An Oncogenic Role for Alternative NF-κB Signaling in DLBCL Revealed upon Deregulated BCL6 Expression

Graphical Abstract

Highlights
- Genetic loss of TRAF3 is associated with alternative NF-κB activation in DLBCL
- Constitutive alternative NF-κB activity promotes B cell and plasma cell hyperplasia
- NF-κB-enforced terminal B cell differentiation is repressed by BCL6 in vivo
- Alternative NF-κB signaling cooperates with BCL6 to induce DLBCL in a mouse model

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In Brief
Zhang et al. report that a sizable fraction of human diffuse large B cell lymphomas (DLBCL) carry genetic lesions activating the alternative NF-κB pathway and often accompanied by BCL6 translocation. Modeling these genetic events in mice, they demonstrate an oncogenic role for the alternative NF-κB pathway in DLBCL pathogenesis.

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An Oncogenic Role for Alternative NF-κB Signaling in DLBCL Revealed upon Deregulated BCL6 Expression

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SUMMARY

Diffuse large B cell lymphoma (DLBCL) is a complex disease comprising diverse subtypes and genetic profiles. Possibly because of the prevalence of genetic alterations activating canonical NF-κB activity, a role for oncogenic lesions that activate the alternative NF-κB pathway in DLBCL has remained elusive. Here, we show that deletion/mutation of TRAF3, a negative regulator of the alternative NF-κB pathway, occurs in ~15% of DLBCLs and that it often coexists with BCL6 translocation, which prevents terminal B cell differentiation. Accordingly, in a mouse model constitutive activation of the alternative NF-κB pathway cooperates with BCL6 deregulation in DLBCL development. This work demonstrates a key oncogenic role for the alternative NF-κB pathway in DLBCL development.

INTRODUCTION

Diffuse large B cell lymphoma (DLBCL), the most common form of non-Hodgkin’s lymphoma, is a genetically, phenotypically, and clinically heterogeneous disease. Various DLBCL subtypes have been revealed by gene expression profile analysis using distinct classification schemes, which is according to their putative cell of origin or the coordinated expression of consensus clusters (Alizadeh et al., 2000; Monti et al., 2005). In the “cell of origin” (COO) classification, two main subtypes of DLBCL have been identified in which transcriptional programs resemble normal B cells at particular developmental stages. These are the germinal center B cell (GCB)-like DLBCL, presumably derived from a GC B cell, and the activated B cell (ABC)-like DLBCL, in which the cell of origin is less clear but may correspond to a cell undergoing plasmacytic differentiation (Lenz and Staudt, 2010; Wright et al., 2003).

Analysis of the coding genome of DLBCL has identified various genetic lesions and revealed their association with the GCB or ABC subtype. Inactivating mutations and deletions of BLIMP1/PRDM1, a key gene in terminal B cell differentiation, are found exclusively in the ABC subtype (~30% of cases) (Man-delbaum et al., 2010; Pasqualucci et al., 2006; Tam et al., 2006). Similarly BCL6 expression is deregulated by chromosomal translocation more frequently in the ABC (~26% of cases) than in the GCB subtype, where BCL6 expression is high a priori (Iqbal et al., 2007; Mandelbaum et al., 2010; Pasqualucci et al., 2011). Interestingly, BCL6 translocations are mutually exclusive with BLIMP1 structural alterations in ABC-DLBCL (Mandelbaum et al., 2010). Given that BCL6 can directly suppress BLIMP1 expression (Tunyaplin et al., 2004), it has been hypothesized that BCL6 translocations represent an alternative mechanism for BLIMP1 inactivation in ABC-DLBCL, although BCL6 controls multiple additional functions in GC B cells (Mandelbaum et al., 2010). Another group of mutations promote constitutive NF-κB
activation, such as those affecting TNFAIP3 (A20), CD79B, and MYD88, predominantly in the ABC subtype (Compagno et al., 2009; Davis et al., 2010; Ngo et al., 2011; Pasqualucci et al., 2011), and CARD11 mutations occurring in both subtypes (Lenz et al., 2008; Pasqualucci et al., 2011).

Notably, NF-κB activating mutations in DLBCLs, including the ones described above, predominantly involve the NF-κB canonical pathway (Compagno et al., 2009; Davis et al., 2010; Lenz et al., 2008; Ngo et al., 2011; Pasqualucci et al., 2011; Staudt, 2010). As a consequence, a role for putative genetic lesions involving the NF-κB alternative pathway remained largely overlooked. Supporting a role of the NF-κB alternative pathway in DLBCL pathogenesis, ~10% of DLBCLs were found to stain positive for NF-κB2 p52 but not NF-κB1 p50 in the nucleus, and another 20% of cases exhibited both NF-κB1 and NF-κB2 nuclear staining (Compagno et al., 2009); furthermore, a recent study revealed that roughly 10% of DLBCLs carry deletions or mutations of TRAF3 or TRAF2 (Pasqualucci et al., 2011). TRAF3 and TRAF2 control the degradation of NF-κB-inducing kinase (NIK) and consequently restrain activation of the alternative NF-κB pathway (Gardam et al., 2008; Häcker et al., 2011; Sasaki et al., 2008).

Although an oncogenic role for constitutive canonical NF-κB activity has been demonstrated in a mouse model of DLBCL (Calado et al., 2010), a functional link between the activation of alternative NF-κB pathway and the pathogenesis of DLBCL remained to be established. In this study, we performed complementary human and mouse studies to investigate mutations activating the alternative NF-κB pathway and concurrent genetic events and developed a genetic system in the mouse to test the role of constitutive alternative NF-κB signaling in the pathogenesis of DLBCL.

RESULTS

TRAF3 Gene Lesions Coexist with BCL6 Translocation in Human DLBCL

Deletions and mutations of TRAF3 have been found in human DLBCLs (Pasqualucci et al., 2011). To have a deeper look into TRAF3 genetic lesions and their distribution in DLBCL subtypes, we analyzed the TRAF3 sequences for the presence of point mutations and deletions in 119 DLBCL samples, including 98 biopsies and 21 cell lines whose phenotypic subtype was known. This analysis revealed missense, frameshift, and nonsense mutations (the two mutations tested being both somatic in origin) in functional domains, which are required for TRAF3 to negatively regulate NIK protein stability (Figure 1A; Annunziata et al., 2007; Häcker et al., 2011; He et al., 2007; Keats et al., 2007). Specifically, we identified one DLBCL case carrying a frameshift mutation (284 fs) and one carrying a nonsense
mutation (R310X), both of which are predicted to disrupt the MATH domain, required for the interaction between TRAF3 and NIK (Häcker et al., 2011; He et al., 2007). One additional DLBCL harbored a missense mutation (H70R) that may alter the function of the RING domain, required for the negative regulation of NIK by TRAF3 (He et al., 2007). Notably, a missense mutation affecting the H70 residue has been previously reported in a multiple myeloma patient (Keats et al., 2007). The present analysis also identified biallelic or monoallelic deletions involving the TRAF3 locus, including two focal homozygous losses that encompass TRAF3 and its neighboring gene RCOR1 (Figure 1B). A similar spectrum of deletions has been observed in human multiple myeloma and was shown to stabilize the NIK protein (Annunziata et al., 2007; Keats et al., 2007). TRAF3 deletions/mutations occurred similarly in GCB and ABC DLBCL (Figure 1C) and significantly correlated with alternative NF-κB cell differentiation (Mandelbaum et al., 2010). While none of the genetic lesions, either of which would presumably disrupt terminal B cell differentiation via inactivating BLIMP1 (Calado et al., 2010), with these observations in mind, we searched whether 2010). With these observations in mind, we searched whether deletions occurred similarly in GCB and ABC DLBCL (Figure 1C) and significantly correlated with alternative NF-κB activation, indicated by nuclear p52 staining (Figure 1D).

We previously observed that constitutive canonical NF-κB activation promotes DLBCL development upon disruption of terminal B cell differentiation via inactivating BLIMP1 (Calado et al., 2010). With these observations in mind, whether TRAF3 mutations in DLBCL associate with BCL6 or BLIMP1 genetic lesions, either of which would presumably disrupt terminal B cell differentiation (Mandelbaum et al., 2010). While none of the 17 DLBCLs carrying TRAF3 deletions/mutations exhibited biallelic BLIMP1 deletion/mutation, 6 of them (35%) had concurrent BCL6 translocation (the small number of cases analyzed did not provide statistical power to assess whether the co-occurrence is significant (Figures 1E and S1).

Taken together, these data show that roughly 15% of DLBCLs carry TRAF3 genetic alterations and that these lesions often coexist with BCL6 translocations.

Impact of Alternative NF-κB Activation and/or BCL6 Deregulation on the GC Reaction

To study the impact of enforced alternative NF-κB activation and BCL6 deregulation on the pathogenesis of DLBCL, we used a system of conditional gain- and/or loss-of-function mutagenesis in mice. Given that DLBCL arises from a GC or post-GC B cell (Shaffer et al., 2012), we decided to perform mutagenesis in GC B cells, using the Cγ1-cre transgene, from which Cre is expressed in B cells at an early stage of the GC reaction (Casola et al., 2006). To induce activation of the alternative NF-κB pathway, we combined this transgene with a ROSA26 allele harboring cDNA encoding NIK, preceded by a loxP flanked STOP cassette (hereafter called NIKstopFL) (Sasaki et al., 2008). NIK expression from the mutant ROSA26 allele is indicated by a GFP reporter controlled by an internal ribosome entry site (Sasaki et al., 2008). For BCL6 deregulated expression, we used an HA-tagged BCL6 transgene inserted into the immunoglobulin (Ig) heavy-chain locus downstream of μ promoter (hereafter called μBcl6), mimicking the observed BCL6/Igh translocation in DLBCL (Cattoretti et al., 2005). To monitor Cre-mediated recombination in cells of compound mutant mice not carrying the NIKstopFL allele, we used a conditional YFP reporter allele in the ROSA26 locus designated YFPstopFL (Srinivas et al., 2001). Mice carrying the Cγ1-cre and YFPstopFL alleles served as controls.

To test the impact of alternative NF-κB pathway activation, alone or together with enforced BCL6 expression, on GC B cell formation, we immunized experimental and control mice with sheep red blood cells (SRBCs). Analysis 10 days after immunization revealed expression of the reporter, GFP or YFP, in the majority of GC B cells, indicating efficient Cre-mediated recombination in mice of all genotypes analyzed (Figure 2A). Control mice and mice with enforced expression of NIK and/or BCL6 also showed similar fractions of GC B cells at day 10 after primary immunization (Figures 2A and 2C). However, at day 21 postimmunization, mice with enforced NIK expression and thus constitutive alternative NF-κB activation alone had a significantly reduced fraction of GC B cells compared with control mice (Figures 2B and 2C), similar to what is seen in mice with constitutive canonical NF-κB activation in GC B cells (Calado et al., 2010). Enforced NIK expression in GC B cells led to increased expression of IRF4, which might, in turn, account for upregulation of BLIMP1 and downregulation of BCL6 in these cells (Figure 2D; Saito et al., 2007; Sciammas et al., 2006). We considered the possibility that the premature termination of the GC reaction in mice with enforced NIK expression might be due to the altered expression of BLIMP1 or BCL6 (Calado et al., 2010; Martins and Calame, 2008; Ye et al., 1997); however, concomitant BLIMP1 deletion (data not shown) or BCL6 enforced expression did not prevent GC early termination in these mice (Figures 2B and 2C). We next looked whether activation of the alternative NF-κB pathway alone or together with enforced BCL6 expression affects physiological processes of GC B cells, such as somatic hypermutation. At day 21 postimmunization, GC B cells from mice with enforced NIK or BCL6 expression alone carried slightly reduced numbers of somatic mutations in their Ig heavy-chain (IgH) V regions, compared with controls, while those from mice with enforced expression of both NIK and BCL6 had significantly fewer mutations (Figure 2E). The reduced somatic mutation load in the latter group is likely due to premature termination of the GC reaction, as similar levels of somatic mutation and AicDA expression were detected in GC B cells from these mice and controls when analyzed at day 10 after immunization, the peak time of the GC reaction (Figures 2C, 2E, and 2F). Collectively, these results demonstrate that constitutive activation of NF-κB signaling through enforced NIK expression negatively impacts the GC reaction and that this effect is independent of BCL6 regulation. Hence, constitutive NF-κB activation is incompatible with the maintenance of a GC B cell phenotype and may in a similar way impact the phenotype of lymphoma cells arising in the context of the GC reaction (see Discussion).

Enforced Activity of the Alternative NF-κB Pathway Enhances B Cell Proliferation and Survival

To evaluate the impact of the constitutive expression of NIK and/or BCL6 on B cell proliferation and survival, we used an in vitro cell culture system where Cre-mediated recombination is induced in B cells upon treatment with anti-CD40 and interleukin 4 (IL-4), mimicking T cell-dependent B cell activation (Calado et al., 2010). NF-κB activation through enforced expression of NIK not only improved survival of ABCs but also increased their proliferation (Figures 3A–3C). Interestingly, in
Figure 2. Impact of Constitutive NIK and BCL6 Expression on the GC Reaction

(A and B) Representative FACS analysis of splenic GC B cells at day 10 (A) and day 21 (B) after primary immunization with SRBCs, respectively. (Upper) The GC B cell population (within the gate; CD19^+Fas^loCD38^lo). (Lower) Reporter expression in GC B cells.

(C) Summary of FACS analysis of GC B cells as in (A) and (B). Black bar represents mean for each genotype of mice at the indicated time points.

(D) Real-time PCR analysis of the expression levels of the indicated genes in reporter-positive GC B cells at day 10 after primary immunization with SRBCs. Values represent normalized levels to HPRT. Data are represented as mean ± SEM.

(E) IgH somatic mutation in reporter-positive GC B cells at day 10 (13 to 16 sequences per mouse from two to three mice per genotype) and day 21 (12–16 sequences per mouse from two to three mice per genotype) after primary immunization with SRBCs. Black bar represents mean. Average mutation frequency at day 21 is shown in graph.

(F) Real-time PCR analysis of AICDA transcript levels in reporter-positive GC B cells at day 10 after primary immunization with SRBCs. Values represent normalized levels to HPRT. Data are represented as mean ± SEM.
this experimental system, concurrent BCL6 expression did not further enhance these effects (Figures 3A–3C). Overall, constitutive NIK expression led to the accumulation of increased numbers of cells in culture, with concomitant BCL6 expression having no additional effect (Figure 3D).

**BCL6 Enforced Expression through a BCL6/IgH Translocation Blocks Plasma Cell Differentiation Induced by Constitutive Alternative NF-κB Signaling**

Enforced expression of NIK in GC B cells promoted transcription of IRF4 (Figure 2D), a key transcription factor for plasma cell differentiation (Klein et al., 2006). Accordingly, SRBC-immunized 

\[ \text{Cy1-cre/+/IuBcl6;NikstopFL} \]

mice displayed a significantly enlarged plasma cell compartment after primary and secondary immunization compared with controls (Figures 4A, 4B, and S2A). In accord with the notion that BCL6 represses BLIMP1 transcription (Figure 2D; Tunyaplin et al., 2004) and the latter is essential for plasma cell differentiation (Martins and Calame, 2008), the plasma cell compartment was significantly reduced in 

\[ \text{Cy1-cre/IuBcl6;NikstopFL} \]

mice compared with 

\[ \text{Cy1-cre/+/NikstopFL} \]

mice (Figures 4A, 4B, and S2A). In line with the in vitro data that enforced alternative NF-κB activity enhances B cell proliferation and survival, while concurrent BCL6 expression has no additive effect (Figure 3), 

\[ \text{Cy1-cre/+/NikstopFL} \]

mice displayed increased numbers of total reporter positive cells (containing both plasma cells and B cells) in spleen compared to controls (Cy1-cre/+/YFPstopFL), and these numbers were not further increased in mice with concurrently enforced expression of BCL6 (Cy1-cre/IuBcl6;NikstopFL mice) (Figures 4A and S2B). Collectively, these data suggest that a major effect of the deregulated BCL6 expression in GC B cells is a block of plasma cell differentiation.

**Mice with Enforced NIK and BCL6 Expression in GC B Cells Display a Shortened Lifespan**

Next, we assessed the role of alternative NF-κB activation with or without concomitant BCL6 deregulation in B cell malignant transformation by monitoring the mice for tumor development over a period of 1.5 years (78 weeks). Mice with enforced expression of NIK displayed a similar lifespan as controls, while 40% of 

\[ \text{Cy1-cre/IuBcl6;NikstopFL} \]

mice died prematurely (Figure 5), consistent with a previous report (Cattoretti et al., 2005). In contrast, all mice with concurrent NIK and BCL6 enforced expression died within the observation period, suggesting a cooperative role (Figure 5).
Plasma Cell Hyperplasia in Mice with Constitutive Alternative NF-κB Signaling

We decided to analyze the Cγ1-cre/+;NikstopFL mice in more detail and sacrificed them at the end of the observation period (1.5 years). Mice with enforced NIK expression displayed enlarged spleens (Figure 6A) and had a significant hyperplasia of both B cells and plasmablasts/plasma cells in spleen and bone marrow compared with age-matched control animals (Figures 6B and S3). Histological analysis revealed dramatically increased numbers of spleen cells expressing the plasma cell marker CD138 and intracellular Ig (Figure 6C). Serum protein electrophoresis further revealed that 8/9 of Cγ1-cre/+;NikstopFL mice displayed a distinct band in the γ-globulin region of the gel (M-spike), in contrast to three of nine of controls (Figure 6D; data not shown), indicative of clonal plasma cell expansion. We conclude that enforced activation of alternative NF-κB signaling promotes B cell hyperplasia, in accord with previous work (Sasaki et al., 2008), as well as an expansion of the plasma cell compartments in spleen and bone marrow.

Alternative NF-κB Signaling Cooperates with Deregulated BCL6 in DLBCL Pathogenesis

Macroscopic examination of terminally ill Cγ1-cre/μBcl6 (6 cases) and Cγ1-cre/μBcl6;NikstopFL (10 cases) mice revealed splenomegaly and lymphadenopathy in all cases (Figure 7A). Histological examination of the enlarged lymphoid organs showed that five of the six Cγ1-cre/μBcl6 mice analyzed had a DLBCL-like disease, characterized by a diffuse growth pattern of large cells, while the remaining mouse had a tumor with a plasmacytoid morphology. Diseased Cγ1-cre/μBcl6;NikstopFL mice on the other hand all showed histological features of DLBCL (Figure 7B; Table S1; data not shown). By flow cytometry, these tumors were all negative for the plasma cell marker CD138 and displayed a mature B cell phenotype (CD19⁺AA4.1⁻IgM⁺ or occasionally IgG⁺; Table S2). Analysis of IgH gene rearrangements by Southern blot revealed that the DLBCLs were of clonal B cell origin and that the same tumor clone was present in both spleen and mesenteric lymph nodes of each mouse examined (Figure 7C), indicative of an aggressive phenotype. We next amplified the rearranged IgH V regions from clonal B cell tumors of Cγ1-cre/μBcl6 and Cγ1-cre/μBcl6;NikstopFL mice (four cases each). Sequence analysis revealed somatically mutated Ig genes in three of the four Cγ1-cre/μBcl6 tumors and two of the four Cγ1-cre/μBcl6;NikstopFL tumors (Table S3), suggesting that a fraction of the tumors derived from GC or post-GC B cells.
The activation of the alternative NF-κB pathway was confirmed in all tumors from Cγ1-cre/μBcl6;NikstopFL mice by the enhanced processing of p100 to p52 on the immunoblot compared with tumors from Cγ1-cre/μBcl6 mice (Figure 7D). In an attempt to subclassify the lymphomas occurring in our mouse cohorts according to the COO classification scheme, we performed immunohistochemistry (IHC) and gene expression profiling (GEP) by RNA sequencing (RNA-seq). By IHC, expression of IRF4/MUM1 usually segregates with ABC-DLBCL, while high levels of BCL6 expression often associate with GCB-DLBCL (Choi et al., 2009; Hans et al., 2004), although a fraction of both canonical and alternative pathways of NF-κB activation (Brown et al., 1995; Traenckner et al., 1995). For this reason we decided to analyze the RNA-seq data of the DLBCLs arising in the compound mutant mice for acquired mutations affecting genes within the canonical NF-κB pathway (for the list of genes, see Supplemental Experimental Procedures). Interestingly, we found such mutations in two of seven DLBCLs arising in Cγ1-cre/μBcl6;NikstopFL mice (Figure S4). More specifically, DLBCLs #773 and #776 harbored the same mutation (R218H) in the MYD88 gene, and increased levels of phospho-IκBz were seen in DLBCL #773 compared with other tumors of the same genotype and normal splenic tissue from a Cγ1-cre/+ mouse (Figure 7D), suggesting a functional role for this mutation (Ngo et al., 2011). We also analyzed RNA-seq data of the DLBCLs arising in mice with enforced BCL6 expression alone for the presence of mutations in genes of both canonical and alternative pathways of NF-κB (for the list of genes, see the Supplemental Experimental Procedures). We found that two of five such DLBCLs had acquired mutations in either the CK1α kinase or CARD11 gene (Figure S4; Bidère et al., 2009; Lenz et al., 2008). DLBCL #1,128 had a mutation in the CK1α kinase domain and displayed elevated phospho-IκBz compared with the normal spleen control (Figure 7D). These observations are consistent with the observed IRF4 expression in this tumor and classification as ABC-DLBCL (Figure 7F). Another Cγ1-cre/μBcl6 derived DLBCL (#603) displayed a mutation in the CARD11 (D401N) coiled-coil domain, to which the CARD11 mutations in human DLBCL are confined (Lenz et al., 2008), and this lymphoma also showed elevated levels of phospho-IκBz compared with the normal spleen control (Figure 7D). Despite canonical NF-κB pathway activation, this tumor was classified as a GCB-DLBCL by GEP profiling. Interestingly, the same exact mutation has been found in a human GCB-DLBCL (Morin et al., 2011). This is consistent with previous observations in human DLBCL, where canonical NF-κB activation could be detected in ~20% of GCB-DLBCLs (Compagno et al., 2009). Collectively, our data show that a fraction of the tumors arising in both Cγ1-cre/μBcl6 and Cγ1-cre/μBcl6;NikstopFL mice has acquired mutations in genes of the canonical NF-κB pathway.
that are also affected in human DLBCL (Compagno et al., 2009; Pasqualucci et al., 2011).

DISCUSSION

A Causal Role of Alternative NF-κB Activation in DLBCL

Recent studies of human DLBCLs have identified various genetic lesions that activate NF-κB through the canonical pathway and revealed their association predominantly with the ABC over the GCB subtype (Compagno et al., 2009; Davis et al., 2010; Kato et al., 2009; Lenz et al., 2008; Ngo et al., 2011; Pasqualucci et al., 2011), in accord with the observation that ABC-DLBCL but not GCB-DLBCL cell lines rely on constitutive canonical NF-κB signaling for survival (Davis et al., 2001; Staudt, 2010). Using a mouse model, we had previously established an oncogenic role for constitutive canonical NF-κB activity in ABC-DLBCL pathogenesis (Calado et al., 2010). In contrast, the question of whether enforced activation of the alternative NF-κB pathway can be functionally involved in DLBCL pathogenesis has not been addressed, despite several observations suggesting a role for this pathway in the disease. Thus, mutations affecting genes (TRAF3 and TRAF2) of the alternative NF-κB pathway have been observed in a subset of human DLBCLs (Pasqualucci et al., 2011); the NF-κB2 gene, encoding the core molecule for this signaling pathway, was originally identified by virtue of its translocation to the IgH locus in a case of DLBCL (Neri et al., 1991), and IHC data revealed nuclear NF-κB2 p52, reflecting activation of the alternative pathway, in a subset of both GCB and ABC-DLBCL (Compagno et al., 2009). While these data demonstrated alternative NF-κB activity in a subset of DLBCLs, a paper by Pham et al. (2011) claimed that in DLBCLs of all subtypes both the canonical and alternative NF-κB pathways are activated through constitutive BAFF-R (BR3) signaling. However, in most DLBCL cell lines analyzed, there was no evidence for robust degradation of p100 to p52.

To clarify these matters and in particular to obtain functional evidence for a contribution of alternative NF-κB signaling to DLBCL pathogenesis, we first extended the analysis of TRAF3 mutations by studying a larger number of DLBCL primary tumors and examining the association of TRAF3 mutations with the ABC or GCB subtype. These analyses demonstrated that biallelic or monoallelic deletions/mutations of TRAF3 occur recurrently in similar fractions (15%) of ABC-DLBCL and GCB-DLBCL and correlate with alternative NF-κB activity in these cases. We then developed a mouse model system that allows conditional activation of the alternative NF-κB pathway in a GC B-cell-restricted manner and found that activation of this pathway, in concert with BCL6 deregulation, leads to the development of DLBCL. This indicates a causal role of deregulated alternative NF-κB signaling in DLBCL pathogenesis. Interestingly, in this scenario, the deregulation of alternative NF-κB activity appears to be required in the context of GC B cell differentiation. Deletion of TRAF3 in mouse B cells from early developmental stages via CD19-cre leads to the formation of B1 and marginal zone B cell lymphomas, not DLBCL (Moore et al., 2012). These tumors resemble human splenic marginal zone lymphoma (SMZL), where inactivating mutations of TRAF3 have also been found.
(Rossi et al., 2011). Together, these observations highlight the importance of ontogenetic timing in the acquisition of oncogenic somatic mutations driving different classes of lymphomas. TRAF3 mutations/deletions are absent in follicular lymphoma, Burkitt lymphoma, and B cell chronic lymphocytic leukemia (data not shown).

Of note, we observed that most Cγ1-cre/IjμBcl6;NikstopFL mice developed DLBCLs of the ABC subtype. This observation is likely related to the fact that activation of the alternative NF-κB pathway interferes with the GC reaction even when BCL6 expression is deregulated and suggests that, in NF-κB positive human GCB-DLBCLs, additional mutation(s) allowing maintenance of the GCB phenotype must exist. Future studies comparing genetic lesions in the GCB versus ABC types of DLBCLs carrying TRAF3 lesions may lead to the identification of those latter events.

**Interference with Terminal B Cell Differentiation Is Required for the Pathogenesis of ABC-DLBCL: A Role for BCL6**

Constitutive NF-κB signaling in B cells promotes their differentiation toward plasma cells through induction of IRF4 (Grumont and Gerondakis, 2000; Klein et al., 2006; Saito et al., 2007; Sciammas et al., 2006), in line with the presence of genetic lesions leading to constitutive NF-κB signaling in multiple myeloma (Annunziata et al., 2007; Keats et al., 2007). The observations that ABC-DLBCLs express some key genes characteristic of a plasmablast (Lenz and Staudt, 2010; Staudt, 2010) suggests that the transformation of an ABC into DLBCL requires interference with terminal B cell differentiation (Lenz and Staudt, 2010; Staudt, 2010). Indeed, mice with specific activation of the canonical NF-κB signaling pathway in
GC B cells developed plasma cell hyperplasia and had an overall normal lifespan, but succumbed to ABC-like DLBCL when B-cell-terminal differentiation was abolished by deletion of Blimp1 (Calado et al., 2010). Similarly, we show here that mice with activation of the alternative NF-κB pathway alone in GC B cells do not succumb to tumors in the timeframe of this study but display overt plasma cell hyperplasia and that the oncogenic role of the alternative NF-κB pathway is revealed upon interference with plasma cell differentiation through enforced BCL6 expression, in accord with the coexistence of TRAF3 and BCL6 mutations in human DLBCL.

Our work indicates that the role of BCL6 in the development of DLBCLs exhibiting constitutive NF-κB signaling is at least in part due to its ability to inhibit BLIMP1 expression, which in turn limits the terminal differentiation of B cells. This is supported by data showing that in the mouse loss of BCL6 cooperates with alternative NF-κB signaling in DLBCL formation (data not shown). However, the fact that human DLBCLs with alternative NF-κB activation are often concurrent with BCL6 translocation but not BLIMP1 inactivation suggests that other functions of BCL6, such as repression of the DNA damage response (Basso and Dalla-Favera, 2010), may also be critically required to complement alternative NF-κB signaling in DLBCL development.

Alternative and Canonical NF-κB Pathway Activation in DLBCLs

While mechanisms of aberrant NF-κB activation in DLBCL can in the major fraction of the cases be attributed to the presence of oncogenic mutations in genes related to the canonical NF-κB pathway (Compagno et al., 2009; Davis et al., 2010; Lenz et al., 2008; Ngo et al., 2011; Pasqualucci et al., 2011), previous data and the present work show that genetic lesions activating the alternative NF-κB pathway occur in up to 15% of DLBCLs. It is worth noting in this context that besides the ~10% DLBCL cases demonstrating nuclear NF-κB activity exclusively for the alternative pathway (indicated by nuclear staining of p52 but not p50) ~20% of DLBCLs display nuclear staining for both p50 and p52 (Compagno et al., 2009), suggesting the activation of both canonical and alternative NF-κB pathways. Indeed, our genetic analysis showed that a fraction (6 of 17) of TRAF3-mutated DLBCLs carries concurrent mutation(s) in genes of the canonical NF-κB pathway. Likewise, while the present mouse model suggests that the alternative pathway can by itself drive DLBCL development if combined with a lesion preventing plasma cell differentiation, it became apparent that in a small fraction (two of seven) of the resulting tumors additional mutations accumulated, which resulted in the activation of canonical NF-κB signaling. We have previously observed redundancy between the canonical and alternative NF-κB pathways to replace BAFF-mediated survival signals in B cells (Sasaki et al., 2006, 2008), and there is evidence in the human that NIK activation can also trigger canonical NF-κB activity (Anunziata et al., 2007; O’Mahony et al., 2000). Given that the NIK allele used in the present study yields only a moderate cell survival advantage, in sharp contrast to a NIK allele lacking the TRAF3 binding site (Sasaki et al., 2008), we speculate that in a NIK-expressing B cell acquisition of an activating canonical NF-κB mutation may confer a further survival advantage, enabling the cell to outcompete its siblings during the clonal evolution of lymphoma. A similar mechanism may operate in human DLBCL pathogenesis. Overall, the current work provides a rationale for the design of therapies targeting the alternative NF-κB pathway in a fraction of DLBCL patients and suggests that for those human DLBCLs that display both canonical and alternative NF-κB mutations (Compagno et al., 2009; Pasqualucci et al., 2011), targeting both arms of NF-κB signaling may be required for therapeutic intervention, as recently demonstrated for multiple myeloma (Fabre et al., 2012).

**EXPERIMENTAL PROCEDURES**

**Sequencing Analysis and High-Density SNP Array Analysis of Human DLBCLs**

One hundred nineteen DLBCL samples, including 98 biopsies (47 GCB and 51 ABC/NC-DLBCLs) and 21 cell lines (14 GCB and 7 ABC-DLBCLs) were analyzed as described previously (Pasqualucci et al., 2011). Oligonucleotides and conditions used for TRAF3 amplification are available upon request.

**Mice, Immunization, and Tumor Cohorts**

C57-cre, Nikfl/fl, Blimp1ff, IκBε, and YFPtm1 alleles have been described (Casola et al., 2006; Cattoretti et al., 2005; Ohinata et al., 2005; Sasaki et al., 2008; Srinivas et al., 2001). B- to 10-week-old mice were immunized intravenously with 1 × 106 SRBCs (Cedarlane) in PBS. Mouse cohorts for tumor development were given monthly antigenic stimulation by SRBC immunization for seven additional times and then monitored twice a week for tumor development and euthanized if signs of tumor development occurred. All animal care and procedures followed NIH guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC 03341) of Harvard University and the Immune Disease Institute.

**Statistical Analysis**

Unless otherwise indicated, data were analyzed using unpaired two-tailed Student’s t test; a p value ≤ 0.05 was considered significant. A single asterisk (*) in the graphs of figures represents p ≤ 0.05. Double asterisks (**) represent p ≤ 0.01, and triple asterisks (***) represent p ≤ 0.001; “ns” stands for not statistically significant, i.e., p > 0.05. Survival curves were compared using the log rank test. Data in text and figures are represented as mean ± SEM unless otherwise indicated.

**ACCESSION NUMBERS**

The data have been deposited to the NCBI GEO and are available under accession numbers GSE65422.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.03.059.

**AUTHOR CONTRIBUTIONS**

B.Z., D.P.C., and K.R. conceived and supervised the study, B.Z., D.P.C., and Z.W. designed, performed, and analyzed the main experiments. L.P. and R.D.-F. were responsible for the human DLBCL analysis. F.W.A. supervised some aspects of the study. S.F., K.K., Y.Q., S.B.K., C.U., S.R., and W.C. performed additional experiments. M.S.-S. and Y.S. generated the NIK transgenic mice and helped conceive the study. B.Z., D.P.C., and K.R. interpreted the results and wrote the paper. All authors read and contributed to the finalization of the paper.
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