

NF κ B activity, function and target gene signatures in primary mediastinal large B-cell lymphoma and diffuse large B-cell lymphoma subtypes

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Abstract

Primary mediastinal large B-cell lymphoma (MLBCL) shares important clinical and molecular features with classical Hodgkin lymphoma, including nuclear localization of the c-REL NF κ B subunit in a pilot series. Herein, we analyzed c-REL subcellular localization in additional primary MLBCLs and characterized NF κ B activity and function in a MLBCL cell line. The new primary MLBCLs had **prominent** c-REL nuclear staining and the MLBCL cell line exhibited high levels of NF κ B binding activity. MLBCL cells expressing a super-repressor form of I κ B α had a markedly higher rate of apoptosis, implicating constitutive NF κ B activity in MLBCL cell survival. The transcriptional profiles of newly diagnosed primary MLBCLs and DLBCLs were then used to characterize the NF κ B target gene signatures of MLBCL and specific DLBCL subtypes. MLBCLs expressed increased levels of NF κ B targets that promote cell survival and favor anti-apoptotic TNF α signaling. In contrast, “ABC-like” DLBCLs had a more restricted, potentially developmentally regulated, NF κ B target gene signature. Of interest, the newly characterized “Host Response” DLBCL subtype had a robust NF κ B target gene signature which partially overlapped that of primary MLBCL. In this large series of primary MLBCLs and DLBCLs, NF κ B activation was not associated with amplification of the c-REL locus, suggesting alternative pathogenetic mechanisms.

Introduction

The large B-cell lymphomas (LBCLs) include several distinct subtypes characterized by specific clinical features and/or transcriptional profiles. In addition to recognized entities such as primary mediastinal large B-cell lymphoma (MLBCL)¹⁻³, there are recently described subtypes of diffuse large B-cell lymphoma (DLBCL) with likely differences in normal cell(s) of origin, genetic bases for transformation and comprehensive transcriptional signatures⁴⁻⁶. With the newly available molecular signatures, it is now possible to evaluate the role of specific survival pathways in discrete LBCL subtypes.

The NF κ B signaling pathway regulates the survival of normal and malignant B-cells by controlling the expression of cell death regulatory genes^{7,8}. The extrinsic apoptotic pathway is triggered by engagement of TNF-family death receptors (TNF receptor 1 and Fas [CD95]), and the intrinsic apoptotic pathway is activated by the translocation of pro-apoptotic BCL2 family members to the mitochondria and subsequent release of cytochrome c⁷. Depending on the cellular context, TNF α signaling and other stimuli (including B-cell receptor, CD40 and Toll receptor engagement) also activate the NF κ B pathway and augment the transcription of NF κ B target genes⁷. These NF κ B target genes enhance cell survival by modulating TNF α signaling, inhibiting FAS-mediated apoptosis and limiting the activity of pro-apoptotic BCL2 family members, in addition to multiple other effects⁷.

Inactive NF κ B heterodimers (primarily c-REL or RELA and NF κ B1 [p50]) reside in the cytoplasm where they are complexed with an inhibitor of kappa B signaling (I κ B)⁷. In response to a variety of signals, I κ B kinase (I κ K) phosphorylates I κ B, resulting in the inhibitor's dissociation from the cytoplasmic NF κ B heterodimer. Thereafter, phosphorylated I κ B is degraded via the proteasome and the freed (active) NF κ B heterodimer translocates to the nucleus where it induces

the transcription of NF κ B target genes ⁷. For this reason, the activity of the NF κ B pathway in a B-cell tumor can be preliminarily assessed by determining the subcellular localization of NF κ B subunits (nuclear vs. cytoplasmic). This type of analysis was previously used to identify the likely role of c-REL containing heterodimers and NF κ B activation in classical Hodgkin's lymphomas (cHL) ⁹.

We recently characterized the transcriptional profile of primary MLBCL and identified important shared features with cHL ². Like Hodgkin Reed-Sternberg (HRS) cells, MLBCLs had low levels of expression of multiple B-cell signaling components and coreceptors ². MLBCLs also had high levels of expression of cytokine pathway components, TNF family members, and extracellular matrix elements previously identified in cHL ². These observations were of particular interest because MLBCL and the most common subtype of cHL (nodular sclerosis) have similar clinical presentations – in younger patients with local/mediastinal tumors characterized by reactive fibrosis.

Given the known role of the NF κ B survival pathway in cHL ⁹⁻¹¹ and the striking similarities between the cHL and MLBCL transcriptional profiles, including upregulation of specific NF κ B target genes, we assessed the subcellular localization of c-REL in a small pilot series of primary MLBCLs ². In almost all cases, the c-REL NF κ B subunit was localized to the nucleus, underscoring the potential role of constitutive NF κ B activation in primary MLBCLs ².

Previous studies also implicate NF κ B signaling in the survival of a subset of DLBCLs. DLBCLs are thought to arise from normal antigen-exposed B-cells that have migrated to or through germinal centers (GC) in secondary lymphoid organs ¹². A series of recent profiling studies highlighted the similarities between subsets of DLBCL and their putative normal B-cell counterparts. These DLBCL subsets shared certain features with normal GC B-cells ("GC-type") or *in vitro* activated peripheral blood B-cells ("ABC-type"); an additional group of tumors ("Type 3" or "Other") could not be classified by cell of origin (COO) ^{4,5}. In associated functional

analyses, DLBCL cell lines with “ABC-type” signatures had high levels of NFκB activity and increased sensitivity to NFκB inhibition, specifically implicating the NFκB survival pathway in this DLBCL subset ¹³.

More recently, our group utilized a large series of newly diagnosed DLBCLs, whole genome arrays and multiple clustering methods to identify 3 robust DLBCL subtypes with unique comprehensive transcriptional signatures -- “Oxidative Phosphorylation”, “B-cell Receptor/Proliferation” and “Host Response” (HR) ⁶. HR tumors have increased expression of T/NK-cell receptor and activation pathway components, complement cascade members, macrophage/dendritic cell markers and inflammatory mediators; these tumors also contain significantly higher numbers of CD2+/CD3+ tumor-infiltrating lymphocytes and interdigitating S100+/GILT+/CD1a-/CD123- dendritic cells ⁶. HR DLBCLs share features of histologically defined T-cell/histiocyte-rich LBCL, including fewer genetic abnormalities, and presentation in younger patients with frequent splenic and bone marrow involvement ⁶. To date, the potential role of NFκB activation in HR tumors and the additional comprehensive clusters (CC) has not been defined.

In LBCLs with likely NFκB activation, the genetic bases for constitutive NFκB signaling are not yet known. In cHL, gains of chromosome 2p12-16 (the c-REL locus) have been associated with the accumulation of nuclear c-REL, suggesting that c-REL amplification leads to increased NFκB activity ⁹. A small number of DLBCLs and MLBCLs also have gains of chromosome 2p12-16, prompting speculation regarding a similar mechanism of NFκB activation in LBCLs ^{14,15}. However, recent studies suggest that c-REL amplification is: 1) more common in “GC-type” DLBCLs than “ABC-type” DLBCLs ⁴; and 2) infrequently associated with nuclear c-REL expression ¹⁶.

Given the likely role of NFκB in promoting normal and malignant B-cell survival, the NFκB pathway includes promising rational therapeutic targets ¹⁷. For these reasons, we have analyzed the role of NFκB activation in MLBCL and DLBCL

subtypes using a combination of c-REL immunolocalization, molecular inhibition of NF κ B in informative cell lines and analyses of NF κ B target gene signatures and c-REL amplification in well-defined primary LBCLs.

Materials and Methods

Cell lines

The Karpas 1106 MLBCL cell line (gift of A. Karpas, Cambridge, UK ¹⁸), two DLBCL cell lines (DHL6 and OCI-LY10; ¹⁹) and a Hodgkin's lymphoma cell line (KM-H2, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cultured at 37°C in 5% CO₂ in RPMI-1640 medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Tissue Culture Biologicals, Tulare, CA).

Immunohistochemistry

Immunohistochemistry (IHC) was performed **on an additional series of primary MLBCLs obtained from the archives of Brigham & Women's Hospital** using 5 μ thick formalin- paraffin-embedded tissue sections. Slides were deparaffinized and pre-treated with 10-mM citrate, pH 6.0 (Zymed, South San Francisco, CA) in a steam pressure cooker (Decloaking Chamber, BioCare Medical, Walnut Creek, CA) and subsequently washed in distilled water. All further steps were performed at room temperature in a hydrated chamber. Slides were pre-treated with Peroxidase Block (DAKO USA, Carpinteria, CA) for 5 minutes to quench endogenous peroxidase activity. Primary rabbit anti-c-Rel antibody (AB-1; 1:1000 dilution; Oncogene Research Products, San Diego, CA) was applied in DAKO diluent (DAKO) for 1 hour. **The specificity of the c-REL antibody was previously confirmed by: 1) immunoblotting of a c-REL positive control cell line and demonstrating reactivity with a single band of appropriate molecular weight (Manufacturer's Information); and 2) detecting nuclear translocation of c-REL in a c-REL positive control cell line stimulated with CD40 ligand ²⁰.** Slides were washed in 50-mM Tris-Cl, pH 7.4 and anti-rabbit horseradish peroxidase-conjugated antibody solution (Envision+ detection kit, DAKO) was applied for 30 minutes. After further washing, immunoperoxidase staining was developed with

DAB chromogen (DAKO) and slides were counterstained with Harris hematoxylin (Polyscientific, Bay Shore, NY). c-REL IHC staining was evaluated using three distinct categories: absent (-), weak to intermediate (+), and intense (++) staining of tumor cell nuclei using an Olympus BX41 microscope with the objective lens of 40 x/0.75 Olympus UPlanFL (Olympus, Melville, NY). **Cytoplasmic staining was used as an internal control and reference to score the intensity of nuclear staining. Tumors were considered to have significant nuclear c-REL if $\geq 40\%$ of tumor cell nuclei exhibited (+) or (++) nuclear c-REL staining.** The pictures were taken using Olympus QColor3 and analyzed with acquisition software QCapture v2.60 (QImaging, Burnaby, BC, Canada) and Adobe Photoshop 6.0 (Adobe, San Jose, CA).

NF κ B activation assay

NF κ B activity was assessed using a colorimetric assay ²¹ that detects binding of cellular p50 (NF κ B1) to immobilized NF κ B- target consensus oligonucleotide sequences (Active Motif, Carlsbad, CA). In brief, 5 ug of each whole cell lysate was added to microwells containing immobilized NF κ B-specific target probes. Following incubation and washing, cellular p50 bound to the immobilized NF κ B target sequences was detected using a p50-specific polyclonal antibody, a HRP-conjugated secondary antibody and colorimetric quantification. NF κ B binding activity in study samples was compared to that of standardized negative and positive controls provided by the manufacturer (2.5 ug of nuclear extract from phorbol 12-*O*-tetradecanoylphorbol-13-acetate (TPA) stimulated Jurkat cells preincubated with NF κ B target sequences [wild-type sequences, negative control and mutated sequences, positive control]).

Molecular cloning and retroviral transduction

An I κ B α super repressor construct in which serines 32 and 36 were replaced with alanines (SR-I κ B α , gift of A. Rabson and C. Gelinas ²²) was used to inhibit NF κ B

activation in Karpas1106 cells. SR-I κ B α , which cannot be phosphorylated by I κ K, remains complexed to the NF κ B heterodimer, inhibiting NF κ B translocation and activation of NF κ B targets. SR-I κ B α was cloned into a modified MSCV vector (Clontech, Palo Alto, CA) that expresses eGFP in a bicistronic manner with the gene of interest ²³. MSCV-eGFP-SR-I κ B α or MSCV-eGFP alone was cotransfected into 293T cells with pKAT (an amphotropic packaging plasmid) and pCMV-VSV-G (a vector encoding the vesicular stomatitis virus G-glycoprotein) using lipofectamine 2000 (Invitrogen, Carlsbad, CA). Supernatants containing retrovirus were harvested at 48 and 72 hrs.

Karpas 1106 cells were washed in phosphate-buffered saline (PBS, Mediatech / Cellgro, Herndon, VA) and resuspended at 1×10^6 cells/ml in fresh media containing Polybrene (final concentration 8 mg/ml, Sigma, St. Louis, MO). 500 μ l of Karpas 1106 was added to individual wells of a 24-well plate and 500 μ l of retroviral supernatant (MSCV-eGFP-SR-I κ B α or vector only) was added thereafter. Cells were then spinoculated at 2000 rpm for 90 min, incubated at 37^oC 5% CO₂ overnight, and subsequently washed and resuspended in fresh medium. GFP expression was analyzed at 24 hrs. and GFP-positive cells were isolated by fluorescence-activated cell sorting (FACS Vantage, Becton Dickinson, San Jose, CA). **Statistical comparisons of NF κ B activity in SR-I κ B α and vector-only transduced cells were performed using a one-sided student's t-test.**

Cell viability and Apoptosis

Proliferation of MSCV-eGFP- SR-I κ B α or vector-only transduced Karpas 1106 cells was assessed by measuring MTS (Promega, Madison, WI) dye absorbance 0, 24, 48, and 72 hrs after cell sorting ¹⁹. **Statistical comparisons of proliferation in SR-I κ B α and vector-only transduced cells were performed using an ANOVA model on the natural logarithm of the data including the cell type (SR-I κ B, empty vector, or parental cells) and time after sorting as variables.** Apoptosis was

assessed using Annexin V conjugated to the red-fluorescent dye, Alexa-568, according to the manufacturer's recommendations (Roche Applied Science, Penzberg, Germany). The percentage of apoptotic cells was defined as the number of cells that co-expressed Annexin V (Alexa 568) and GFP within the entire GFP+ cell population.

Microarray analysis: Description of dataset

The expression of NF κ B target genes in primary large cell lymphomas was evaluated using a recently described dataset of 176 DLBCL and 34 MLBCL transcriptional profiles^{2,6} (URL:<http://www.broad.mit.edu/cgi-bin/cancer/datasets>). All primary DLBCL and MLBCL tumor specimens were nodal or mediastinal biopsies from newly diagnosed, previously untreated patients. The dataset included the top 15,000 genes from the Affymetrix U133A/U133B arrays, ranked using a *median absolute deviation* (MAD) variation filter across all samples². The DLBCL tumor samples were previously assigned to the developmentally related "cell-of-origin" (COO) categories "Germinal Center B-cell-like" (GCB), "Activated B-cell-like" (ABC), and "Other" using linear predictive scores^{5,6}. The DLBCL tumor samples were also assigned to the more recently described comprehensive consensus clusters, "Oxidative Phosphorylation" (OXP), "B-cell receptor signaling/ proliferation" (BCR), and "Host Response" (HR)⁶.

Curation of microarray-based NF κ B target gene sets

Three NF κ B target gene sets were selected for detailed analysis based on the gene sets' relevance to the biology of normal and malignant B-cells: (Geneset 1) NF κ B target genes that were downregulated in Hodgkin's lymphoma cell lines following introduction of NF κ B super-repressor I κ B Δ N¹¹; (Geneset 2) Previously described NF κ B target genes that were differentially expressed at specific stages of normal B-cell development and/or in DLBCLs with "ABC" features^{13,24} (Geneset 3) NF κ B target genes that were downregulated after siRNA silencing of

REL-A (p65) in TNF α -stimulated HeLa cells²⁵. The complete gene sets and their associated microarray probe sets are included in the Supplementary Information.

Enrichment test for NF κ B genes in MLBCL.

Gene Set Enrichment Analysis (GSEA)^{2,6,26} was performed as previously described using the above-mentioned NF κ B target gene sets and the MLBCL and DLBCL array data. Enrichment was assessed by: 1) ranking the 15,000 genes with respect to the phenotype “MLBCL vs. DLBCL”; 2) locating the represented members of a given NF κ B target gene set within the ranked gene list; 3) measuring the proximity of the gene set to the overexpressed end of the ranked list with a Kolmogorov-Smirnoff (KS) score (with a higher score corresponding to a higher proximity); and 4) comparing the observed KS score to the distribution of 100 permuted KS scores for all gene sets. The p-values were obtained by pooling the permuted KS scores for all the gene sets tested, and by locating the observed KS scores within the resulting permutation distribution (Supplementary information).

Supervised analysis in the space of NF κ B target genes

The above-mentioned sets of NF κ B target genes were combined for additional supervised analyses in specific lymphoma subsets (MLBCL vs. DLBCL, GCB vs. non-GCB DLBCL, ABC vs non-ABC DLBCL, HR vs non-HR DLBCL). For inclusion in the supervised analysis, NF κ B target genes had to be represented in the 15,000-gene dataset. 64 of the 68 NF κ B target genes met these criteria (Supplementary Information). NF κ B target genes correlating with the class distinction of interest (i.e. MLBCL vs. DLBCL, “ABC” DLBCL vs non-ABC DLBCL, HR or non-HR DLBCL) were identified by ranking the target genes according to their signal-to-noise ratio (SNR) based on medians. Thereafter, the observed values in the data were compared with the 99th percentile of the permutation distribution (1000 permutations, Supplementary Information). In addition, the fold-

difference in expression of a given NF κ B target was calculated by dividing the median expression value for the class of interest by the median expression value of the comparison group (i.e. MLBCL/DLBCL). NF κ B target genes which met the 99th percentile of the permutation distribution and exhibited a $\geq 30\%$ difference in median expression values were considered to be differentially expressed (Supplementary Information).

The same method was used to assess differential expression of NF κ B target genes in an independent set of 38 MLBCL and 26 DLBCL with available cDNA microarray (“lymphochip”) profiles (URL: <http://lmpp.nih.gov/PMBL/>). 92% (59/64) of the combined set of NF κ B target genes included in the 15,000 gene dataset were also represented on the lymphochip platform (Supplementary Information and Ref. ³).

Quantitative PCR analysis of c-REL amplification

Quantitative PCR was used to measure *c-REL* copy numbers in genomic DNA isolated using a published method ²⁷. In brief, real-time PCR was performed using the ABI Prism 7700 sequence detector system (Applied Biosystems, Foster City, CA). *C-REL* copy numbers were compared to those of two control genes (beta-2-microglobulin, albumin), which map to loci that are rarely involved in chromosomal gains or losses (primer sequences and reaction conditions available upon request). On each 96-well reaction plate, a dilution series of normal human genomic spleen DNA was used as reference. Further, each reaction plate included a negative control (normal human genomic DNA, Applied Biosystems, Foster City, CA), and two positive controls (genomic DNA isolated from two Hodgkin’s lymphoma cell lines with known *c-REL* amplification, KM-H2 and L-428 ²⁸, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). All measurements were performed in triplicate. Cases with a ratio of *cREL*/beta-2-microglobulin and *c-REL*/albumin copy number >2 were considered positive for *c-REL* amplification.

Results

Confirmation of nuclear subcellular localization of c-REL in MLBCL

To confirm and extend our original observations regarding c-REL nuclear localization in MLBCL, we analyzed a new series of primary tumors with a robust c-REL immunohistochemical assay. As before, there was **prominent** c-REL nuclear staining in 7/7 new primary MLBCLs (Fig. 1). c-REL staining intensity and subcellular localization were more variable in DLBCLs, consistent with recent observations¹⁶.

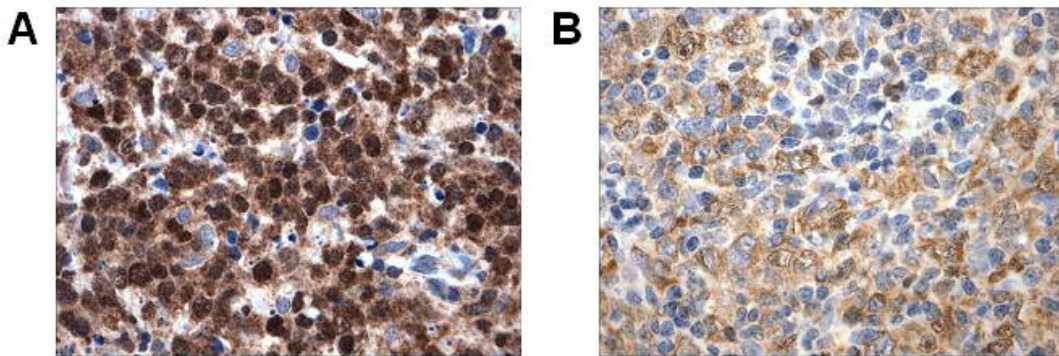


Fig. 1 : Subcellular localization of c-REL in MLBCL. A) Prominent intense c-REL nuclear staining in a representative primary MLBCL. B) Variable c-REL staining intensity and subcellular localization in a DLBCL. Original magnification 400X.

NF κ B activity is required for cell survival of a MLBCL cell line

After demonstrating c-REL nuclear staining in the additional primary MLBCLs, we sought functional evidence of NF κ B activation in a MLBCL cell line (Karpas 1106). Initially, NF κ B activity in Karpas 1106 was measured and compared to that in lymphoma cell lines with previously reported high or low levels of NF κ B activity (Hodgkin's lymphoma line [KM-H2] and DLBCL line [OCI-Ly10], high and

DLBCL line [DHL6], low). Like KMH2 and OCI-Ly10, the Karpas 1106 MLBCL cell line had high levels of constitutive NF κ B activity, \approx 4-fold greater than that of DHL6 cells (Fig. 2A).

We next assessed the consequences of NF κ B inhibition on the survival and proliferation of Karpas 1106 MLBCL cells. The MLBCL cells were transduced with a retrovirus encoding GFP and the super-repressor form of I κ B α (SR-I κ B α) or GFP alone. Thereafter, GFP-expressing SR-I κ B α and vector-only MLBCL cells were isolated by FACS and analyzed for NF κ B activity. As expected, SR-I κ B α MLBCL cells had \approx six-fold less NF κ B activity than cells expressing the vector alone ($p < .0001$, one-sided student's t-test, Fig. 2B). SR-I κ B α MLBCL cells had a \approx 4-fold higher rate of apoptosis (Annexin V expression) than cells expressing the vector alone (SR-I κ B α 54% apoptosis vs. vector-only 14% apoptosis, Figs. 2B [right panel] and C). SR-I κ B α MLBCL cells also failed to proliferate in culture whereas vector-only cells grew at the same rate as parental Karpas 1106 cells (Fig. 2D). **The difference between SR-I κ B transduced MLBCL cells and either empty vector expressing or parental cells was significant ($p < 0.0001$ in each case), whereas there was no difference between empty vector and parental cells ($p = N.S.$).** Taken together, these data indicate that constitutive NF κ B activity is essential for the survival of this MLBCL cell line.

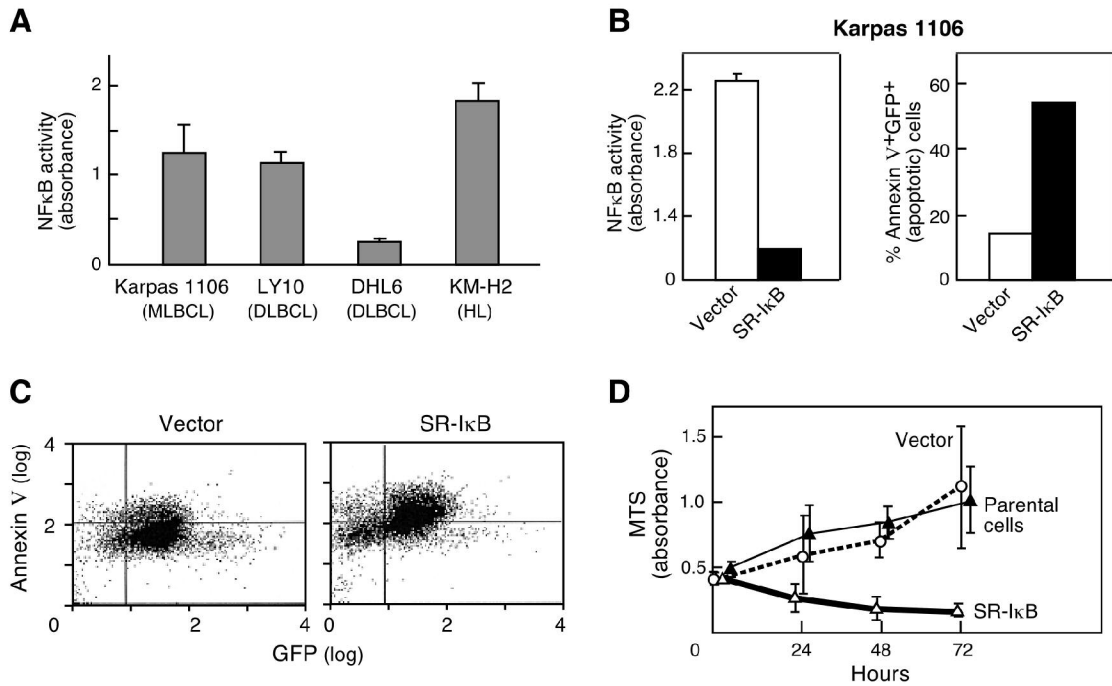


Fig. 2: NF κ B activity and inhibition in a MLBCL cell line. A) NF κ B DNA-binding activity of the MLBCL cell line, Karpas 1106, two DLBCL cell lines, OCI LY10 and DHL6, and a Hodgkin's lymphoma cell line, KM-H2. NF κ B DNA binding activity was measured using a colorimetric assay. *Measurements were performed in triplicates.* B) NF κ B activity and apoptosis in MLBCLs expressing an I κ B α super-repressor. Left panel) NF κ B DNA-binding activity in MLBCL cells transduced with MSCV-eGFP (vector) alone or MSCV-eGFP-SR-I κ B α . *Measurements were performed in triplicates. NF κ B activity was significantly lower in SR-I κ B α cells than in vector - only cells ($p < .0001$, one-sided student's t-test).* Right panel) The apoptotic fraction (percentage Annexin V+ GFP+) of cells transduced with MSCV-eGFP-SR-I κ B α is much higher than that of cells transduced with vector alone. C) Apoptosis in MLBCL cells expressing an I κ B super-repressor. GFP-positive cells expressing vector alone or MSCV-eGFP-SR-I κ B α were analyzed for expression of Annexin V (Alexa 568). GFP, x-axis, Annexin V, y-axis. D) Proliferation of MLBCL cells expressing an I κ B α super-repressor. The proliferation (MTS absorbance) of parental and GFP+ vector only and MSCV-eGFP-SR-I κ B α transduced MLBCL cells was measured at 0, 24, 48 and 72 hours *and assessed with an ANOVA model which included the type of treatment and time of measurement. The difference between SR-I κ B α transduced MLBCL cells and either vector - only or parental cells was significant ($p < .0001$ in each case), whereas there was no difference between empty vector and parental cells ($p = N.S.$). The measurements were performed in triplicates. All experiments (A-D) were performed 3-4 times with comparable results; representative experiments are shown.*

The primary MLBCL signature is enriched for NF κ B target genes

After demonstrating the importance of NF κ B activity for the proliferation and viability of a MLBCL cell line, we assessed the consequences of NF κ B activation in our series of primary MLBCLs with available transcriptional profiles. Specifically, we asked whether previously defined, co-regulated sets of NF κ B target genes were upregulated in primary MLBCLs (as compared with DLBCLs) using gene set enrichment analysis (GSEA). Three independently defined sets of NF κ B target genes with likely biologic relevance in MLBCL were used: two series of target genes responsive to NF κ B inhibition in Hodgkin's lymphoma cell lines and TNF-stimulated adherent cells, respectively^{11,25}; and an additional series of previously described NF κ B targets that were differentially expressed in *in vitro* activated normal peripheral blood B-cells and "ABC-like" DLBCLs^{13,24}. The primary MLBCL signature was significantly enriched for the credentialed NF κ B targets from Hodgkin's lymphoma cell lines (Geneset 1, $p = .05$) and the likely NF κ B targets in normal activated B-cells and "ABC-like" DLBCLs (Geneset 2, $p = .02$). In addition, there was a trend toward more abundant TNF-induced NF κ B target transcripts in primary MBLCLs (Geneset 3, $p = .08$).

MLBCL are characterized by a broad NF κ B activation signature

Given the partial overlap between the above-mentioned series of NF κ B target genes, we then combined the NF κ B target gene sets for more detailed analyses of NF κ B activation signatures in primary MLBCLs and previously defined subsets of DLBCL. A large series of NF κ B targets were expressed at significantly higher levels in MLBCL than DLBCL (Fig. 3A), including genes regulating cell viability. To compare the NF κ B target gene signature of MLBCL with that of “ABC-like” DLBCLs, we also identified the differentially expressed NF κ B target genes in “ABC-like” vs. “non-ABC-like” DLBCL (Fig. 3B). Not surprisingly, the “ABC-like” DLBCLs in our series had more abundant expression of previously described ABC NF κ B targets including BCL-2, IRF4, cyclin D2 and CD44. However, there was a much smaller series of abundant NF κ B targets in ABC-like DLBCLs and only limited overlap with the NF κ B activation signature in primary MLBCLs (compare Figs. 3A, B and D).

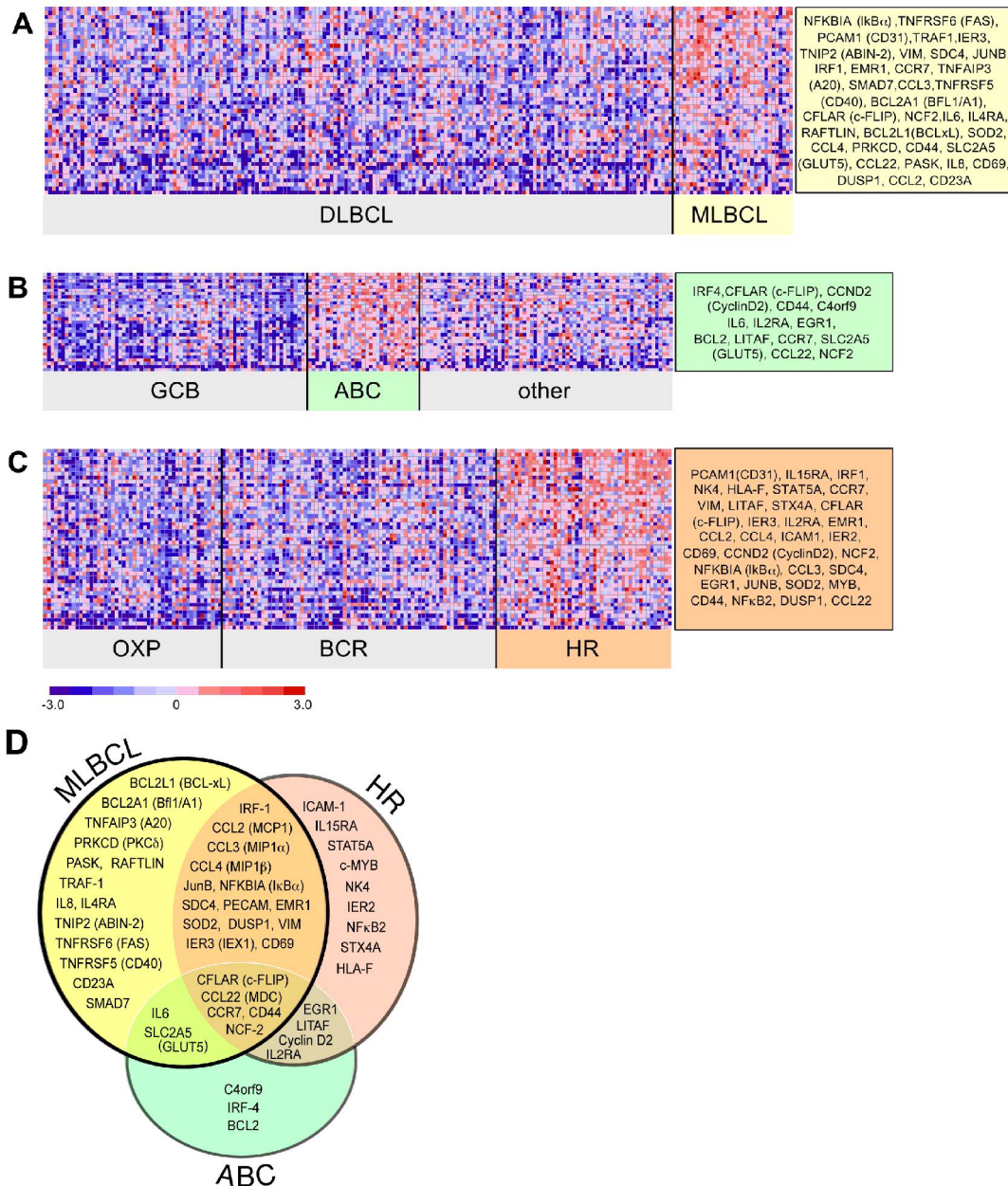


Fig. 3: Differential expression of $\text{NF}\kappa\text{B}$ target genes in large B-cell lymphoma subtypes. $\text{NF}\kappa\text{B}$ target genes that met the 99th percentile of the permutation distribution and exhibited a $\geq 30\%$ difference in median expression values were considered to be differentially expressed. Differentially expressed $\text{NF}\kappa\text{B}$ target genes in: A) primary MLBCL vs. DLBCL; B) “ABC-like” DLBCLs vs. “GCB-like” and “Other” DLBCLs; and C) “HR” DLBCLs vs. “OxPhos” and “BCR/Proliferation” DLBCLs. D) Comparison of the $\text{NF}\kappa\text{B}$ target gene signatures from primary MLBCL, “HR” DLBCLs and “ABC-like” DLBCLs.

Confirmation of NF κ B activation signature in MLBCL in independent dataset

We next assessed the reproducibility of the identified NF κ B target gene signatures in MLBCL and DLBCL subsets using an independent series of primary tumors (38 MLBCLs and 13 “ABC-like” and 13 “GC-like” DLBCLs) with **publicly** available transcriptional profiles ³. This independent series of primary MLBCLs also had significantly higher expression of 79% of the NF κ B target genes that were identified in our series and represented on both platforms (compare Figs. 3A and 4A and Supplementary Information). Within the independent series of DLBCLs, tumors with ABC features also had significantly higher expression of many of the identified NF κ B targets in our series (compare Figs. 3B and 4B and Supplementary Information).

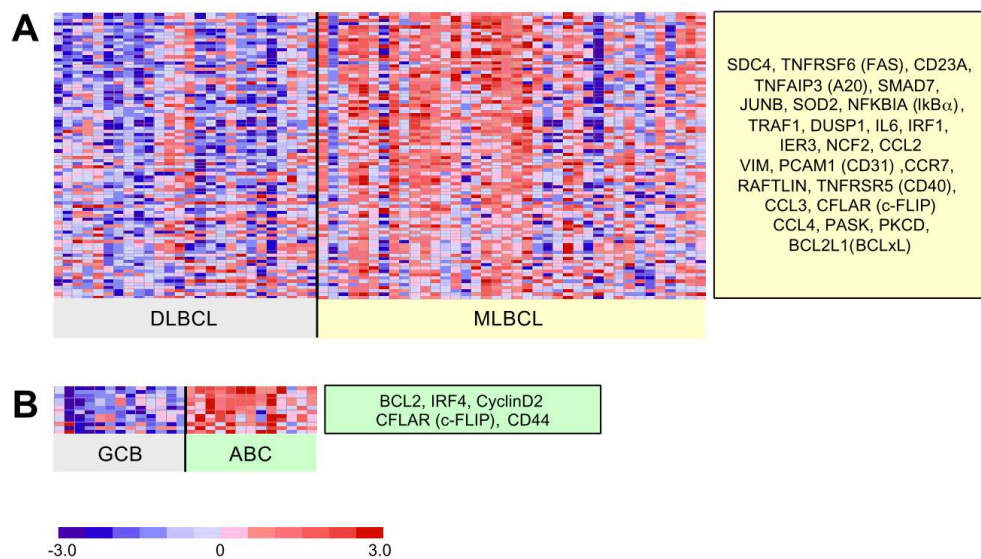


Fig. 4: Differential expression of NF κ B target genes in an independent series of primary MLBCLs and DLBCLs (Ref. ³). Differentially expressed NF κ B target genes were defined as in Fig. 3. A) MLBCLs vs. DLBCLs. (This independent series of primary MLBCLs had significantly higher expression of 79% (26/33 of the NF κ B target genes that were identified in our series and represented on both platforms); and B) “GC-type” vs “ABC-like” DLBCLs. *The independent data set includes all primary MLBCLs (38 tumors) and DLBCLs (26 tumors, 13 “GC-type” and 13 “ABC-like”) that were made available at <http://lmpp.nih.gov/PMBL/> (Supplementary Information of Ref. ³).*

Amplification of the c-REL locus is associated with high c-REL transcript abundance, but not with a NFκB activation signature

A small number of DLBCLs and primary MLBCLs have amplification of the c-REL locus, raising the possibility that higher c-REL copy numbers increase c-REL transcript abundance and augment NFκB activity. To address this issue in our lymphoma series, we analyzed c-REL copy numbers in the primary tumors and compared REL amplification and transcript abundance with the identified NFκB activation signature. As expected, primary DLBCLs with an amplified c-REL locus (15/127 examined tumors) had significantly more abundant c-REL transcripts (2.5X higher, $p < .001$, Fig. 5A). c-REL amplification was somewhat more common in DLBCLs with germinal center (GC) features, as previously described (% REL amplification in “GC” vs. “non-GC” DLBCLs, $p = .06$, Fig. 5B) ⁴. Consistent with this observation, GC-like DLBCLs also had more abundant c-REL transcripts than non-GC DLBCL (1.7X higher c-REL transcripts, $p < .005$, Fig. 5B). Although c-REL transcripts were more abundant in GC-like DLBCLs, these tumors did not exhibit upregulation of the identified NFκB target genes (Fig. 3B) and primary GC-like DLBCLs had largely cytoplasmic c-REL expression (4/6 tumors cytoplasmic only, 2/6 tumors cytoplasmic with < 5% nuclear staining, data not shown). In addition, GC-like DLBCL cell lines had low levels of NFκB activity (Fig. 2A and ¹³). Conversely, our series of MLBCLs had evidence of NFκB activation although c-REL amplification was rare (1/34 tumors) and c-REL transcripts were not increased (Figs. 3A and 5B). Taken together, the data indicate that in LBCLs, increased abundance of c-REL per se does not translate into increased NFκB activity and NFκB activation is not primarily dependent on c-REL transcript abundance.

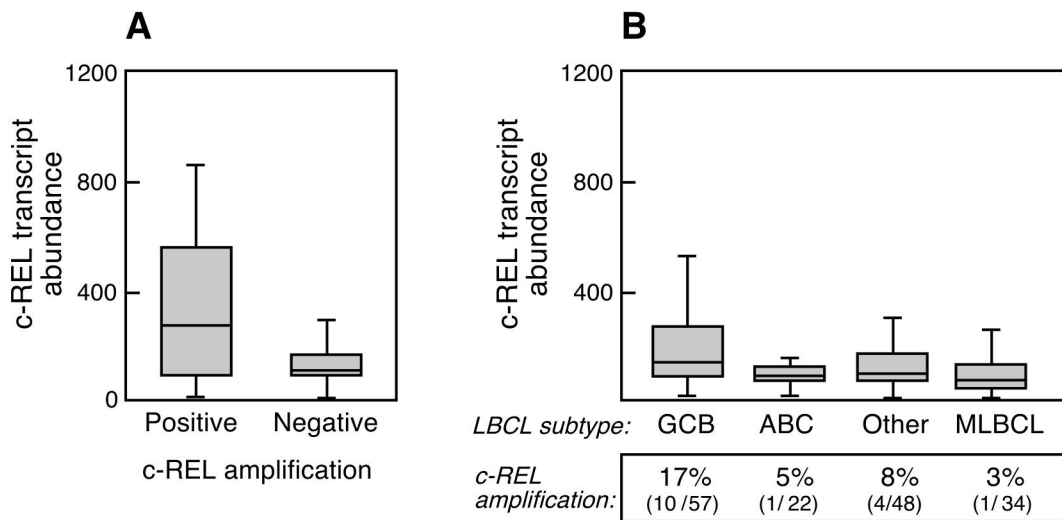


Fig. 5: *c-REL* amplification and transcript abundance in large B-cell lymphoma subtypes. A) *c-REL* transcript abundance in primary DLBCLs with an amplified *c-REL* locus (positive, 15/127 examined tumors) or no *c-REL* amplification (negative, 112/127 examined tumors). B) *c-REL* transcript abundance and *c-REL* amplification in DLBCLs arranged by cell of origin (“GCB”, “ABC” and “Other”) and primary MLBCL.

NF κ B-activation signature in HRS-type lymphomas

In addition to recognized LBCL subtypes (such as MLBCL) and DLBCLs with shared developmental features (“ABC-“ and “GC-like” tumors), DLBCL subsets with highly reproducible, comprehensive transcriptional profiles have been identified (“OxPhos”, “BCR/Proliferation”, “Host Response (HR)”, ⁶). To evaluate the potential role of the NF κ B pathway in these newly described DLBCL subtypes, we compared NF κ B target gene expression in each group of tumors. Of interest, HR tumors had increased expression of a broad series of NF κ B targets which partially overlapped those expressed by primary MLBCLs (compare Figs. 3A, C and D). To more specifically define the NF κ B target gene signature in HR DLBCLs, we performed GSEA with the individual NF κ B target lists in HR vs. non-HR tumors. The HR signature was significantly enriched for

TNF-induced NF κ B targets ($p = .01$) but not the other NF κ B target gene sets. These data suggest that the consequences of NF κ B activation may vary in tumors with different developmental features and specific microenvironmental signals (Fig. 3D).

Discussion

Herein, we confirmed the nuclear accumulation of c-REL-containing NF κ B heterodimers in an additional series of primary MLBCLs and directly implicated NF κ B in the survival of a MLBCL cell line. As in Hodgkin's lymphoma, the nuclear localization of c-REL may prove to be a useful diagnostic feature in primary MLBCL ²⁹. The direct evidence of constitutive NF κ B activation and NF κ B-dependent survival of a MLBCL cell line also indicates that this pathway may be a promising rational therapeutic target.

In addition, we characterized the NF κ B target gene signature of primary MLBCLs and compared their signature with that of additional LBCL subtypes ("ABC-like" DLBCLs and HR tumors). Primary MLBCLs expressed increased levels of NF κ B targets which promote TNF α -induced cell survival (TRAF1 ⁷, BFL1/A1 ^{30,31}, PKCdelta ³², SOD2 ³³) and regulate TNF α signaling (A20 ³⁴ and ABIN2 ³⁵) (Figs. 3A and D). In addition to BFL1/A1, MLBCL NF κ B target genes include another antiapoptotic BCL2 family member, BCLxL ⁷. Primary MLBCLs also had increased expression of the critical NF κ B target and key inhibitor of FAS-mediated apoptosis and caspase 8, c-FLIP (Figs. 3A and D) ^{36,37}. These results are of particular interest given the documented role of c-FLIP-mediated resistance to death receptor-induced apoptosis in Hodgkin's Reed-Sternberg cells ^{38,39}. Like Hodgkin's lymphoma, primary MLBCLs also express increased levels of the NF κ B target and AP-1 transcription factor, JUN-B ⁴⁰.

Although primary "ABC-like" DLBCLs had a more restricted NF κ B target gene signature than primary MLBCLs, "ABC-like" tumors also had more abundant c-FLIP transcripts (Figs. 3B and D). Whereas primary MLBCLs expressed increased levels of BFL1/A1 and BCLxL, ABC-like DLBCLs had more abundant expression of a different NF κ B target and antiapoptotic BCL2 family member, BCL2. Modulators of TNF α -induced cell survival, inflammatory cytokines and

adhesion molecules that were part of the MLBCL NF κ B target signature were not seen in “ABC-like” DLBCL (Figs. 3B and D). As expected, several of the NF κ B targets that were more abundant in ABC-type DLBCLs (IRF-4, BCL2, cyclin D2) were also expressed at high levels in normal “*in vitro* activated” B-cells ²⁴, suggesting that these targets may be developmentally regulated in this LBCL subset.

In contrast to primary MLBCLs, “ABC-like” DLBCLs did not exhibit consistent nuclear localization of c-REL-containing heterodimers, prompting speculation that additional NF κ B heterodimers may be active in these tumors. Consistent with this possibility, “ABC-like” DLBCL cell lines were previously reported to have p50/REL-A DNA binding activity in addition to p50/c-REL heterodimers ¹³. Given the unique characteristics of the “ABC-like” NF κ B target gene signature, these data suggest that NF κ B activation may be associated with different combinations of NF κ B heterodimers and signaling molecules in discrete LBCL subtypes.

In addition to characterizing the NF κ B target gene signatures of primary MLBCL and ABC-type DLBCLs, we identified a robust NF κ B target gene signature in “HR” DLBCLs. “HR” DLBCLs and primary MLBCLs share certain features including a brisk host inflammatory infiltrate and increased expression of associated NF κ B target genes (CCL2 (MCP1), CCL3 (MIP1 α), SDC4 ⁴¹, PECAM ⁴², SOD2 ³³, Fig. 3D). Like primary MLBCLs and “ABC-like” DLBCLs, HR DLBCLs also had increased expression of c-FLIP, underscoring its potential importance in each tumor type. Consistent with the specific characteristics of the T/NK-cell rich inflammatory infiltrate in HR DLBCLs, these tumors also had increased expression of NF κ B targets potentially derived from infiltrating activated T/NK cells (IL15R ⁴³ and NK4, Fig. 3D).

Given the prominent host inflammatory responses in primary HR DLBCLs and MLBCLs, it is of interest that the LBCLs express NF κ B targets which may limit

the effectiveness of host immune responses (HLA-F and CCL22, Fig. 3D). Although HLA-F has limited tissue distribution, this atypical HLA class 1 molecule is expressed on B lymphoblastoid and monocytoid cell lines⁴⁴. Recent reports suggest that HLA-F interacts with the inhibitory counter-receptors, ILT2 and ILT4, potentially limiting associated T and NK cell responses^{45,46}. The chemokine, CCL22 (MDC), which is expressed by macrophages, dendritic cells and certain tumors (including Hodgkin's lymphoma), attracts CCR4+ T regulatory cells into secondary lymphoid organs and areas of inflammation and limits host anti-tumor immune responses⁴⁷⁻⁴⁹.

After implicating the NF κ B cell survival pathway in primary MLBCLs and characterizing the NF κ B target gene signatures in primary MLBCLs and "ABC-like" and HR DLBCLs, we assessed the role of c-REL amplification in these tumors. Consistent with recent reports⁴, we found that c-REL amplification was more common in "GC-like" DLBCLs than in LCL subtypes with evidence of NF κ B activation. In our series, only 1 of 34 primary MLBCLs had amplification of the c-REL locus, although all analyzed tumors had nuclear accumulation of c-REL and NF κ B target gene upregulation. Taken together, these data suggest that amplification of the c-REL locus is not the pathogenetic mechanism associated with NF κ B activity in LBCL subtypes and that additional gene(s) at 2p12-16 may contribute to the observed "GC" subtype.

The current studies define a role for NF κ B-mediated tumor cell survival in primary MLBCL and identify an overlapping NF κ B target gene signature in the newly characterized HR DLBCLs. In addition, the studies delineate important differences between the shared NF κ B target gene signature in primary MLBCLs and HR tumors and the more restricted, potentially developmental NF κ B signature in ABC-type DLBCLs. In addition to providing specific insights regarding similarities and differences in NF κ B signaling in these tumors, the data

will guide our attempts to pharmacologically manipulate the NF κ B pathway in these diseases⁵⁰.

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