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De Silva, NS, Anderson, MM, Carette, A et al. (4 more authors) (2016) Transcription factors of the alternative NF- κ B pathway are required for germinal center B-cell development. *Proceedings of the National Academy of Sciences*, 113 (32). pp. 9063-9068. ISSN 0027-8424

<https://doi.org/10.1073/pnas.1602728113>

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Classification: Biological Sciences (major); Immunology and Inflammation (minor)

The transcription factors of the alternative NF- κ B pathway are required for germinal center B-cell development

Short title: NF- κ B subunits in late B-cell development

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Keywords: Germinal center B cell; Plasma cell; NF- κ B transcription factors

ABSTRACT

The NF- κ B signaling cascade relays external signals essential for B-cell growth and survival. This cascade is frequently hijacked by cancers that arise from the malignant transformation of germinal center (GC) B cells, underscoring the importance of deciphering the function of NF- κ B in these cells. The NF- κ B signaling cascade is comprised of two branches, the canonical and alternative NF- κ B pathways, mediated by distinct transcription factors. The expression and function of the transcription factors of the alternative pathway, RELB and NF- κ B2, in late B-cell development is incompletely understood. Using conditional deletion of *relb* and *nfkb2* in GC B cells, we here report that ablation of both RELB and NF- κ B2, but not of the single transcription factors, resulted in the collapse of established GCs. RELB/NF- κ B2 deficiency in GC B cells was associated with impaired cell cycle entry and reduced expression of the cell-surface receptor ICOSL that promotes optimal interactions between B and T cells. Analysis of human tonsillar tissue revealed that plasma cells and their precursors in the GC expressed high levels of NF- κ B2 relative to surrounding lymphocytes. In accordance, deletion of *nfkb2* in murine GC B cells resulted in a dramatic reduction of antigen-specific antibody-secreting cells, while deletion of *relb* had no effect. These results demonstrate that the transcription factors of the alternative NF- κ B pathway control distinct stages of late B-cell development, which may have implications for B-cell malignancies that aberrantly activate this pathway.

SIGNIFICANCE

In many human B-cell cancers, a complex signaling cascade called nuclear factor- κ B (NF- κ B) is abnormally activated by genetic mutations. The uncontrolled activity of NF- κ B due to genetic mutations promotes the formation B-cell tumors. The NF- κ B cascade is comprised of two distinct pathways. We here define the role of one of these routes, called the alternative NF- κ B pathway, in the normal cells from which these B-cell tumors are derived, namely germinal center B cells or plasma cells. We found that the inactivation of the alternative NF- κ B pathway led to the loss of germinal center B cells and impaired plasma cell development. Understanding the role of this pathway in normal cells may provide important insights into how aberrant activation promotes B-cell tumors.

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INTRODUCTION

During T-cell-dependent immune responses, B cells diversify their antigen-receptors by somatic hypermutation (SHM) of the immunoglobulin variable region (IgV) genes (1). SHM and selection of B cells with increased antigen-affinity occurs within GCs. The efficiency of the GC reaction is enhanced by topological and temporal segregation of proliferation and SHM within the dark zone (DZ) and antigen-selection within the light zone (LZ) (2-4). Recirculation of GC B cells between these zones result in the generation of high-affinity, often isotype-switched memory B cells and plasma cells (PCs) (2-5). The GC reaction is critical for immunity; however, errors during SHM and class-switch recombination can lead to genetic aberrations that promote lymphomagenesis (6, 7). Recently, genetic mutations resulting in constitutive activation of the NF- κ B signaling cascade were identified in a large fraction of GC-derived B-cell lymphomas and multiple myeloma (MM) (8-16).

Activation of NF- κ B signaling results in the transcription of NF- κ B target genes that regulate many cellular processes including cell survival and proliferation (17, 18). The NF- κ B-signaling cascade comprises two branches, the canonical and alternative (or noncanonical) NF- κ B pathways, which activate specific NF- κ B transcription factor subunits that occur mainly as heterodimers. Canonical NF- κ B pathway activation leads to the nuclear translocation of c-REL, RELA and p50, while alternative pathway activation causes nuclear translocation of RELB and p52. In normal cells, NF- κ B activation is transient and tightly controlled. Conversely, constitutive NF- κ B activation due to genetic alterations in NF- κ B pathway components is pathogenic (8, 9). Mutations affecting multiple different NF- κ B signaling components have been identified in several GC-derived B-cell malignancies which can lead to the constitutive activation of the canonical and/or alternative NF- κ B pathways (8-16). The selection of these mutations implies

that NF- κ B-signaling may have an important biological role during normal GC B-cell development that is “hijacked” in tumors (7, 8).

Distinguishing the functions of the canonical and alternative NF- κ B pathways by studying upstream regulators may be complicated by the possibility of pathway crosstalk. Therefore, focusing on the downstream transcription factor subunits may help to clarify the specific roles of the separate NF- κ B pathways. Towards this aim, early work on human lymphoid tissue revealed that nuclear translocation of canonical NF- κ B subunits within GCs occurred only within a subset of cells in the LZ (19). By ablating the canonical NF- κ B transcription factors c-REL or RELA specifically in GC B cells, we recently showed that c-REL was essential for GC maintenance, whereas RELA was required for PC development (20). The expression, activation status and function of the alternative NF- κ B transcription factors RELB and p52 in GC B cells remain largely unknown. Due to the diverse functions of the alternative NF- κ B pathway in a range of cell types, mice with constitutional knockout of either *relb* or *nfkb2* (the gene encoding the p100/p52 precursor, referred to as NF- κ B2, from which p52 is generated upon activation) have severe defects in lymphoid organization (21-23), thus hampering the analysis of GC B-cell development in these mice. We here determined the expression pattern of the alternative NF- κ B subunits in human lymphoid tissue and investigated their roles during GC B-cell development *in vivo* by crossing conditional *relb* and/or *nfkb2* alleles to mice that express Cre-recombinase in GC B-cells. We found that RELB and NF- κ B2 were jointly required to maintain the GC B-cell reaction while the development of antigen-specific PCs was impaired upon deletion of only *nfkb2* in GC B-cells.

RESULTS

Expression and activation of alternative NF- κ B subunits in human GC B cells. The expression and activation of the alternative NF- κ B subunits in GC B cells has not been

investigated. Since CD40-stimulation strongly activates both NF- κ B pathways (24, 25), the CD40-CD40L interaction occurring between LZ B cells and T-follicular helper cells (Tfh cells) is expected to activate alternative NF- κ B signaling. Indeed, Western-blot analysis of human tonsillar GC B cells cultured on CD40L-expressing fibroblasts demonstrated p100→p52 processing (Fig. 1A, left) and thus alternative pathway activation. This was accompanied by the downregulation of the GC master regulator BCL6, an event believed to occur during LZ selection (7), and resulted in nuclear translocation of p52 along with the canonical subunit p50 (Fig. 1A, right). In accordance with the *in vitro* findings, nuclear translocation of p52 could be observed *in vivo* in tonsillar GCs within LZ B cells by immunofluorescence (IF) analysis (Fig. 1B). Thus, nuclear translocation of p52 was detected within a small subset of LZ B cells and therefore suggests that the alternative NF- κ B pathway may have a functional role in LZ B cells.

Interestingly, we observed strong staining of p100/p52 in PCs localizing in the tonsillar subepithelium that were identified by staining for the major PC regulator BLIMP1 (26, 27), relative to lymphocytes at the border of the subepithelium (Fig. 1C, top). The same staining pattern was observed in BLIMP1⁺ PC precursors in the LZ of tonsillar GCs (Fig. 1C, bottom). These observations may point towards a potential role of the alternative NF- κ B pathway in the development of normal PCs and their precursors in the GC. The large amount of NF- κ B2 in the cytoplasm may predispose BLIMP1⁺ plasma cell precursors and plasma cells to undergo strong signaling via the alternative NF- κ B pathway upon stimulation. Alternative pathway activation was observed via strong p100→p52 processing in two MM cell lines (Fig. 1D), and to a lesser extent in a cell line corresponding to diffuse large B-cell lymphoma (DLBCL), where mutations leading to activation of the alternative pathway have been observed in a subset of cell lines and primary cases (13, 15). Of note, Western-blot analysis revealed that the canonical NF- κ B subunit c-REL was expressed at dramatically lower levels in the MM lines compared to the DLBCL lines (Fig. 1D). A low expression of c-REL relative to surrounding lymphocytes appears

to also be a feature of normal PC precursors in the LZ (Fig. 1E, right), as identified by strong staining for IRF4 which at high expression levels promotes PC differentiation along with BLIMP1 (27, 28). In contrast, cytoplasmic p100/p52 expression is increased in several IRF4⁺ cells (Fig. 1E, left), similar to the corresponding BLIMP1 staining (Fig. 1c, bottom). Collectively, these data suggest that relative to mature B cells, PCs and their precursors in the GC are characterized by a distinct expression pattern of NF- κ B subunits; high expression of NF- κ B2 and low expression of c-REL.

Combined GC B cell-specific deletion of *relb* and *nfkb2* impairs the GC reaction. To determine the *in vivo* role of RELB and NF- κ B2 during GC B-cell development, we crossed conditional *relb* and *nfkb2* alleles (29) to C γ 1-Cre mice (30), either alone or in combination, to delete the genes in GC B cells. Expression of C γ 1-Cre is induced upon T cell-dependent immunization, resulting in the Cre-mediated deletion of loxP-flanked genes in the majority of GC B cells (30). *relb*^{fl/fl}C γ 1-Cre, *nfkb2*^{fl/fl}C γ 1-Cre or *relb*^{fl/fl}*nfkb2*^{fl/fl}C γ 1-Cre mice and the corresponding heterozygous and C γ 1-Cre control mice were immunized with sheep red blood cells (SRBCs) to induce a robust GC response. 14d following immunization, the fractions of splenic CD95^{hi}PNA^{hi} GC B cells in *relb*^{fl/fl}C γ 1-Cre and *nfkb2*^{fl/fl}C γ 1-Cre were not significantly different from those in the controls (Fig. 2A, left&middle). In contrast, the fraction of splenic GC B cells in *relb*^{fl/fl}*nfkb2*^{fl/fl}C γ 1-Cre mice was markedly reduced in comparison to *relb*^{fl/+}*nfkb2*^{fl/+}C γ 1-Cre and C γ 1-Cre mice 14d post-immunization (Fig. 2A, right). In accordance, immunohistochemistry (IHC) revealed reduced BCL6⁺ GCs within B-cell follicles in *relb*^{fl/fl}*nfkb2*^{fl/fl}C γ 1-Cre mice compared to controls at d14 post-immunization (Fig. 2B). Together, these findings demonstrate that single ablation of either RELB or NF- κ B2 in GC B cells had no significant impact on the GC reaction. Instead, combined ablation of RELB and NF- κ B2 in GC B

cells strongly impaired the GC reaction, demonstrating that both subunits of the alternative NF- κ B pathway are required for GC maintenance.

To define the temporal kinetics of the impaired GC reaction observed in *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre mice, we determined the fractions of splenic GC B cells at various time-points following immunization with SRBCs. 7d post-immunization, the fraction of GC B cells in *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre mice was comparable to that observed in the controls (SI Appendix, Fig. S1A). Since the conditional *relb* and *nfkb2* alleles were constructed such that Cre-mediated recombination of *loxP*-sites is accompanied by expression of an enhanced-GFP (eGFP) (29), it was possible to trace the gene-deleted GC B cells by flow cytometry for eGFP expression. Analysis for eGFP expression in GC B cells from *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre and *relb^{fl/+}nfkb2^{fl/+}*Cy1-Cre mice revealed single peaks of expression (SI Appendix, Fig. S1A, bottom right), indicating that the vast majority of GC B cells have deleted the *relb* and *nfkb2* alleles at d7. GC B-cell development therefore occurred normally in *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre mice up to d7 of the GC reaction, after which GC B cells were progressively lost.

To investigate the possibility of a selective loss of a particular GC B-cell subpopulation, we determined the fractions of CXCR4^{hi}CD86^{lo} DZ and CXCR4^{lo}CD86^{hi} LZ B-cell fractions (31) over time (SI Appendix, Fig. S1B). Statistically significant differences in the DZ and LZ B-cell fractions between *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre and Cy1-Cre control mice were observed; however, these differences were minor and do not point towards a preferential loss of a specific GC B-cell subpopulation. Thus, these data suggest that RELB and NF- κ B2 are required for the maintenance of both DZ and LZ subpopulations past d7 of the GC reaction.

Identification of genes controlled by the alternative NF- κ B subunits RELB and NF- κ B2 in GC B cells. To identify the biological programs controlled by RELB and NF- κ B2 that are required for GC maintenance, we isolated GC B cells from *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre and Cy1-Cre

control mice 7d post-immunization and conducted an RNA-seq analysis. We reasoned that gene expression changes that ultimately contribute to the loss of *relb/nfkb2*-deleted GC B cells at later time-points would already be detectable in these cells at d7. Splenic eGFP⁺ GC B cells were flow cytometrically sorted from two *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre mice, and GC B cells were isolated from three Cy1-Cre mice 7d post-immunization with SRBCs, and subjected to RNA-seq analysis. Reduced transcript counts of the *relb* and *nfkb2* genes were identified in GC B cells from *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre compared to Cy1-Cre mice (SI Appendix, Fig. S2A), and the *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre and Cy1-Cre samples clustered into two separate groups in an unsupervised analysis (SI Appendix, Fig. S2B). Since a monoclonal antibody was available for the surface molecule CD36, a putative fatty acid translocase (32), we could confirm reduced protein expression of CD36 on eGFP⁺ GC B cells from *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre mice in comparison to GC B cells from Cy1-Cre mice and eGFP⁺ GC B cells from *relb^{fl/+}nfkb2^{fl/+}*Cy1-Cre mice (Fig. 3A). Together, these observations validate the robustness of the RNA-seq data set.

Differentially expressed sequence analysis (DESeq) of RELB/NF- κ B2-proficient vs. RELB/NF- κ B2-deficient GC B cells identified 59 transcripts with greater than 2.5-fold reduced expression and 84 transcripts with greater than 2.5-fold increased expression in the *relb/nfkb2*-deleted B cells at a significance threshold of $p < 0.01$ (for the identity of the corresponding genes, fold-change and p values, see Datasets 1&2). Transcripts with reduced expression could be assigned to functional categories, with genes involved in the immune response and metabolism representing the largest categories (SI Appendix, Fig. S2C; for the identity of the genes, see Dataset 1). The metabolism category was largely comprised of two groups of genes with presumptive roles in either protein or lipid metabolism.

We next used gene set enrichment analysis (GSEA) (33) to further investigate the gene expression changes among the genotypes. The largest group of signatures enriched in the control cells vs. RELB/NF- κ B2-deficient cells was associated with cell cycle regulation (Fig. 3B

and Dataset 3), suggesting that RELB/NF- κ B2 may control genes involved in proliferation. To test this possibility, we cultured RELB/NF- κ B2-deficient B cells purified from *relb^{fl/fl} nfbk2^{fl/fl}* CD19-Cre mice and control B cells isolated from CD19-Cre mice with CD40 and IL-4, a combination that provides a strong proliferative signal and activates the alternative pathway via CD40. By analyzing the proliferation profile of these cells at d3 of stimulation, we found that a significantly smaller fraction of eGFP⁺RELB/NF- κ B2-deficient B cells entered the cell cycle in comparison to control B cells (Fig. 3C). Of note, the *relb/nfkb2*-deleted B cells that were able to enter the cell cycle appeared to proliferate slightly more than controls. Together, the data suggest that RELB/NF- κ B2-deficient B-cells have a reduced ability to enter the cell cycle, which may contribute to the loss of RELB/NF- κ B2-deficient GC B cells over time that we observed *in vivo*.

To investigate whether RELB/NF- κ B2-deficient GC B cells proliferate less than WT GC B cells *in vivo*, we assessed BrdU incorporation at day 10. At day 10, a reduction in *relb/nfkb2*-deleted vs. WT GC B cells is already evident (SI Appendix, Fig. S3A). We chose this time-point for analysis since it is in-between day 7 (when *relb/nfkb2*-deleted GC B cells are present at normal frequencies; SI Appendix, Fig. S1A) and day 14 (when *relb/nfkb2*-deleted GC B cells are greatly reduced; Fig. 2). We observed a trend towards decreased BrdU-incorporation in *relb/nfkb2*-deleted vs. WT GC B cells identified as CD19⁺GL7^{hi} cells (SI Appendix, Fig. S3C, for gating strategy see SI Appendix, Fig. S3B). We believe these differences are minor since the percentage of GFP⁺ cells is variable between different mice (SI Appendix, Fig. S3D), which could reflect counterselection against *relb/nfkb2*-deleted GC B cells. Since the BrdU protocol involves fixation, we were unable to specifically measure BrdU incorporation in GFP⁺ GC B cells, which could dilute the actual difference in the fraction of cells that have incorporated BrdU.

The second largest group of signatures identified in the GSEA analysis was associated with the metabolism of proteins (Fig. 3B). In addition, when we compared our RNA-seq data set to a library of normal and pathological lymphoid gene expression signatures (34), five signatures

were found to be enriched in RELB/NF- κ B2-proficient vs. deficient GC B cells (SI Appendix, Fig. S4), including a ribosomal protein signature and two X box-binding protein-1 (XBP1)-associated gene expression signatures. XBP-1 is required for the unfolded protein response and is essential for the development of PCs capable of secreting large amounts of antibodies (26, 27). This suggests that in GC B cells, the alternative NF- κ B subunits may be required to set up a program that allows for the efficient production of proteins and facilitates antibody secretion, presumably in GC B cells destined to become plasmablasts (see below).

Finally, we have previously shown that deletion of the gene encoding the canonical NF- κ B subunit c-REL (*rel*) in GC B cells leads to the involution of GCs (20) similar to what we observed upon *relb/nfkb2* deletion, suggesting that c-REL and the alternative NF- κ B subunits exert non-redundant functions during the GC reaction. In support of this notion, genes with reduced expression in *relb/nfkb2* or *rel*-deleted GC B cells vs. controls were found to be largely mutually exclusive (Fig. 3D), indicating that the different canonical and alternative NF- κ B subunits control distinct transcriptional programs within the same GC context.

RELB/NF- κ B2-deficient GC B cells have reduced cell-surface expression of ICOSL. The interaction between inducible T-cell co-stimulator (ICOS), expressed on Tfh cells, and ICOS ligand (ICOSL), expressed on GC B cells, promotes the selection of high-affinity B cells (35). The expression of ICOSL is regulated by the alternative NF- κ B subunits in response to B-cell activating factor (BAFF) receptor (BAFF-R)-stimulation (36) and also CD40-stimulation (29) in murine B cells. To determine the extent to which the deletion of *relb* and *nfkb2* in GC B cells affects ICOSL expression on GC B cells *in vivo*, we stained splenic mononuclear cells from *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre and Cy1-Cre control mice for ICOSL and GC markers 10d following SRBC immunization. eGFP⁺RELB/NF- κ B2-deficient GC B-cells from *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre mice showed a slight but significant reduction in the surface expression of ICOSL compared to WT

GC B-cells and eGFP⁻RELB/NF-κB2-proficient GC B cells from the same mice (Fig. 3E; SI Appendix, Fig. S2D). Reduced cell-surface expression of ICOSL on RELB/NF-κB2-deficient GC B cells may impair optimal GC B cell–Tfh cell interactions within the GC, which may contribute to their gradual disappearance after d7 of the GC reaction.

Deletion of *nfkb2* in GC B cells impairs the development of antigen-specific PCs. The combined deletion of *relb* and *nfkb2* resulted in the involution of established GCs and, as expected, PCs in *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre mice were also reduced compared to *relb^{fl/+}nfkb2^{fl/+}*Cy1-Cre and Cy1-Cre mice (SI Appendix, Fig. S5). The deletion of *relb* or *nfkb2* alone in GC B cells did not however affect GC B-cell maintenance upon SRBC immunization (Fig. 2A) or immunization with 4-hydroxy-3-nitrophenyl-acetyl coupled to keyhole limpet hemocyanin (NP-KLH) (SI Appendix, Fig. S6). Therefore, to determine whether RELB or NF-κB2 are required for the generation of antigen-specific PCs in the GC reaction *in vivo*, we immunized *relb^{fl/fl}*Cy1-Cre or *nfkb2^{fl/fl}*Cy1-Cre and the corresponding control mice with NP-KLH and performed ELISA and ELISPOT analysis. 28 days post-immunization, we found that whereas deletion of *relb* in GC B cells did not significantly reduce NP-specific IgG1 serum levels or the number of NP-specific IgG1 antibody-secreting cells (ASCs) in the spleen and bone marrow (Fig. 4A), GC-specific deletion of *nfkb2* led to a ~3-fold reduction in NP-specific IgG1 serum levels and an 8-10-fold reduction in ASCs compared to the control mice (Fig. 4B). This defect does not appear to be due to the loss of plasma cells, as we were able to detect eGFP⁺, and therefore *nfkb2*-deleted, CD138⁺ plasma cells (see SI Appendix, Fig. S7&8). While the basis for this observation remains to be determined, these results provide functional evidence for a biological role of NF-κB2, which is highly expressed in PCs and their precursors, in the development of PCs that cannot be complemented by other NF-κB subunits.

DISCUSSION

In agreement with previous work using bone-marrow chimeras (21, 23), we found that GC B-cell development proceeds normally in mice with GC B cell-specific ablation of either RELB or NF- κ B2 alone. In contrast, combined ablation of RELB and NF- κ B2 resulted in the progressive loss of GC B cells. Therefore, RELB and NF- κ B2 are jointly required for the maintenance of the GC reaction.

Among the alternative NF- κ B subunits, only RELB is a transcriptional activator. It was therefore perhaps surprising to observe that ablation of RELB alone did not impair the GC reaction, revealing redundancy between RELB and NF- κ B2 in GC B cells. This redundancy in the absence of either subunit may be explained by dimerization of the remaining transcription factor with subunits of the canonical NF- κ B pathway (37). It is clear, however, that redundancy does not exist between the canonical and alternative NF- κ B pathways, since the GC maintenance defect observed in the combined absence of RELB and NF- κ B2 was not compensated for by canonical NF- κ B subunits.

Evidence suggests that CD40-stimulation by Tfh cells leads to activation of both the canonical and alternative NF- κ B pathways in LZ B cells. An additional signal that may activate the alternative pathway in LZ B cells is stimulation by BAFF (38). Recent work provides evidence that Tfh cells secrete BAFF locally to adjacent LZ B cells (39). Of note, while abolishing BAFF secretion by Tfh cells impaired the selection of high-affinity GC B cells, it had no impact on the maintenance of the GC reaction. This finding suggests that the inability of LZ B cells to transmit signals through the BAFF-R is unlikely to contribute to the loss of GCs observed upon GC B cell-specific deletion of *relb* and *nfkb2*. Since follicular dendritic cells may contribute to BAFF production in the GC (40), the conclusive determination of the function of BAFF signaling during the GC reaction would therefore require conditional deletion of the BAFF-R in GC B cells.

GCs are believed to reach maturity at ~d7 of the GC reaction, the time-point at which DZ/LZ polarization has been established and when selection of high-affinity GC B-cell mutants, followed by cyclic reentry, is initiated (2, 3). It is clear that continuous or periodic signals are required for the maintenance of mature GCs, as the involution of established GCs has been observed upon inhibition of CD40-signaling (41). Via specific conditional gene deletion within GC B cells it has been shown that c-MYC, c-REL and NF- κ B-induced kinase (NIK), an upstream regulator of the alternative NF- κ B pathway that can also activate the canonical pathway (42, 43), are all required for the maintenance of established GCs (20, 44-47). We here demonstrated that RELB and NF- κ B2 have a similarly critical role in this process.

It is becoming increasingly clear that individual NF- κ B subunits have divergent roles in GC and post-GC development. In the case of the canonical subunits, RELA is dispensable for the GC reaction but promotes PC development, whereas c-REL is required for GC maintenance (20) similar to what we demonstrated here for the alternative NF- κ B subunits RELB and NF- κ B2. Interestingly, however, gene expression profiling analysis revealed that the genes controlled by c-REL and RELB/NF- κ B2 are largely distinct. This finding suggests that the respective transcription factors regulate complementary biological programs that are independently required for the GC reaction to persist over time. Impaired cell proliferation and reduced cell-surface expression of ICOSL on LZ B cells may contribute to the progressive loss of RELB/NF- κ B2-deficient GC B cells. LZ B cells undergoing selection receive signals from Tfh cells that promote their survival and license cyclic reentry and division in the DZ. Our results suggest that LZ B cells lacking the alternative subunits may respond improperly to these signals, resulting in fewer cells reentering the cell cycle and seeding the GC over time. In addition, reduced cell-surface expression of ICOSL could lead to suboptimal interactions with Tfh cells, further depriving these cells of critical signals necessary for GC maintenance.

Several observations suggest a biological role for the alternative NF- κ B pathway in PCs. Our finding of strong protein expression of the NF- κ B2 subunit in tonsillar PCs is in accordance with a gene expression profile analysis that reported an upregulation of mRNA encoding NF- κ B2 and RELB in human tonsillar and bone marrow PCs relative to other B-cell subsets (48). Moreover, murine plasmacytoma lines were characterized by the nuclear translocation of RELB/p52 (49). These observations are supported by the *in vivo* data reported here demonstrating a requirement for NF- κ B2 in PC development. The results of our GSEA analysis on RELB/NF- κ B2-deficient GC B cells raise the intriguing possibility that the alternative NF- κ B pathway may have a role in establishing a genetic program that facilitates the production of high amounts of antibodies in GC B cells destined to develop into PCs.

MATERIAL AND METHODS

Mice. The conditional *relb* and *nfkb2* alleles, Cy1-Cre and CD19-cre mice have been described (29, 30, 50). Mice were housed and treated in compliance with the guidelines of Columbia University. The animal protocol was approved by the institution's IACUC. Mice were immunized with SRBCs or NP-KLH in complete Freund's adjuvant as described (20).

Cell culture. Discarded leftovers from routine tonsillectomies performed on children at Columbia-Presbyterian Medical Center were obtained. IRB approval was obtained for all procedures. Consent was not required since all patient identifiers were deidentified and specimens anonymized before use. Human GC B cells were isolated as described (51). Human GC B-cells and the CD40L expressing mouse feeder cell lines (52) were cultured in RPMI/10% FBS. P3HR1, SUDHL2 and JJN3 lines were cultured in Iscove's-Modified Dulbecco's Medium (IMDM)/10% FBS. U266 was cultured in IMDM/20% FBS, LY10 in IMDM with 15% human

serum (New York Blood Center). Murine B cells were purified and cultured with CD40 and/or IL-4 as described (20).

Immunoblot analysis. Cell lines or human GC B cells were subjected to immunoblot analysis as described (20). For antibodies used, see Table S1.

Flow cytometry. Spleen cell suspensions were stained and analyzed as described (20). For antibodies used, see Table S1. The CellTrace Violet Proliferation Kit (Thermo Fisher) was used for cell trace experiments. For the analysis of BrdU incorporation in GC B cells *in vivo*, mice were injected with 2 mg of BrdU and sacrificed 6h later. Staining for BrdU was conducted using the APC-BrdU kit (Becton Dickinson).

Histology and immunohistochemistry. Spleen-sections (4 μ m) were H&E-stained for morphological evaluation. Immunohistochemical staining analysis was performed as described (20). For antibodies used, see Table S1.

Immunofluorescence. For single-cell staining, cells were spun onto slides using a cytocentrifuge and fixed in 10% formalin for 20m followed by 20m of methanol fixation. Nuclear permeabilization was achieved via incubation with 0.2% triton/PBS. Cytospin slides were incubated with primary antibodies overnight followed by incubation with a Cy3-conjugated antibody. Slides were counterstained with DAPI (Molecular Probes). Images were acquired with an Eclipse E400 microscope (Nikon). Tissue sections were prepared and stained as described (51). For antibodies used, see Table S1.

Gene expression analysis. B cells were isolated from spleens of *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre and Cy1-Cre mice as described (20). eGFP⁺CD95^{hi}PNA^{hi} GC B cells were flow-cytometrically sorted from splenic B-cells of *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre mice and GC B cells were sorted from splenic B

cells of C γ 1-Cre mice. Total RNA was isolated using the Nucleospin RNA XS-isolation kit (Macherey-Nagel). NY Genome Center amplified RNA using the NuGEN Ovation RNA-Seq System V2 prior to RNA-sequencing. 35-40 million 2x50 bp paired-end reads were sequenced per sample on an HiSeq2500 (Illumina). DESeq analysis identified differentially expressed genes. Genes identified via RNA-seq analysis with reduced expression in RELB/NF- κ B2-deficient GC B cells (Dataset 1) and genes identified via DNA microarray analysis with reduced expression in c-REL-deficient GC B cells (20) were compared after filtering out genes identified via the RNA-seq analysis that were not represented on the microarray. The overlap between the data sets was determined using Venny 2.1.0 available at <http://bioinfogp.cnb.csic.es/tools/venny/index.html>. GSEA (33) was used to identify signatures enriched in control *vs. relb/nfkb2*-deleted GC B cells. We screened the collection of signatures under the category CP:REACTOME, CP:KEGG, CP:BIOCARTA, BP:GO, MF:GO, CC:GO and signatures from a library of normal and pathological lymphoid gene expression signatures (34) to determine significant enrichment (FDR<25%, P \leq 0.05).

ELISA and ELISPOT. ELISA and ELISPOT-analysis for NP-specific IgG1 or NP-specific IgG1 antibody-secreting cells, respectively, was conducted as described (20).

ACKNOWLEDGEMENTS

We thank L. Pasqualucci and members of the Klein lab for insights and discussion, J. Zhang, M. Cato and R. Rickert for helpful experimental suggestions, A. Holmes for bioinformatics support and Q. Shen and H. Tang for advice on staining procedures. We are grateful to Y.-J. Liu for providing the CD40L feeder cell line. This work was supported by NCI/NIH grant R01-CA157660 to U.K., a grant from the Stewart Trust Foundation (USA), the HICCC, a Cancer Biology Training Program fellowship (NCI/NIH 19 grant 5T32-CA009503-26) to N.S.D. and a fellowship of the German Research Council (DFG) to N.H.

Author contributions: N.S.D. and U.K. designed research; N.S.D. performed most of the experiments; M.M.A. and K.S. performed mouse husbandry and genotyping; A.C. performed IF analysis; N.H. performed experiments; G.B. performed histologic evaluation; and N.S.D. and U.K. wrote the paper.

The authors declare no conflict of interest.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE77374).

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FIGURE LEGENDS

Figure 1. Expression and activation of alternative NF- κ B subunits in normal and transformed human GC B cells and PCs.

(A) Human tonsillar GC B cells *ex vivo* or following 24h of co-culture on CD40L expressing feeders were subjected to Western blot analysis for p100/p52 and BCL6 (left) and IF analysis for p105/p50 and p100/p52 (right, red) and DAPI (blue). (B) IF analysis of tonsil sections for p100/p52 and DAPI or CD20 in the GC light zone (LZ). (C) IF analysis of tonsil sections for p100/p52, BLIMP1 and DAPI in the subepithelium and GC LZ. (D) Western-blot analysis of DLBCL and MM cell lines for p100/p52, RELB, c-REL, RELA and p105/p50. (E) IF analysis of tonsil sections for IRF4 and NF- κ B subunits (either p100/p52 or c-REL) in the GC LZ.

Figure 2. Combined GC B cell-specific deletion of *relb* and *nfkb2* impairs the GC reaction.

(A) *relb*^{fl/fl}Cy1-Cre, *nfkb2*^{fl/fl}Cy1-Cre or *relb*^{fl/fl}*nfkb2*^{fl/fl}Cy1-Cre mice and the corresponding heterozygous and Cy1-Cre control mice were analyzed by flow cytometry 14d following immunization with SRBCs for CD95^{hi}PNA^{hi} splenic GC B cells. Summary of the frequencies of GC B cells (bottom). Each symbol represents a mouse. Statistical significance was determined by Student's *t* test (*, *P*<0.05; **, *P*<0.01). Data are shown as mean \pm standard deviation (SD). (B) Spleen sections from the indicated genotypes were analyzed for the expression of BCL6 and IgM via IHC.

Figure 3. Identification of genes controlled by the alternative NF- κ B subunits RELB and NF- κ B2 in GC B cells.

(A) *relb*^{fl/fl}*nfkb2*^{fl/fl}Cy1-Cre mice and the corresponding heterozygous and Cy1-Cre control mice were analyzed via flow cytometry 8-12d following immunization with SRBCs for the expression of CD36 on CD95^{hi}CD38^{lo} GC B cells. Summary of the corresponding median fluorescence intensities (MFI) (bottom). (B) GSEA was used to identify gene signatures that were enriched in GC B cells from Cy1-Cre vs. *relb*^{fl/fl}*nfkb2*^{fl/fl}Cy1-Cre mice. (Top) Representative example of a cell-cycle regulation signature. (Bottom) Gene sets showing

significant enrichment were grouped into functional categories. For the identity of the gene sets, see Dataset 3. (C) CellTrace Violet dilution in CD40+IL-4-stimulated B cells of the indicated genotypes (d3). (Top) Representative examples. Gates on the right identify non-dividing cells, gates on the left cells that have undergone divisions. (Bottom) Summary of the results. (D) Venn diagram showing the overlap of genes with reduced expression in RELB/NF- κ B2 or c-REL-deficient GC B cells vs. controls. (E) *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre mice and Cy1-Cre control mice were analyzed via flow cytometry 10d following immunization with SRBCs for the expression of ICOSL on CD95^{hi}CD38^{lo} GC B cells. Summary of the corresponding MFI in GC B cells from Cy1-Cre mice and eGFP⁺ and eGFP⁻ GC B cells from *relb^{fl/fl}nfkb2^{fl/fl}*Cy1-Cre mice (right). (A, C, E) Each symbol represents a mouse. Statistical significance was determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$). Data are shown as mean \pm SD.

Figure 4. Deletion of *nfkb2* in GC B cells impairs the development of antigen-specific PCs. (A) *relb^{fl/fl}*Cy1-Cre and (B) *nfkb2^{fl/fl}*Cy1-Cre and the corresponding heterozygous and Cy1-Cre control mice were analyzed for NP₉-specific IgG1 levels via ELISA (top left) and NP₂₅-specific IgG1 ASCs via ELISPOT (bottom) 28d following immunization with NP-KLH. Summary of the frequencies of NP₂₅-specific IgG1 ASCs (right). Each symbol represents a mouse. Statistical significance was determined by Student's *t* test (*, $P < 0.05$; ***, $P < 0.001$). Data are shown as mean \pm SD.