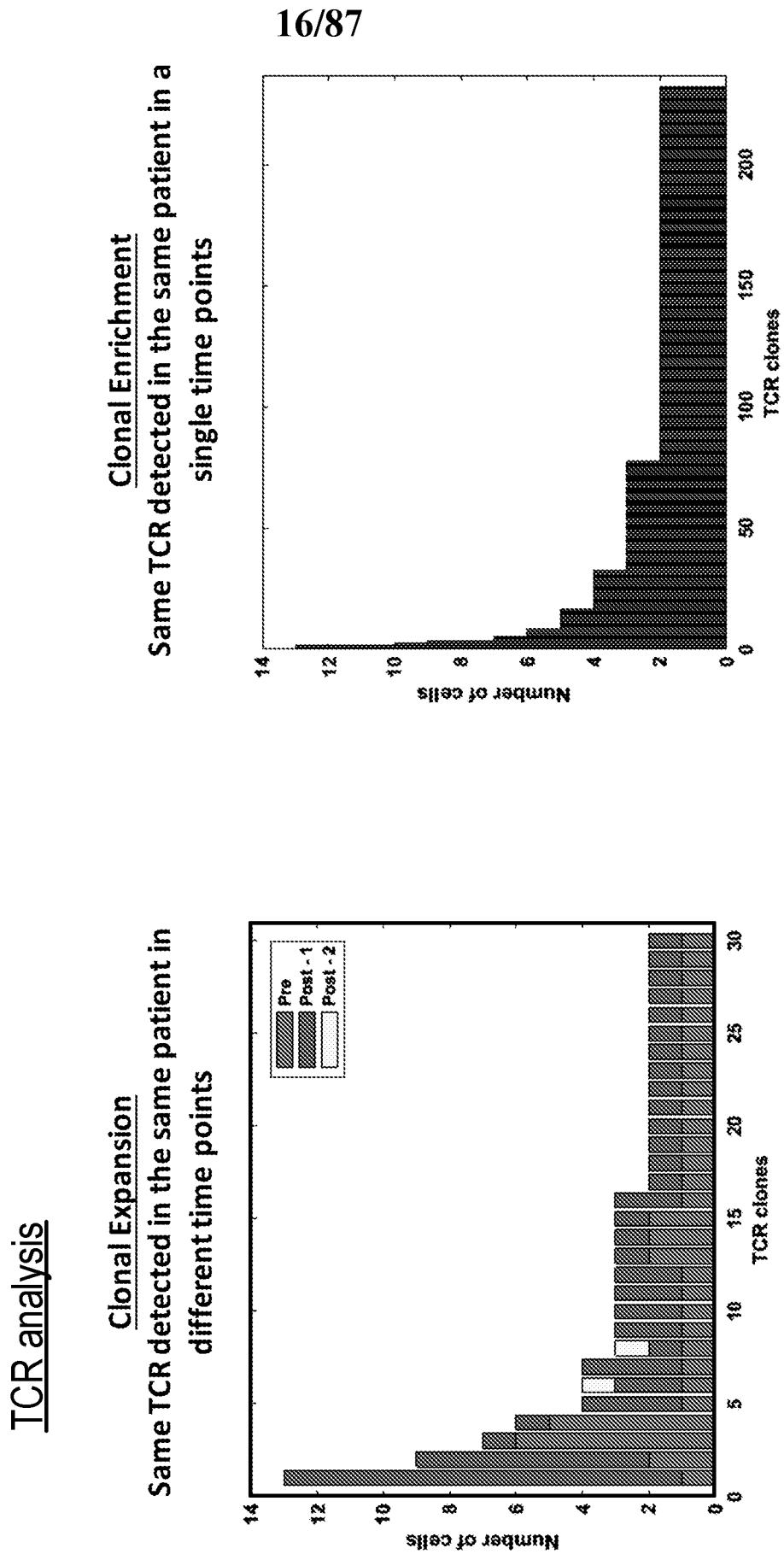


FIG. 16

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$\delta\gamma$ T-cells are Enriched in DN

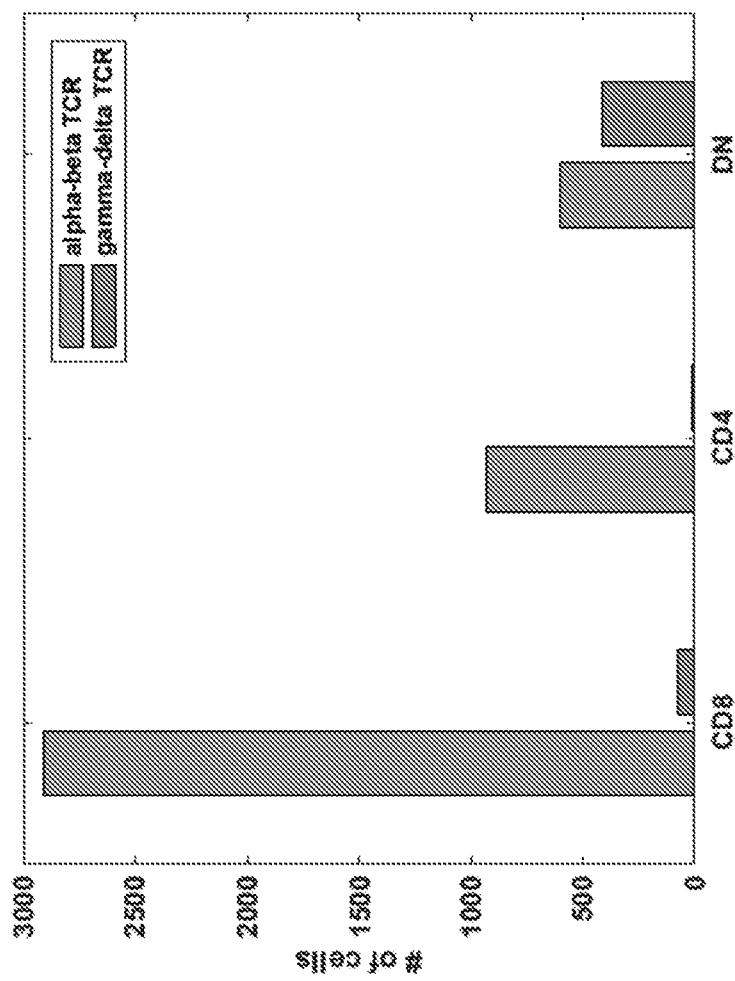


FIG. 17

Sy T-cells

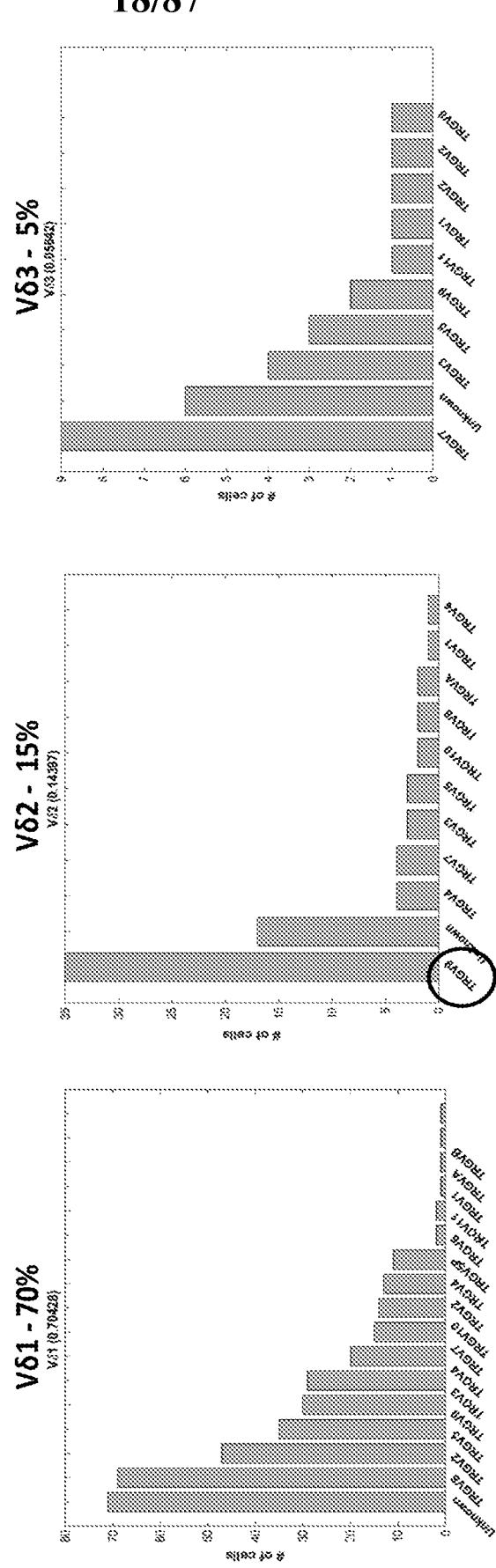
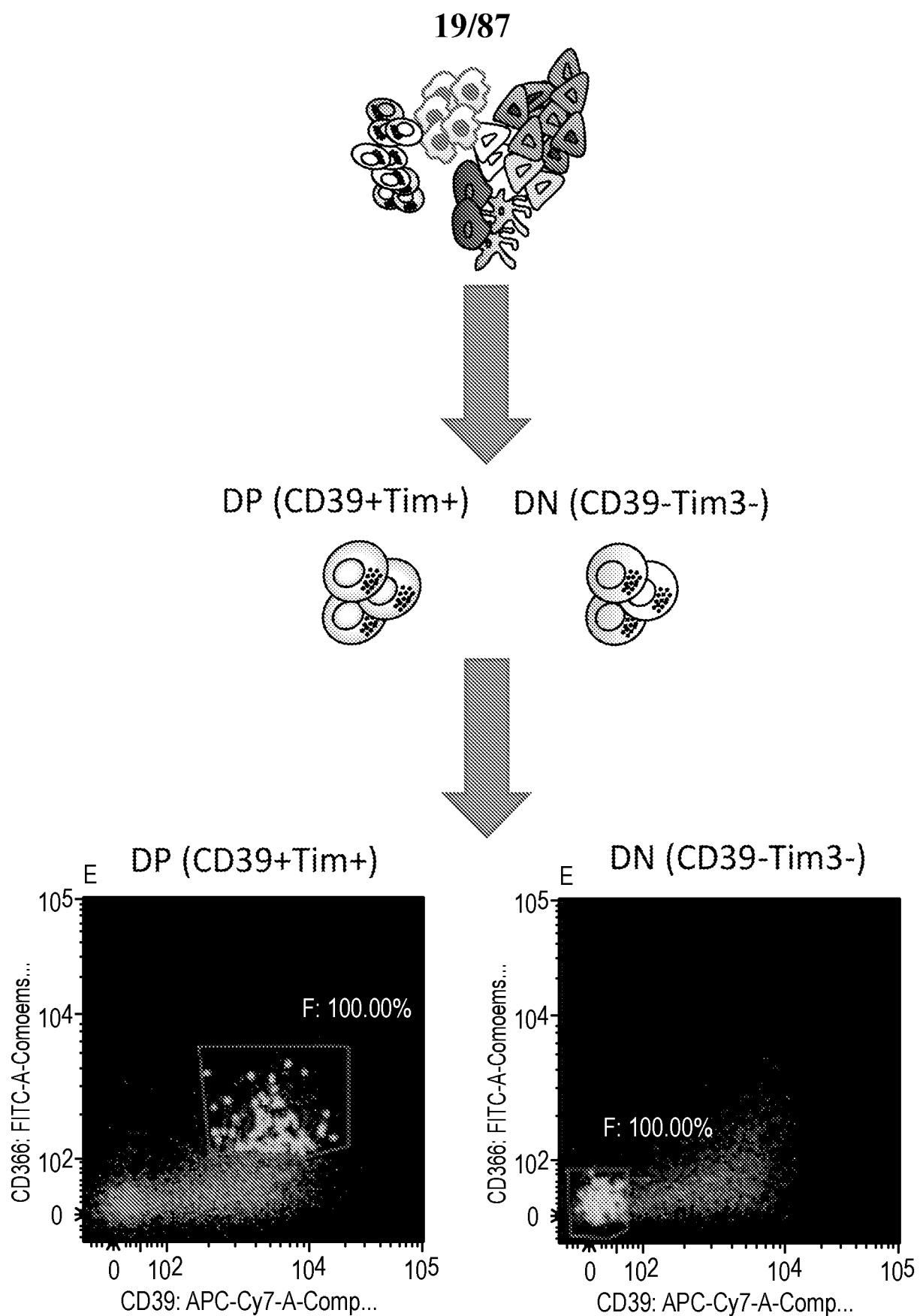


FIG. 18

**FIG. 19**

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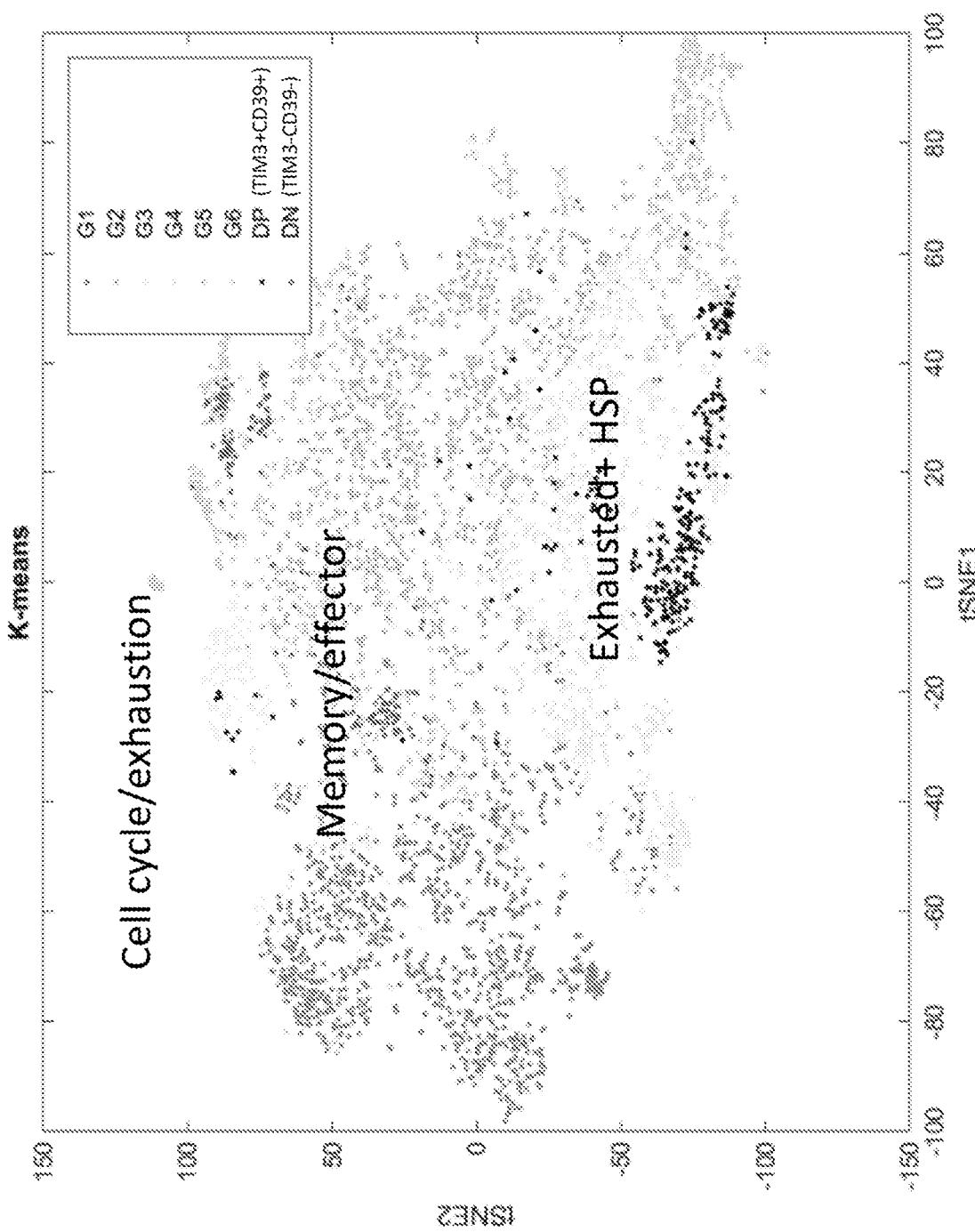


FIG. 20

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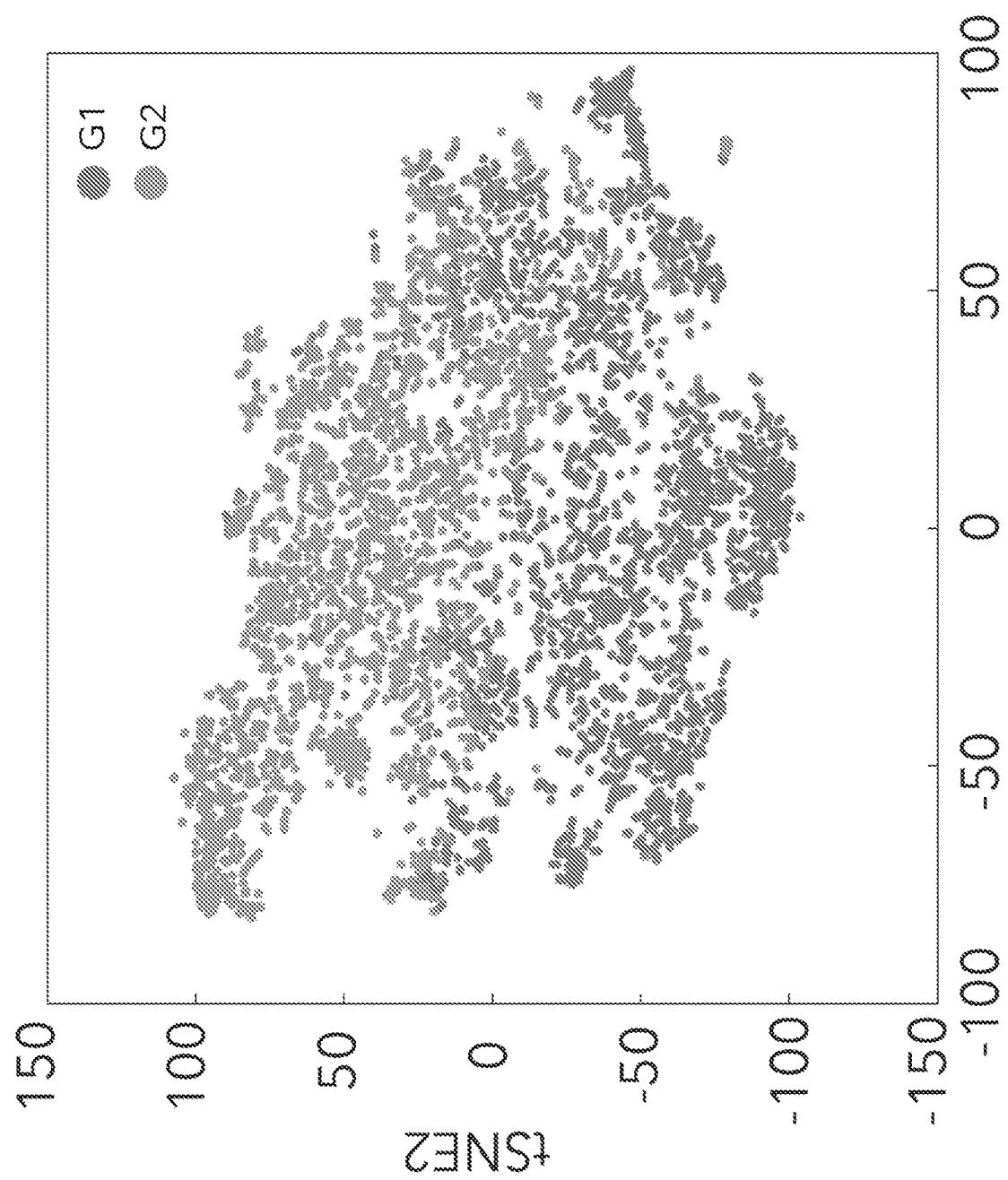


FIG. 21A

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b

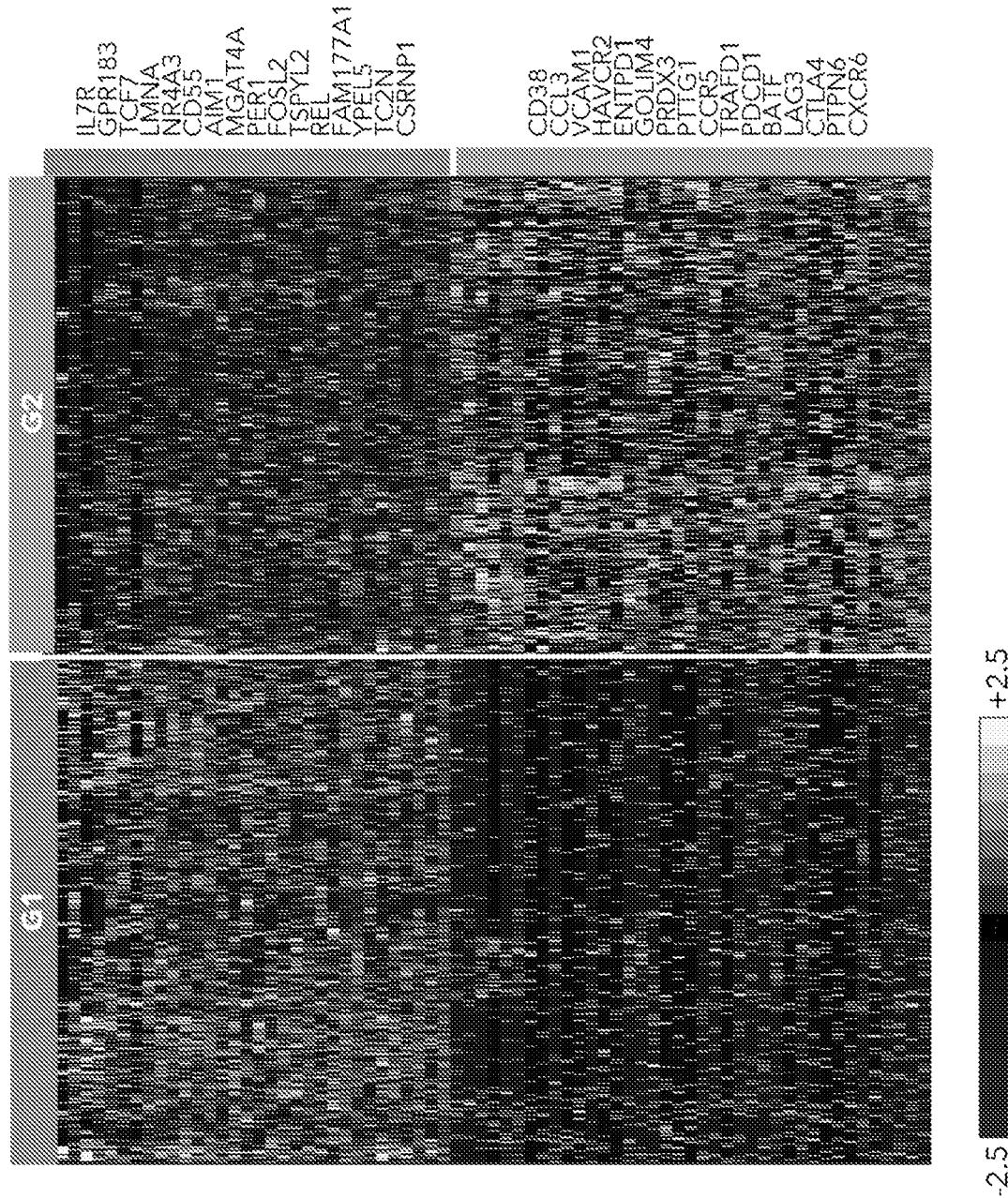


FIG. 21B

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C

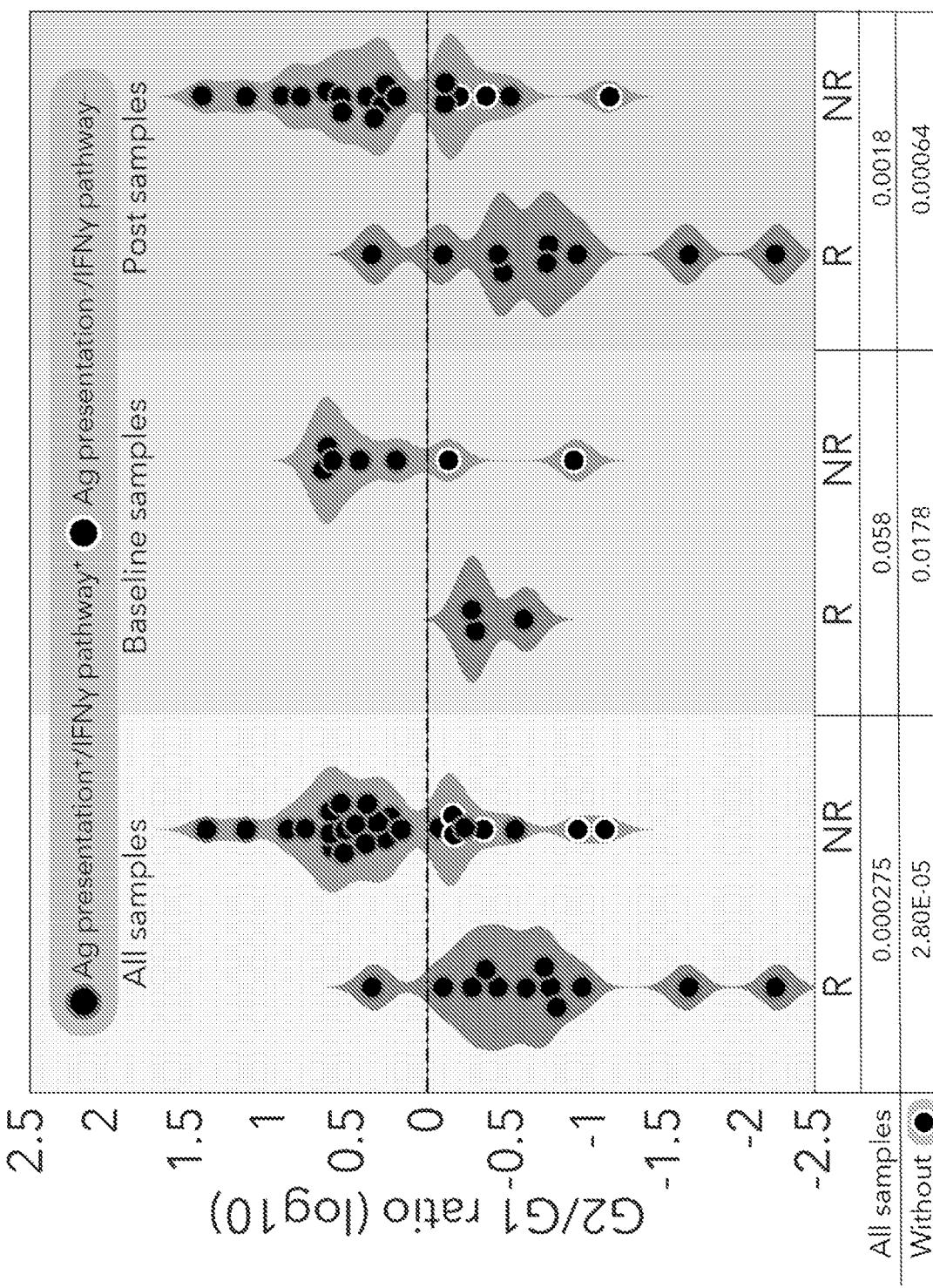
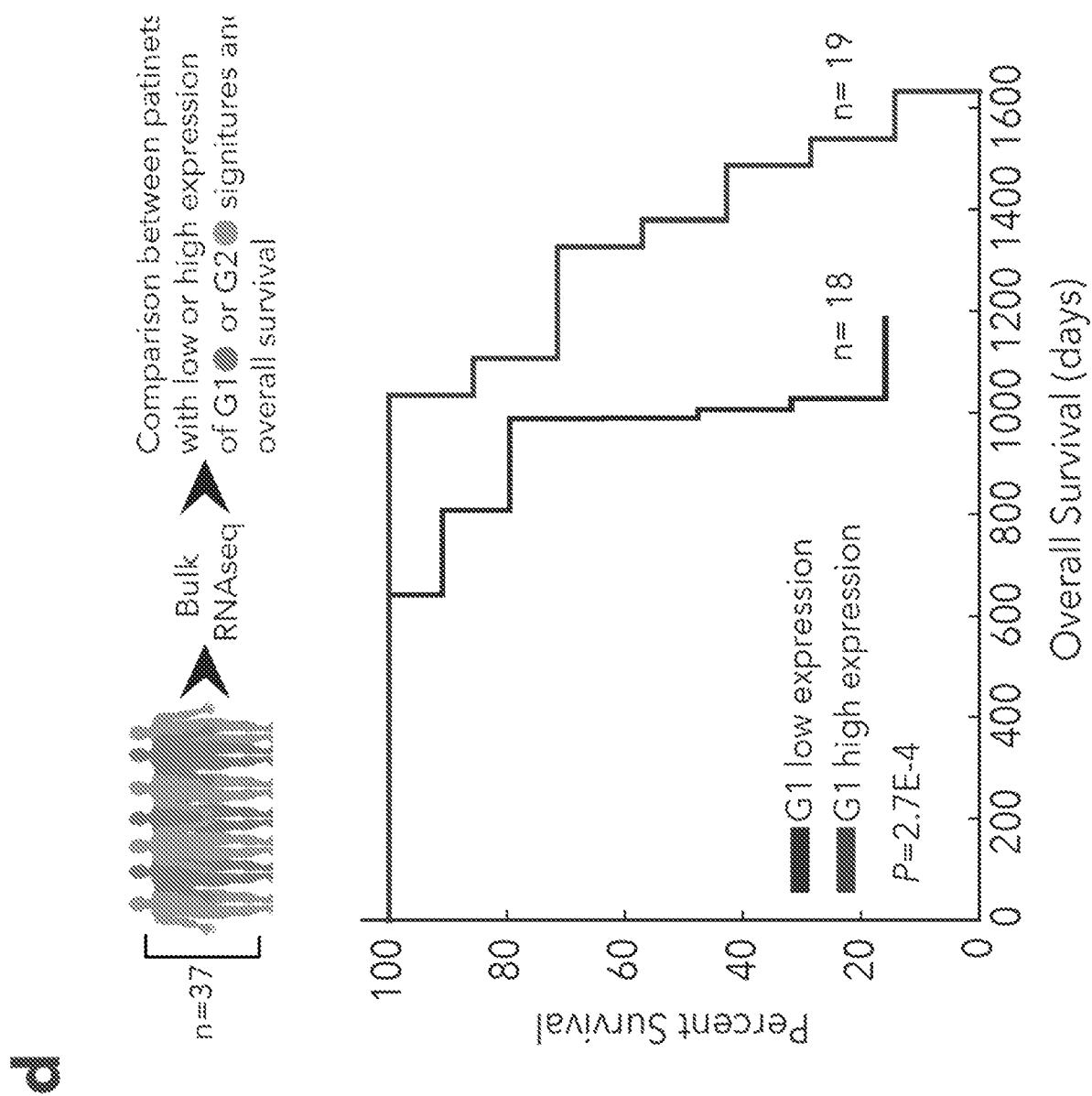
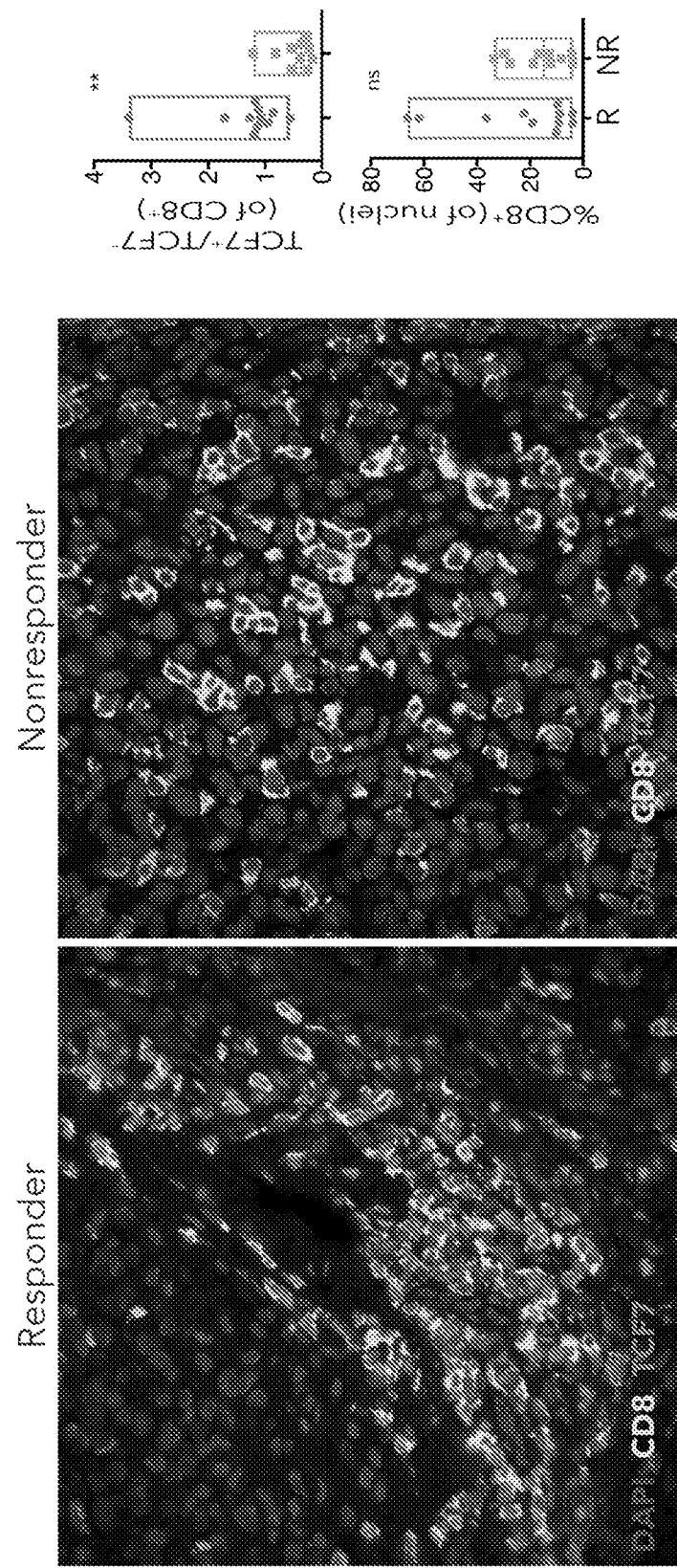


FIG. 21C

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**FIG. 21D**

25/87**FIG. 21E**

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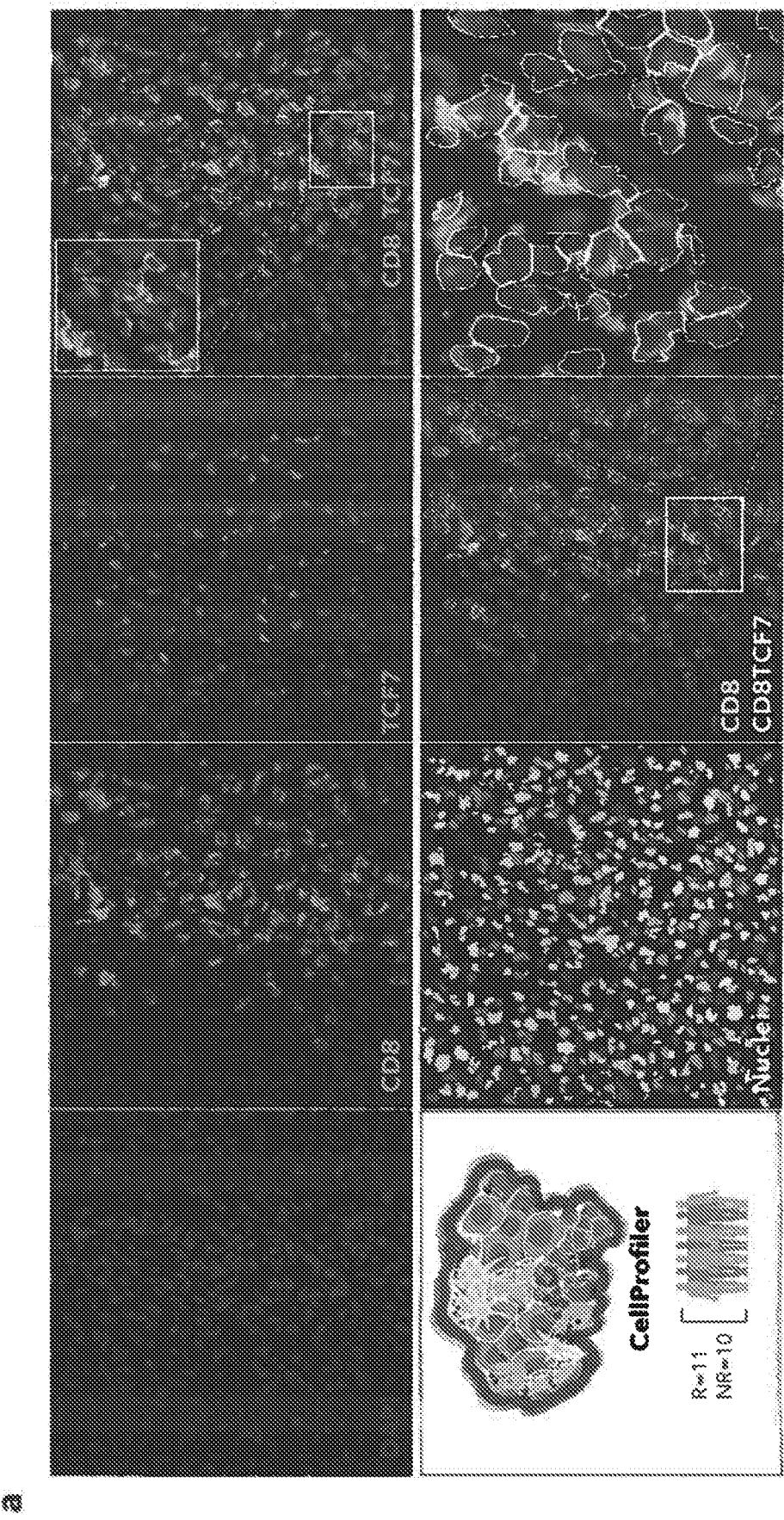
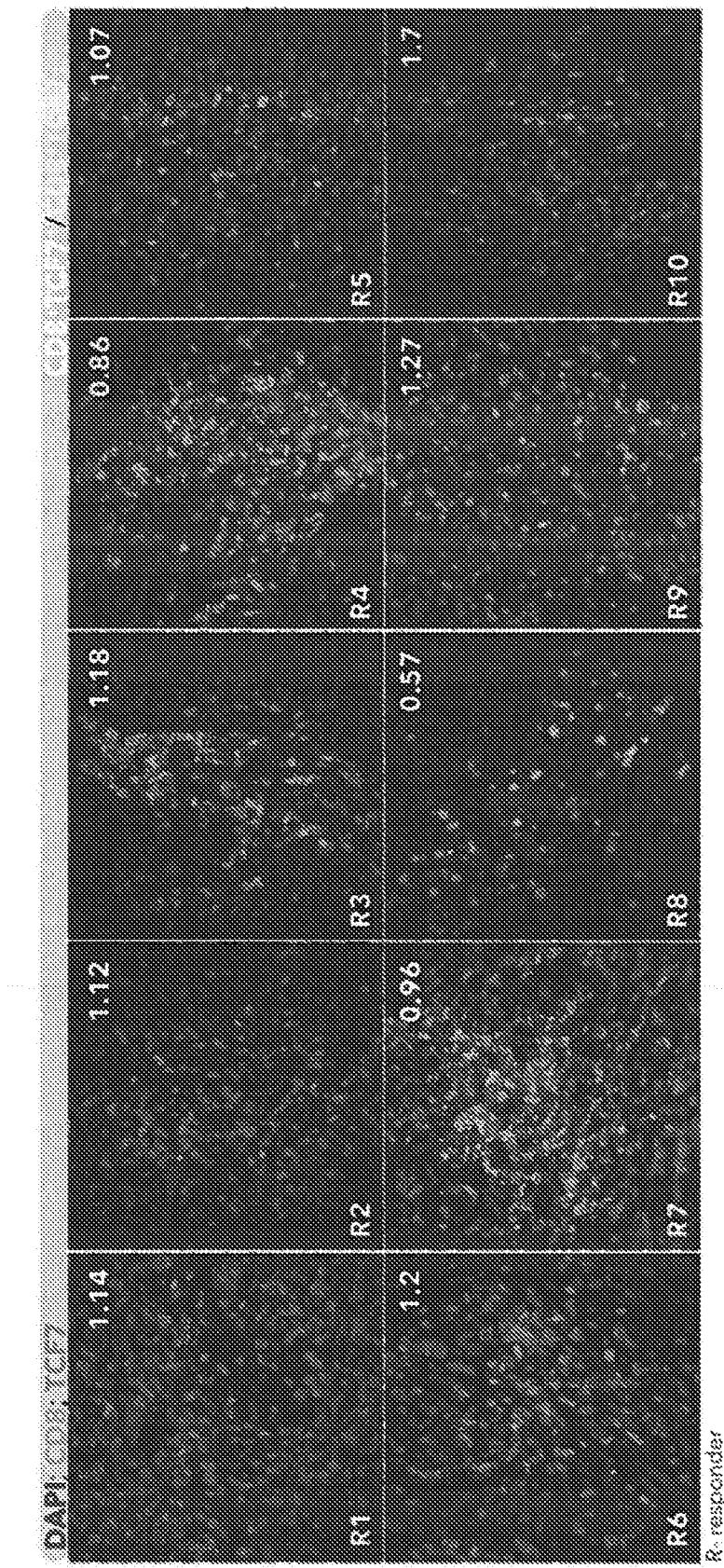


FIG. 22A

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b

FIG. 22B

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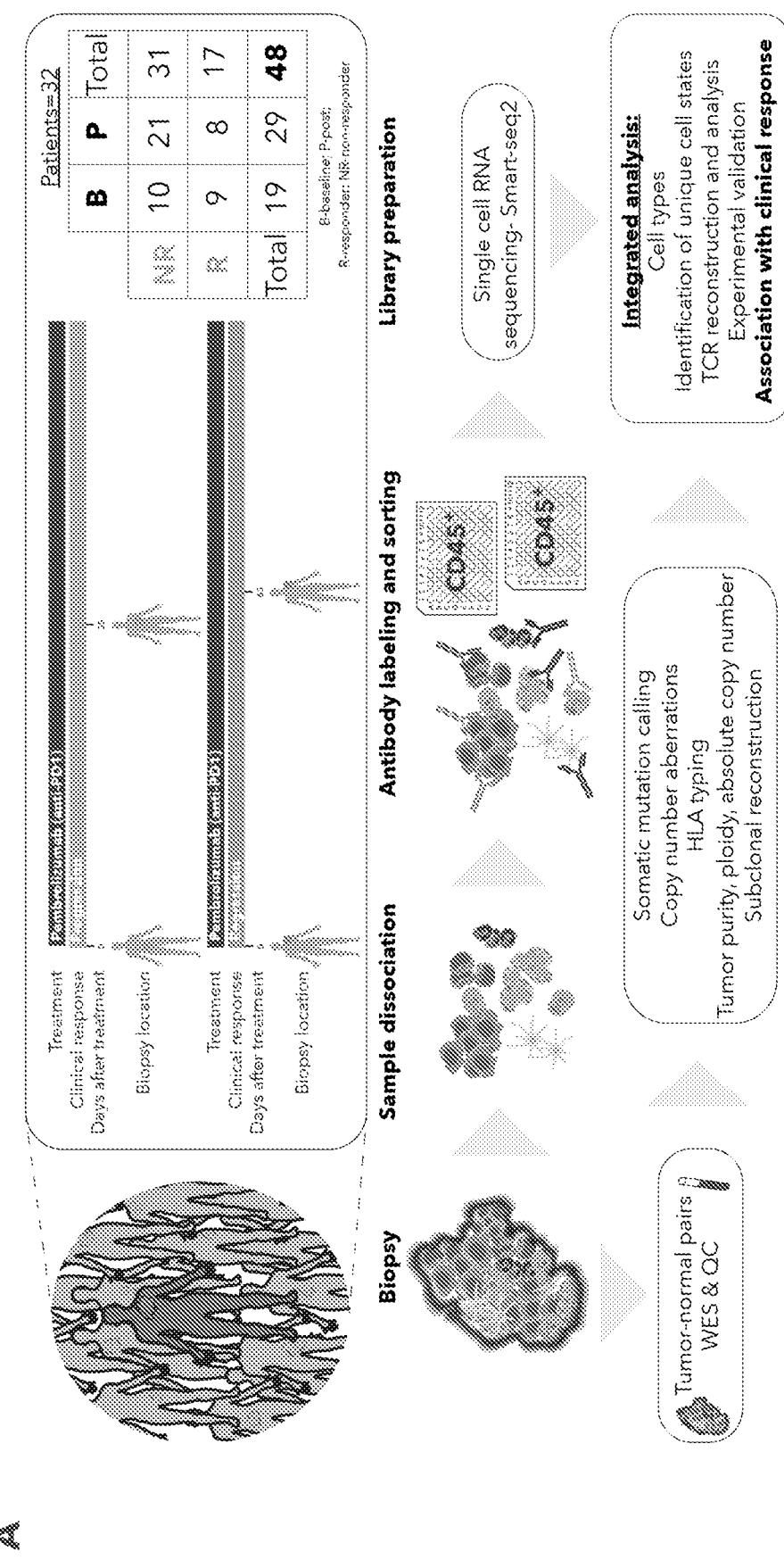
NR1	NR2	NR3	NR4	NR5	NR6	NR7	NR8	NR9	NR10
0.17	0.53	0.39	0.24	0.23	0.23	0.23	0.23	0.23	0.23

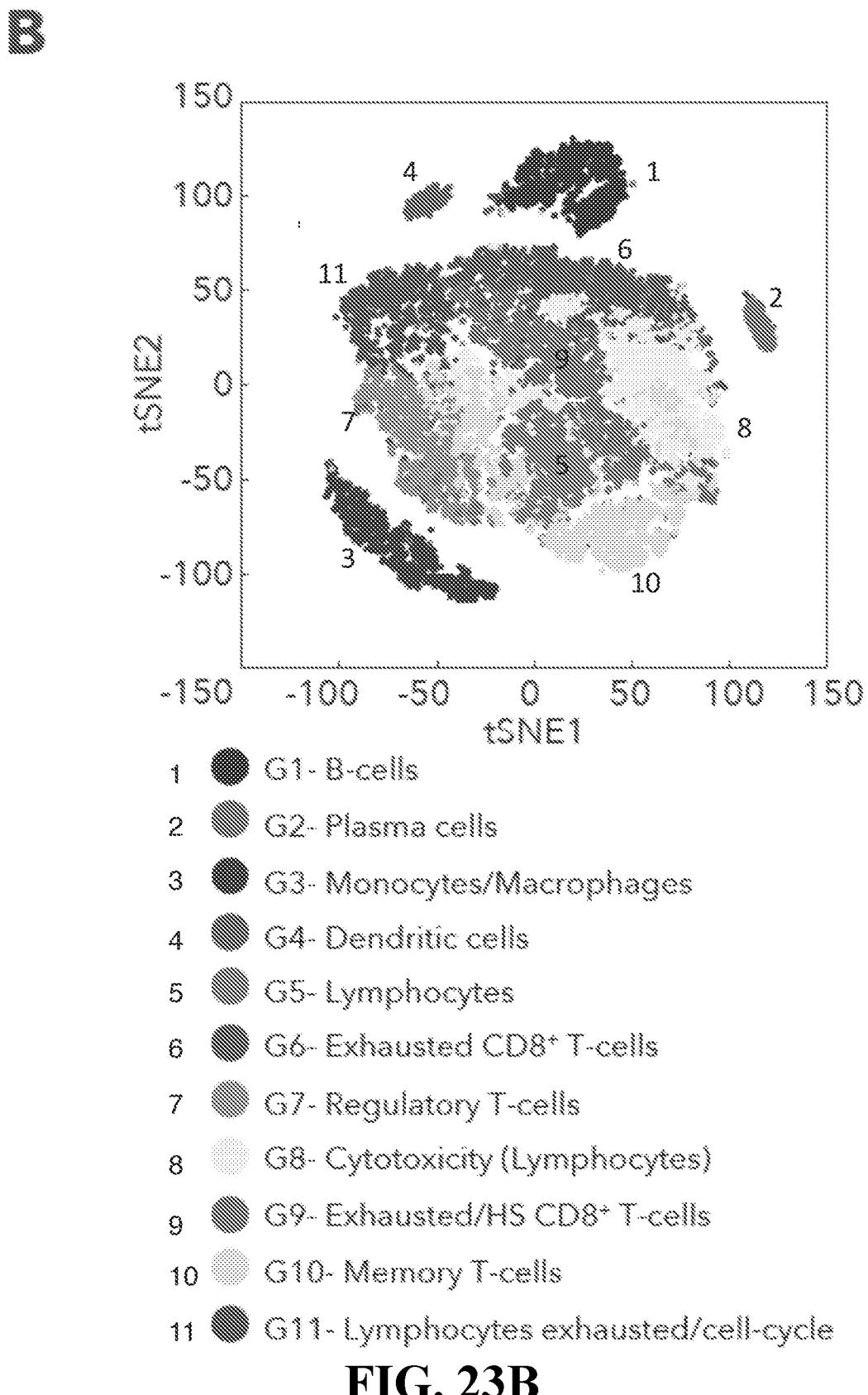
NR=nonresponder

C

FIG. 22C

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**FIG. 23A**

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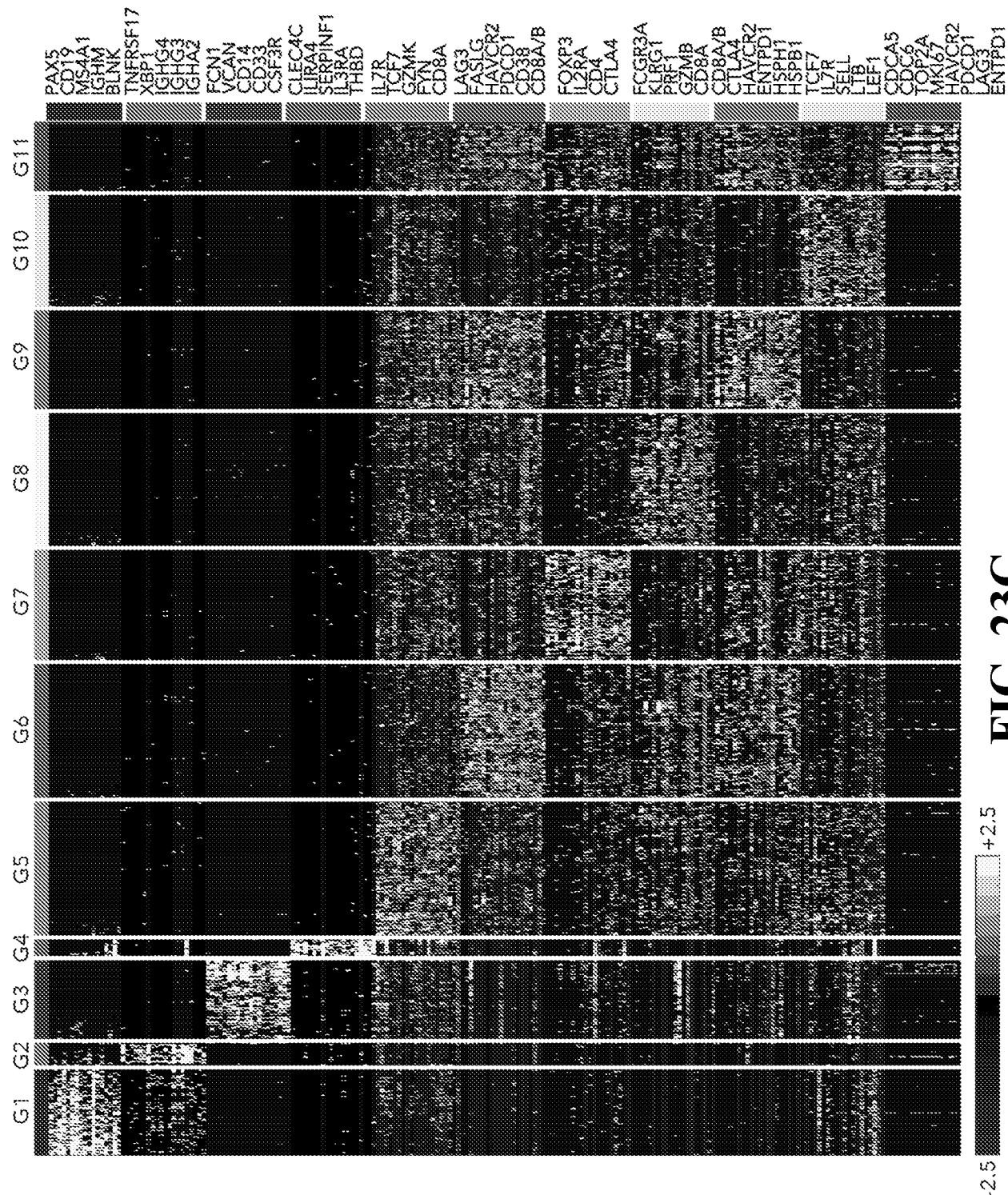
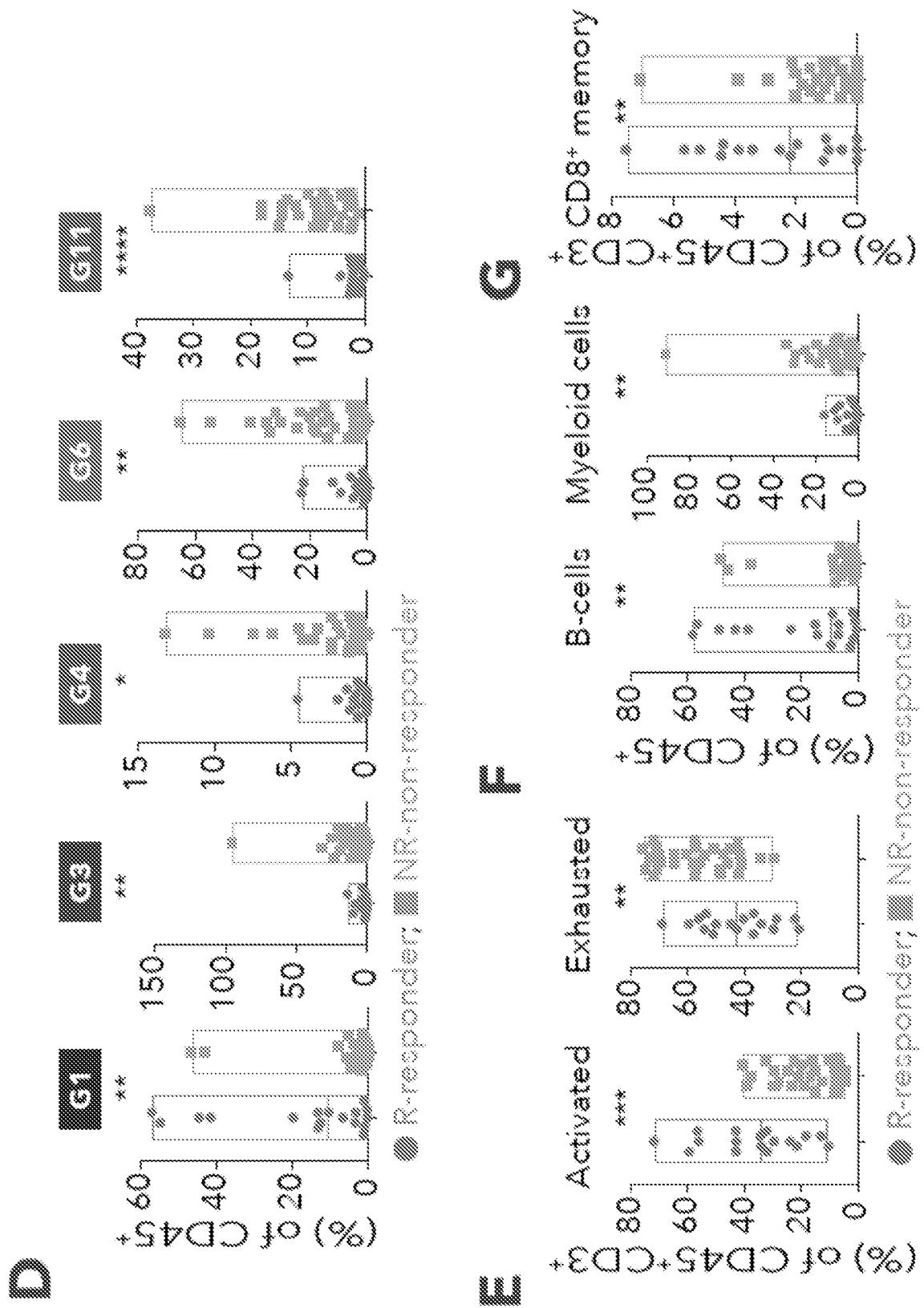


FIG. 23C

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**FIG. 23D-23G**

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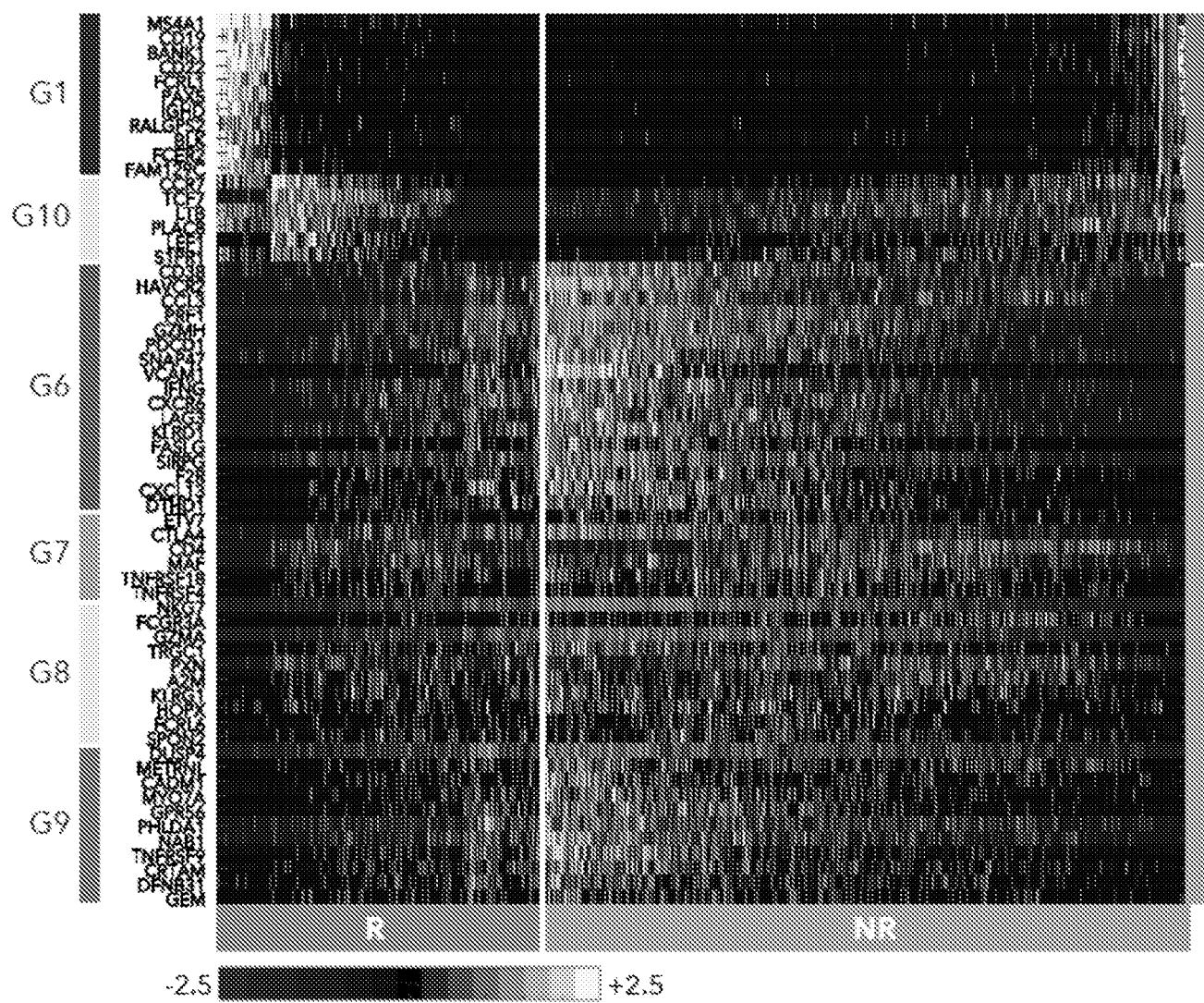


FIG. 23H

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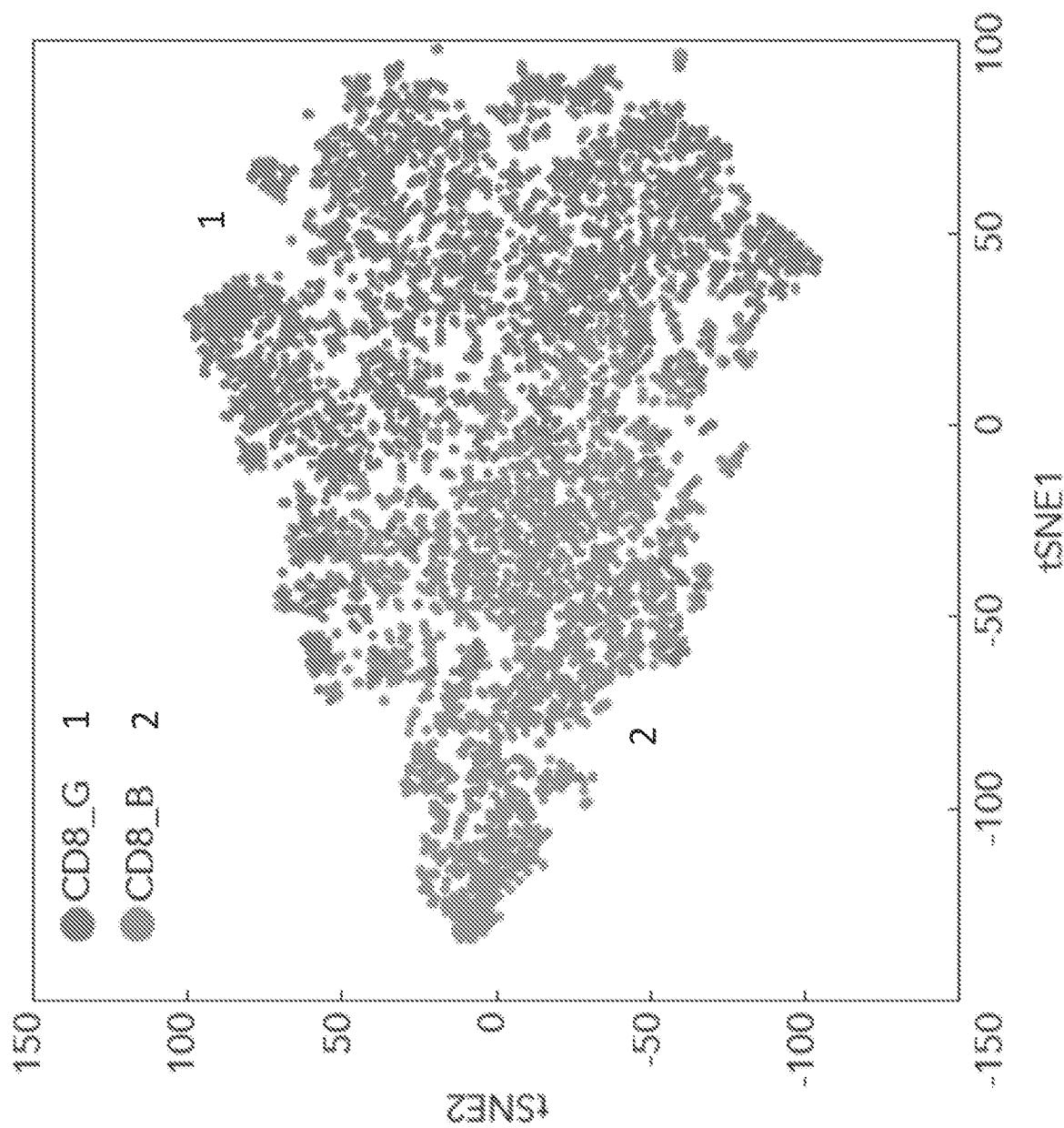


FIG. 24A

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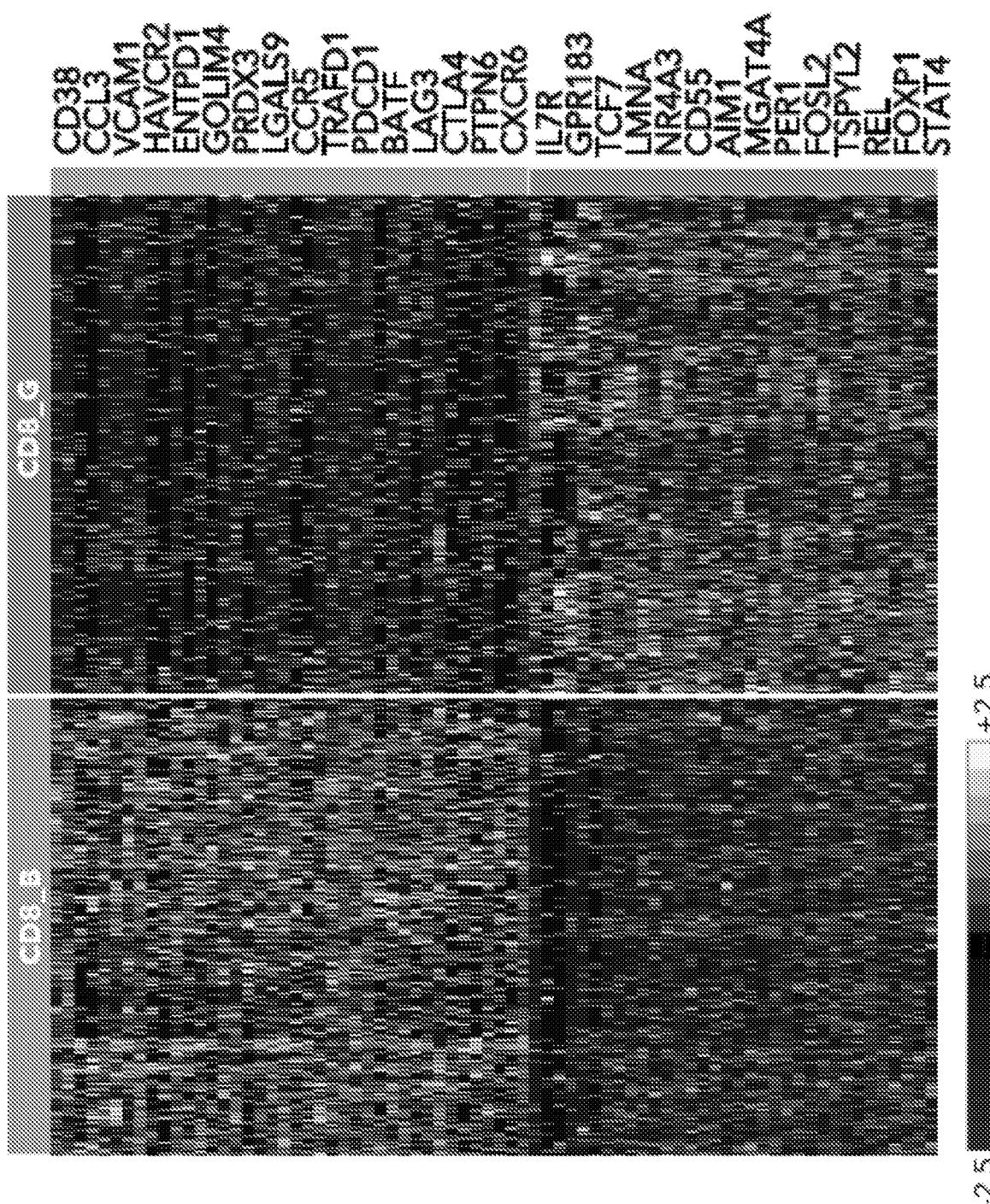
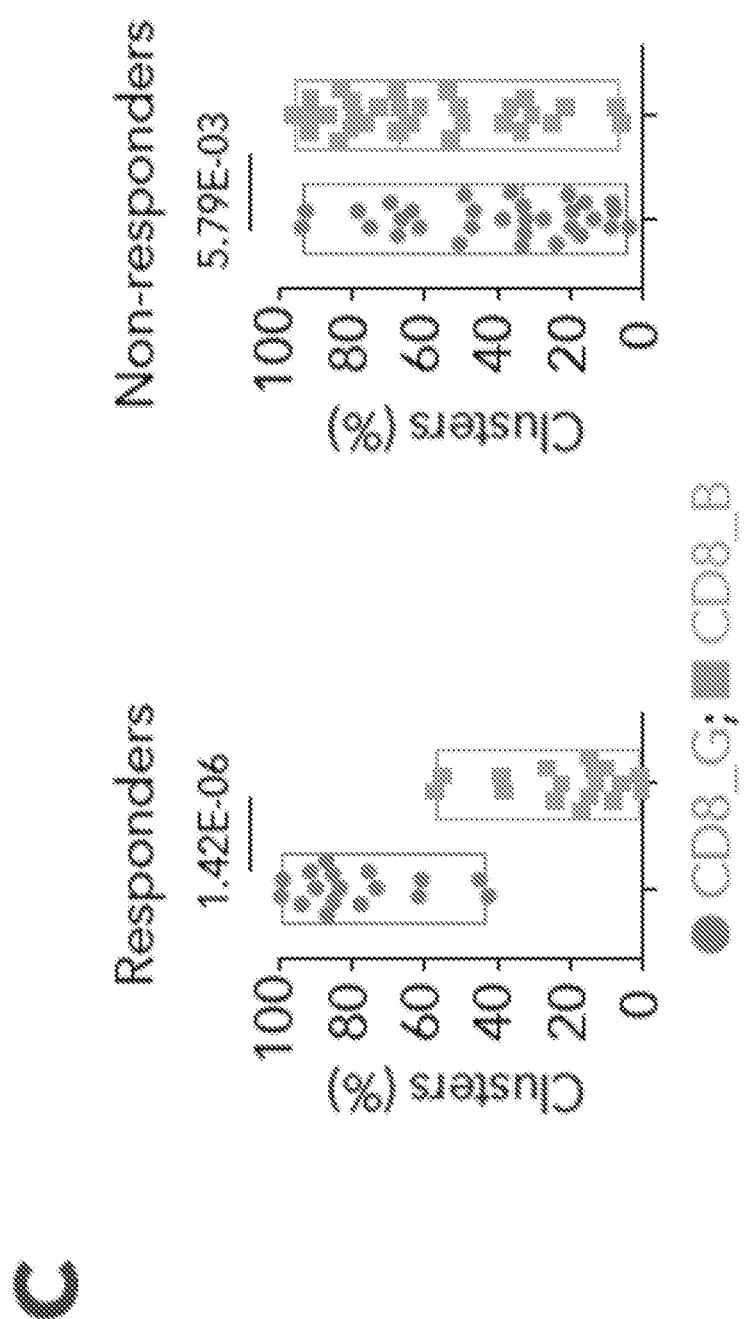


FIG. 24B

36/87**FIG. 24C**

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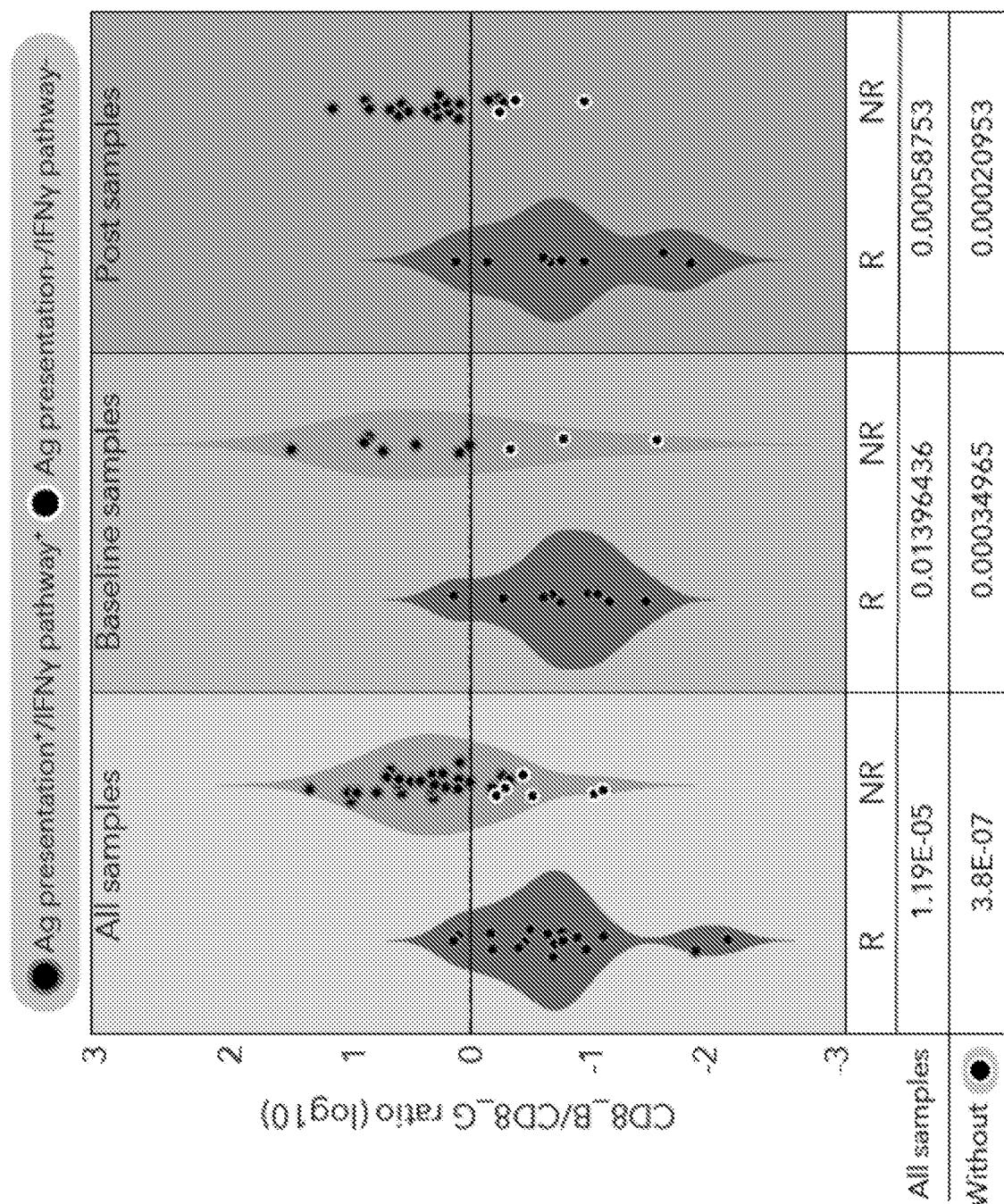


FIG. 24D

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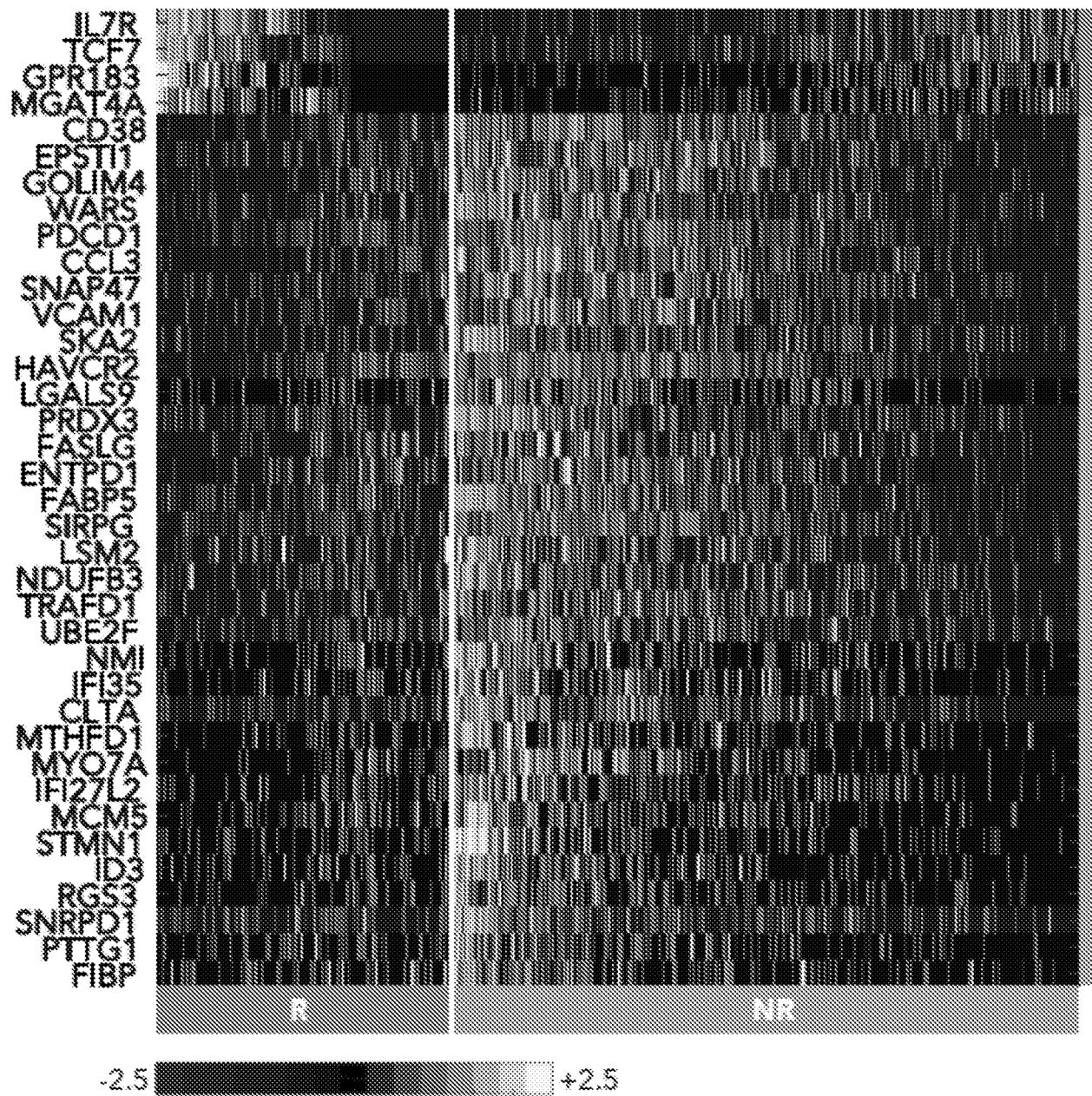


FIG. 24E

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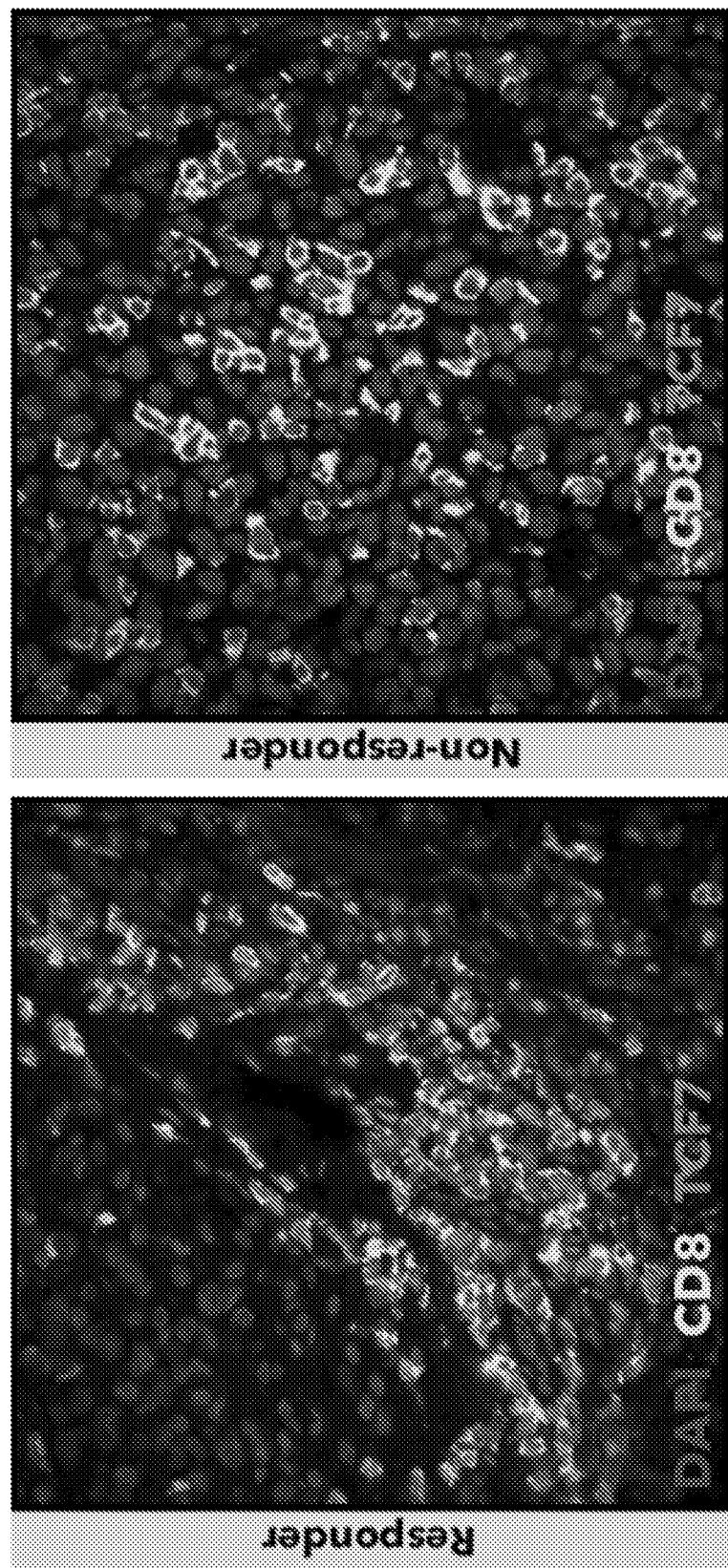


FIG. 24F

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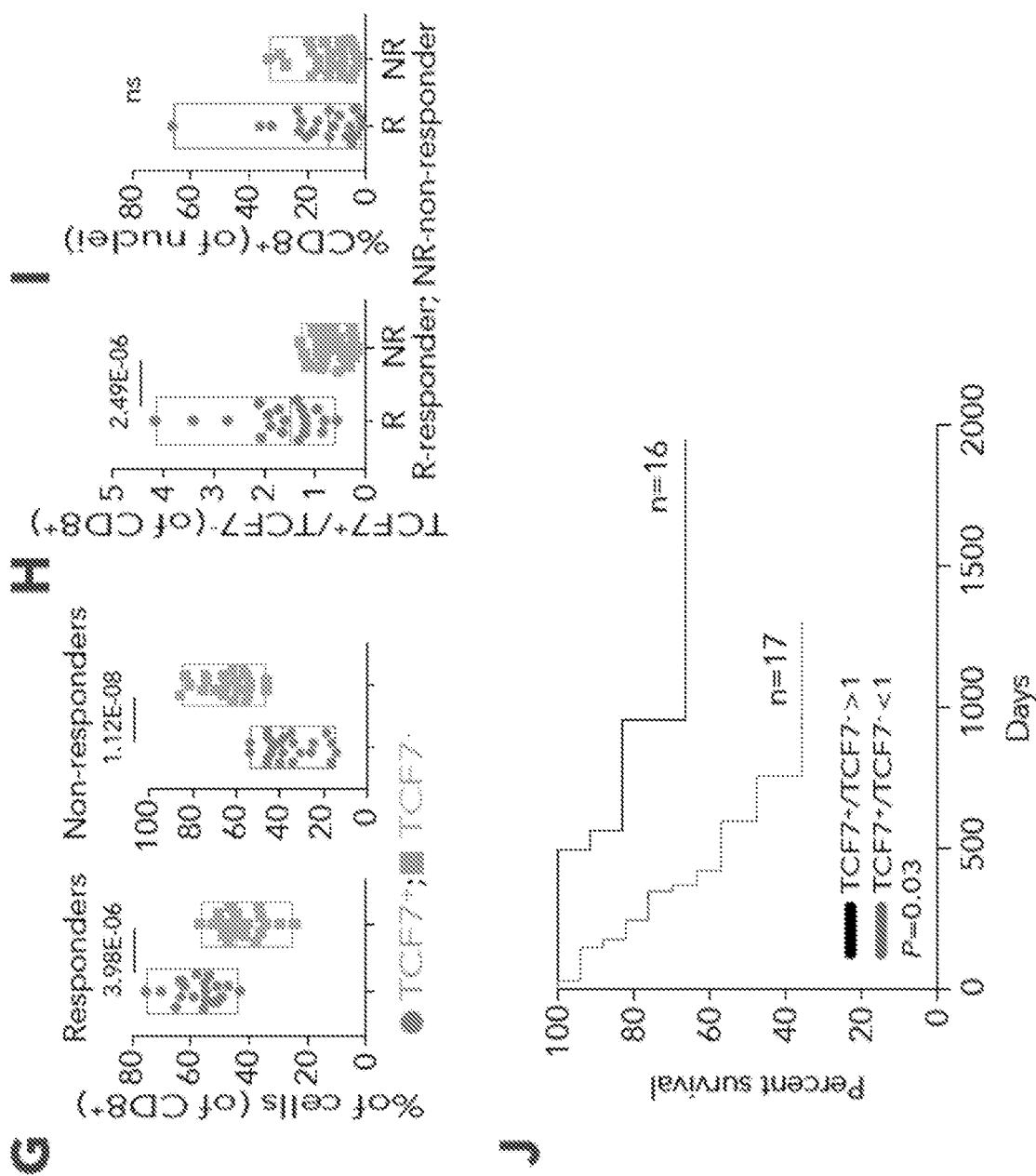


FIG. 24G-24J

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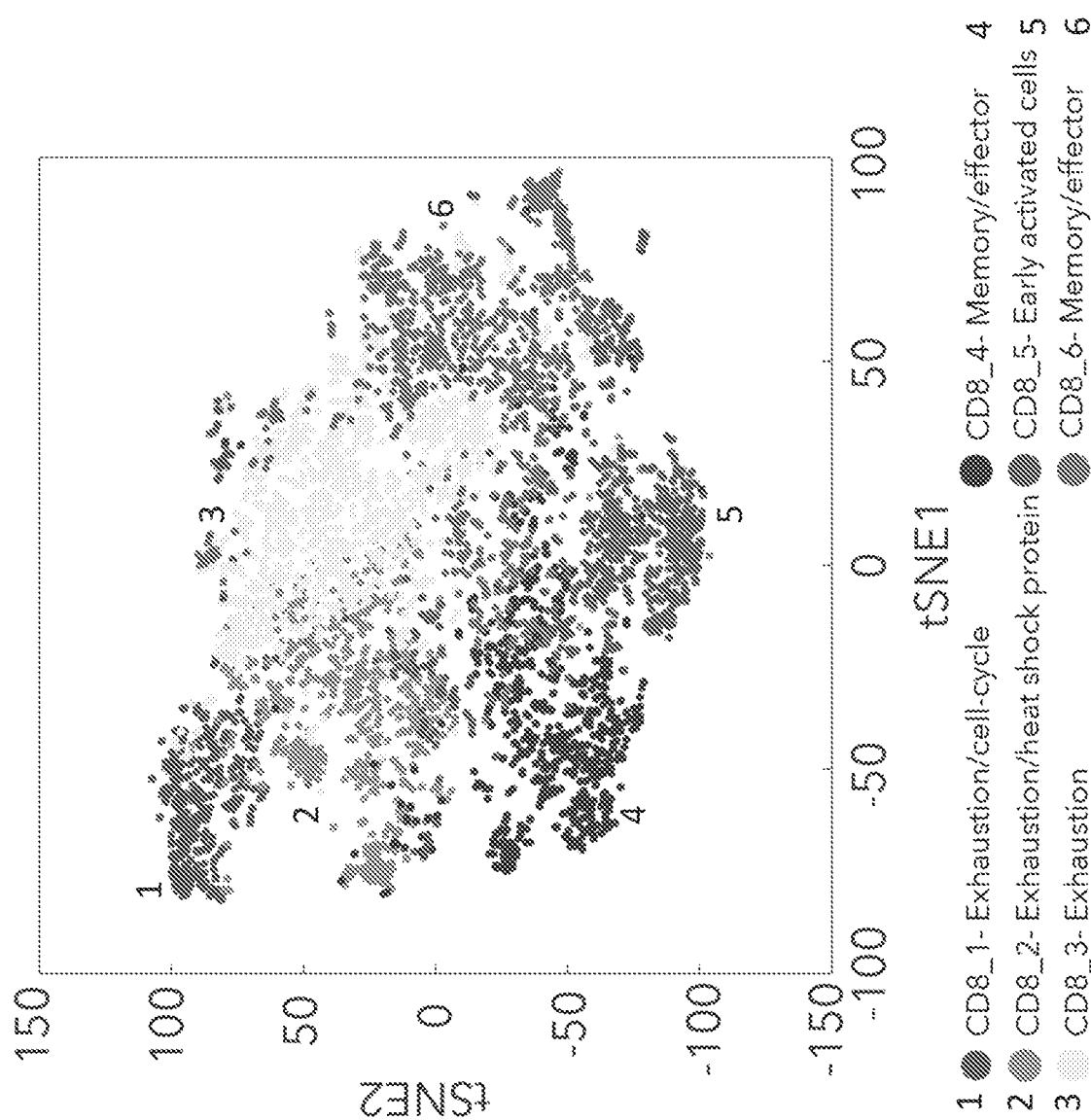


FIG. 25A

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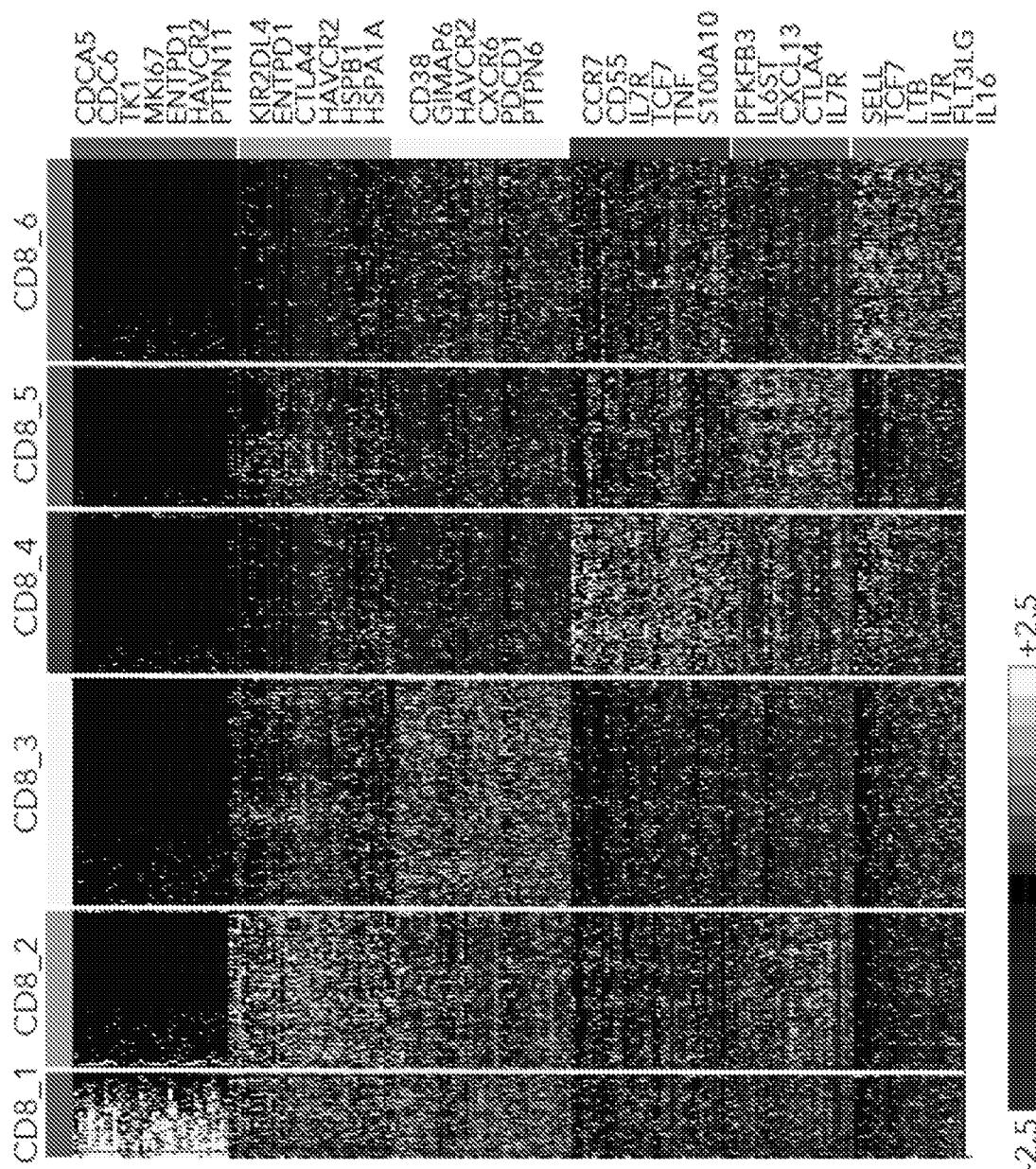


FIG. 25B

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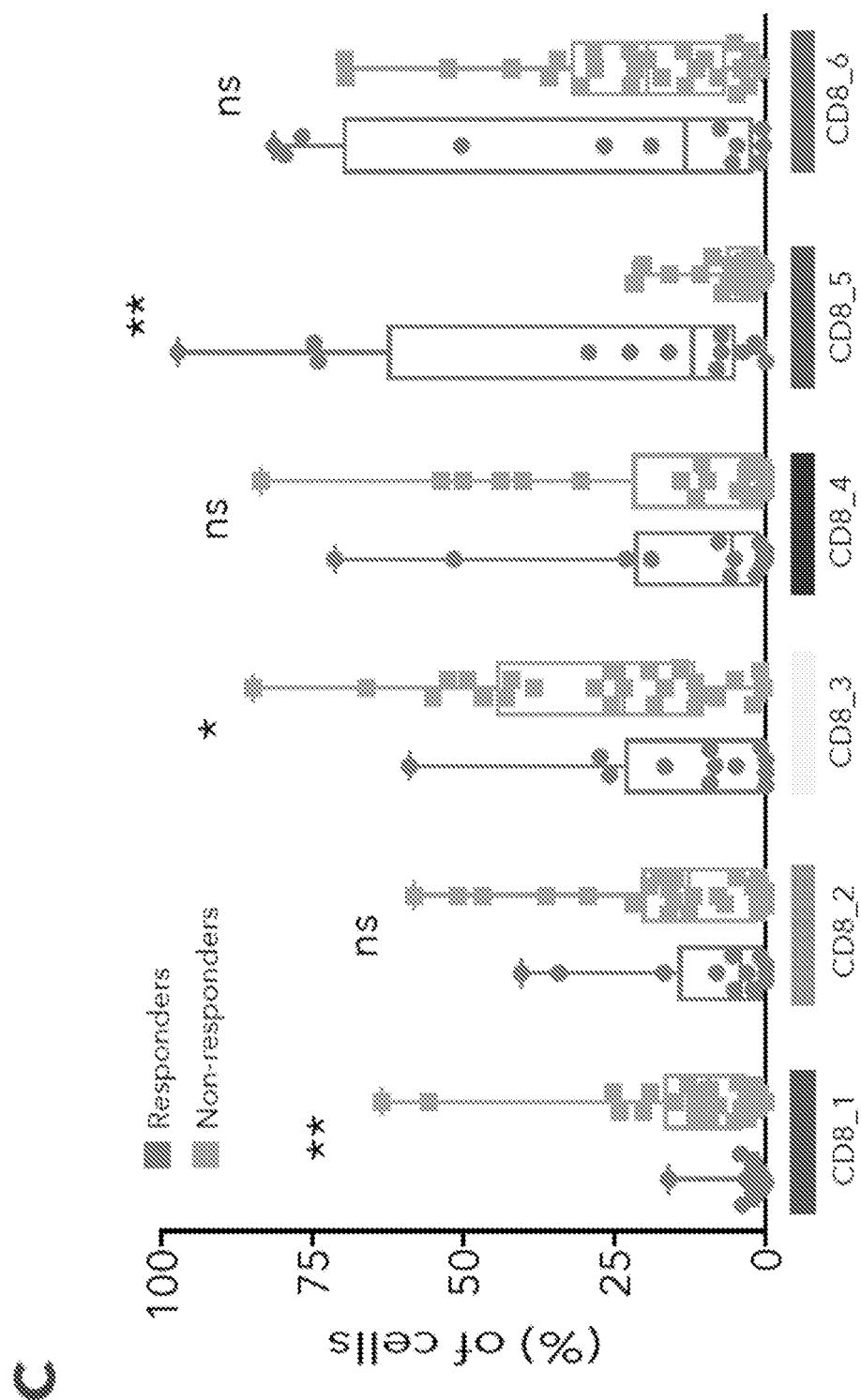
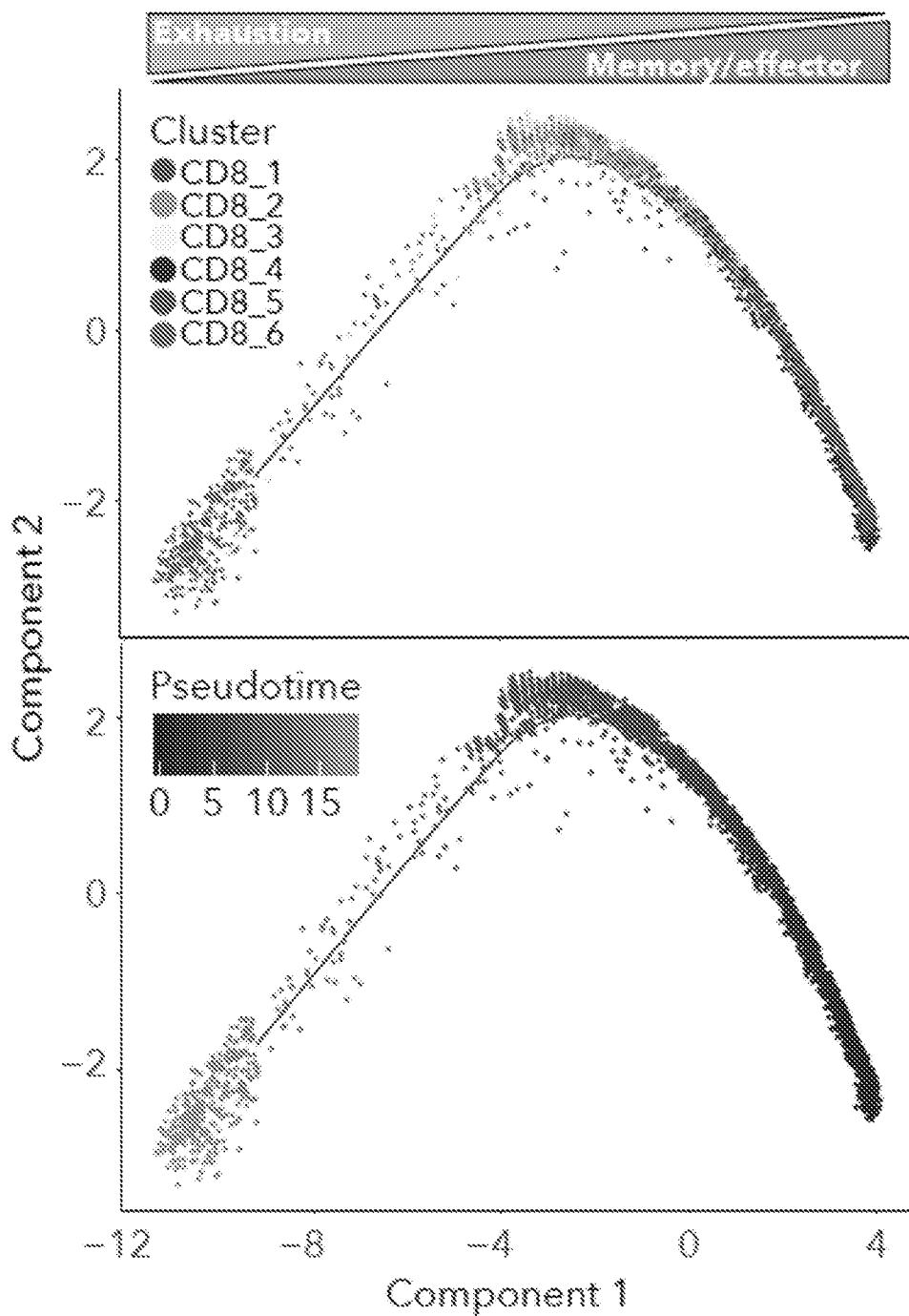


FIG. 25C

44/87**D****FIG. 25D**

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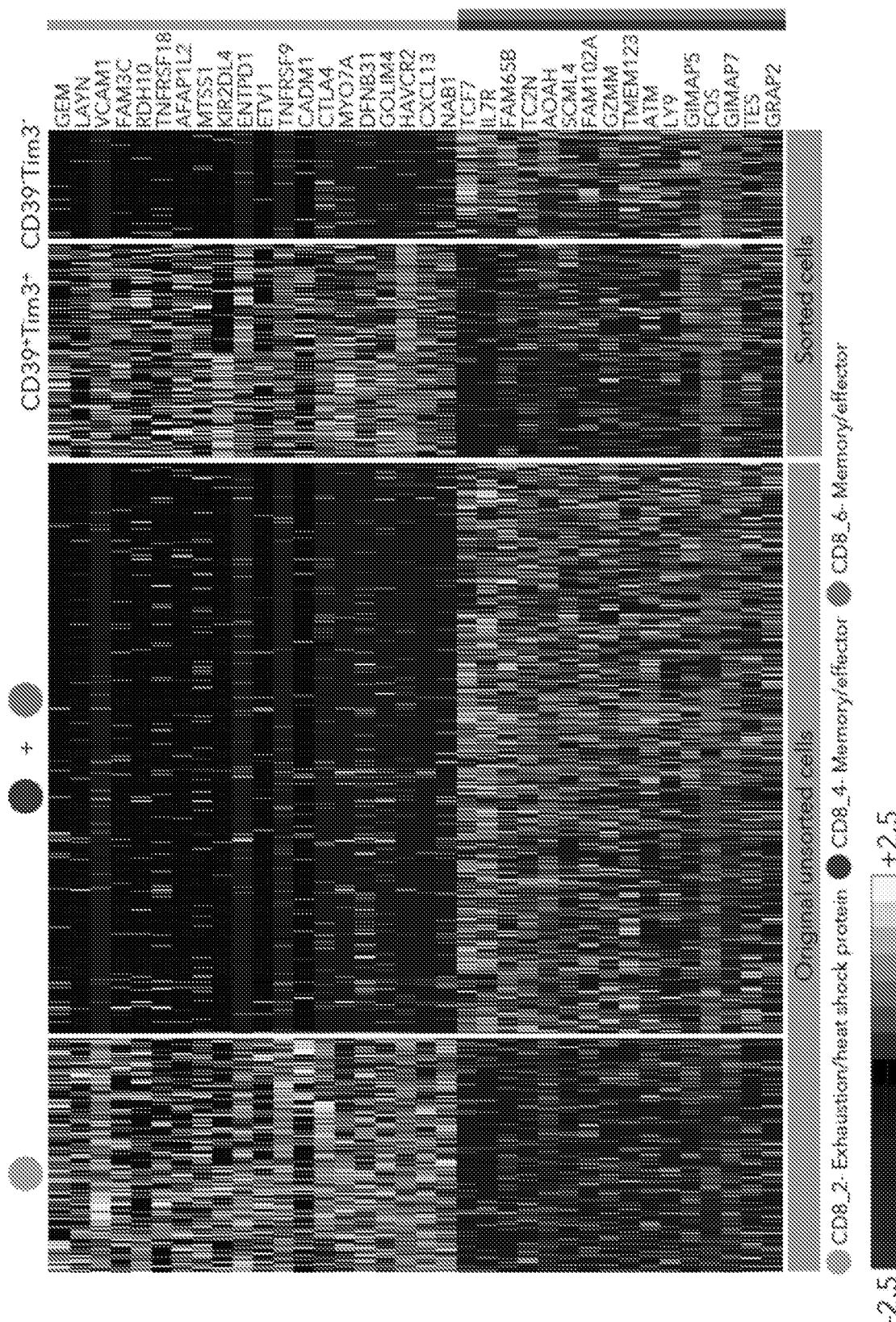
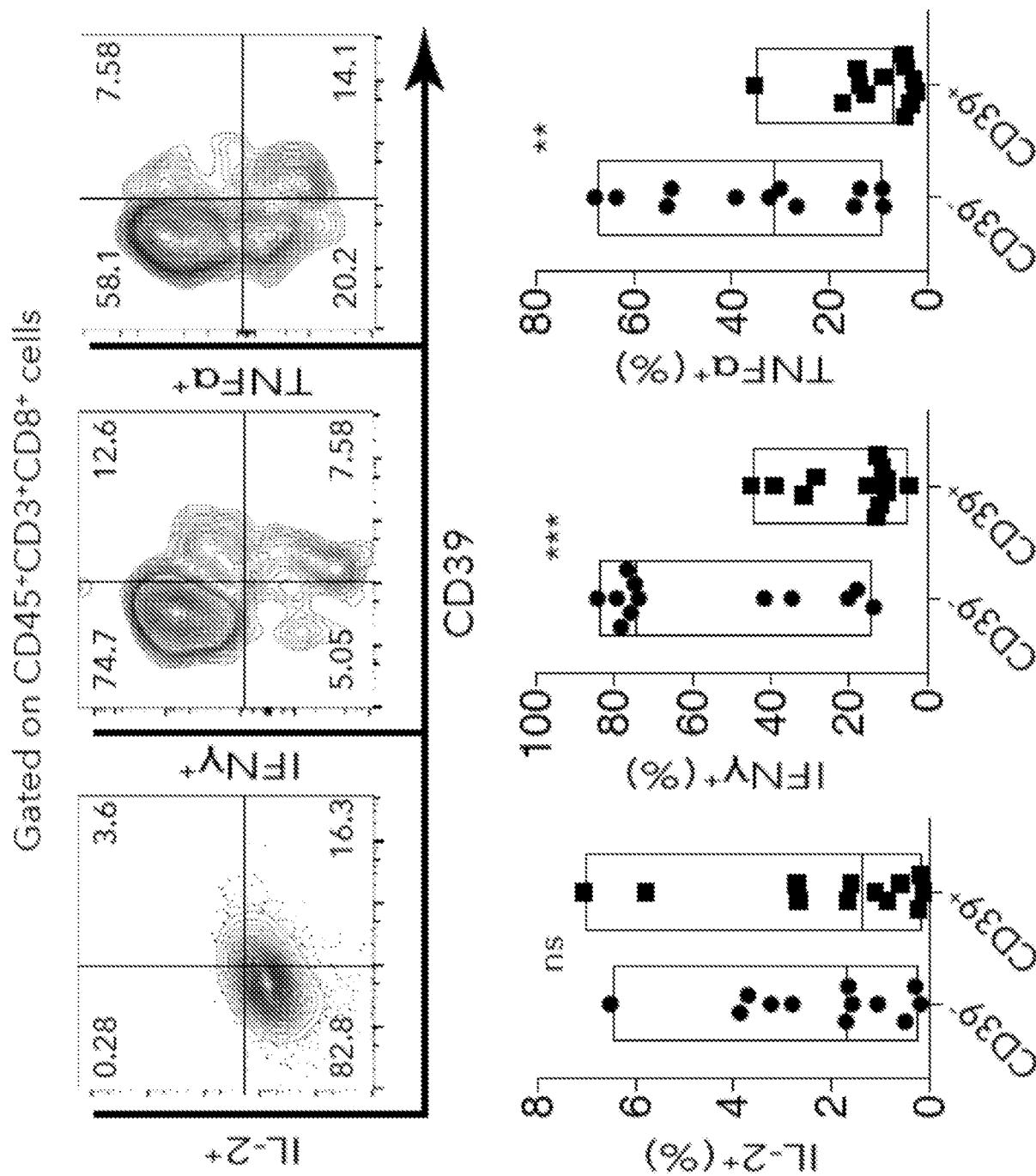
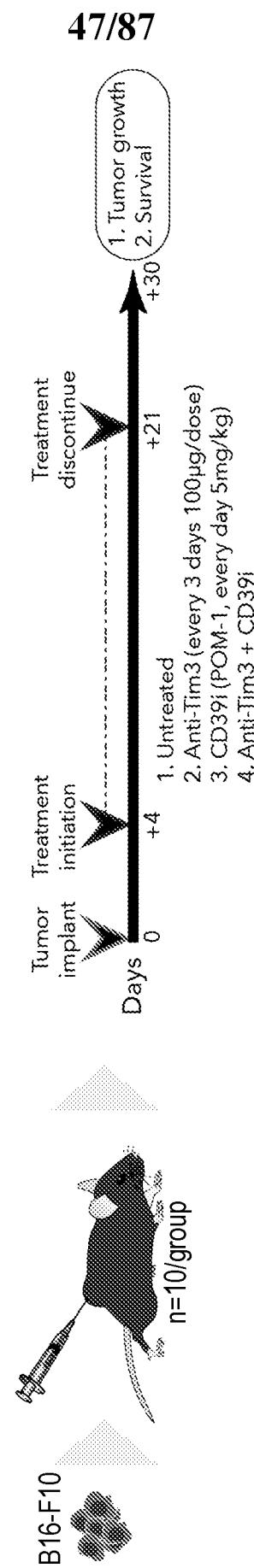


FIG. 26A

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**FIG. 26B**

**FIG. 26C**

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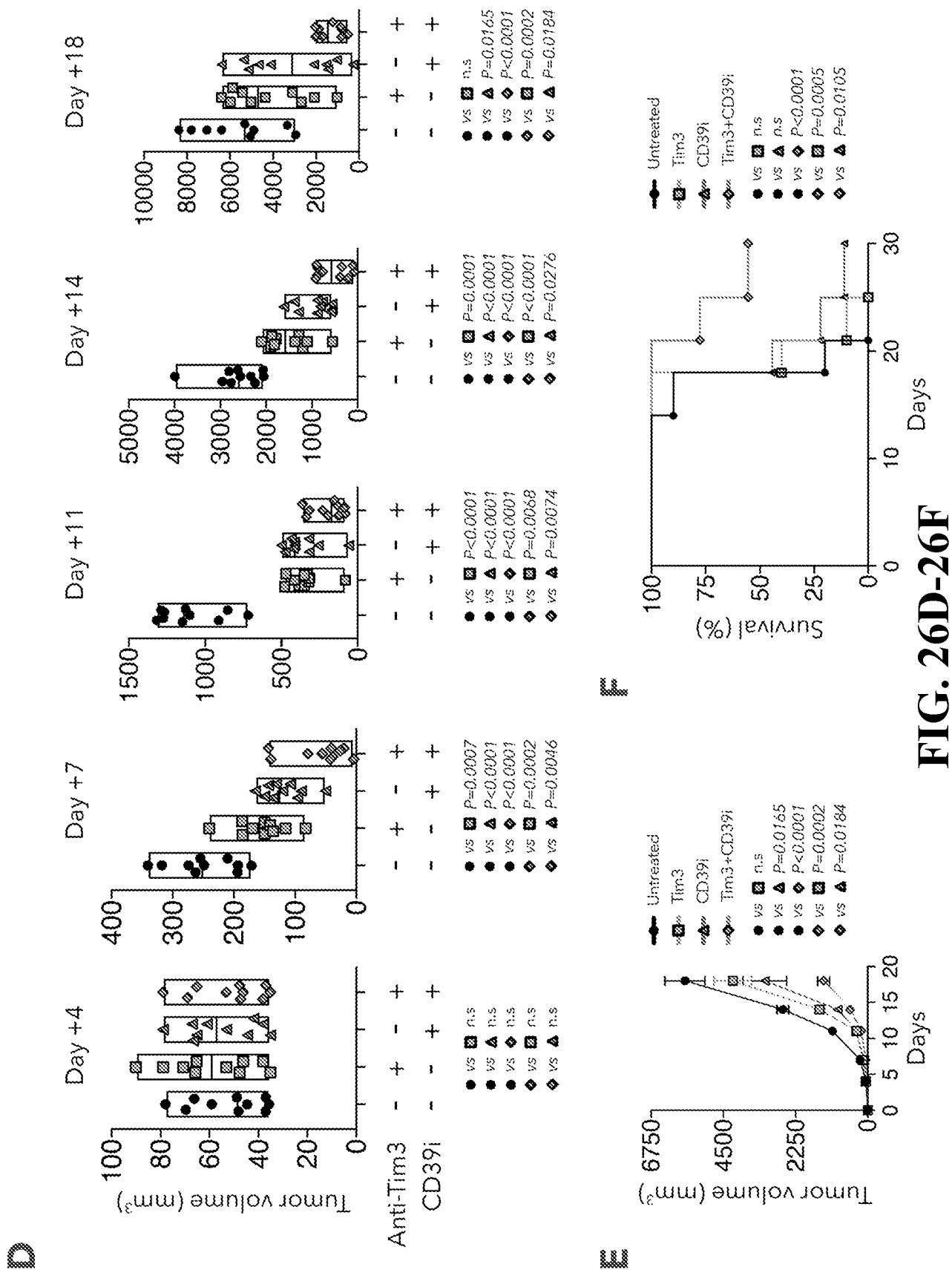


FIG. 26D-26F

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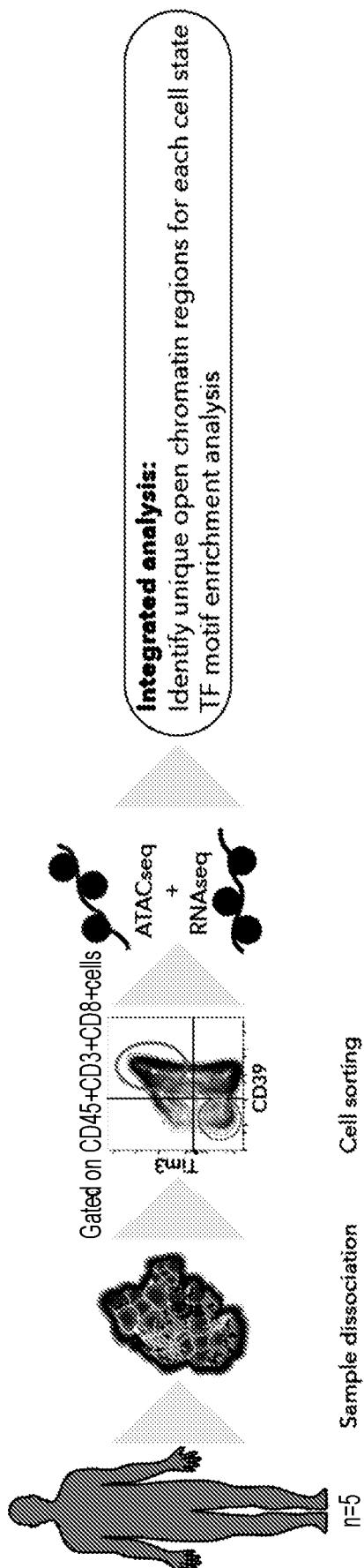
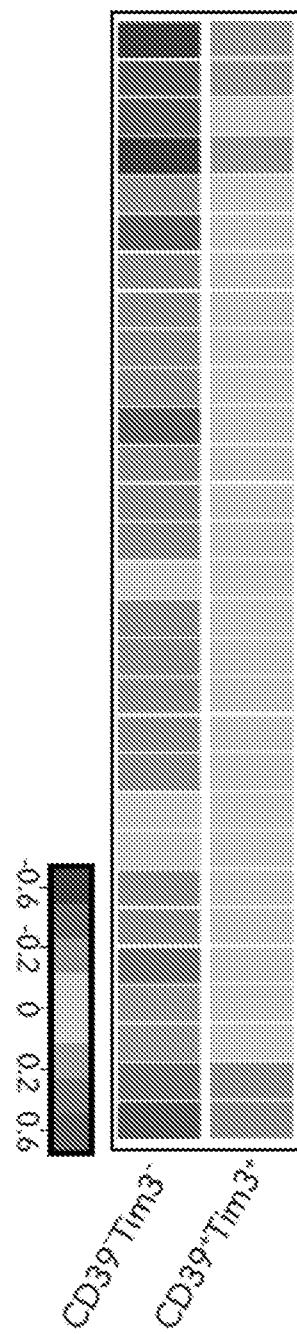
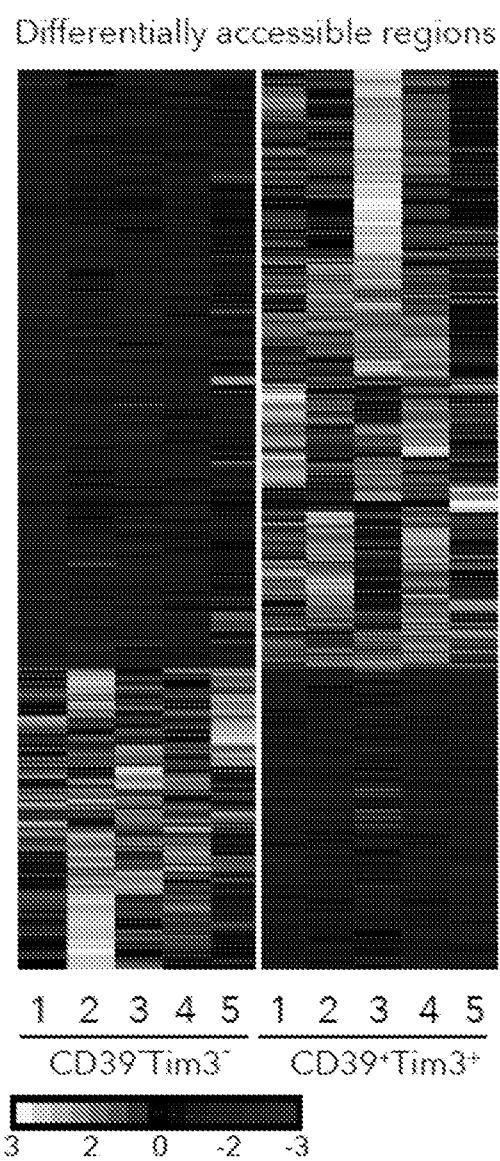


FIG. 27A

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B**C****FIG. 27B-27C**

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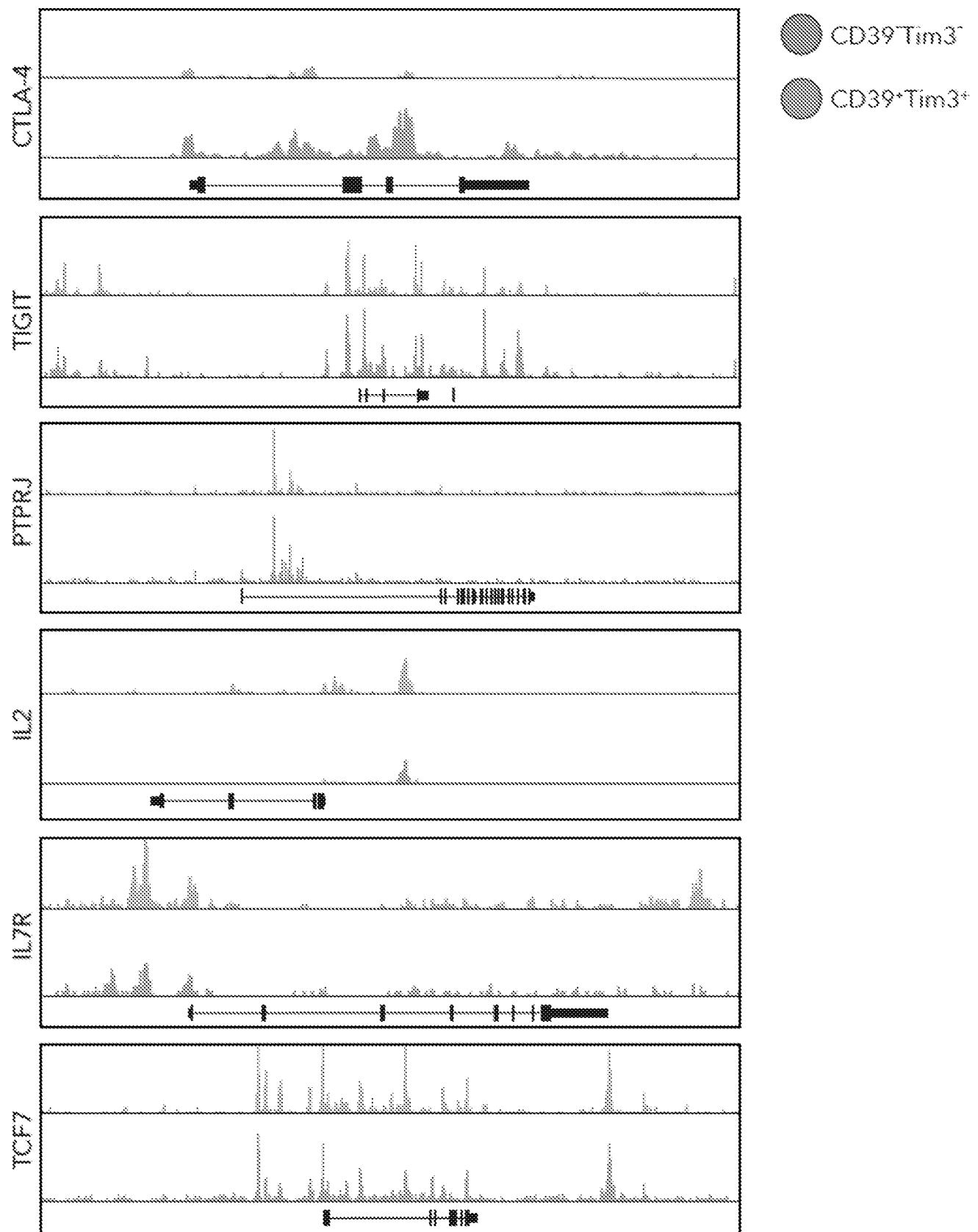


FIG. 27D

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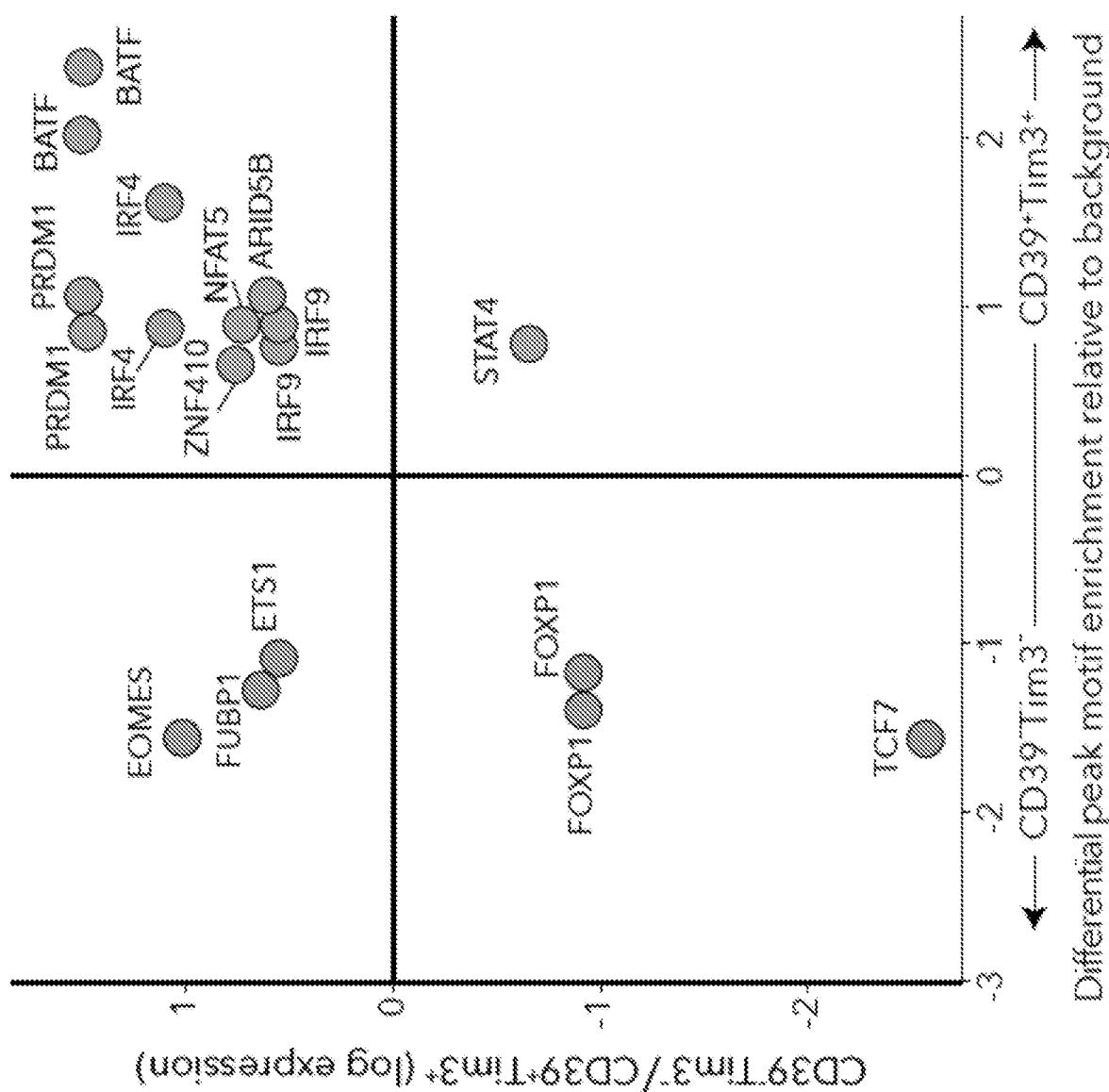
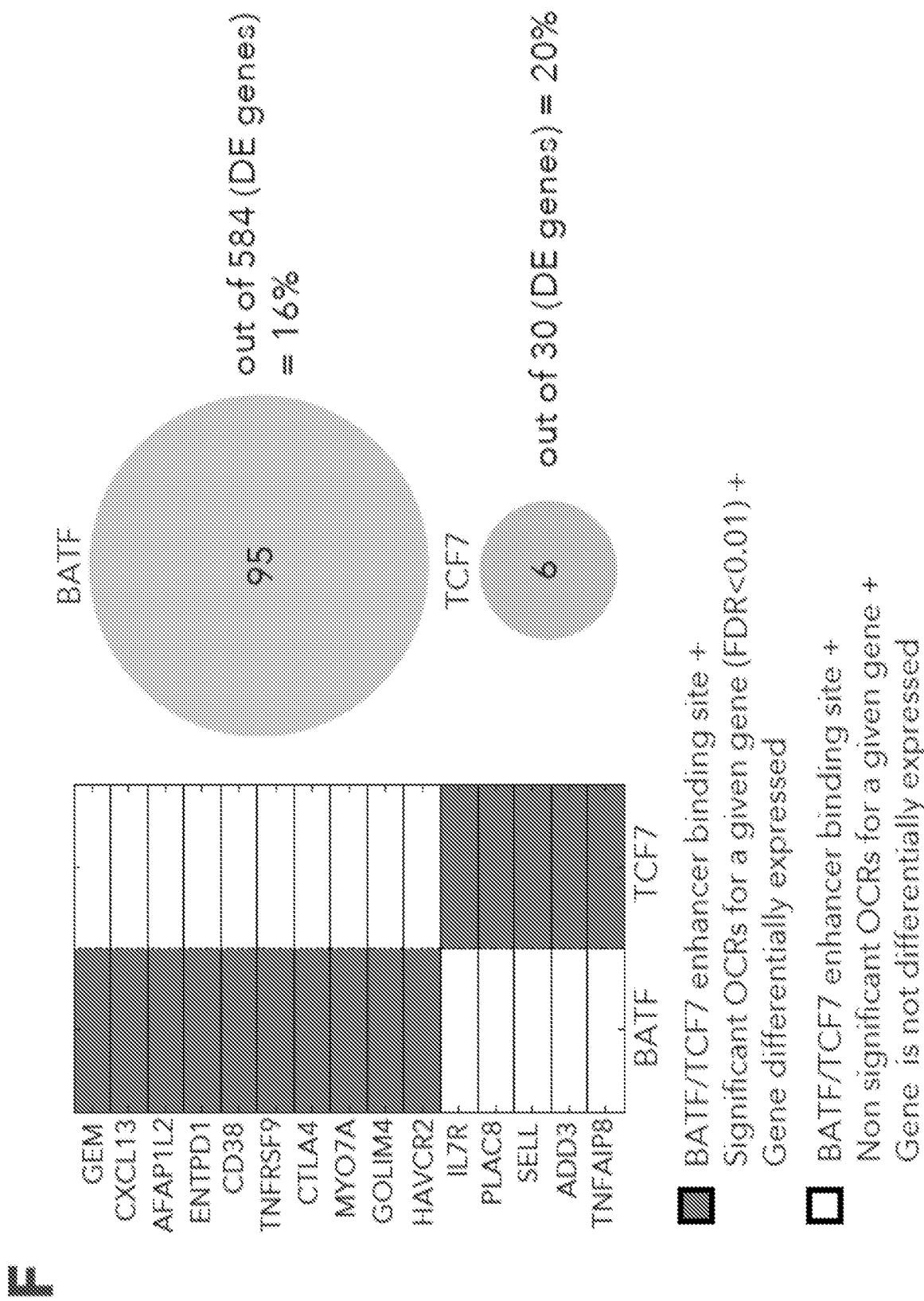
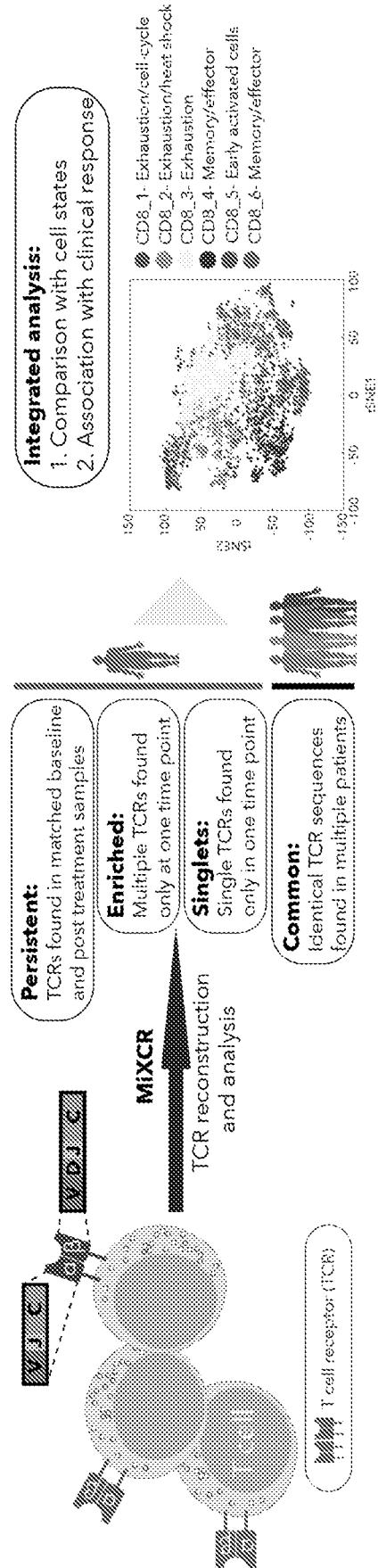


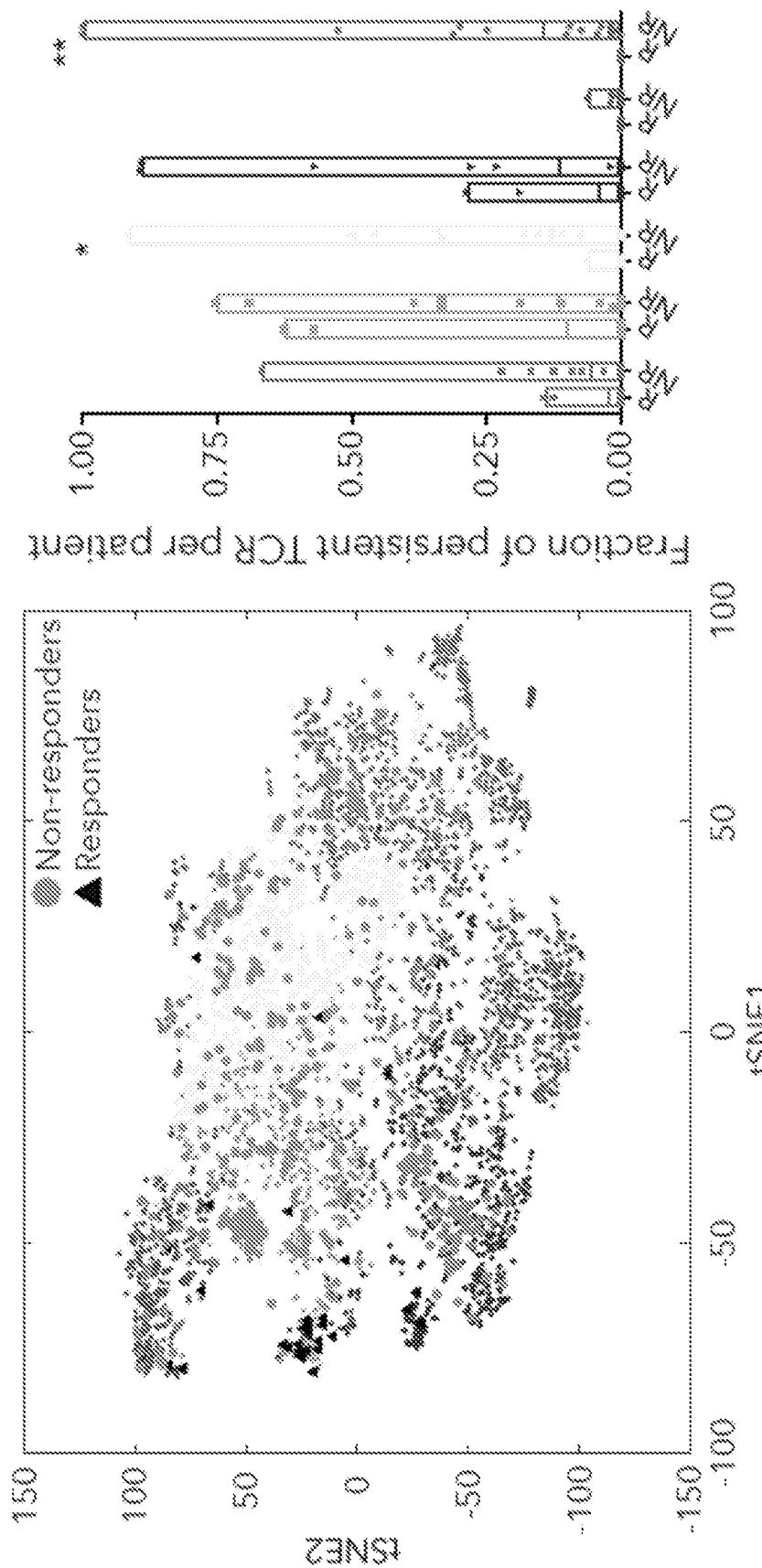
FIG. 27E

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**FIG. 27F**

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**FIG. 28A**

55/87**FIG. 28B**

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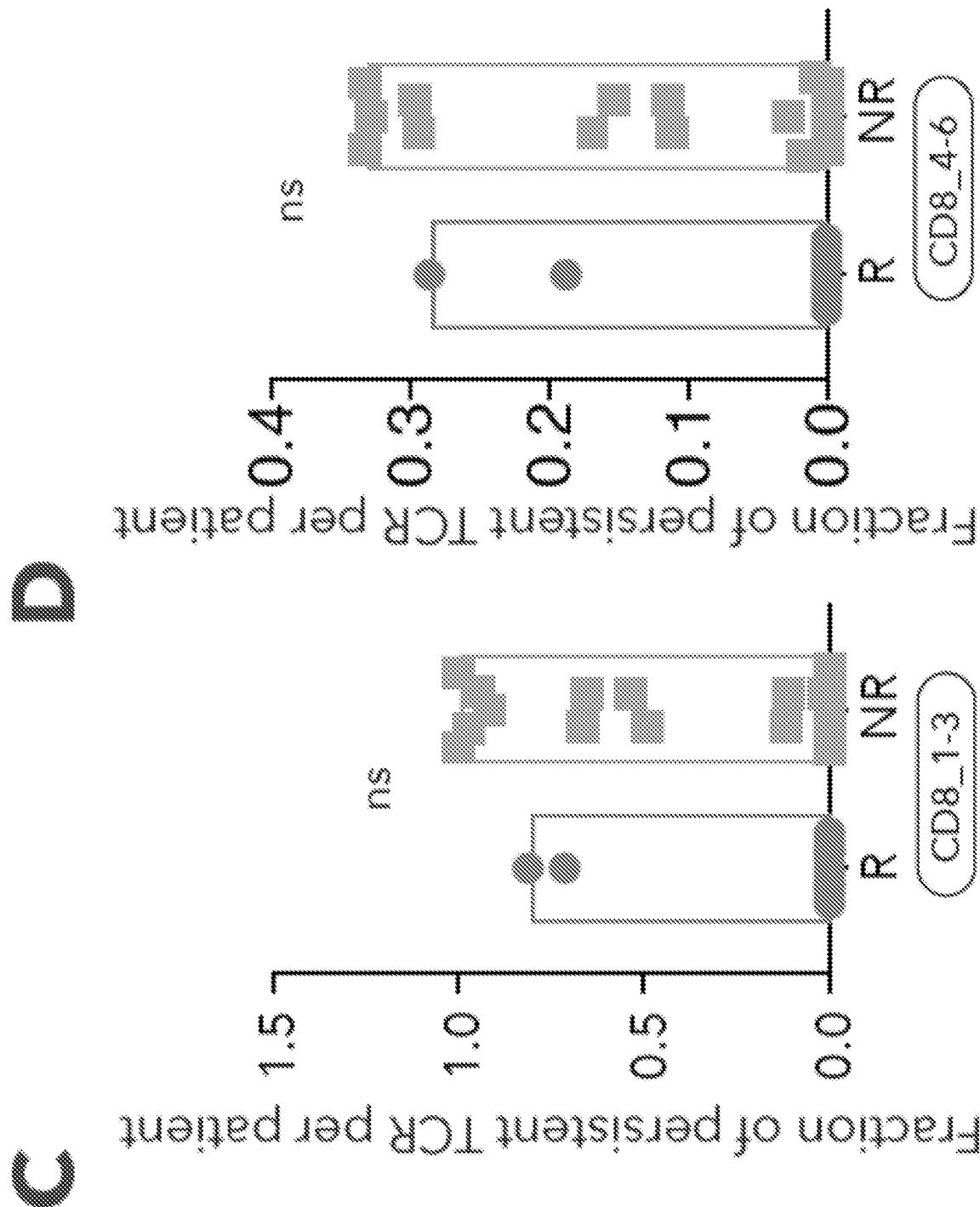


FIG. 28C-28D

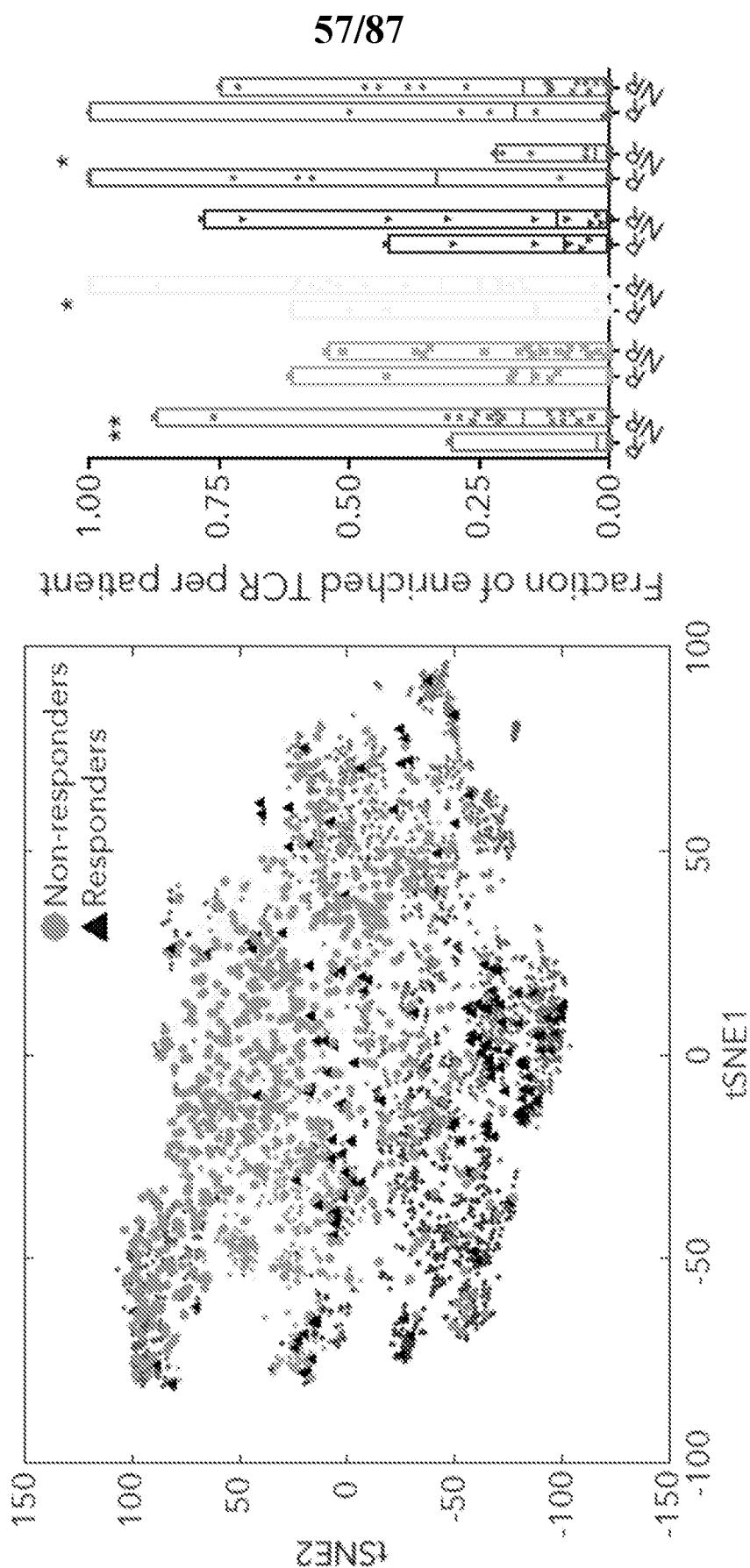


FIG. 28E

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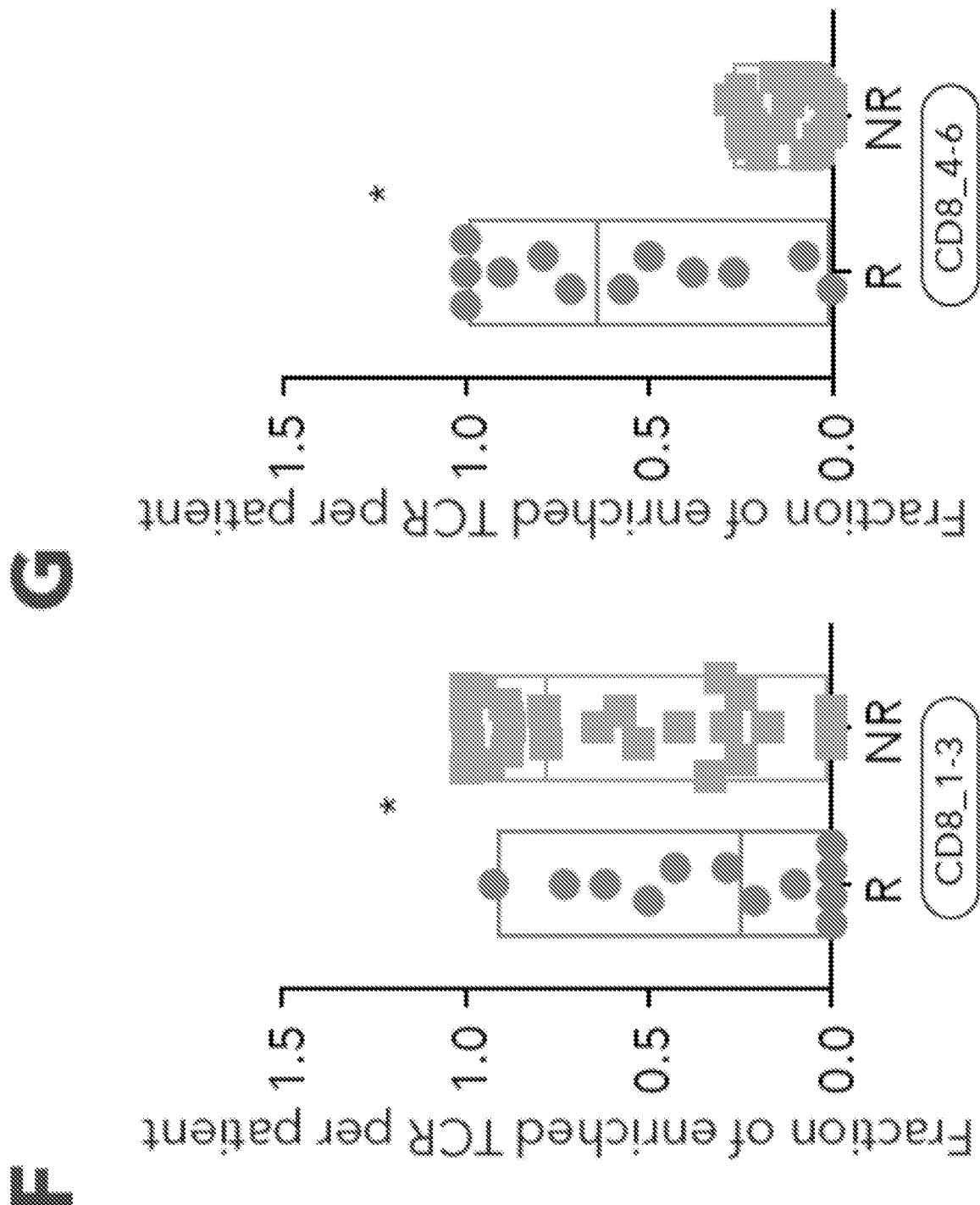


FIG. 28F-28G

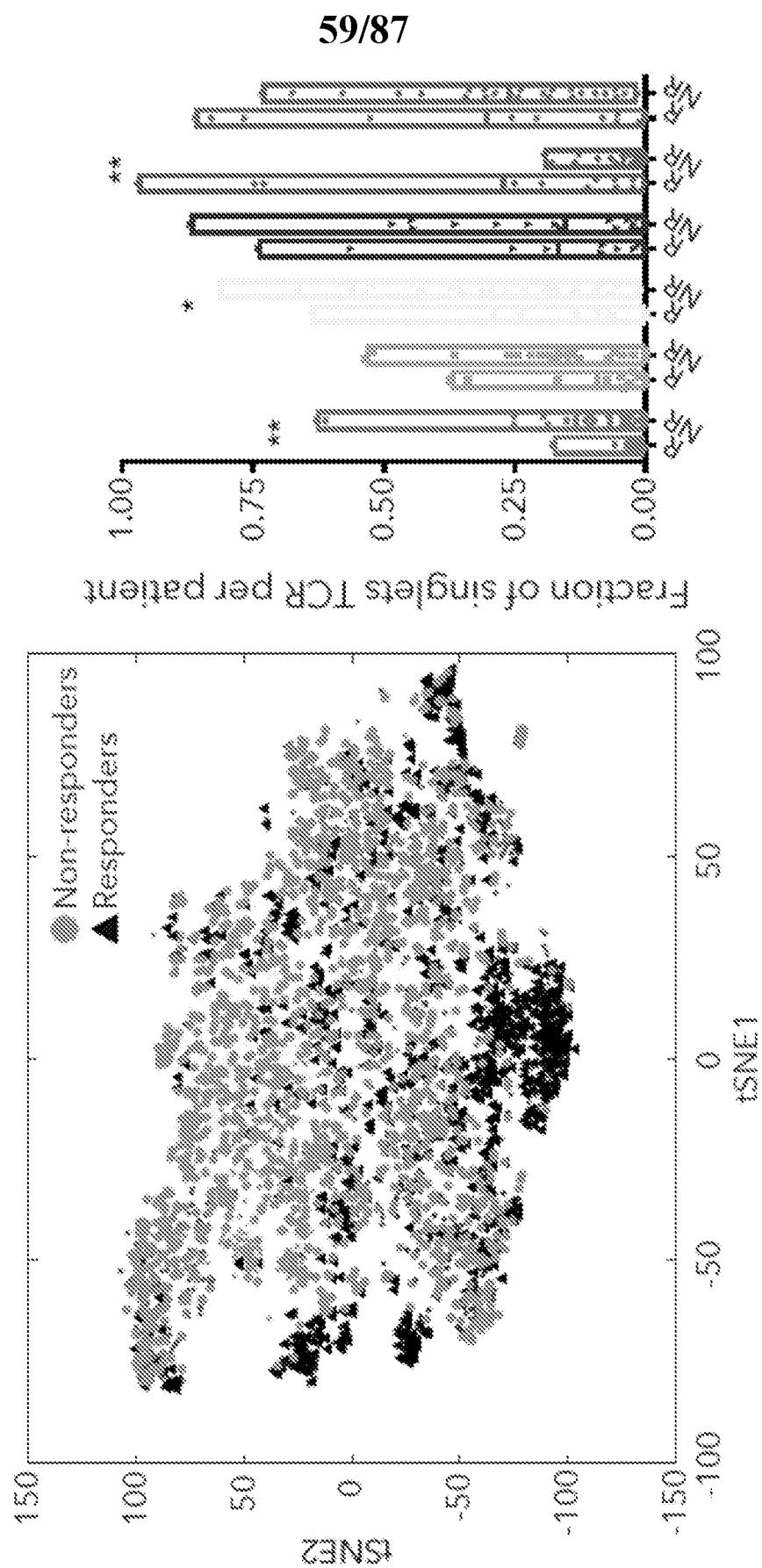


FIG. 28H

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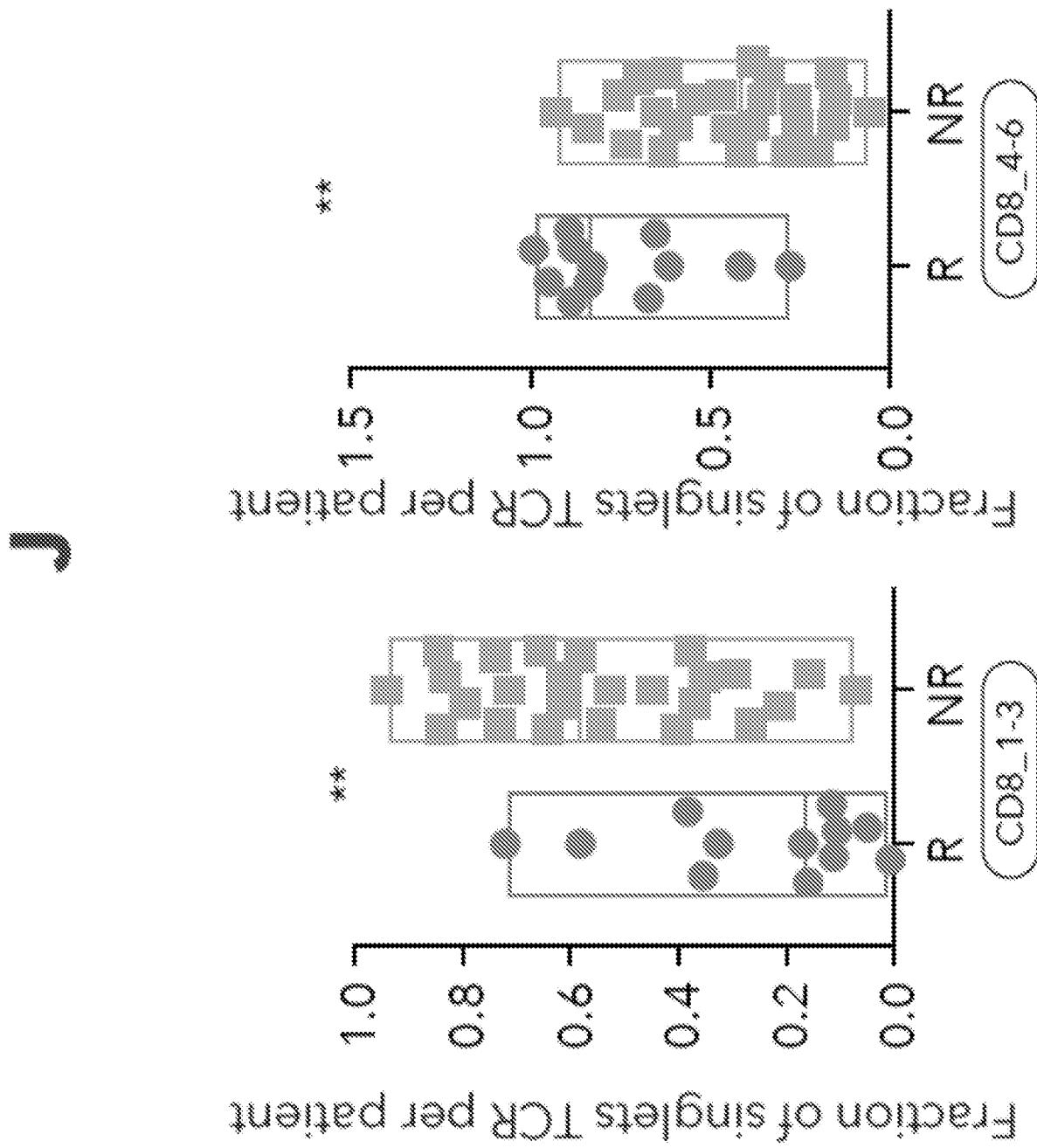
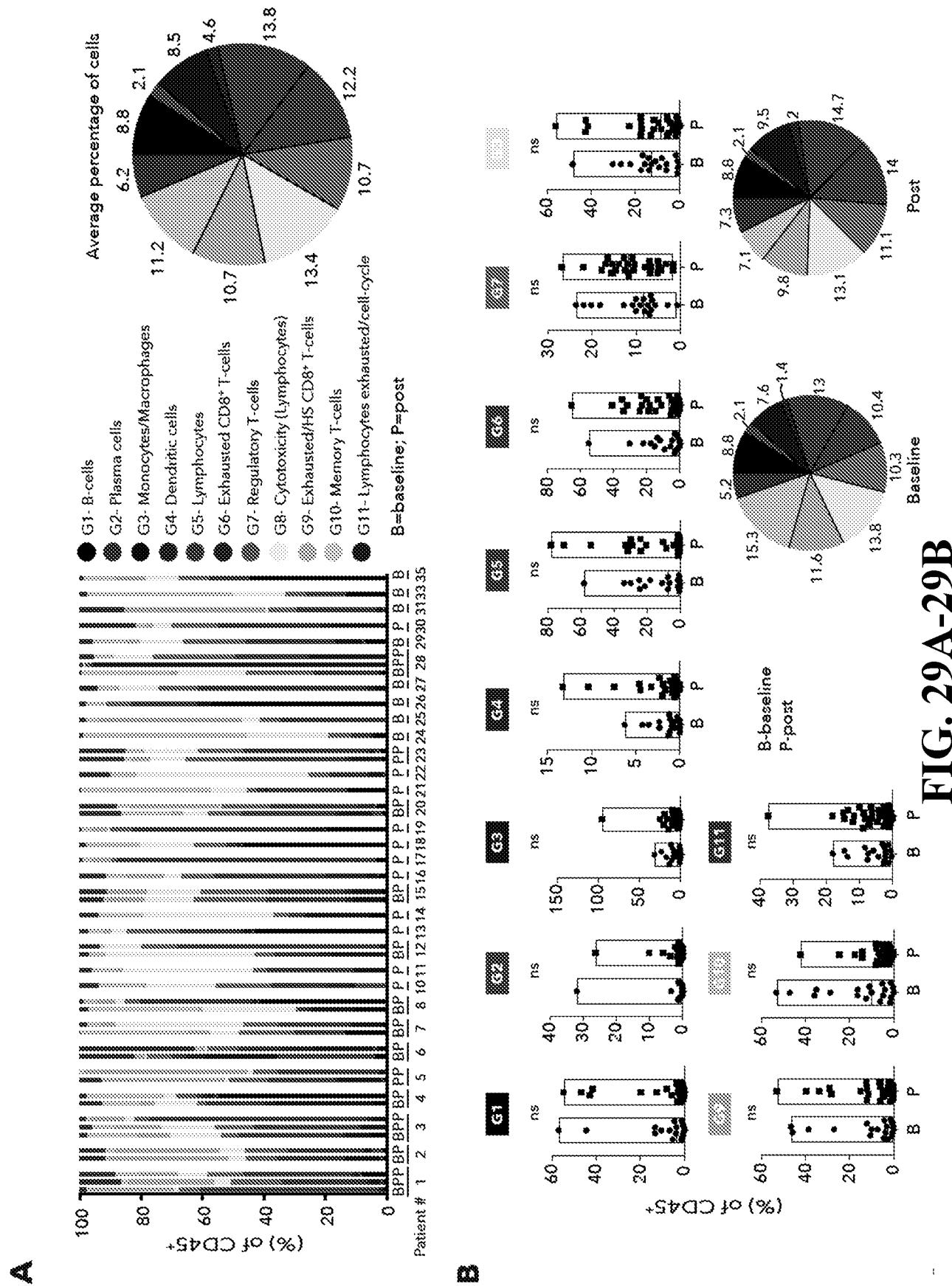


FIG. 28I-28J

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**FIG. 29A-29B**

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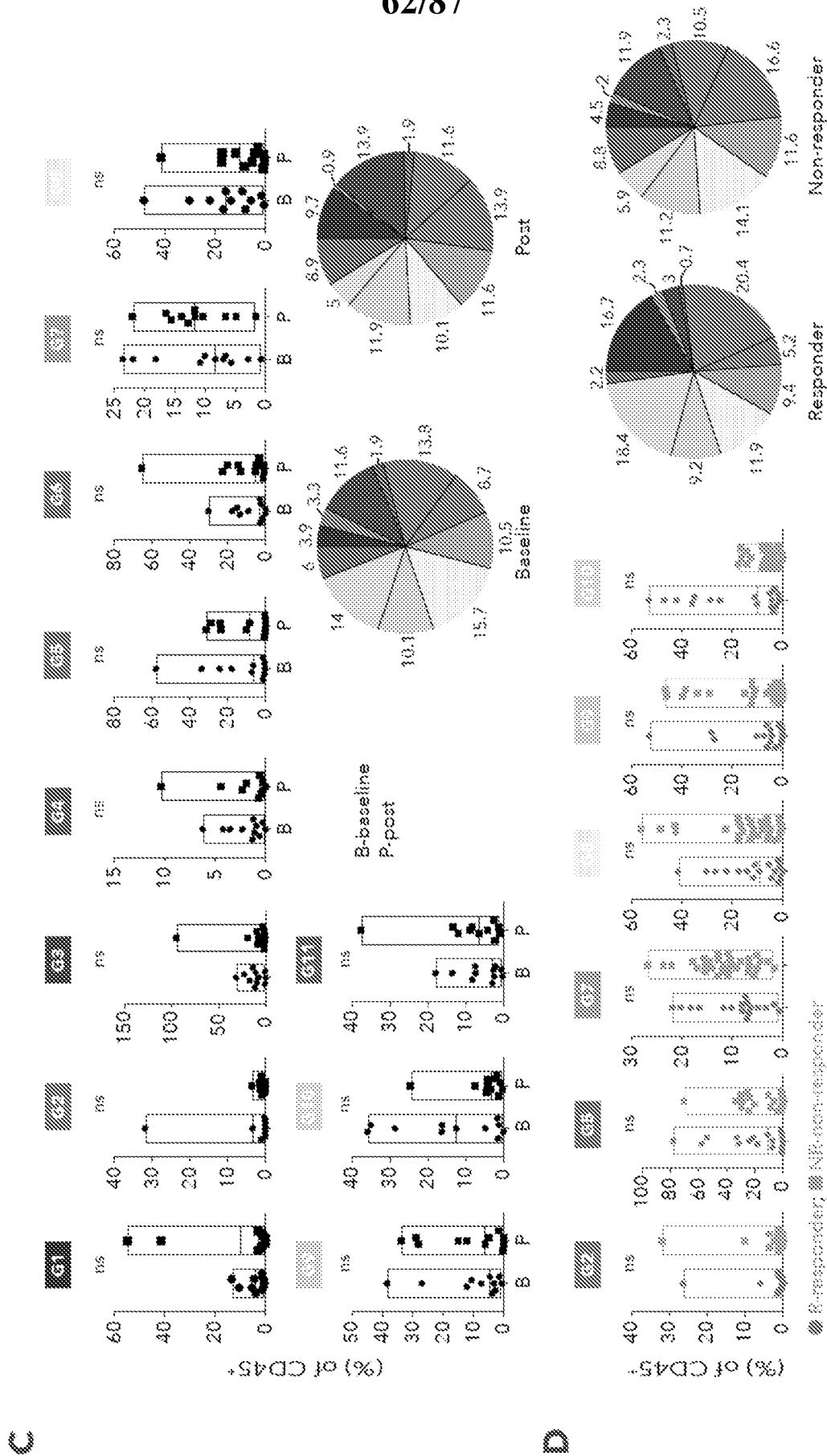


FIG. 29C-29D

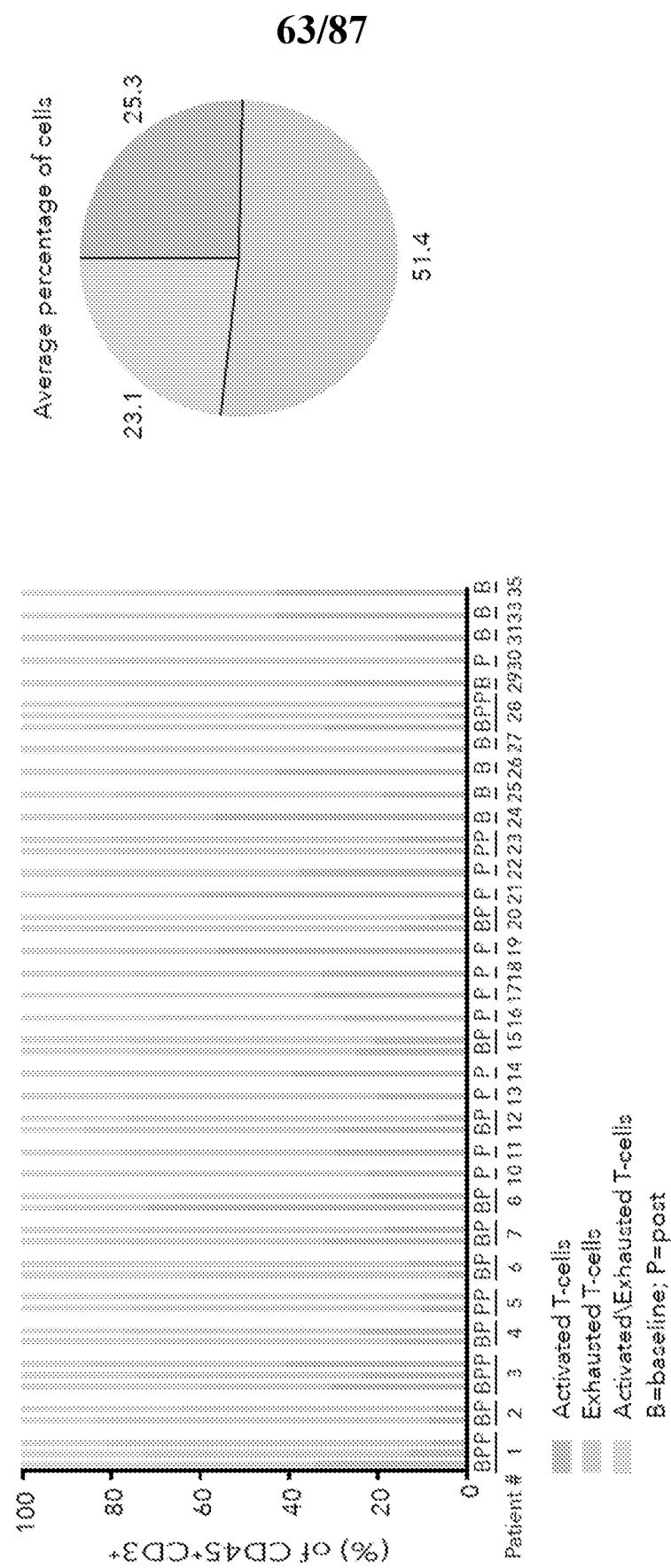


FIG. 30A

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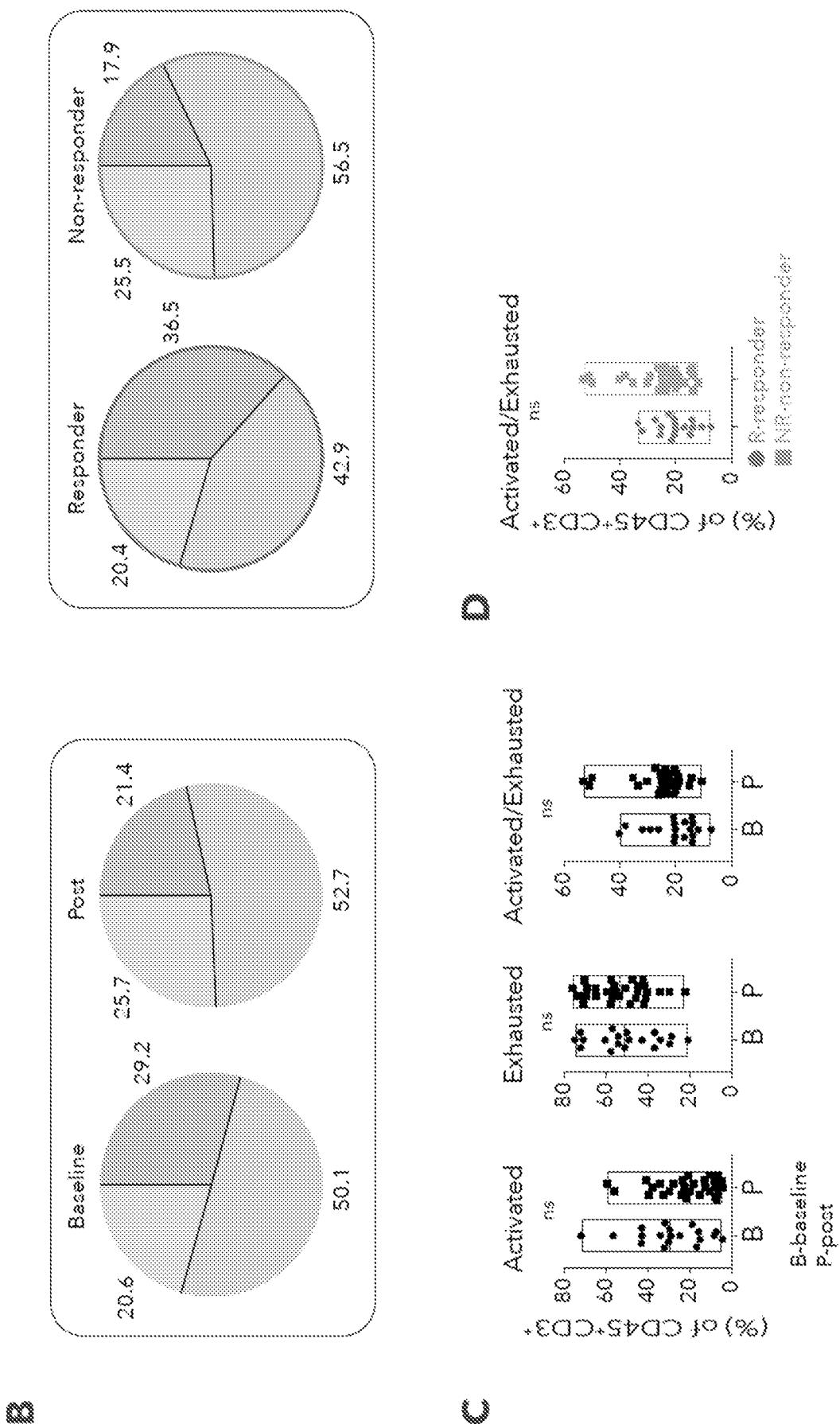
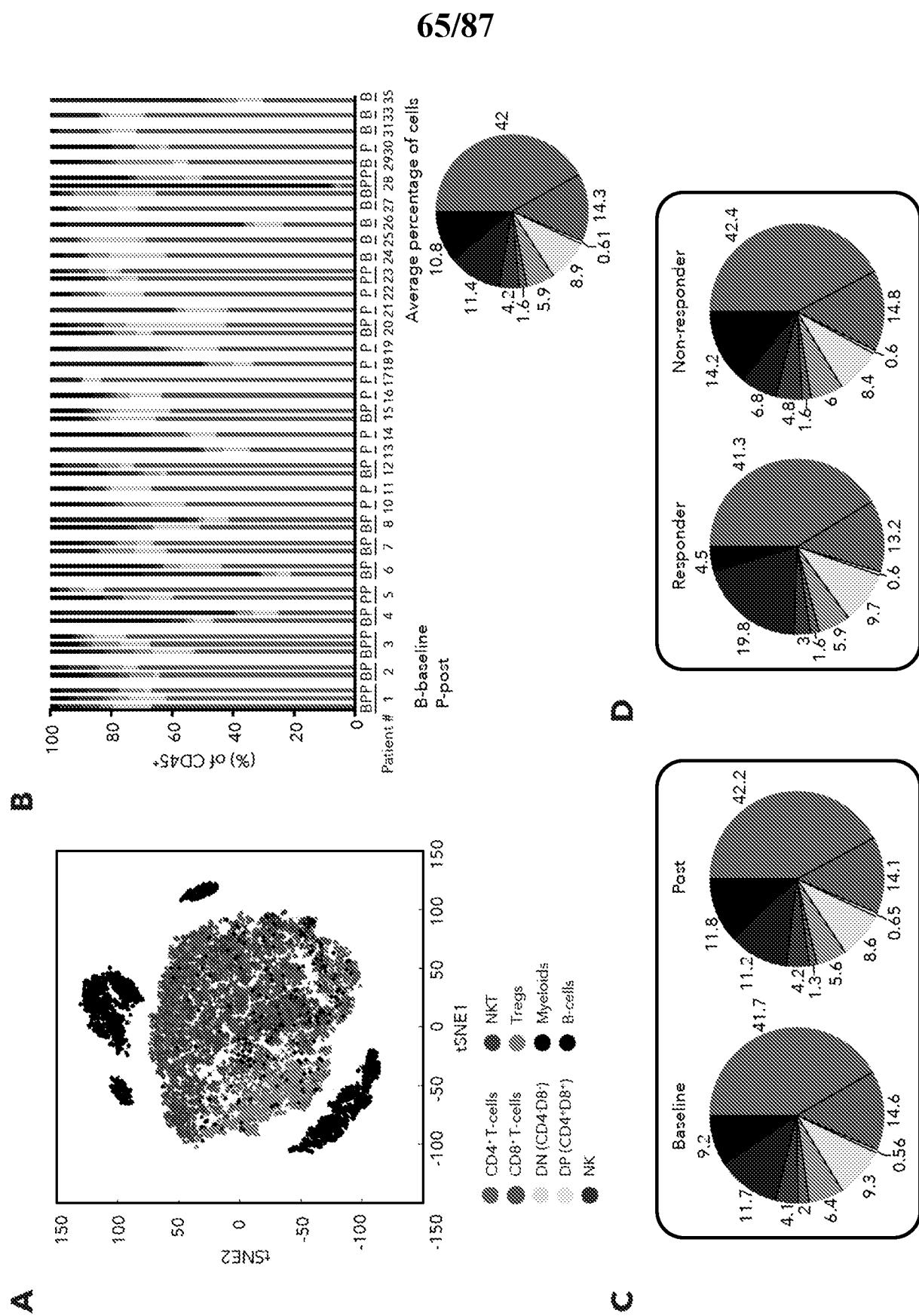


FIG. 30B-30D

**FIG. 31A-31D**

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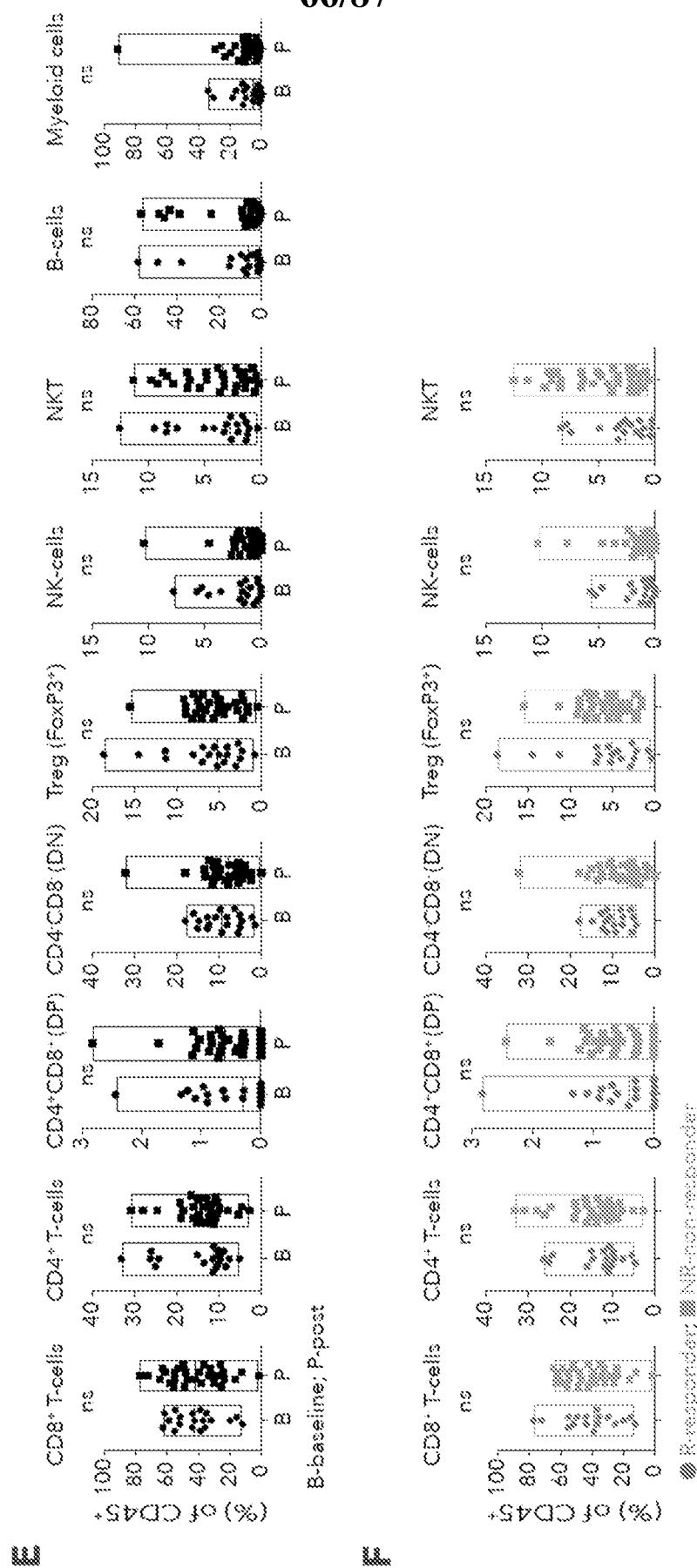
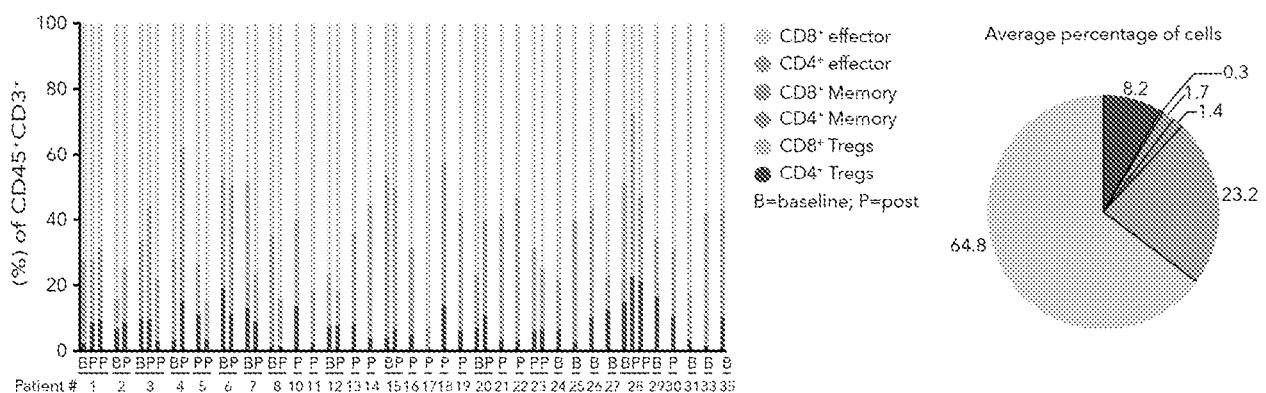
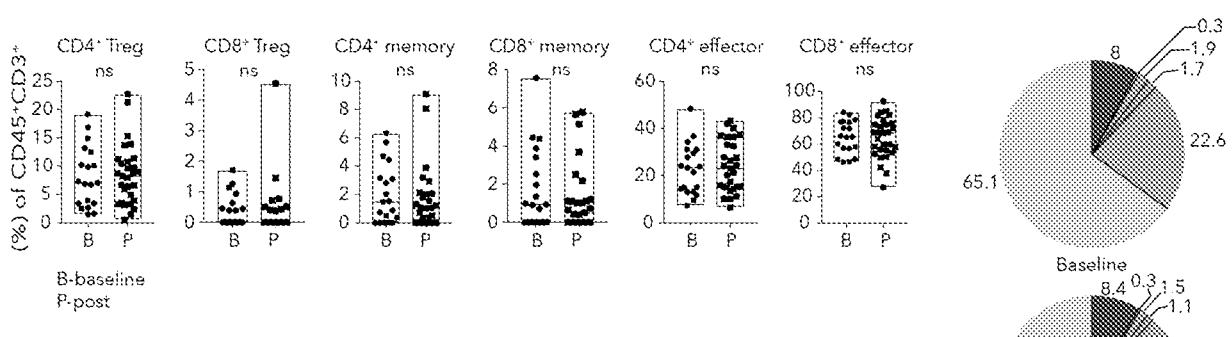
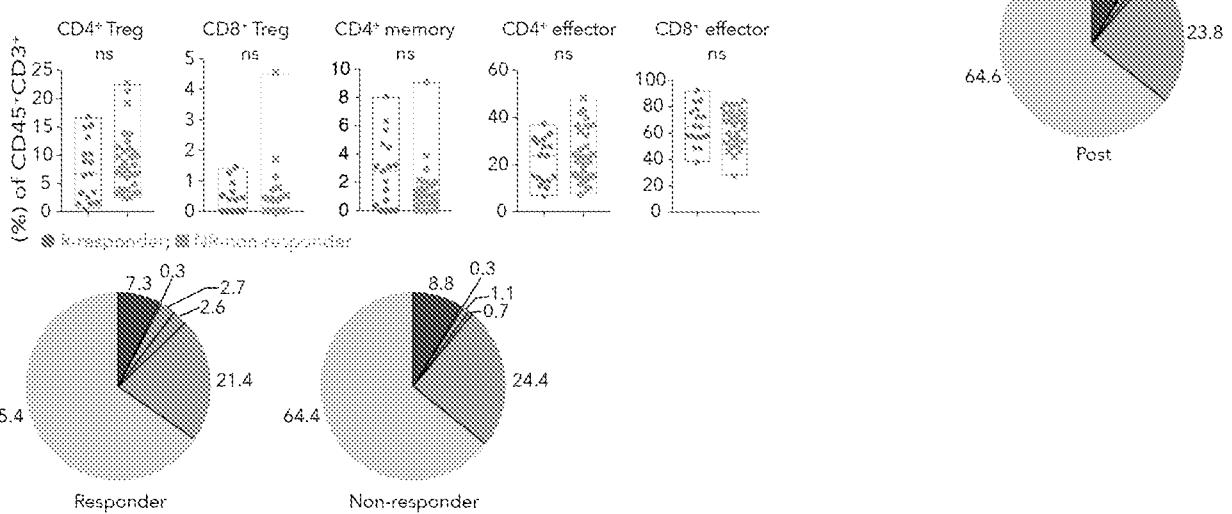


FIG. 31E-31F

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A**B****C****FIG. 32A-32C**

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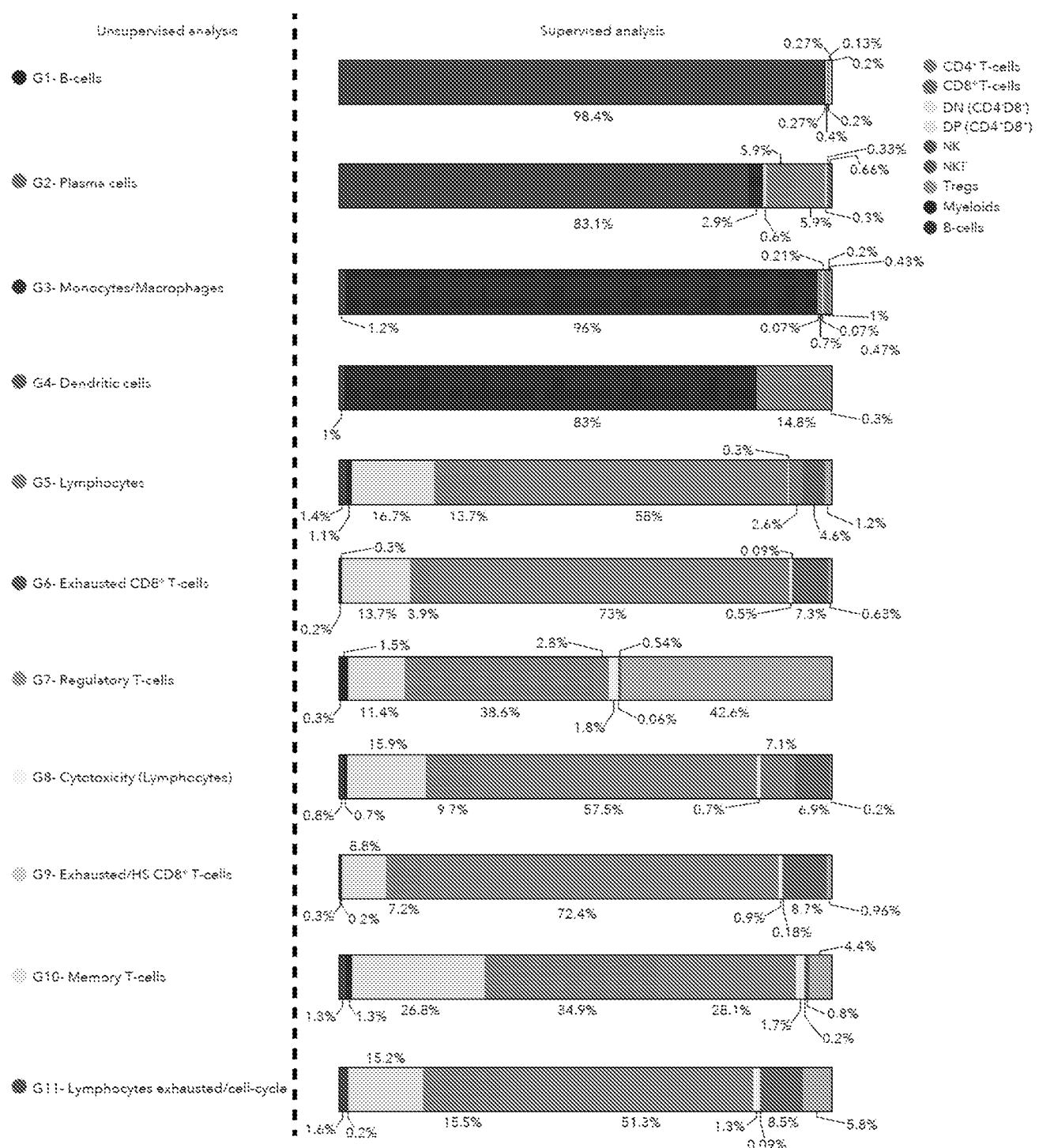
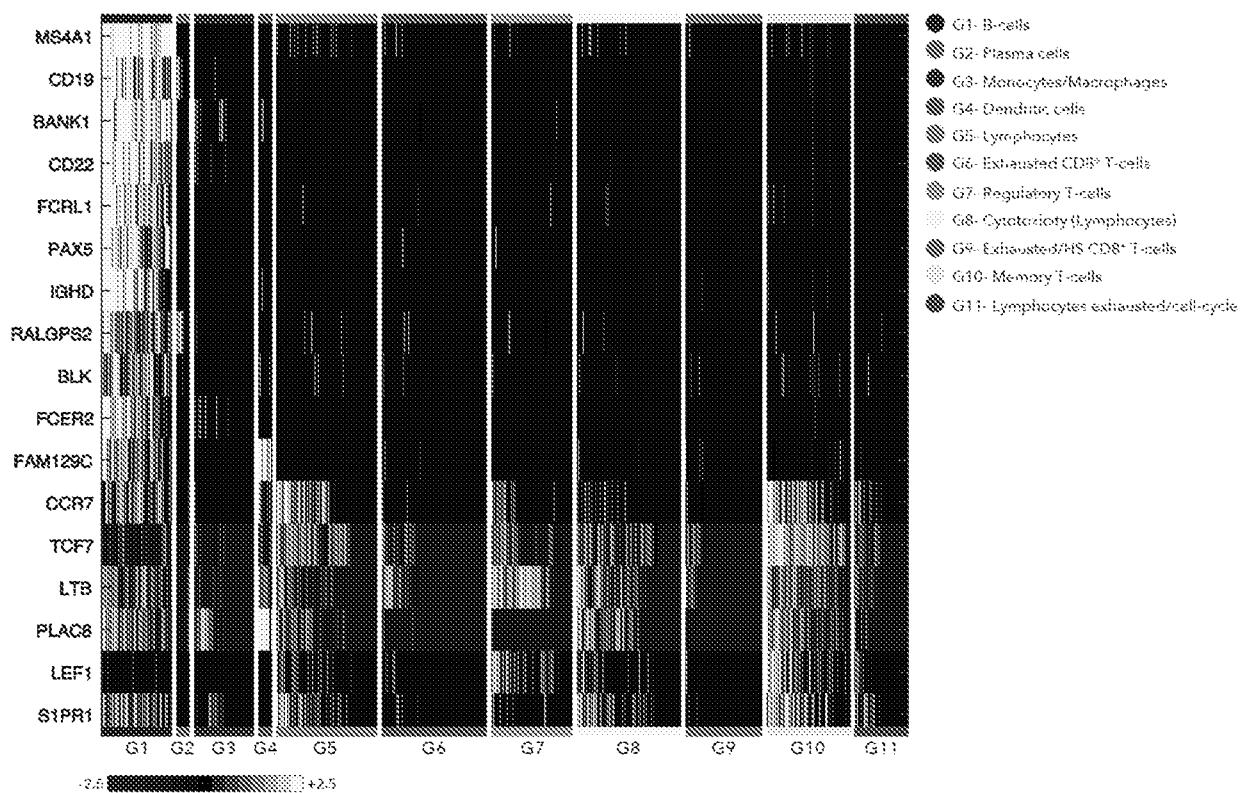


FIG. 33

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A



B

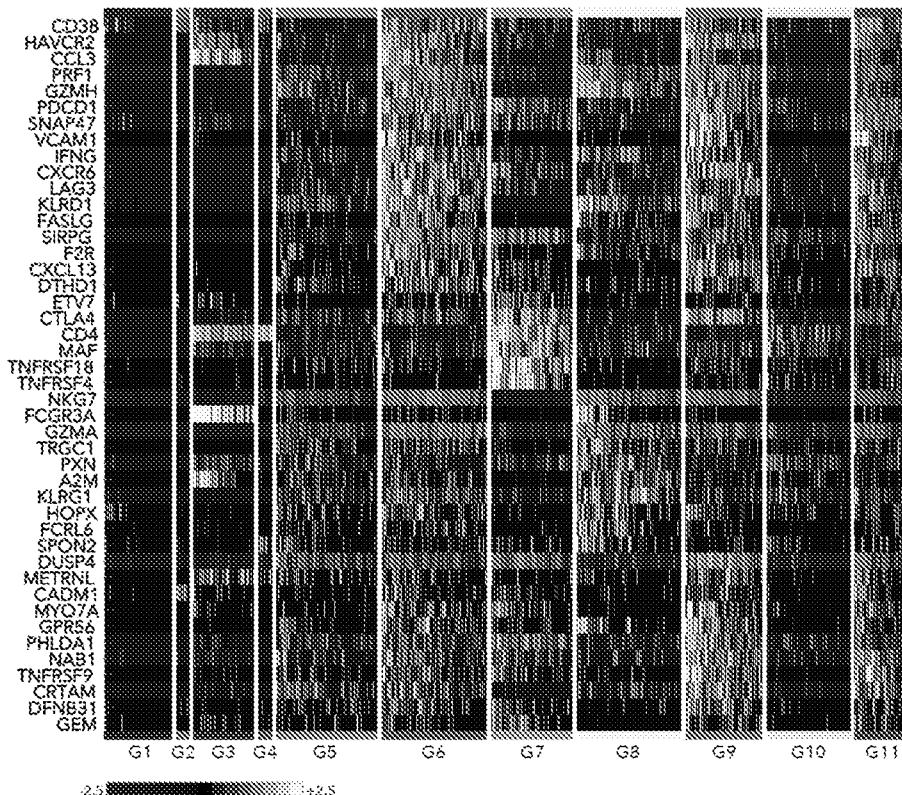


FIG. 34A-34B

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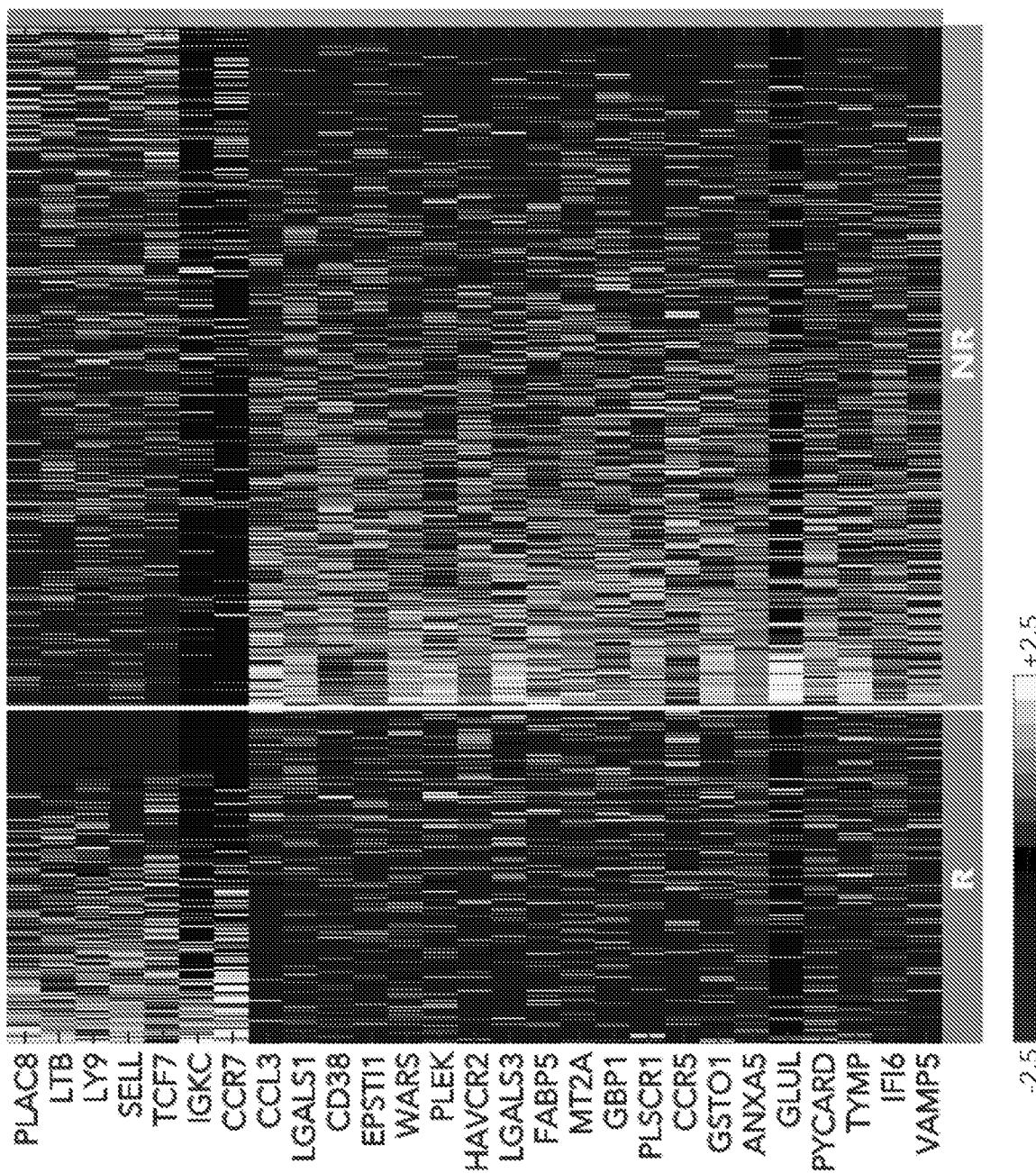


FIG. 35

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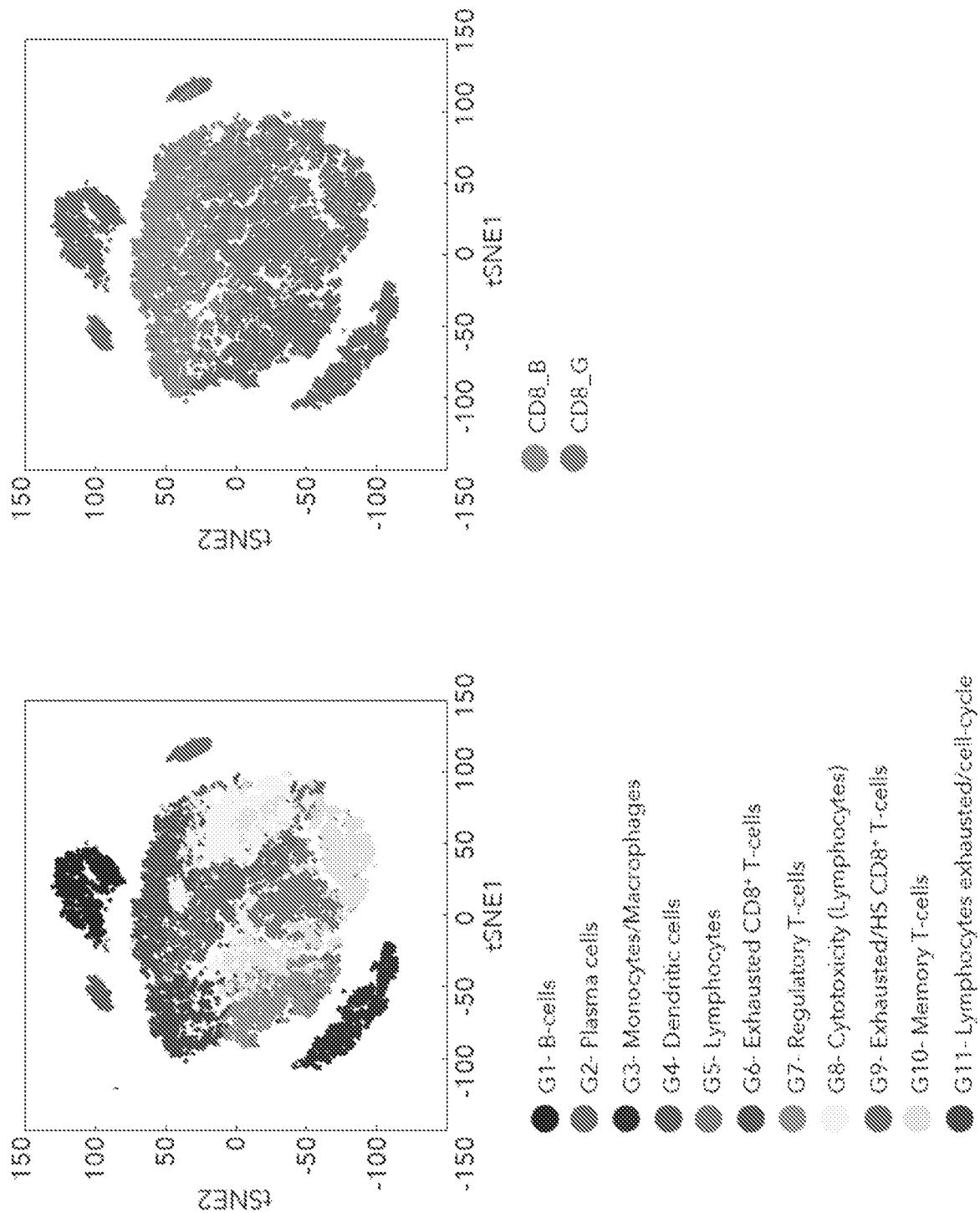
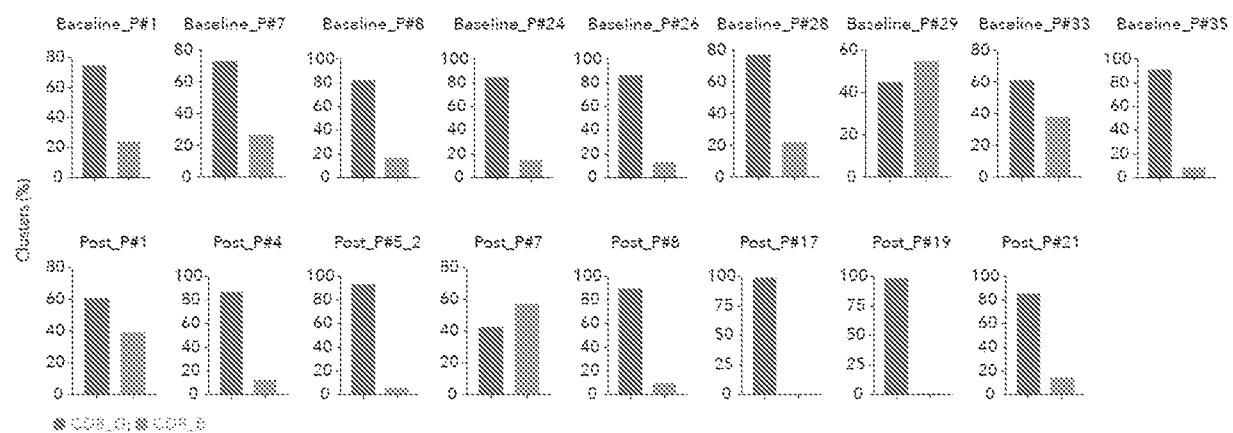


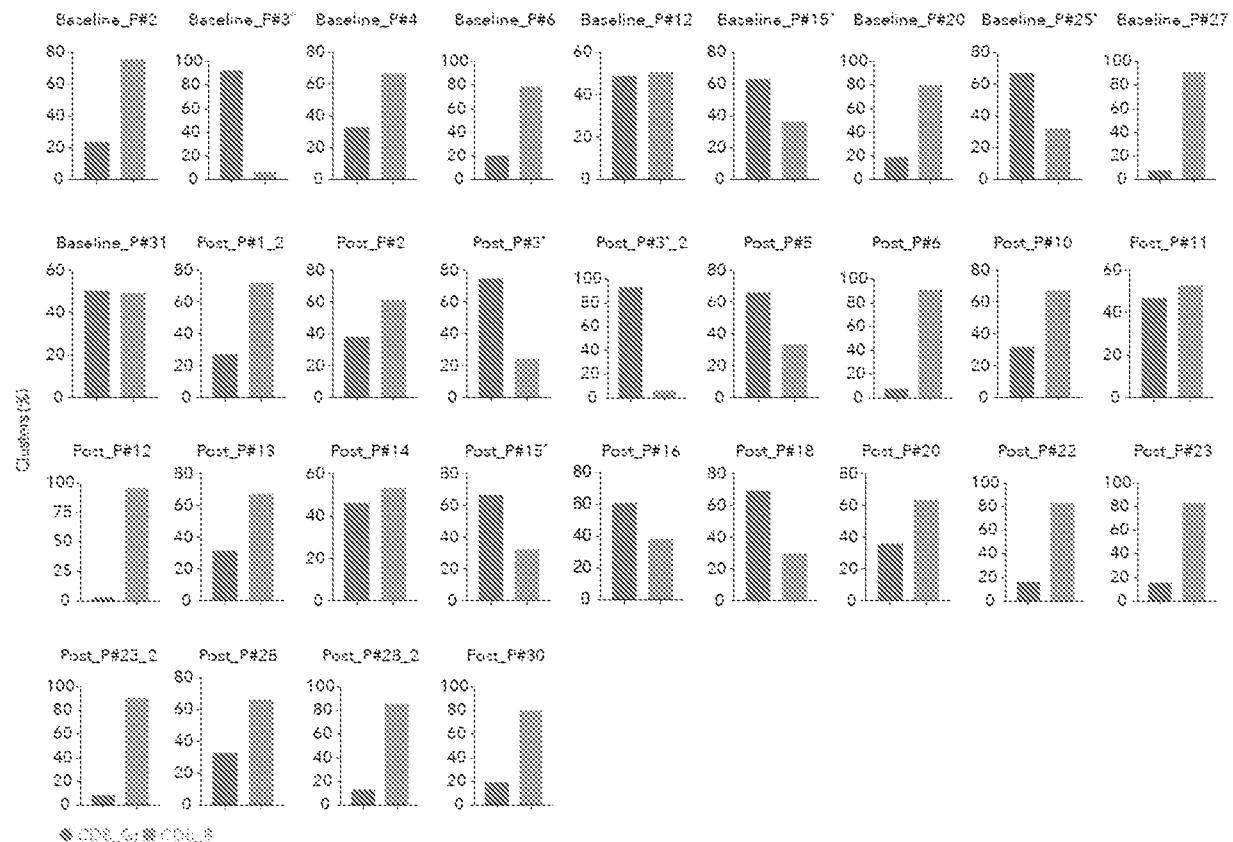
FIG. 36

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A



B



C

Responder Non-responder

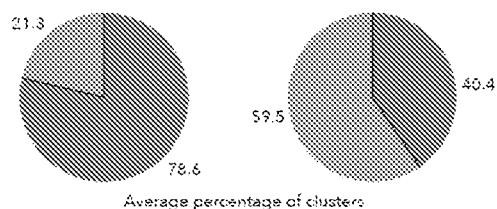
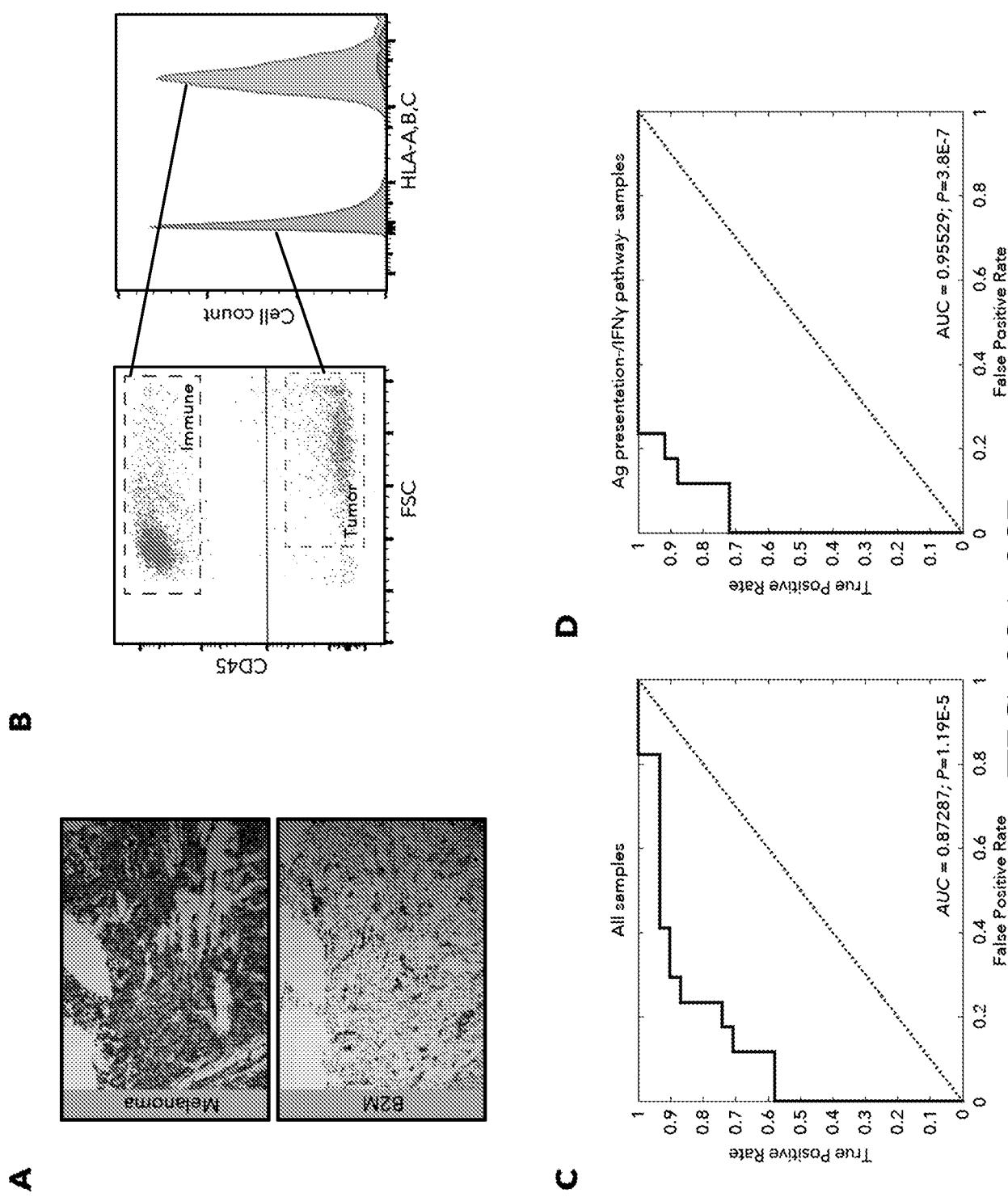
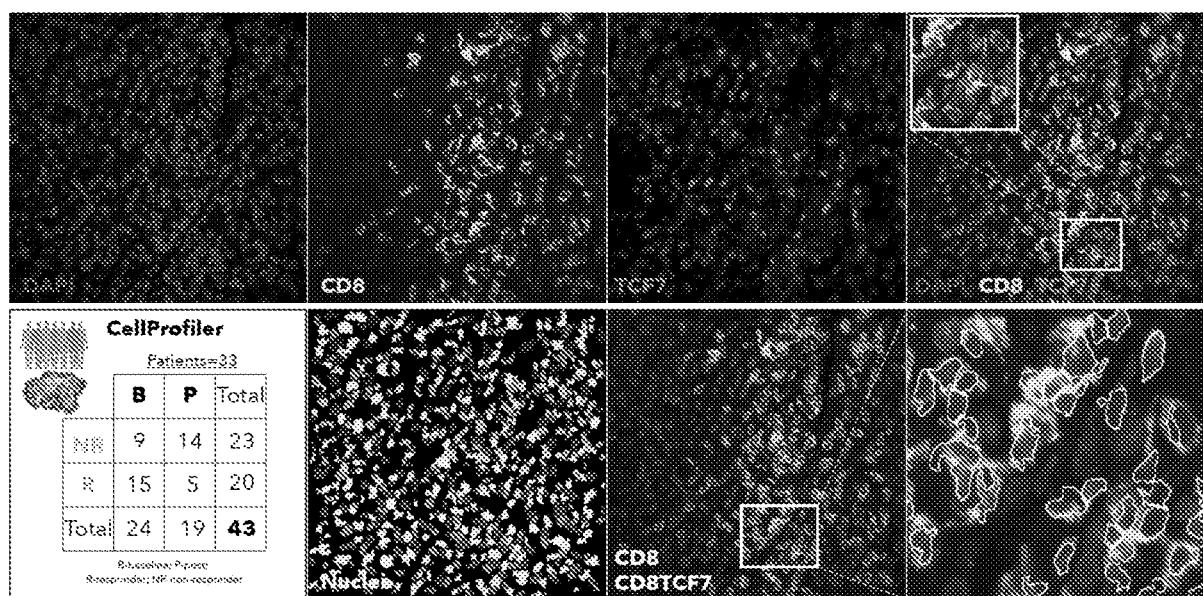
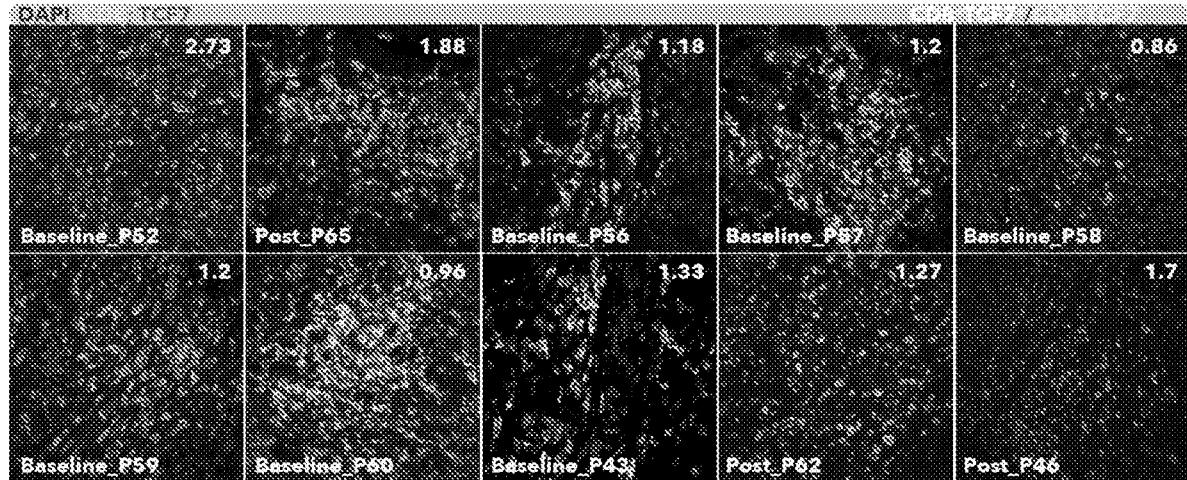
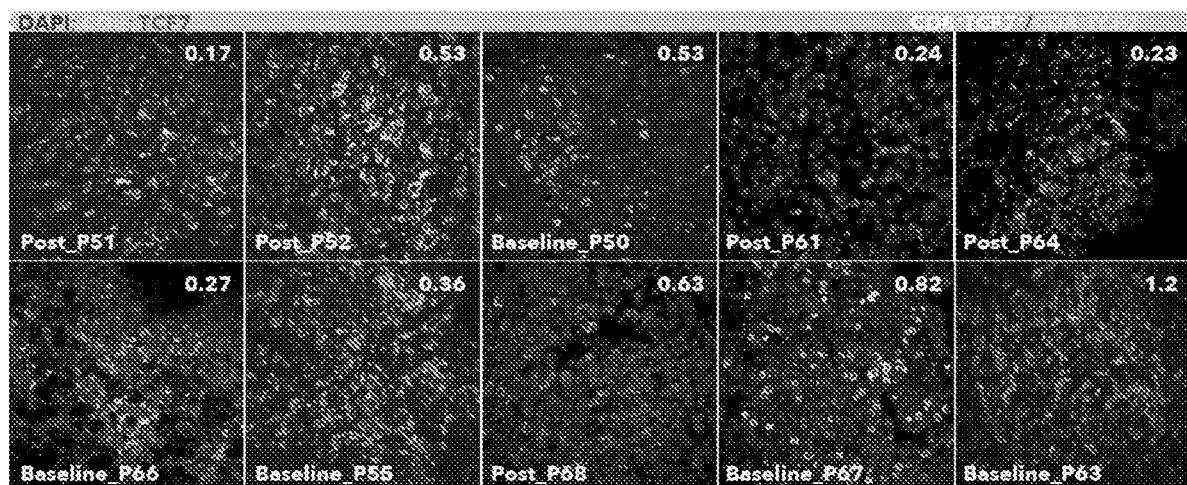


FIG. 37A-37C

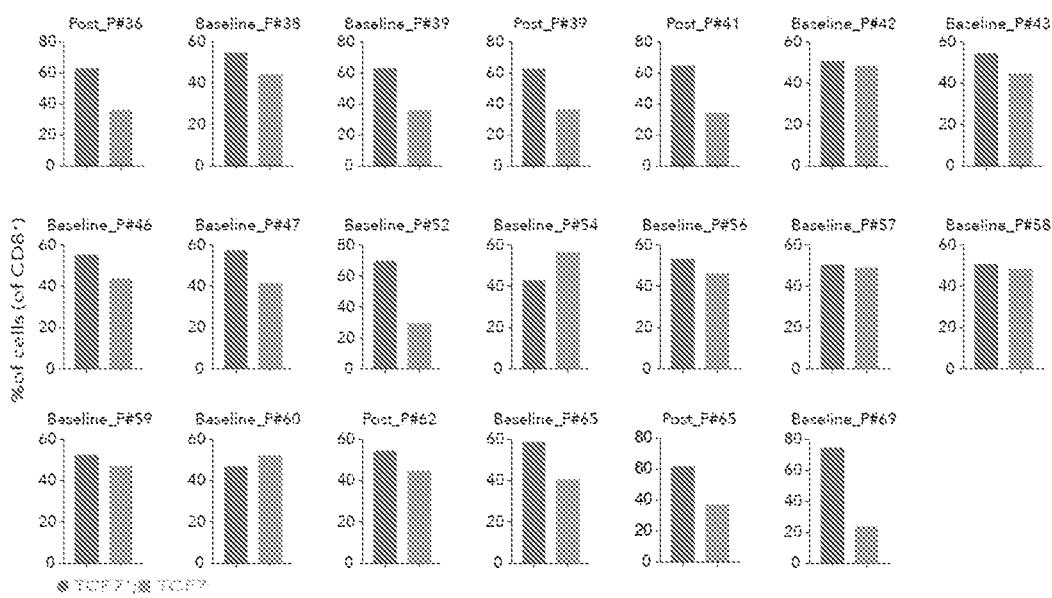
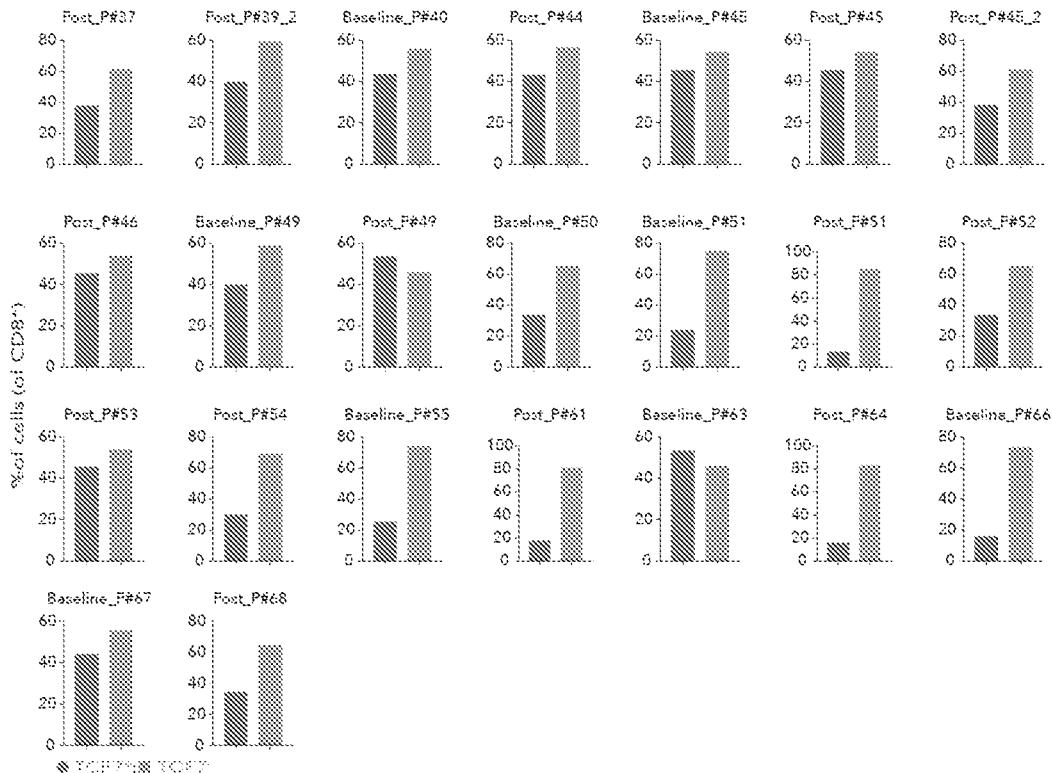
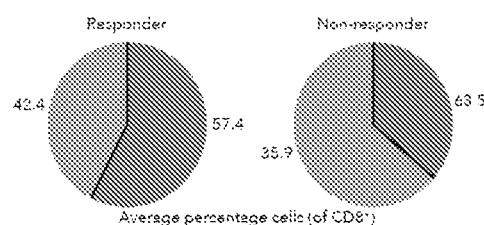
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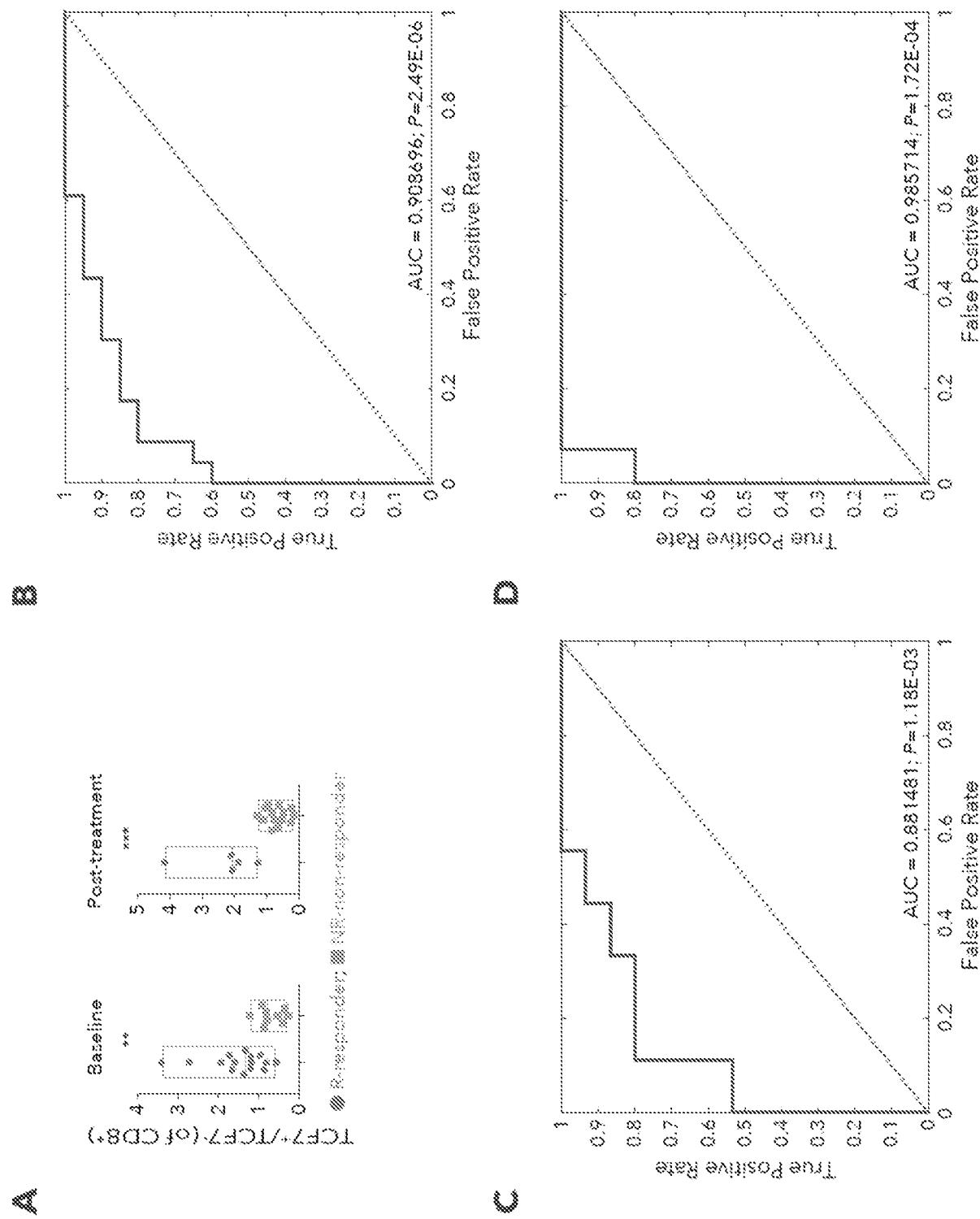
A**B****C****FIG. 39A-39C**

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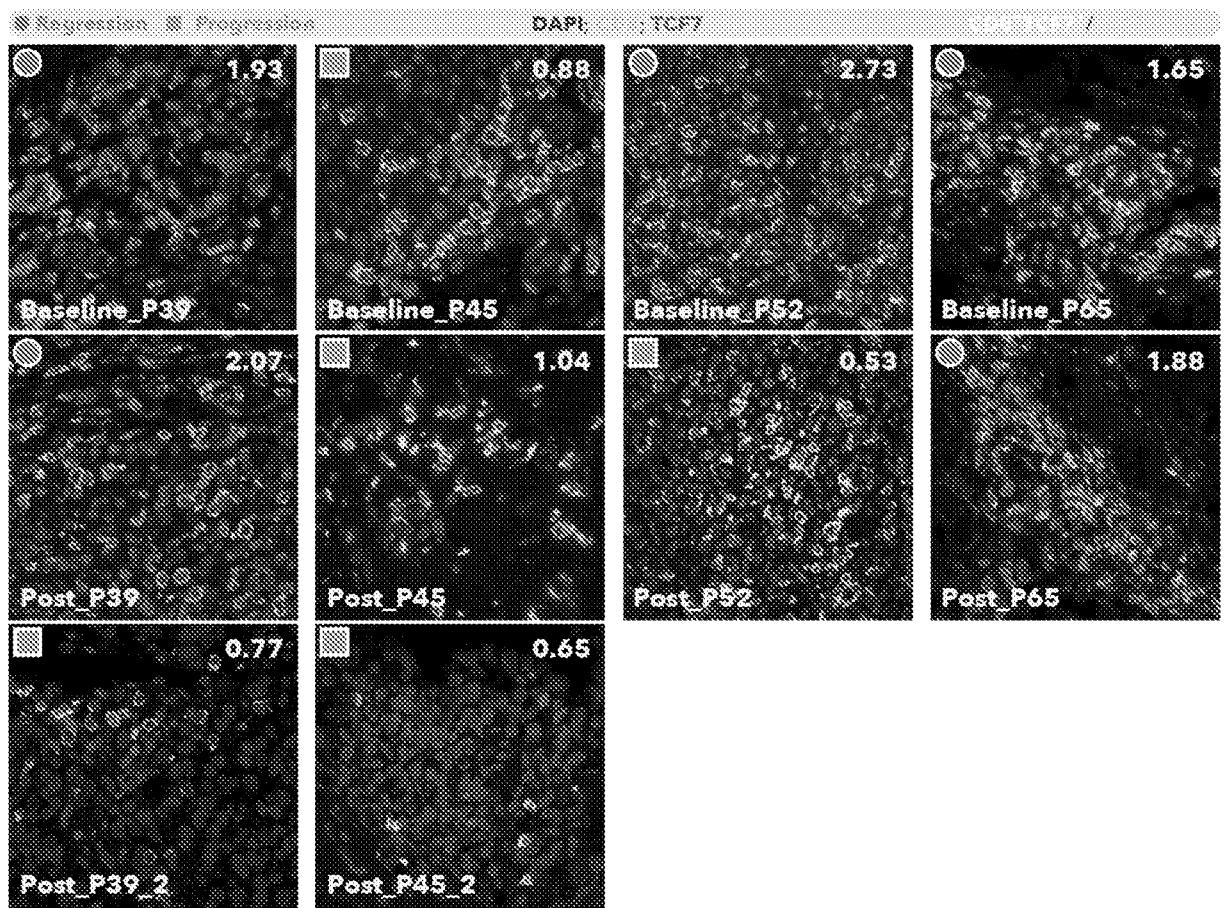
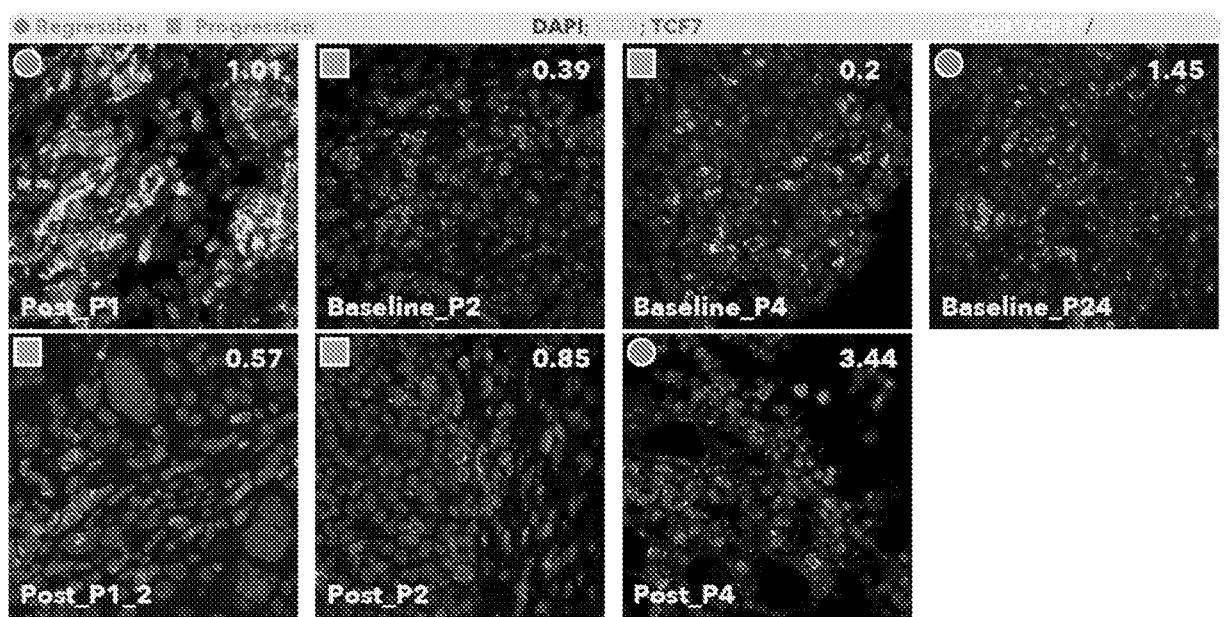
A**B****C****FIG. 40A-40C**

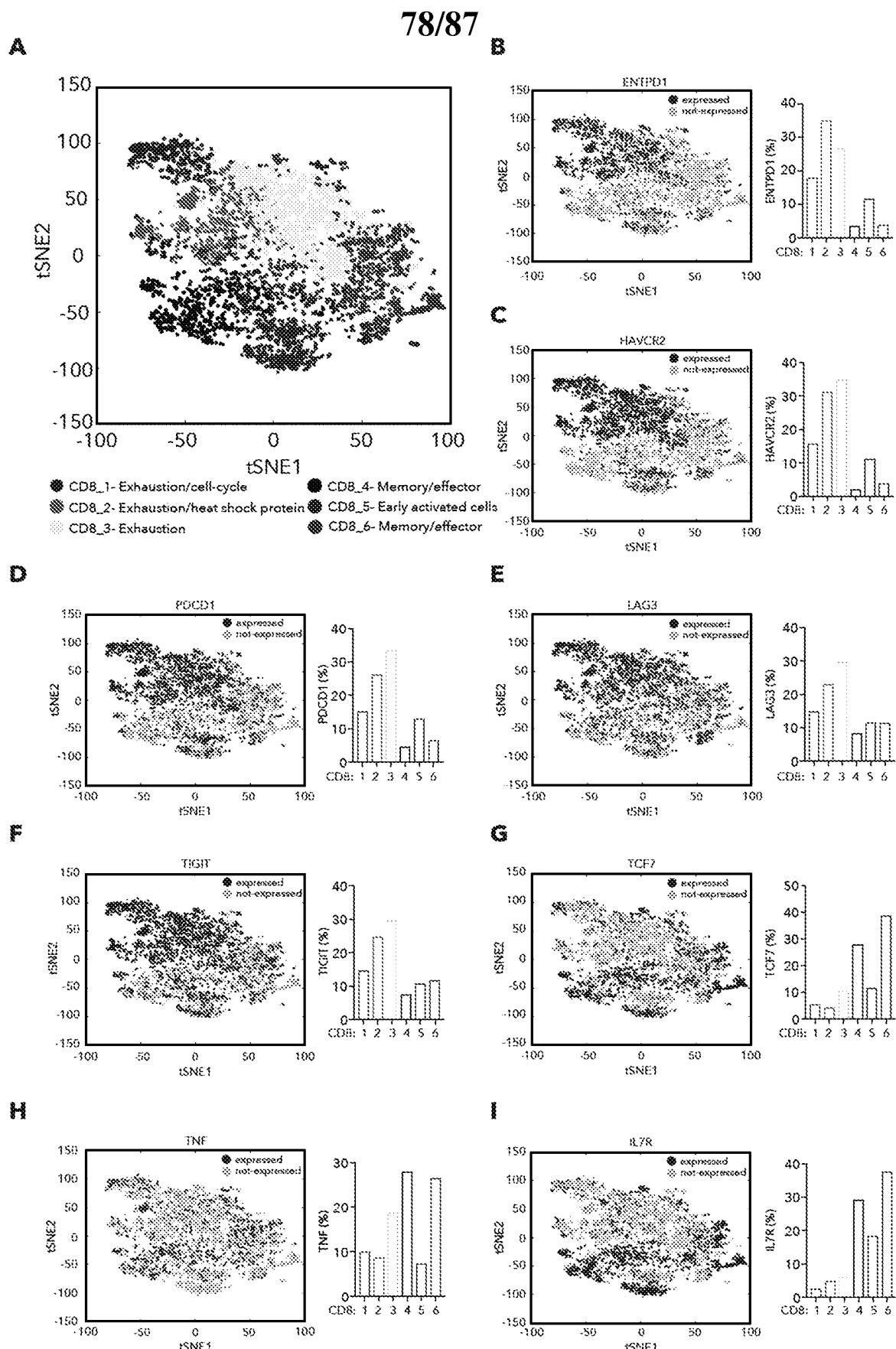
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**FIG. 41A-41D**

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A**B****FIG. 42A-42B**

**FIG. 43A-43I**

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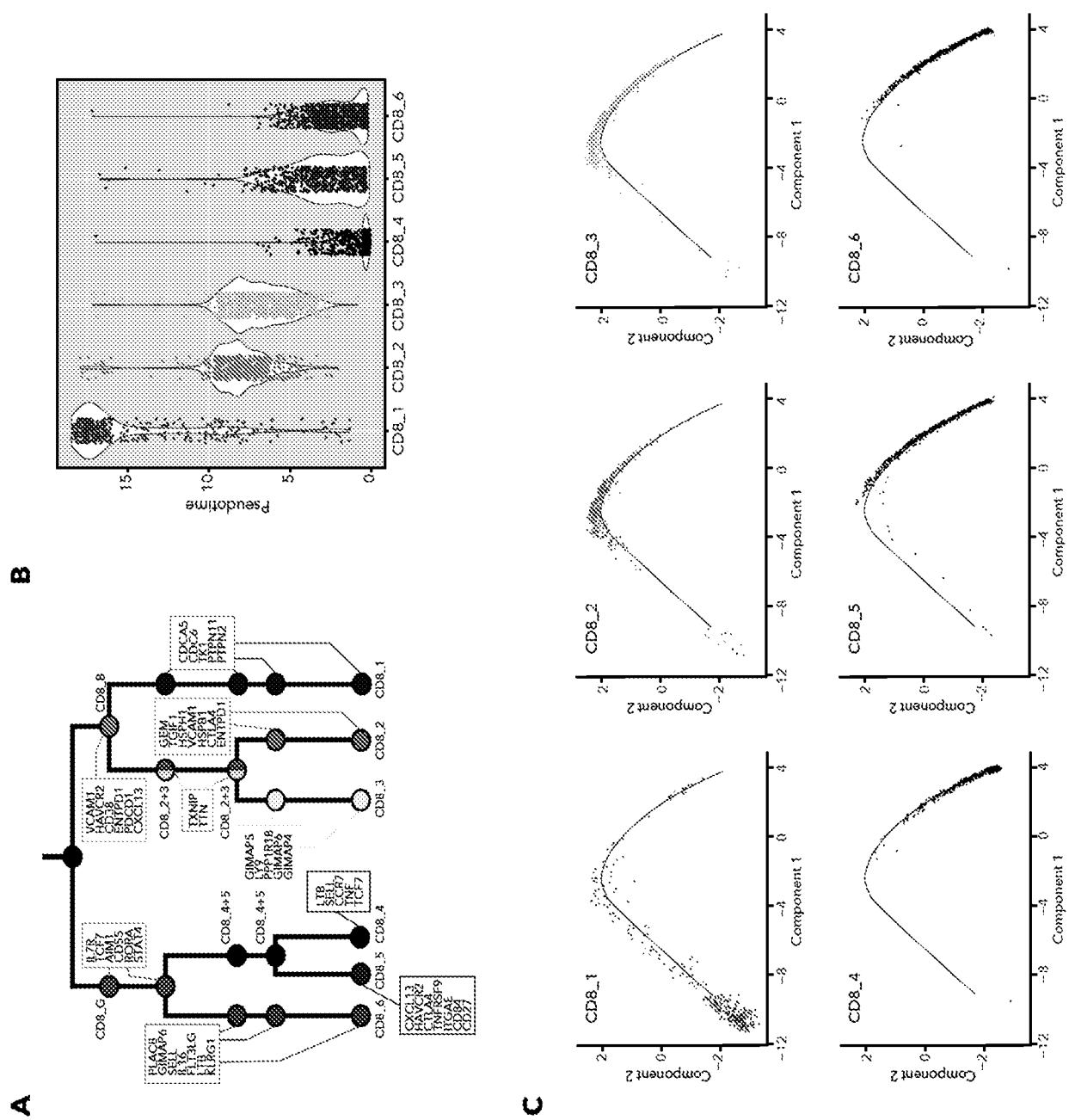
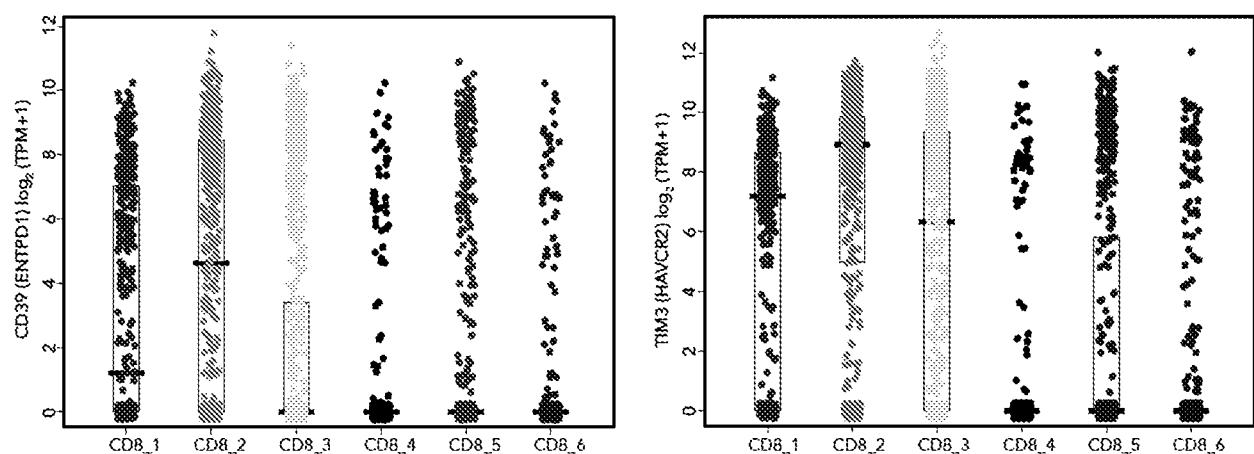
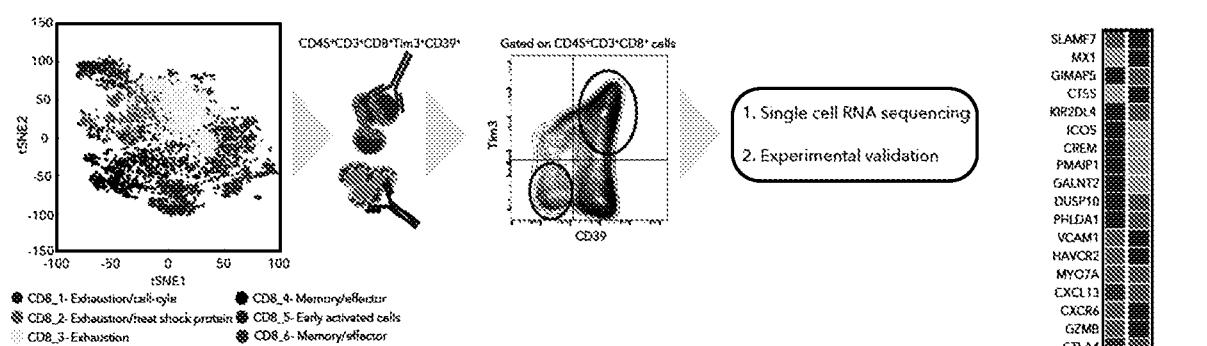
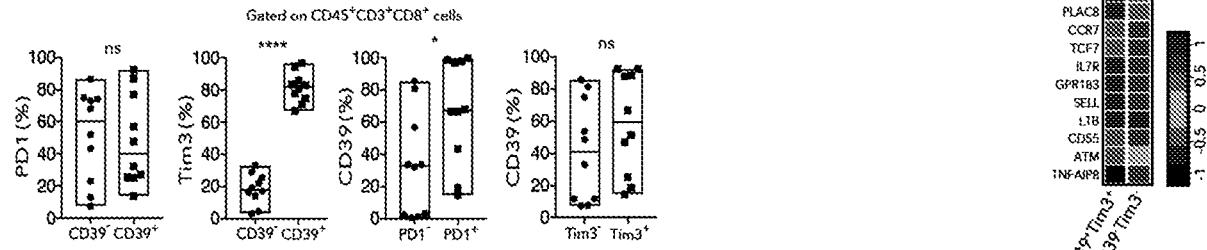
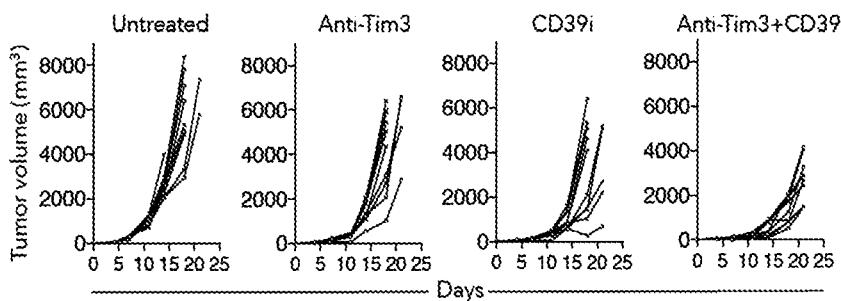
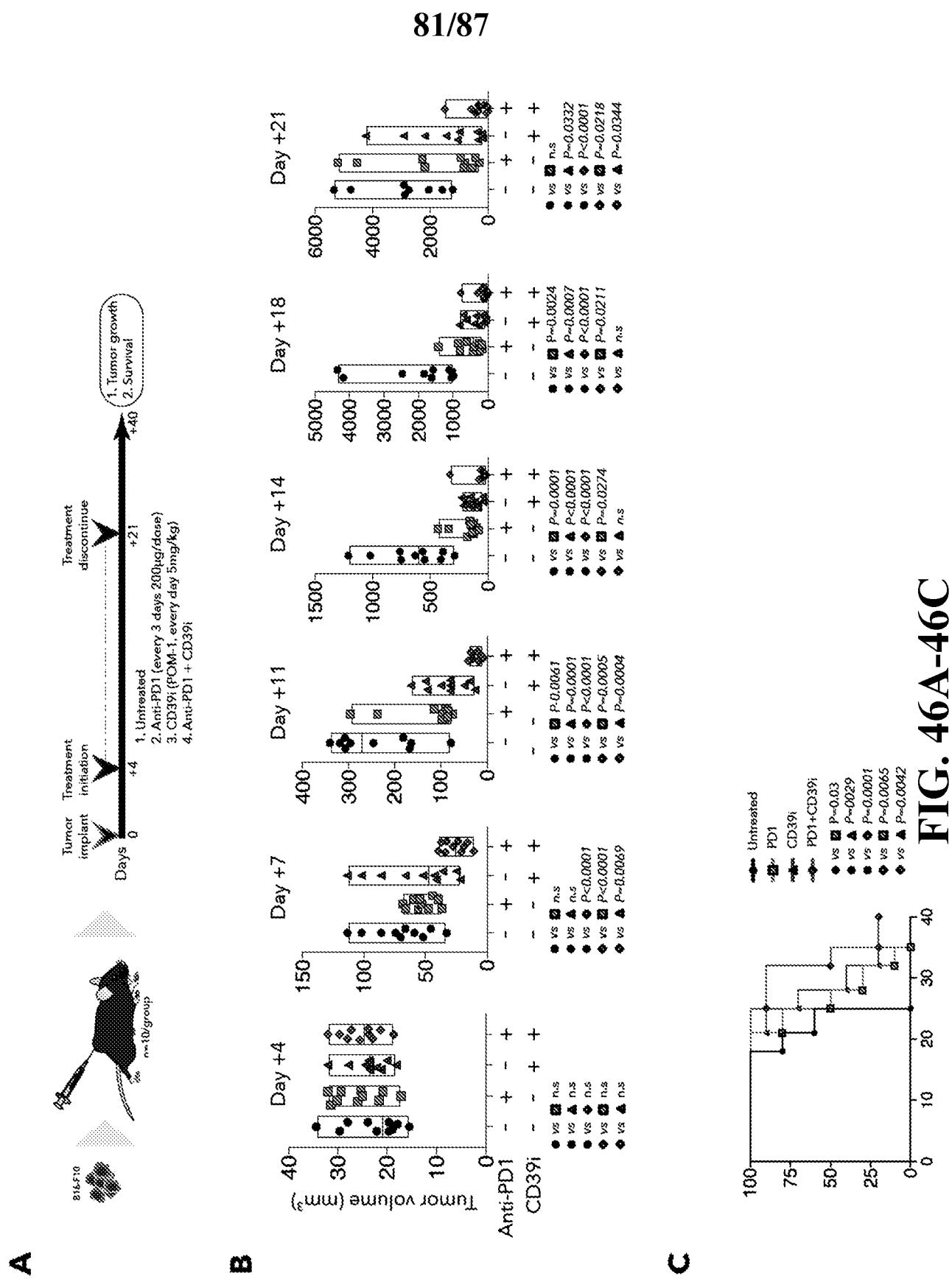


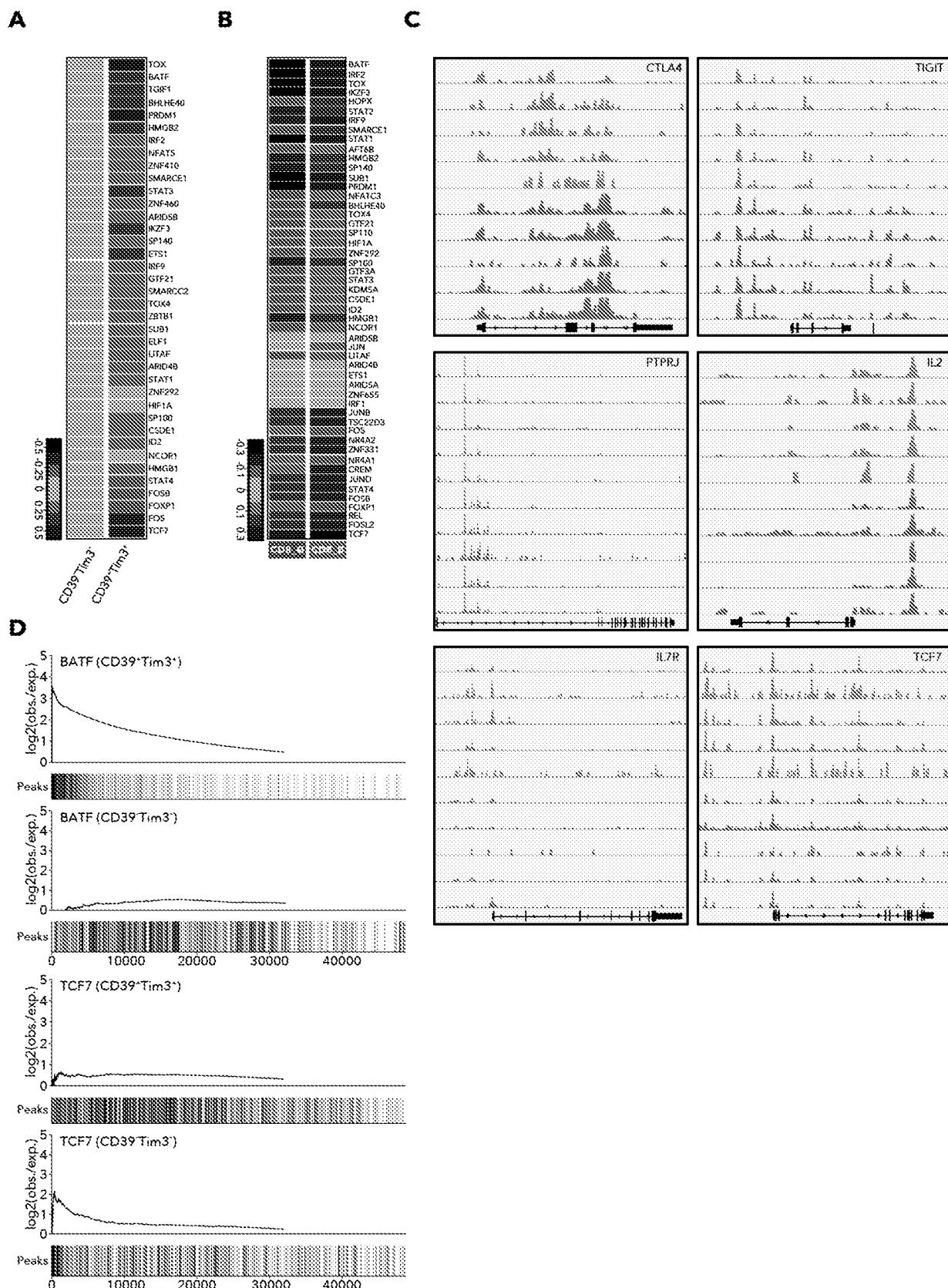
FIG. 44A-44C

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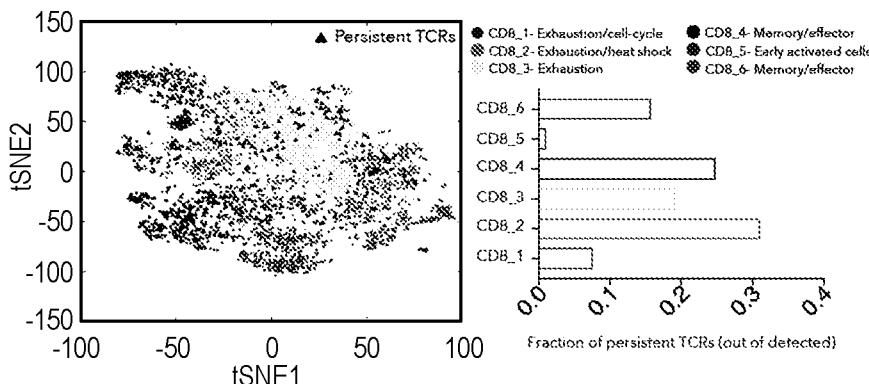
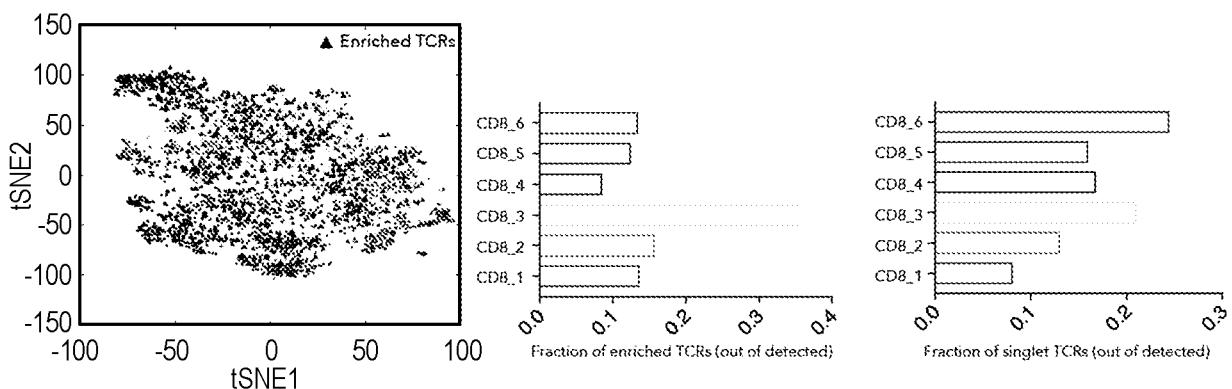
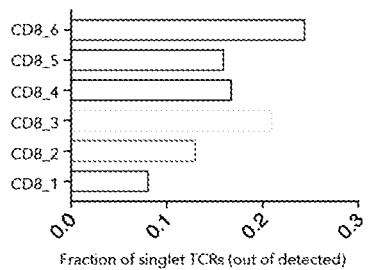
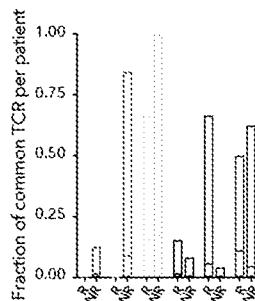
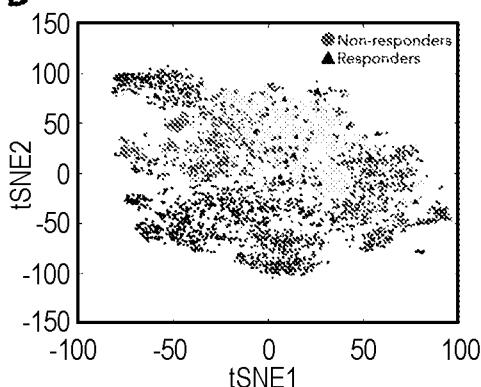
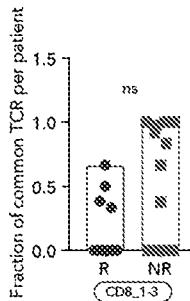
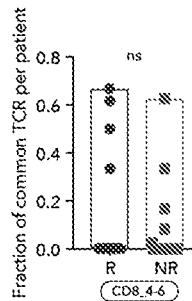
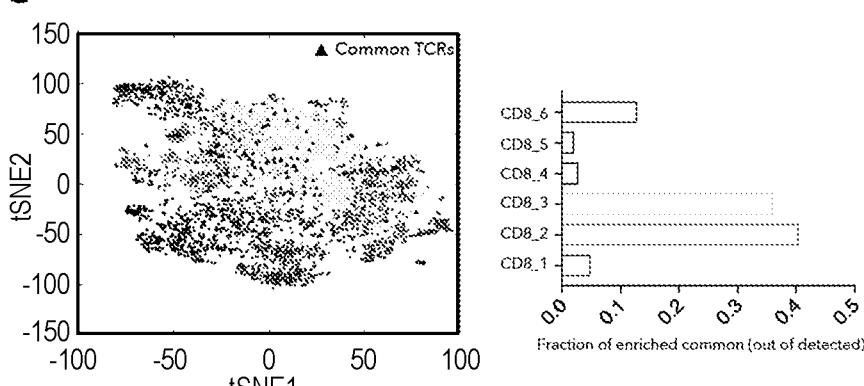
A**B****D****E****FIG. 45A-45E**



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**FIG. 47A-47D**

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A**B****C****D****E****F****G****FIG. 48A-48G**

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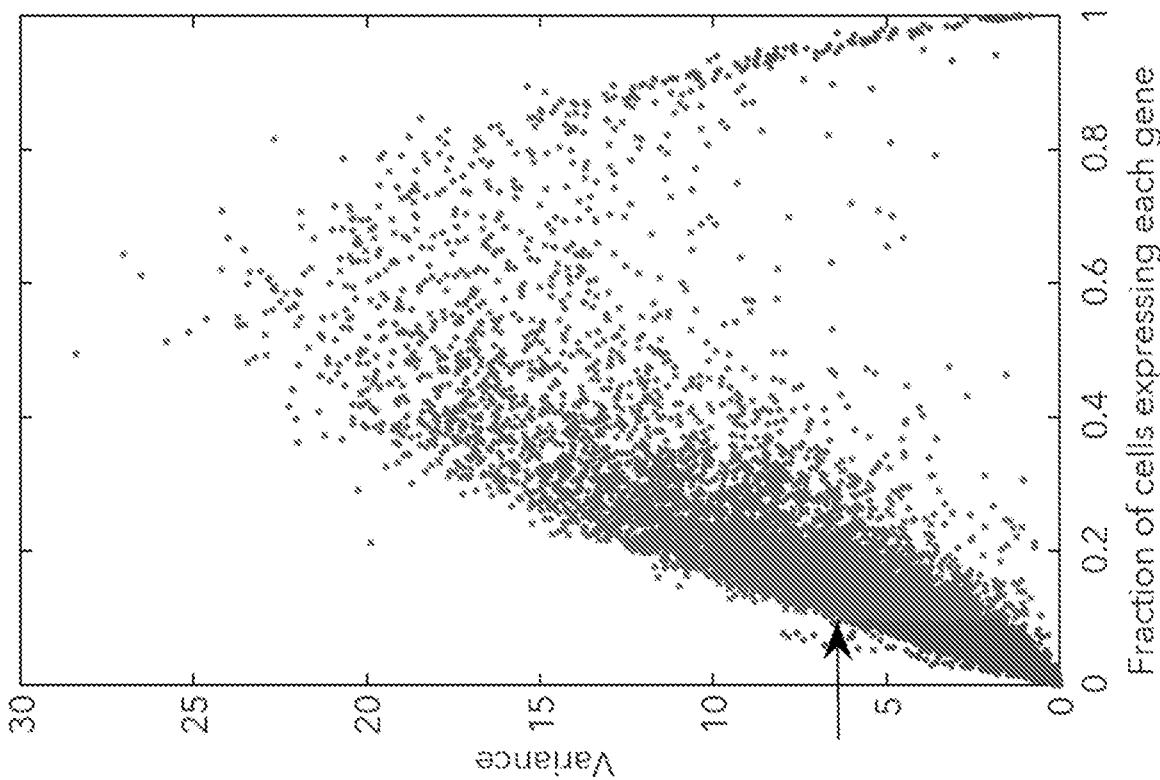
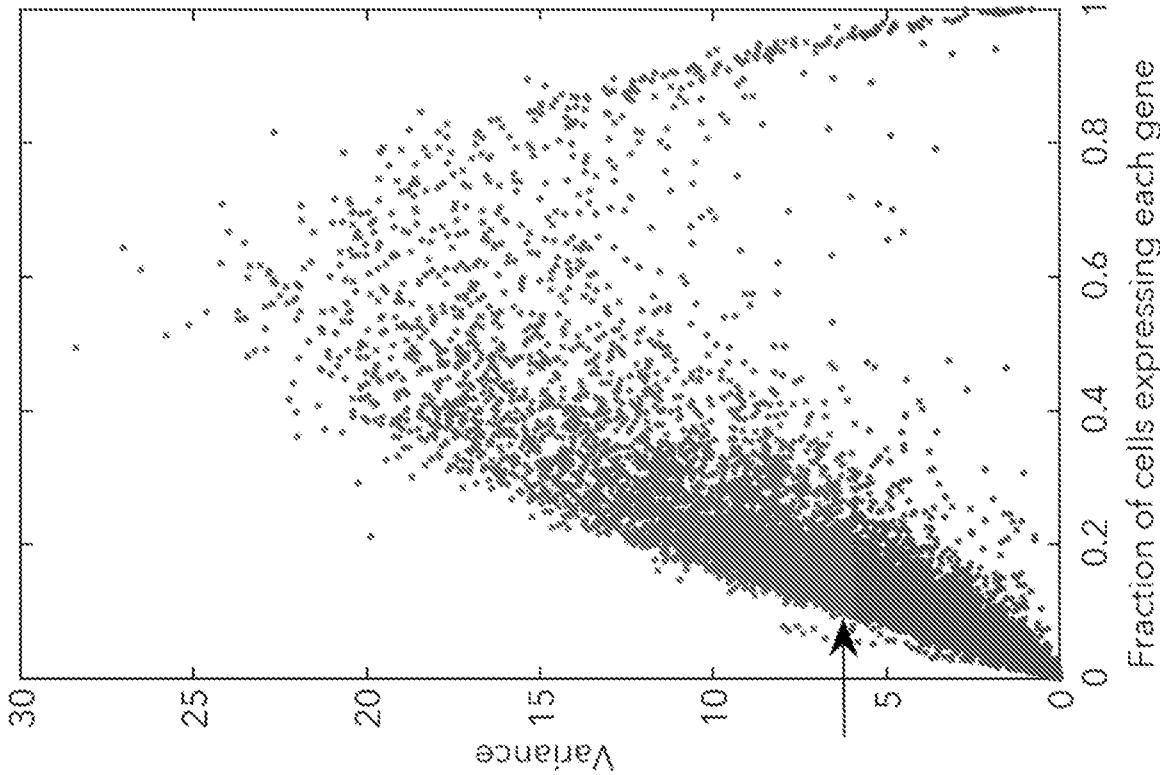
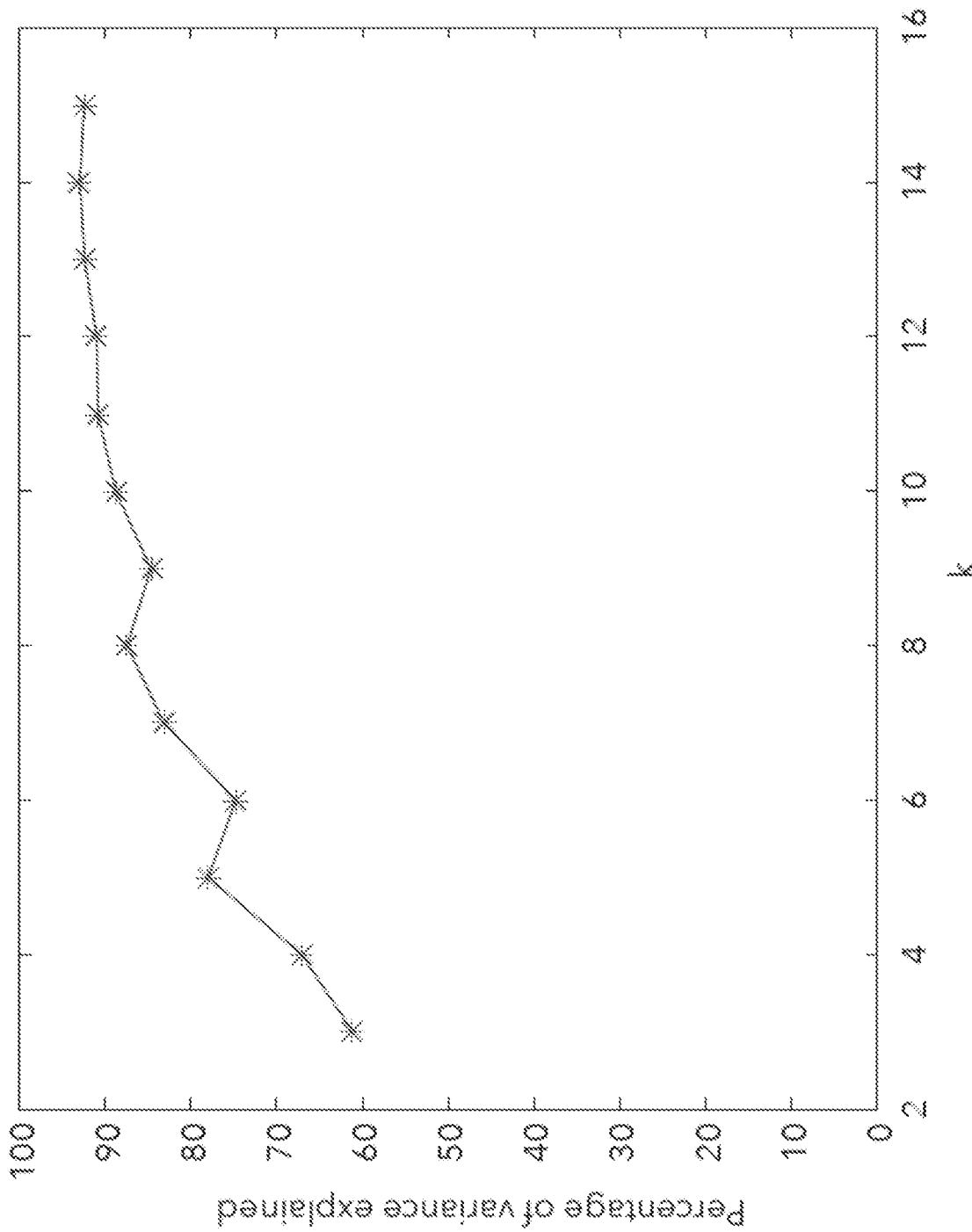
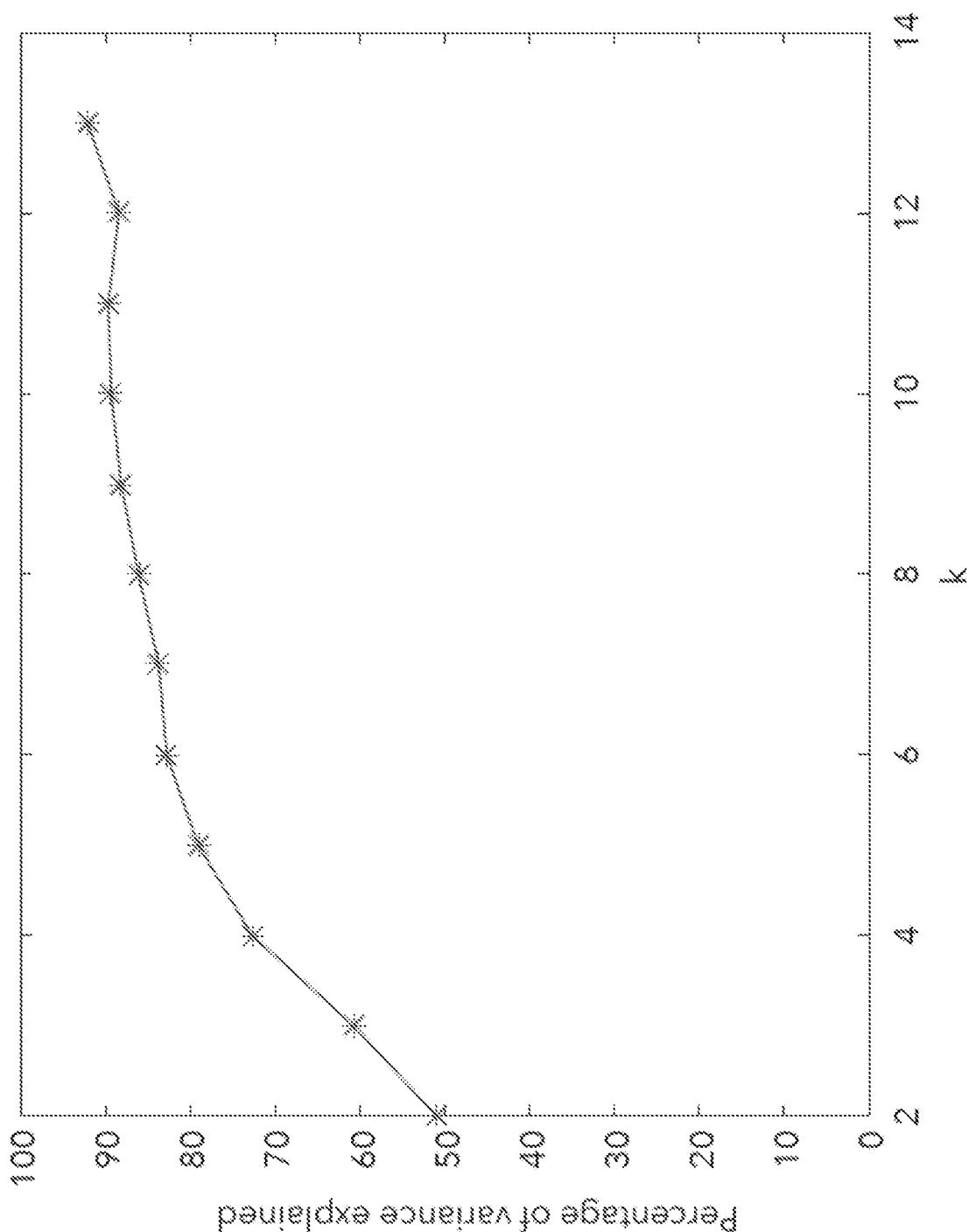
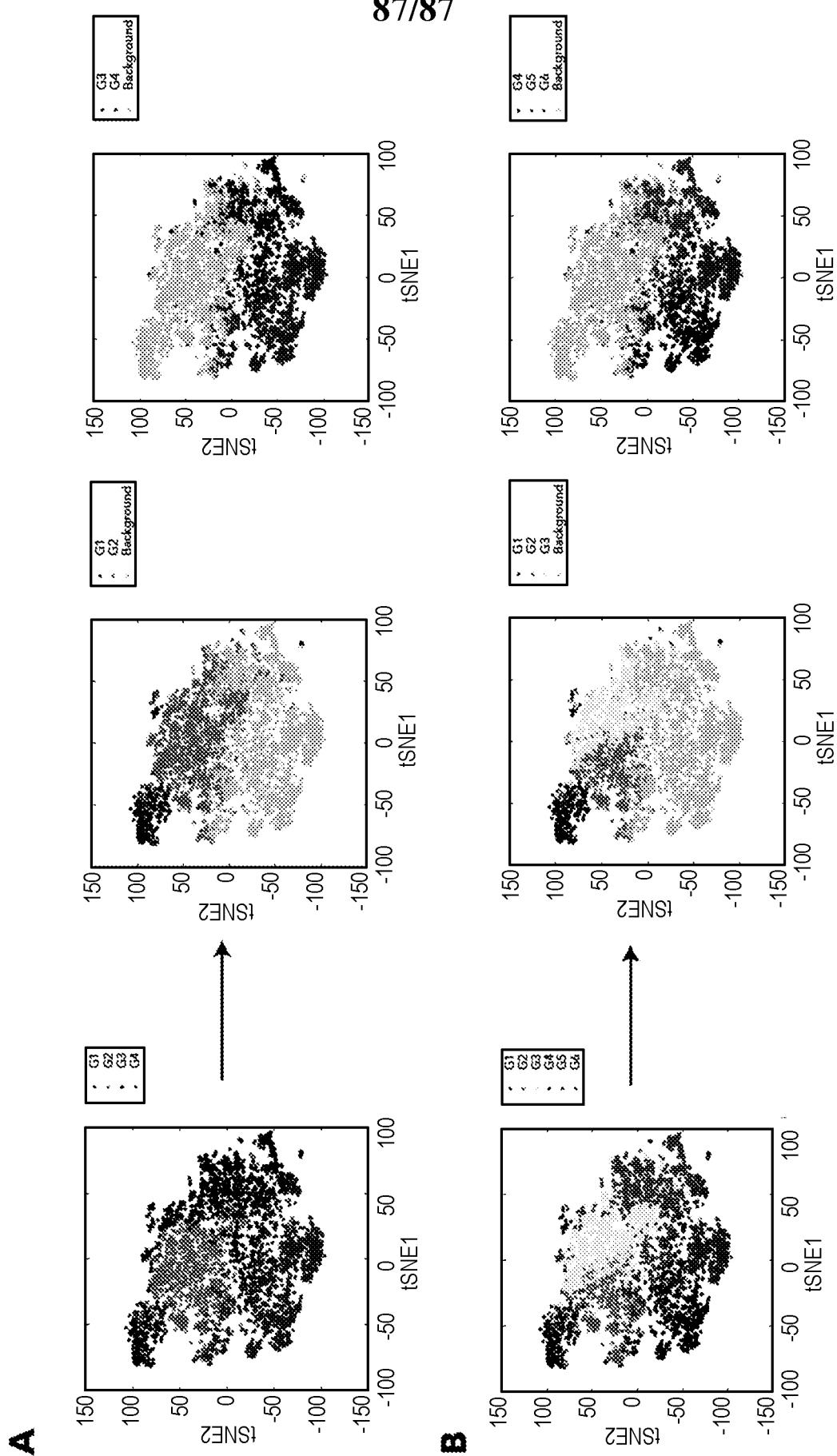


FIG. 49

85/87**FIG. 50**

86/87**FIG. 51**

87/87**FIG. 52A-52B**