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

Friedrich Feuerhake[1], Jeffery L. Kutok[2], Stefano Monti[3], Wen Chen[1], Ann S. LaCasce[1], Giorgio Cattoretti[4], Paul Kurtin [5], Geraldine S. Pinkus[2], Laurence de Leval[6], Nancy L. Harris[6], Kerry J. Savage[1], Donna Neuberg[7] Thomas M. Habermann[8], Riccardo Dalla-Favera[4], Todd R. Golub[1,9], Jon C. Aster[2], Margaret A. Shipp[1]

[1]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA; [2]Department of Pathology, Brigham and Women's Hospital, Boston, MA; [3]The Broad Institute, Cambridge, MA; [4]Institute for Cancer Genetics, Columbia University, New York, NY; [5]Department of Pathology, Mayo Clinic, Rochester, MN; [6]Department of Pathology, Massachusetts General Hospital, Boston, MA; [7]Department of Biostatistics, Dana-Farber Cancer Institute, Boston, MA; [8]Division of Hematology and Department of Medicine, Mayo Clinic, Rochester, MN; [9]Department of Pediatric Oncology, Dana-Farber Cancer Institute and Howard Hughes Medical Institute, Boston, MA

Running title:	NFκB in MLBCL and DLBCL
Scientific heading:	Neoplasia
Word count:	4,749
Abstract	197

Corresponding author: M. Shipp, Dana-Farber Cancer Institute, 44 Binney Street., Boston, MA 02115, 617-632-3874 (phone), 617-632-4734 (fax), margaret_shipp@dfci.harvard.edu

Key words: NFκB, MLBCL, DLBCL, profiling, c-REL

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 NFκB subunit in a pilot series. Herein, we analyzed c-REL subcellular localization in additional primary MLBCLs and characterized NFκB 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 NFκB binding activity. MLBCL cells expressing a super-repressor form of IκBα had a markedly higher rate of apoptosis, implicating constitutive NFκB activity in MLBCL cell survival. The transcriptional profiles of newly diagnosed primary MLBCLs and DLBCLs were then used to characterize the NFκB target gene signatures of MLBCL and specific DLBCL subtypes. MLBCLs expressed increased levels of NFκB targets that promote cell survival and favor anti-apoptotic TNFα signaling. In contrast, "ABC-like" DLBCLs had a more restricted, potentially developmentally regulated, NFκB target gene signature. Of interest, the newly characterized "Host Response" DLBCL subtype had a robust NFκB target gene signature which partially overlapped that of primary MLBCL. In this large series of primary MLBCLs and DLBCLs, NFκB 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 signatures [4-6]. With the newly available molecular signatures, it is now possible to evaluate the role of specific survival pathways in discrete LBCL subtypes.

The NFκB 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 c [7]. Depending on the cellular context, TNFα signaling and other stimuli (including B-cell receptor, CD40 and Toll receptor engagement) also activate the NFκB pathway and augment the transcription of NFκB target genes [7]. These NFκB target genes enhance cell survival by modulating TNFα signaling, inhibiting FAS-mediated apoptosis and limiting the activity of pro-apoptotic BCL2 family members, in addition to multiple other effects [7].

Inactive NFκB heterodimers (primarily c-REL or RELA and NFκB1 [p50]) reside in the cytoplasm where they are complexed with an inhibitor of kappa B signaling (IκB) [7]. In response to a variety of signals, Ikappa kinase (IκK) phosphorylates IκB, resulting in the inhibitor's dissociation from the cytoplasmic NFκB heterodimer. Thereafter, phosphorylated IκB is degraded via the proteosome and the freed (active) NFκB heterodimer translocates to the nucleus where it induces

the transcription of NFκB target genes [7]. For this reason, the activity of the NFκB pathway in a B-cell tumor can be preliminarily assessed by determining the subcellular localization of NFκB subunits (nuclear vs. cytoplasmic). This type of analysis was previously used to identify the likely role of c-REL containing heterodimers and NFκB 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 NFκB survival pathway in cHL [9-11] and the striking similarities between the cHL and MLBCL transcriptional profiles, including upregulation of specific NFκB 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 NFκB subunit was localized to the nucleus, underscoring the potential role of constitutive NFκB activation in primary MLBCLs [2].

Previous studies also implicate NFκB 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 NFκB in informative cell lines and analyses of NFκB 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 5μ 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).

NFκB activation assay

NFκB activity was assessed using a colorimetric assay [21] that detects binding of cellular p50 (NFκB1) to immobilized NFκB- target consensus oligonucleotide sequences (Active Motif,Carlsbad, CA). In brief, 5 ug of each whole cell lysate was added to microwells containing immobilized NFκB-specific target probes. Following incubation and washing, cellular p50 bound to the immobilized NFκB target sequences was detected using a p50-specific polyclonal antibody, a HRP-conjugated secondary antibody and colorimetric quantification. NFκB 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 NFκB target sequences [wild-type sequences, negative control and mutated sequences, positive control]).

Molecular cloning and retroviral transduction

An IκBα super repressor construct in which serines 32 and 36 were replaced with alanines (SR-IκBα, gift of A. Rabson and C. Gelinas [22]) was used to inhibit NFκB

activation in Karpas1106 cells. SR-IκBα, which cannot be phosphorylated by IκK, remains complexed to the NFκB heterodimer, inhibiting NFκB translocation and activation of NFκB targets. SR-IκBα 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-IκBα 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 x 10^6 cells/ml in fresh media containing Polybrene (final concentration 8 mg/ml, Sigma, St. Louis, MO). 500 μl of Karpas 1106 was added added to individual wells of a 24-well plate and 500 μl of retroviral supernatant (MSCV-eGFP-SR-IκBα or vector only) was added thereafter. Cells were then spinoculated at 2000 rpm for 90 min, incubated at 37^{o}C 5% C02 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 NFκB activity in SR-IκBα and vector-only transduced cells were performed using a one-sided student's t-test.

Cell viability and Apoptosis

Proliferation of MSCV-eGFP- SR-IκBα 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-IκBα and vector-only transduced cells were performed using an ANOVA model on the natural logarithm of the data including the cell type (SR-IκB, 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 NFκB 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 samples [2]. 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 NFκB target gene sets

Three NFκB 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) NFκB target genes that were downregulated in Hodgkin's lymphoma cell lines following introduction of NFκB super-repressor IκBΔN [11]; (Geneset 2) Previously described NFκB 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) NFκB target genes that were downregulated after siRNA silencing of

REL-A (p65) in TNFα-stimulated HeLa cells [25]. The complete gene sets and their associated microarray probe sets are included in the Supplementary Information.

Enrichment test for NFκB genes in MLBCL.

Gene Set Enrichment Analysis (GSEA) [2,6,26] was performed as previously described using the above-mentioned NFκB 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 NFκB 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 NFκB target genes

The above-mentioned sets of NFκB 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, NFκB target genes had to be represented in the 15,000-gene dataset. 64 of the 68 NFkB target genes met these criteria (Supplementary Information). NFκB 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 NFκB 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). NFκB target genes which met the 99th percentile of the permutation distribution and exhibited a ≥ 30% difference in median expression values were considered to be differentially expressed (Supplementary Information).

The same method was used to assess differential expression of NFκB target genes in an independent set of 38 MLBCL and 26 DLBCL with available cDNA microarray ("lymphochip") profiles (URL: http://llmpp.nih.gov/PMBL/). 92% (59/64) of the combined set of NFκB 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 observations [16].

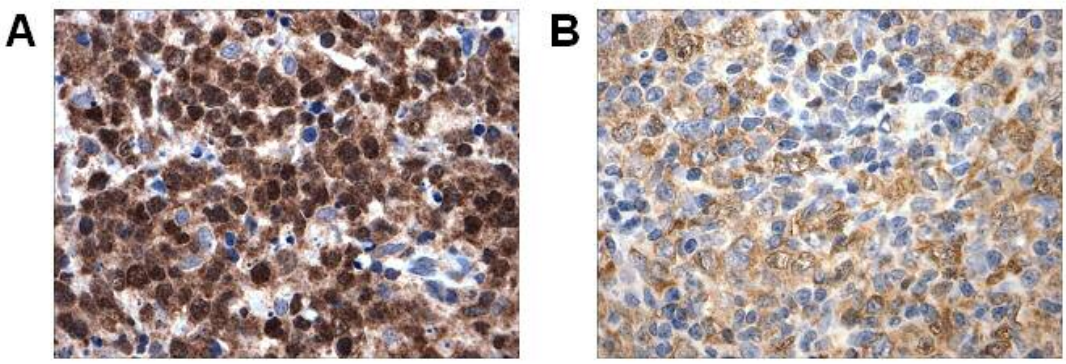

Fig. 1 : *Subcellular localization of c-REL in MLBCL. A) Prominent intense c-REL nuclear staining in a representative primary MLBCL. B) Variable c-REL staining intensity and subcellular localization in a DLBCL. Original magnification 400X.*

NFκB 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 NFκB activation in a MLBCL cell line (Karpas 1106). Initially, NFκB activity in Karpas 1106 was measured and compared to that in lymphoma cell lines with previously reported high or low levels of NFκB 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 NFκB activity, ≈ 4-fold greater than that of DHL6 cells (Fig. 2A).

We next assessed the consequences of NFκB 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 IκBα (SR-IκBα) or GFP alone. Thereafter, GFP-expressing SR-IκBα and vector-only MLBCL cells were isolated by FACS and analyzed for NFκB activity. As expected, SR-IκBα MLBCL cells had ≈ six-fold less NFκB activity than cells expressing the vector alone ($p<.0001$,one-sided student's t-test, Fig. 2B). SR-IκBα MLBCL cells had a ≈ 4-fold higher rate of apoptosis (Annexin V expression) than cells expressing the vector alone (SR-IκBα 54% apoptosis vs. vector-only 14% apoptosis, Figs. 2B [right panel] and C). SR-IκBα 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-IκB 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 NFκB activity is essential for the survival of this MLBCL cell line.

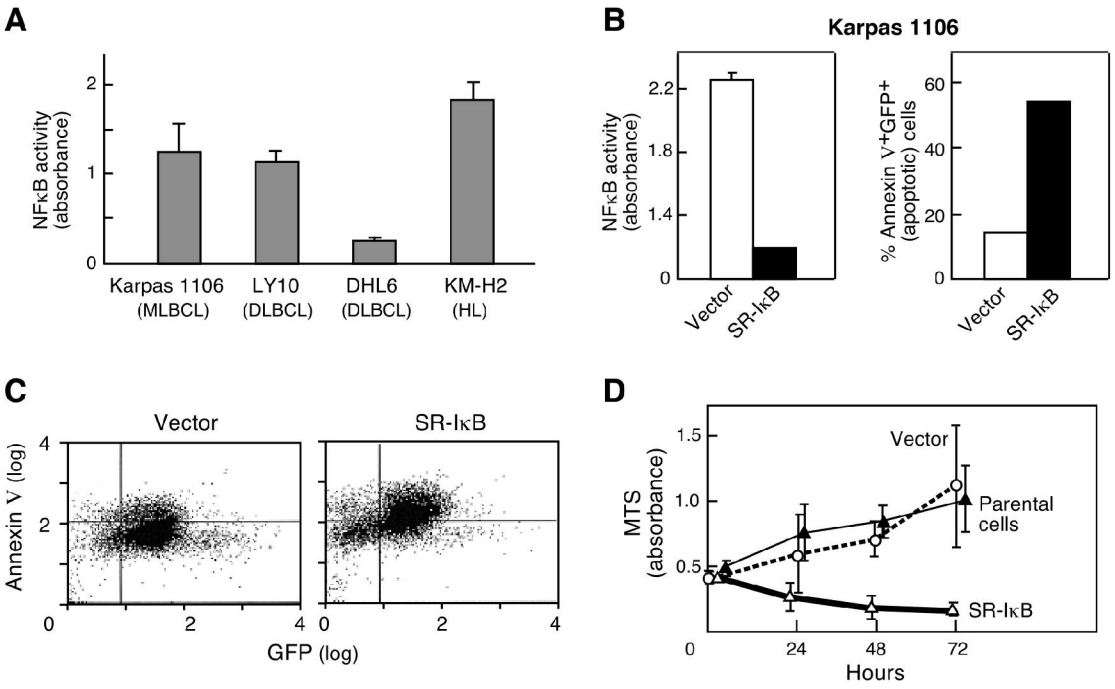

Fig. 2: *NFκB activity and inhibition in a MLBCL cell line. A) NFκB DNA-binding activity of the MLBCL cell line, Karpas 1106, two DLBCL cell lines, OCI LY10 and DHL6, and a Hodgkin's lymphoma cell line, KM-H2. NFκB DNA binding activity was measured using a colorimetric assay. Measurements were performed in triplicates. B) NFκB activity and apoptosis in MLBCLs expressing an IκBα super-repressor. Left panel) NFκB DNA-binding activity in MLBCL cells transduced with MSCV-eGFP (vector) alone or MSCV-eGFP-SR-IκBα. Measurements were performed in triplicates. NFκB activity was significantly lower in SR-IκBα 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-IκBα is much higher than that of cells transduced with vector alone. C) Apoptosis in MLBCL cells expressing an IκB super-repressor. GFP-positive cells expressing vector alone or MSCV-eGFP-SR-IκBα were analyzed for expression of Annexin V (Alexa 568). GFP, x-axis, Annexin V, y-axis. D) Proliferation of MLBCL cells expressing an IκBα super-repressor. The proliferation (MTS absorbance) of parental and GFP+ vector only and MSCV-eGFP-SR-IκBα 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-IκBα 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 NFκB target genes

After demonstrating the importance of NFκB activity for the proliferation and viability of a MLBCL cell line, we assessed the consequences of NFκB activation in our series of primary MLBCLs with available transcriptional profiles. Specifically, we asked whether previously defined, co-regulated sets of NFκB target genes were upregulated in primary MLBCLs (as compared with DLBCLs) using gene set enrichment analysis (GSEA). Three independently defined sets of NFκB target genes with likely biologic relevance in MLBCL were used: two series of target genes responsive to NFκB inhibition in Hodgkin's lymphoma cell lines and TNF-stimulated adherent cells, respectively [11,25]; and an additional series of previously described NFκB 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 NFκB targets from Hodgkin's lymphoma cell lines (Geneset 1, $p = .05$) and the likely NFκB 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 NFκB target transcripts in primary MBLCLs (Geneset 3, $p = .08$).

MLBCL are characterized by a broad NFκB activation signature

Given the partial overlap between the above-mentioned series of NFκB target genes, we then combined the NFκB target gene sets for more detailed analyses of NFκB activation signatures in primary MLBCLs and previously defined subsets of DLBCL. A large series of NFκB targets were expressed at significantly higher levels in MLBCL than DLBCL (Fig. 3A), including genes regulating cell viability To compare the NFκB target gene signature of MLBCL with that of "ABC-like" DLBCLs, we also identified the differentially expressed NFκB 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 NFκB targets including BCL-2, IRF4, cyclin D2 and CD44. However, there was a much smaller series of abundant NFκB targets in ABC-like DLBCLs and only limited overlap with the NFκB activation signature in primary MLBCLs (compare Figs. 3A, B and D).

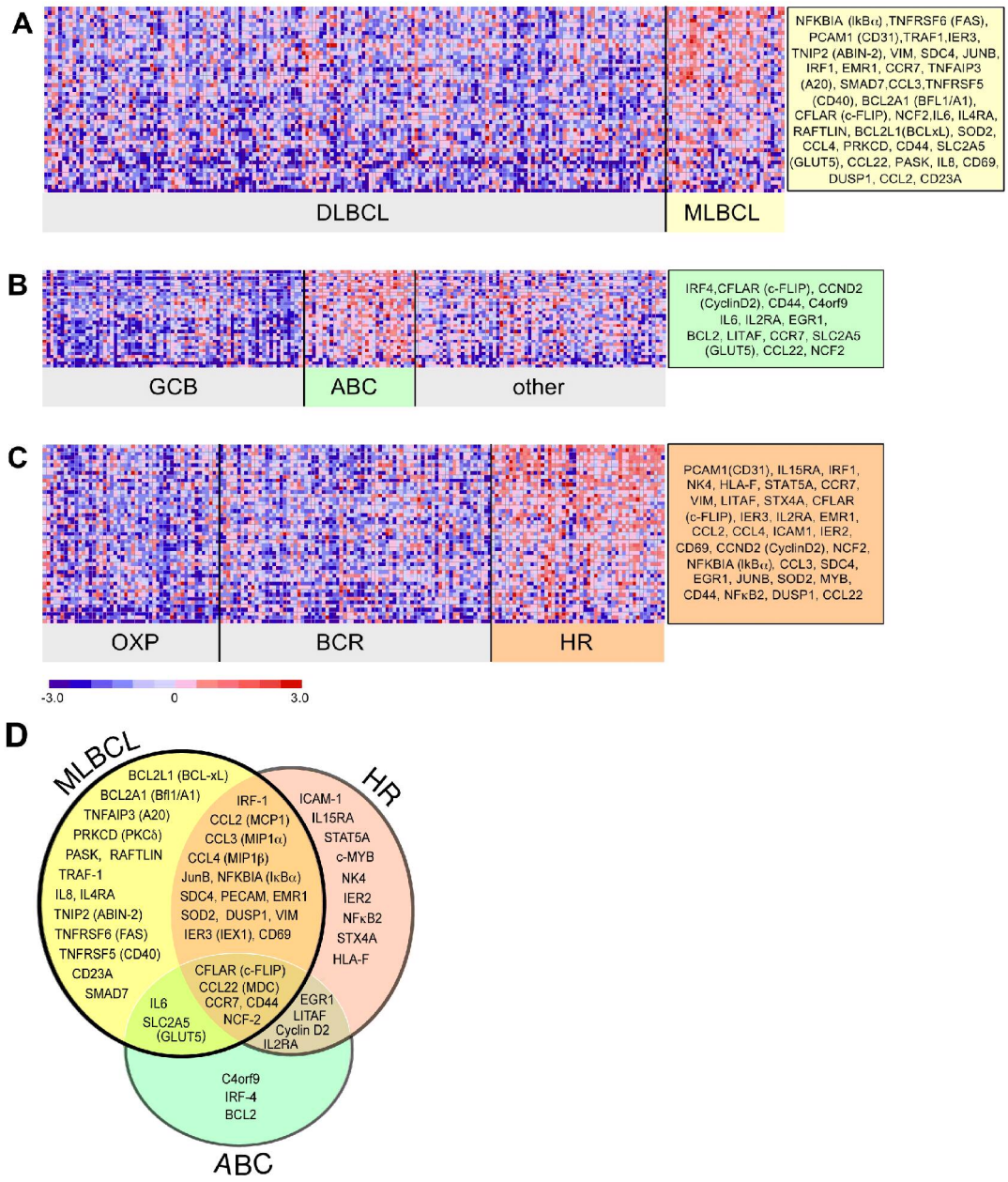

***Fig. 3**:Differential expression of NFκB target genes in large B-cell lymphoma subtypes. NFκB target genes that met the 99th percentile of the permutation distribution and exhibited a ≥ 30% difference in median expression values were considered to be differentially expressed. Differentially expressed NFκB 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 NFκB target gene signatures from primary MLBCL, "HR" DLBCLs and "ABC-like" DLBCLs.*

Confirmation of NFκB activation signature in MLBCL in independent dataset

We next assessed the reproducibility of the identified NFκB 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 NFκB 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 NFκB targets in our series (compare Figs. 3B and 4B and Supplementary Information).

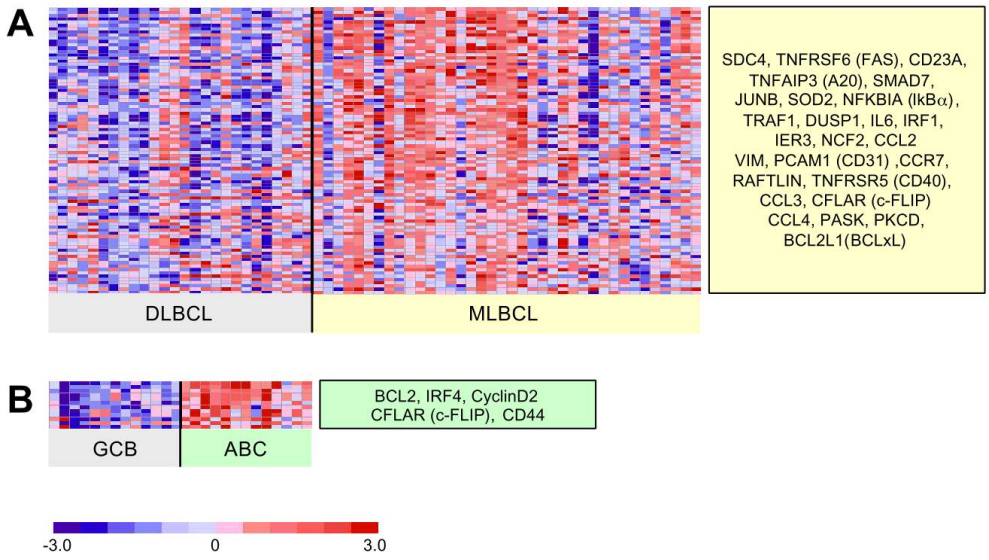

***Fig. 4**: Differential expression of NFκB target genes in an independent series of primary MLBCLs and DLBCLs (Ref. [3]). Differentially expressed NFκB target genes were defined as in Fig. 3. A) MLBCLs vs. DLBCLs. (This independent series of primary MLBCLs had significantly higher expression of 79% (26/33 of the NFκB 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://llmpp.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.

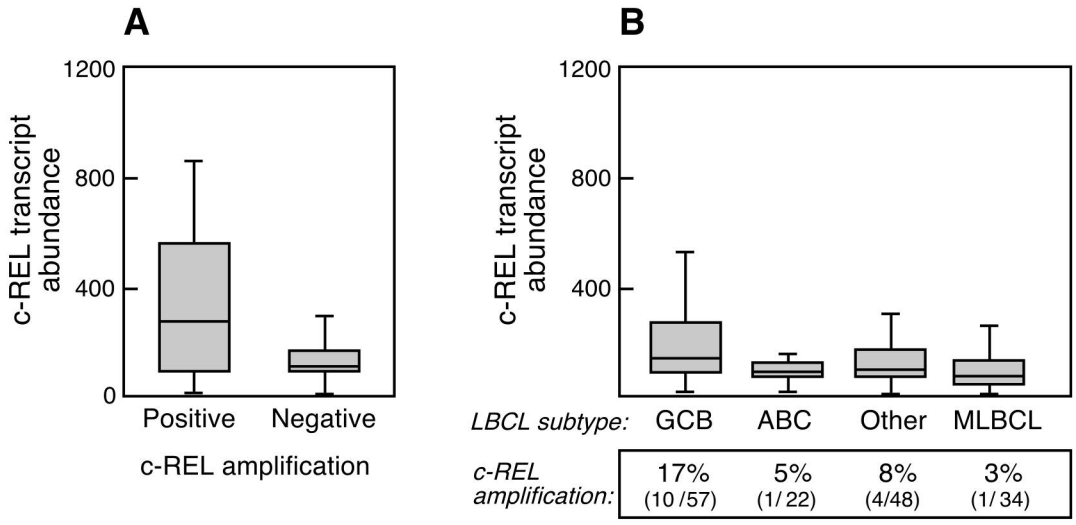

Fig. 5: *c-REL amplification and transcript abundance in large B-cell lymphoma subtypes. A) c-REL transcript abundance in primary DLBCLs with an amplified c-REL locus (positive, 15/127 examined tumors) or no c-REL amplification (negative, 112/127 examined tumors). B) c-REL transcript abundance and c-REL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

NFκB-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 NFκB pathway in these newly described DLBCL subtypes, we compared NFκB target gene expression in each group of tumors. Of interest, HR tumors had increased expression of a broad series of NFκB targets which partially overlapped those expressed by primary MLBCLs (compare Figs. 3A, C and D). To more specifically define the NFκB target gene signature in HR DLBCLs, we performed GSEA with the individual NFκB target lists in HR vs. non-HR tumors. The HR signature was significantly enriched for

TNF-induced NFκB targets ($p = .01$) but not the other NFκB target gene sets. These data suggest that the consequences of NFκB 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 NFκB heterodimers in an additional series of primary MLBCLs and directly implicated NFκB 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 NFκB activation and NFκB-dependent survival of a MLBCL cell line also indicates that this pathway may be a promising rational therapeutic target.

In addition, we characterized the NFκB 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 NFκB targets which promote TNFα-induced cell survival (TRAF1 [7], BFL1/A1 [30,31], PKCdelta [32], SOD2 [33]) and regulate TNFα signaling (A20 [34] and ABIN2 [35]) (Figs. 3A and D). In addition to BFL1/A1, MLBCL NFκB target genes include another antiapoptotic BCL2 family member, BCLxL [7]. Primary MLBCLs also had increased expression of the critical NFκB 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 NFκB target and AP-1 transcription factor, JUN-B [40].

Although primary "ABC-like" DLBCLs had a more restricted NFκB 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 NFκB target and antiapoptotic BCL2 family member, BCL2. Modulators of TNFα-induced cell survival, inflammatory cytokines and

adhesion molecules that were part of the MLBCL NFκB target signature were not seen in "ABC-like" DLBCL (Figs. 3B and D). As expected, several of the NFκB 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 NFκB 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" NFκB target gene signature, these data suggest that NFκB activation may be associated with different combinations of NFκB heterodimers and signaling molecules in discrete LBCL subtypes.

In addition to characterizing the NFκB target gene signatures of primary MLBCL and ABC-type DLBCLs, we identified a robust NFκB 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 NFκB target genes (CCL2 (MCP1), CCL3 (MIP1α), 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 NFκB 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 NFκB 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 lines [44]. 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 responses [47-49].

After implicating the NFκB cell survival pathway in primary MLBCLs and characterizing the NFκB 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 reports [4], we found that c-REL amplification was more common in "GC-like" DLBCLs than in LCL subtypes with evidence of NFκB 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 NFκB target gene upregulation. Taken together, these data suggest that amplification of the c-REL locus is not the pathogenetic mechanism associated with NFκB 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 NFκB-mediated tumor cell survival in primary MLBCL and identify an overlapping NFκB target gene signature in the newly characterized HR DLBCLs. In addition, the studies delineate important differences between the shared NFκB target gene signature in primary MLBCLs and HR tumors and the more restricted, potentially developmental NFκB signature in ABC-type DLBCLs. In addition to providing specific insights regarding similarities and differences in NFκB signaling in these tumors, the data

will guide our attempts to pharmacologically manipulate the NFκB pathway in these diseases [50].

References

1. Lazzarino M, Orlandi E, Paulli M, Boveri E, Morra E, Brusamolino E, Kindl S, Rosso R, Astori C, Buonanno MC. Primary mediastinal B-cell lymphoma with sclerosis: an aggressive tumor with distinctive clinical and pathologic features. J Clin Oncol. 1993;11:2306-2313

2. Savage K, Monti S, Kutok J, Cattoretti G, Neuberg D, de Leval L, Kurtin P, Dal Cin P, Ladd C, Feuerhake F, RCT A, Li S, Salles G, Berger F, Jing W, Pinkus G, Habermann TM, Dalla-Favera R, Harris N, Aster JC, Golub TR, Shipp MA. The molecular signature of mediastinal large B-cell lymphoma differs from that of other diffuse large B-cell lymphomas and shares features with classical Hodgkin lymphoma. Plenary Paper. Blood. 2003;102:3871-3879

3. Rosenwald A, Wright G, Leroy K, Yu X, Gaulard P, Gascoyne RD, Chan WC, Zhao T, Haioun C, Greiner T, Weisenburger D, Lynch J, Vose JM, Armitage JO, Smeland E, Kvaloy S, Holte H, Delabie J, Campo E, Montserrat E, Lopez-Guillermo A, Ott G, Muller-Hermelink H, Connors J, Braziel R, Grogan T, Fisher R, Miller T, LeBlanc M, Chiorazzi M, Zhao H, Yang L, Powell J, Wilson W, Jaffe E, Simon R, Klausner R, Staudt L. Molecular diagnosis of primary mediastinal B cell lymphoma identifies a clinically favorable subgroup of diffuse large B cell lymphoma related to Hodgkin lymphoma. J Exp Med. 2003;198:851-862

4. Rosenwald A, Wright G, Chan WC, Connors JM, Campo E, R.I. F, Gascoyne RD, Muller-Hermelink HK, Smeland EB, Staudt LM. The use of molecular profiling to predict survival after chemotherapy for diffuse large B-cell lymphoma. N Engl J Med. 2002;346:1937-1947

5. Wright G, Tan B, Rosenwald A, Hurt E, Wiestner A, Staudt L. A gene expression-based method to diagnose clinically distinct subgroups of diffuse large B cell lymphoma. PNAS. 2003;100:9991-9996

6. Monti S, Savage KJ, Kutok J, Feuerhake F, Kurtin P, M. M, Wu B, Pasqualucci L, Neuberg D, Aguiar RCT, Dal Cin P, Ladd C, Pinkus GS, Salles G, Harris NL, Dalla-Favera R, Habermann TM, Aster JC, Golub TR, Shipp MA. Molecular profiling of diffuse large B-cell lymphoma identifies robust subtypes

including one characterized by host inflammatory response. Plenary Paper. Blood. 2005;105:1851-1861

7. Karin M, Lin A. NF-kappaB at the crossroads of life and death. Nat Immunol. 2002;3:221-227

8. Karin M, Cao Y, Greten FR, Li ZW. NF-kappaB in cancer: from innocent bystander to major culprit. Nat Rev Cancer. 2002;2:301-310

9. Barth TF, Martin-Subero JI, Joos S, Menz CK, Hasel C, Mechtersheimer G, Parwaresch RM, Lichter P, Siebert R, Moller P. Gains of 2p involving the REL locus correlate with nuclear c-Rel protein accumulation in neoplastic cells of classical Hodgkin lymphoma. Blood. 2003;101:3681-3686

10. Hinz M, Loser P, Mathas S, Krappmann D, Dorken B, Scheidereit C, Eichten A, Heder A, Strauss M, Shaffer AL, Rosenwald A, Hurt EM, Giltnane JM, Lam LT, Pickeral OK, Staudt LM, Zhou A, Scoggin S, Gaynor RB, Williams NS. Constitutive NF-kappaB maintains high expression of a characteristic gene network, including CD40, CD86, and a set of antiapoptotic genes in Hodgkin/Reed-Sternberg cells. Blood. 2001;97:2798-2807

11. Hinz M, Lemke P, Anagnostopoulos I, Hacker C, Krappmann D, Mathas S, Dorken B, Zenke M, Stein H, Scheidereit C. Nuclear factor kappaB-dependent gene expression profiling of Hodgkin's disease tumor cells, pathogenetic significance, and link to constitutive signal transducer and activator of transcription 5a activity. J Exp Med. 2002;196:605-617

12. Kuppers R, Klein U, Hansmann M-L, Rajewsky K. Cellular origin of human B-cell lymphomas. N Engl J Med. 1999;341:1520-1529

13. Davis RE, Brown KD, Siebenlist U, Staudt LM. Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. J Exp Med. 2001;194:1861-1874.

14. Joos S, Otano-Joos M, Ziegler S, Bruderlein S, du Manoir S, Bentz M, Moller P, Lichter P. Primary mediastinal (thymic) B-cell lymphoma is characterized by gains of chromosomal material including 9P and amplification of the REL gene. Blood. 1996;87:1571-1578

15. Houldsworth J, Matthew S, Rao P, Dyomina K, Louis D, Parsa N, Offit K, Chaganti R. REL proto-oncogene is frequently amplified in extranodal diffuse large cell lymphoma. Blood. 1996;87:25-29

16. Houldsworth J, Olshen AB, Cattoretti G, Donnelly GB, Teruya-Feldstein J, Qin J, Palanisamy N, Shen Y, Dyomina K, Petlakh M, Pan Q, Zelenetz AD, Dalla-Favera R, Chaganti RS. Relationship between REL amplification, REL function, and clinical and biologic features in diffuse large B-cell lymphomas. Blood. 2004;103:1862-1868

17. Karin M, Yamamoto Y, Wang M. The IKK NF-kB system: A treasure trove for drug development. Nat Rev Drug Discovery. 2004;3:17-26

18. Copie-Bergman C, Boulland ML, Dehoulle C, Moller P, Farcet JP, Dyer MJ, Haioun C, Romeo PH, Gaulard P, Leroy K. Interleukin 4-induced gene 1 is activated in primary mediastinal large B-cell lymphoma. Blood. 2003;101:2756-2761

19. Smith PG, Wang F, Wilkinson KN, Savage KJ, Klein U, Neuberg DS, Bollag G, Shipp MA, Aguiar RC. The phosphodiesterase PDE4B limits cAMP associated, PI3K-AKT dependent, apoptosis in diffuse large B-cell lymphoma.
T. Blood. 2004;102:3871-3879

20. Basso K KU, Niu H, Stolovitzky GA, Tu Y, Califano A, Cattoretti G, Dalla-Favera R. Tracking CD40 signaling during germinal center development. Blood. 2004;104:4088-4096

21. Renard P, Ernest I, Houbion A, Art M, Le Calvez H, Raes M, Remacle J. Development of a sensitive multi-well colorimetric assay for active NFkappaB. Nucleic Acids Res. 2001;29:E21

22. Suh J, Payvandi F, Edelstein LC, Amenta PS, Zong WX, Gelinas C, Rabson AB. Mechanisms of constitutive NF-kappaB activation in human prostate cancer cells. Prostate. 2002;52:183-200

23. Weng AP, Nam Y, Wolfe MS, Pear WS, Griffin JD, Blacklow SC, Aster JC. Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. Mol Cell Biol. 2003;23:655-664

24. Shaffer AL, Rosenwald A, Hurt EM, Giltnane JM, Lam LT, Pickeral OK, Staudt LM. Signatures of the immune response. Immunity. 2001;15:375-385

25. Zhou A, Scoggin S, Gaynor RB, Williams NS. Identification of NF-kappa B-regulated genes induced by TNFalpha utilizing expression profiling and RNA interference. Oncogene. 2003;22:2054-2064

26. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Maurila E, Houstis N, Daly JJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschlorn JN, Altshuler D, Groop LC. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet. 2003;34:267-273

27. Goff L, Neat M, Crawley C, Jones L, Jones E, Lister T, Gupta R. The use of real-time quantitative polymerase chain reaction and comparative genomic hybridization to identify amplification of the REL gene in follicular lymphoma. Br J Haematol. 2000;111:618-625

28. Joos S, Granzow M, Holtgreve-Grez H, Siebert R, Harder L, Martin-Subero JI, Wolf J, Adamowicz M, Barth TF, Lichter P, Jauch A. Hodgkin's lymphoma cell lines are characterized by frequent aberrations on chromosomes 2p and 9p including REL and JAK2. Int J Cancer. 2003;103:489-495

29. Rodig SJ, Savage KJ, Pinkus GS, Shipp MA, Aster JC, Kutok JL. TRAF-1 expression and c-Rel activation distinguish classical Hodgkin lymphoma from other morphologically or immunophenotypically similar forms of malignant lymphoma. Am J Surg Pathol. 2005;29:196-203

30. Werner A, de Vries E, Tait S, Bontjer I, Borst J. BCL-2 family member BFL-1/A1 sequesters truncated bid to inhibit its collaboration with pro-apoptotic BAK or BAX. J Biol Chem. 2002;277:22781-22788

31. Zong W, Edelstein L, Chen C, Bash J, Gelinas C. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-kappaB that blocks TNFalpha-induced apoptosis. Genes & Devel. 1999;13:382-387

32. Kilpatrick LE, Lee JY, Haines KM, Campbell DE, Sullivan KE, Korchak HM. A role for PKC-delta and PI 3-kinase in TNF-alpha-mediated antiapoptotic signaling in the human neutrophil. Am J Physiol Cell Physiol. 2002;283:C48-C57

33. Dhar SK, Lynn BC, Daosukho C, St. Clair DK. Identification of nucleophosmin as an NF-κB co-activator for the induction of the human SOD2 gene. J Biol Chem. 2004;279:28209-28219

34. Wertz IE, O'Rourke KM, Zhou H, Eby M, Aravind L, Seshagirl S, Wu P, Wiesmann C, Baker R, Boone DL, Ma A, Koonin EV, Dixit VM. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-κB signalling. Nature. 2004;430:694-699

35. Beyaert R, Heyninck K, Van Huffel S. A20 and A20-binding proteins as cellular inhibitors of nuclear factor-kappa B-dependent gene expression and apoptosis. Biochem Pharmacol. 2000;60:1143-1151

36. Micheau O, Lens S, Gaide O, Alevizopoulos K, Tschopp J. NF-κB signals induce the expression of c-FLIP. Mol Cell Biol. 2001;21:5299-5305

37. Thome M, Tschopp J. Regulation of lymphocyte proliferation and death by FLIP. Nat Rev. 2001;1:50-58

38. Mathas S, Lietz A, Anagnostopoulos I, Hummel F, Wiesner B, Janz M, Jundt F, Hirsch B, Johrens-Leder K, Vornlocher H-P, Bommert K, Stein H, Dorken B. c-FLIP mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor-induced apoptosis. J Exp Med. 2004;199:1041-1052

39. Dutton A, O'Neil JD, Milner AE, Reynolds GM, Starczynski J, Crocker J, Young LS, Murray PG. Expression of the cellular FLICE-inhibitory protein (c-FLIP) protects Hodgkin's lymphoma cells from autonomous Fas-mediated death. PNAS. 2004;101:6611-6616

40. Mathas S, Hinz M, Anagnostopoulos I, Krappmann D, Lietz A, Jundt F, Bommert K, Mechta-Grigoriou F, Stein H, Dorken B, Scheidereit C, Hopken UE, Foss HD, Meyer D, Leder K, Lipp M. Aberrantly expressed c-Jun and JunB are a hallmark of Hodgkin lymphoma cells, stimulate proliferation and synergize with NF-kappa B. Embo J. 2002;21:4104-4113

41. Thodeti CK, Albrechtsen R, Grauslund M, Asmar M, Larsson C, Takada Y, Mercurio AM, Couchman JR, Wewer UM. ADAM12/syndecan-4 signaling promotes B1 integrin-dependent cell spreading through protein kinase C alpha and RhoA. J Biol Chem. 2003;278:9576-9584

42. Jackson DE. The unfolding tale of PECAM-1. FEBS Letters. 2003;540:7-14

43. Vamosi G, Bodnar A, Vereb G, Jenei A, Goldman CK, Langowski J, Toth K, Matyus L, Szollosi J, Waldmann TA, Damjanovich S. IL-2 and IL-15 receptor alpha-subunits are coexpressed in a supramolecular receptor cluster in lipid rafts of T cells. PNAS. 2004;101:11082-11087

44. Lee N, Geraghty DE. HLA-F surface expression on B cell and monocyte cell lines is partially independent from tapasin and completely independent from TAP1. J Immunol. 2003;171:5264-5271

45. Ishitani A, Sageshima N, Lee N, Dorofeeva N, Hatake K, Marquardt H, Geraghty DE. Protein expresion and peptide binding suggest unique and interacting functional roles for HLA-E, F, and G in maternal-placental immune recognition. J Immunol. 2003;171:1376-1384

46. Lepin EJM, Bastin JM, Allan DSJ, Roncador G, Braud VM, Mason DY, van der Merwe PA, McMichael AJ, Bell JI, Powis SH, O'Callaghan CA. Functional characterization of HLA-F and binding of HLA-F tetramers to ILT2 and ILT4 receptors. Eur J Immunol. 2000;30:3552-3561

47. Iellem A, Mariani M, Lang R, Recalde H, Panina-Bordignon P, Sinigaglia F, D'Ambrosio D. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4+ CD25+ regulatory T cells. J Exp Med. 2001;194:847-853

48. Maggio EM, Van Den Berg A, Visser L, Diepstra A, Kluiver J, Emmens R, Poppema S. Common and differential chemokine expression patterns in RS cells of NLP, EBV positive and negative classical Hodgkin lymphomas. Int J Cancer. 2002;99:665-672

49. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis MI, Knutson KL, Chen L, Zou W. Specific

recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med. 2004;10:942-949

50. Lam LT, Davis RE, Pierce J, Hepperle M, Xu Y, Hottelet M, Nong Y, Wen D, Adams J, Dang L, Staudt LM. Small molecule inhibitors of IkappaB kinase are selectively toxic for subgroups of diffuse large B-cell lymphoma defined by gene expression profiling. Clin Cancer Res. 2005;11:28-40