Brief Definitive Report

B Cells Lacking RelB Are Defective in Proliferative Responses, but Undergo Normal B Cell Maturation to Ig Secretion and Ig Class Switching

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Summary

A number of distinct functional abnormalities have been observed in B cells derived from p50/NF-κB or c-*rel* knockout mice. RelB, another member of the NF-κB/Rel family of transcription factors, is expressed during the latter stages of B cell maturation and can bind to regulatory sites within the Ig heavy chain locus. Therefore, we tested the ability of B cells from *relB* knockout mice ($relB^{-/-}$) to proliferate, undergo maturation to IgM secretion, and switch to the expression of downstream Ig isotypes in response to distinct activators including LPS, anti-CD40 mAb or CD40 ligand, and/or dextran anti-IgD antibodies in combination with various cytokines, including IL-4, IL-5, IFN-γ, and TGF-β. B cells lacking RelB showed up to 4-fold reductions in DNA synthesis in response to LPS, CD40, and membrane Ig-dependent activation relative to controls. However, $relB^{-/-}$ B cells were comparable to control B cells in their ability to undergo maturation to IgM secretion and switch to the expression of IgG3, IgG1, IgG2b, IgG2a, IgE, and/or IgA under all activation conditions tested. Thus, RelB, like c-Rel and p50/NF-κB, plays a role in B cell proliferation. However, in contrast to c-Rel and p50/NF-κB, it is not critically involved in maturation to Ig secretion or expression of Ig isotypes.

The NF-κB/Rel family of transcription factors, which includes p50 (and its precursor p105), p52 (and its precursor p100), RelA, RelB, and c-Rel, has been implicated in the rapid regulation of a wide array of genes involved in inflammation and immunity (1–3). These transcription factors are expressed in most cell types in an inactive cytoplasmic form and can be induced by a wide range of stimuli leading to degradation of the inhibitors, collectively termed IκB (4, 5). The NF-κB/Rel members form either homodimers or various combinations of heterodimers, possessing distinct binding affinities for particular promoters and enhancers. After cellular activation, IκB is rapidly degraded allowing NF-κB/Rel dimers to enter the nucleus and bind to κB consensus sequences, which are located in the promoters and enhancers of multiple genes. In this manner NF-κB/Rel family members can participate in the transcriptional regulation of that gene. Activation of normal murine B cells through membrane (m)Ig crosslinking, CD40 activation, or with LPS leads to rapid induction of NF-κB (6–8). Further, normal B cells and B cell tumors demonstrate a patterned expression of NF-κB/Rel family members during ontogeny and upon activation (9). Thus, pre-B cells express mainly p50 and p65, mature B cells p50 and c-Rel, and plasmacytoma lines or LPS-activated B cells express p52 and RelB along with the others. Indeed, the late expression of RelB has led to the suggestion that it may play a role in B cell maturation to Ig secretion.

Although the precise role of the NF-κB/Rel family members in normal B cell function is largely unknown, multiple NF-κB/Rel binding sites have been identified in the Ig heavy chain locus. Thus, p50 binding sites have been identified in the germline promoters ("I" regions) of the constant heavy (C_H) genes encoding γ3 (10), ε (11), γ1, and α (Lin, S.C., and J. Stavnezer, manuscript submitted for publication). In addition, multiple p50 binding sites have been identified in the switch regions for γ3, γ1, and γ2b, particularly at sites of switch recombination (12–14). A recent study has also identified functionally relevant κB binding sites in a pair of Ig heavy chain enhancers located 3′ to the constant heavy gene for α (i.e., 3′αE [hs1,2] and 3′α-hs4). Specifically, 3′αE (hs1,2) was found to bind RelB, c-Rel, and p50, whereas 3′α-hs4 contained specific binding sites for RelB, p50, and p52 (15).

Several recent studies have demonstrated striking functional deficits in B cells from knockout mice deficient in either c-Rel or p50. Thus, B cells from c-Rel-deficient mice were defective in DNA synthesis in response to LPS, CD40 ligand, or mIg cross-linking, relative to control B

cells (16). A different functional phenotype was observed for B cells derived from p50 knockout mice. In this case, $p50^{-/-}$ B cells were markedly defective in their proliferative response to LPS, but showed comparable responses to mIg crosslinking or CD40 activation, compared to control B cells (17, 18). $p50^{-/-}$ B cells also demonstrated selective defects in B cell maturation to IgM secretion, germline C_H transcription, and Ig class switching (18).

RelB-deficient mice exhibit a number of abnormalities in their hematopoietic system including a reduction in the number of thymic epithelial and dendritic cells and myeloid hyperplasia (19, 20). These abnormalities are associated with a mixed inflammatory cell infiltration of several organs and splenomegaly due to extramedullary hematopoiesis. However, although T and B cell development in *relB*$^{-/-}$ mice appears grossly normal, the functional capacity of B cells derived from relB knockout mice have not been studied. In light of RelB binding to regulatory sites in the Ig heavy chain locus and the induction of RelB expression during late phases of B cell maturation we tested the capacity of highly purified *relB*$^{-/-}$ B cells to proliferate, undergo maturation to Ig secretion, and switch to the expression of downstream Ig isotypes in response to multiple distinct stimuli.

Materials and Methods

Mice. Mice with a targeted disruption of the *relB* gene were originally established from *relB*$^{-/+}$ mice of (I29/Sv × C57BL6/J) background and were subsequently maintained by heterozygote intercrosses (19). *Nur77/N10*-transgenic mice lack mature T cells due to the expression of a *Nur77/N10* cDNA under the control of the T cell-specific mouse proximal *lck* promoter and the human CD2 gene locus control region (21). RelB-deficient mice that also express the *Nur77/N10* transgene are described elsewhere (Weih, F., S.K. Durham, D.S. Barton, W.C. Sha, D. Baltimore, and R. Bravo, manuscript submitted for publication). The first set of experiments were performed using *relB*$^{-/-}$ and *relB*$^{+/+}$ mice. The second and third set of experiments were performed with *relB*$^{-/-}$ *Nur77/N10* and *relB*$^{-/+}$*Nur77/N10* mice. The latter mice were used in addition to the former since they demonstrated markedly reduced pathology due to the *relB*$^{-/-}$ phenotype in the absence of mature T cells. Results from both sets of experiments were found to be essentially equivalent. To minimize potential contributions from non-B cells due to differences in background genes between the different strains, small resting *relB*$^{-/-}$ and *relB*$^{+/+}$ B cells were purified by electronic cell sorting from splenocytes pooled from multiple littermates. All animals were housed and bred within the same room in the Veterinary Sciences Department of the Bristol-Myers Squibb Pharmaceutical Research Institute. The absence of pathogens was assessed by extensive periodic, comprehensive serology and histopathology of sentinel animals housed within the same room.

Reagents. Hδ^a/1 (monoclonal mouse IgG2b [b allotype] anti-mouse IgD [a allotype]) and AF3 (monoclonal mouse IgG2a [a allotype] anti-mouse IgD [b allotype]) antibodies were purified from ascites. Dextran-conjugated Hδ^a/1 and AF3 antibodies ($\alpha\delta$-dex) were prepared by conjugation of the respective mAbs, to high molecular weight dextran (2×10^6 M.W.) as previously described (22). The concentration of dextran-conjugated antibodies that is noted in the text reflects only the anti-Ig antibody concentrations and not that of the entire dextran conjugate. Membrane-bound (m) CD40L was prepared from Sf9 insect cells infected with a CD40L-containing recombinant baculovirus vector (23). Anti-CD40 mAb (3/23, rat IgG2a anti-mouse CD40 mAb) (24) was a kind gift from G.G.B. Klaus (National Institute for Medical Research, London, England) and was purified from ascites. *Escherichia coli* LPS serotype 0111:B4, phenol-extracted was purchased from Sigma (St. Louis, MO). Recombinant murine IL-4, IL-5, IFN-γ, and human TGF-β2 were kind gifts from Alan Levine (Case Western Reserve University School of Medicine, Cleveland, OH), Richard Hodes (National Institutes of Health, Bethesda, MD), Genentech, Inc. (South San Francisco, CA), and Wendy Waegell (Celtrix Pharmaceuticals, Santa Clara, CA), respectively.

Preparation and Culture of B Cells. Single cell suspensions were made from spleen and RBCs were lysed with ACK lysing buffer. Cells were stained with polyclonal goat anti-mouse IgM-PE (Southern Biotechnology Associates, Birmingham, AL) and monoclonal FITC-rat anti-mouse CD3ϵ (PharMingen, San Diego, CA). Small, resting B cells (IgM$^+$CD3$^-$, low forward and side scatter profile) were obtained by electronic cell sorting on an EPICS Elite cytometer (Coulter Corp, Hialeah, FL). Reanalysis of sorted cells immediately after isolation showed B cell purities of 97–99%. Sorted cells were cultured immediately after their isolation.

Measurement of DNA Synthesis by [^{3}H]TdR Incorporation. B cells were cultured for 72 h in a final volume of 0.2 ml in complete RPMI in flat bottom 96-well trays (Costar, Cambridge, MA). [^{3}H]TdR (1 μCi) (Amersham Corp., Arlington Heights, IL) with a specific activity of 20 Ci/mmol was added to the cultures for an additional 18 h. Cultured cells were then harvested onto glass fiber filter paper with an LKB Wallac (Turku, Finland) 1295-001 cell harvester. Specific incorporation of [^{3}H]TdR was analyzed by scintillation spectroscopy and results are expressed as the arithmetic mean ± SEM of triplicate cultures.

Quantitation of Secreted Ig Isotype Concentrations in Culture SN. Ig isotype concentrations were measured by an ELISA assay. For determination of concentrations of secreted IgM, IgG3, (IgG1, IgG2b, IgG2a), and IgA in culture SN, Immulon 2, 96-well flat-bottomed ELISA plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with unlabeled affinity-purified polyclonal goat anti-mouse IgM, IgG3, IgG, and IgA antibodies (10 μg/ml) (Southern Biotechnology Associates, Birmingham, AL), respectively. Plates were then washed, blocked with FBS-containing buffer, and incubated with various dilutions of culture SN and standards. After washing, plates were incubated with alkaline phosphatase-conjugated affinity-purified, polyclonal goat anti-mouse IgM, IgG3, IgG1, IgG2b, IgG2a, and IgA antibodies (100 ng/ml) (Southern Biotechnology Associates) as indicated, washed again, and a fluorescent product was generated by cleavage of exogenous 4-methyl umbilliferyl phosphate (MUP) (Sigma) by the plate-bound alkaline phosphatase-conjugated antibodies. For determination of IgE concentrations, a similar procedure was followed except that plates were coated with monoclonal rat IgG2a anti-mouse IgE (clone EM95) (10 μg/ml) (purified from ascites, and a kind gift of Dr. Fred Finkelman, University of Cincinnati, Cincinnati, OH), followed by samples and standards, then monoclonal biotin-rat IgG1 anti-mouse IgE (clone R35-92) (250 ng/ml) (PharMingen), then streptavidin-alkaline phosphatase (PharMingen) (100 ng/ml), then MUP. Fluorescence was quantitated on a 3 M FluoroFAST 96 fluorometer (Mountain View, CA) and fluorescence units were converted to Ig concentrations by interpola-

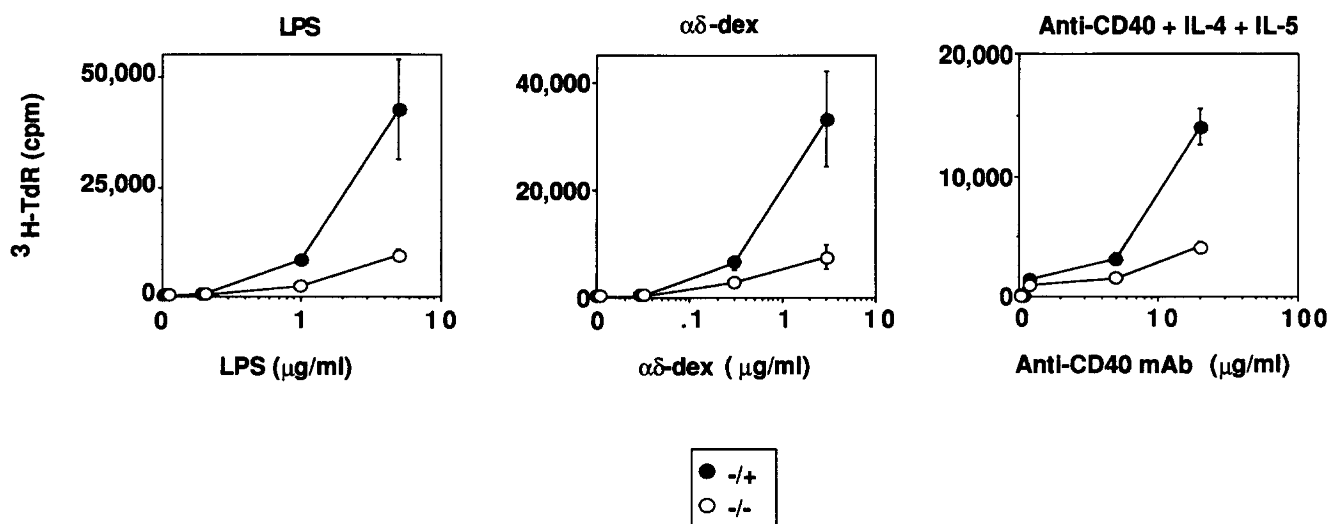

Figure 1. B cells lacking RelB show modest defects in DNA synthesis relative to controls. Sort-purified resting B cells from *relB*$^{-/-}$ and control mice were cultured for 72 h at 1 × 10^5 cells/ml in the presence of LPS (0.2–20 μg/ml), αδ-dex (0.03–3 ng/ml), or anti-CD40 mAb (1.25–20 μg/ml) + IL-4 (3,000 U/ml) + IL-5 (150 U/ml). [^{3}H]TdR was added overnight and cells were then harvested for determination of [^{3}H]TdR incorporation. Values shown represent the means of triplicate cultures ± SEM. Similar results were obtained in two additional sets of experiments.

tion from standard curves that were determined with known concentrations of purified myeloma Ig. Each assay system showed no significant cross-reactivity or interference from other Ig isotypes (IgM, IgD, IgG3, IgG1, IgG2b, IgG2a, IgE, and IgA) found in the culture supernatants.

Results and Discussion

B Cells Lacking RelB Exhibit Defects in DNA Synthesis in Response to LPS, mIg or CD40 Signaling. Activation of normal murine B cells with LPS or through engagement of CD40 or membrane (m)Ig all induce B cell mitogenesis, with further augmentation in response to IL-4 + IL-5, although the signal transduction pathways mediating this event are distinct. Further, B cells activated through these modalities show distinctive patterns of Ig secretion and Ig isotype switching in response to specific patterns of stimuli (25). In this context, and in light of distinctive roles for p50 and c-Rel in murine B cell proliferation, we determined whether any of these mitogenic pathways were dependent upon the expression of RelB. Highly purified resting B cells from *relB*$^{-/-}$ and control mice, were obtained by electronic cell sorting and stimulated for 72 h with various concentrations of LPS, αδ-dex or anti-CD40 mAb plus IL-4 + IL-5. Comparable results were obtained in three independent experiments which demonstrated up to 4-fold reductions in DNA synthesis in B cells lacking RelB compared to controls in response to any of the 3 activation modalities (Fig. 1). These results differ in several ways from those obtained using B cells from *c-rel*$^{-/-}$ or *p50*$^{-/-}$ mice. *c-rel*$^{-/-}$ B cells show a more profound defect in proliferation in response to mIg- or CD40-mediated signaling, although, like *relB*$^{-/-}$ B cells, exhibited only a partial proliferative response to LPS, relative to control B cells (16). By contrast, *p50*$^{-/-}$ B cells showed a more profound proliferative defect in response to LPS, a partial reduction upon CD40 activation, and normal DNA synthesis after mIg crosslinking relative to control B cells (17, 18). Thus, although *relB*$^{-/-}$, *c-rel*$^{-/-}$, and *p50*$^{-/-}$ B cells all demonstrate defects in DNA synthesis they appear to differ (*i*) in the severity of the defect and (*ii*) in their capacity to respond to different mitogenic signals.

B Cells Lacking RelB Show Normal Maturation of Ig Secretion and Switching to Downstream Ig Isotypes. A previous study indicated that RelB is expressed late during B cell maturation (9). This suggested that RelB could regulate B cell maturation to Ig secretion. Further, RelB binding sites have been identified in both 3′αE (hs1,2) and 3′α-hs4 (15). B cells from mice made genetically deficient in 3′αE (hs1,2) showed selective defects in germline C_H transcription and consequent Ig isotype switching (26), implicating 3′αE (hs1,2) in transcriptional regulation of C_H genes in germline. Indeed, the 3′αE (hs1,2) also contains p50 binding sites (15), and B cells from *p50*$^{-/-}$ mice demonstrate a pattern of germline C_H expression similar to B cells from mice lacking 3′αE (hs1,2) (15, 21). The ability of LPS and anti-Ig to regulate 3′αE (hs1,2) activity in normal B cells (27) further indicates that this enhancer is active during B cell activation. Thus, we tested the relative ability of B cells lacking RelB to undergo B cell maturation to IgM secretion and switch to the expression of downstream Ig isotypes in response to various combinations of B cell activators and cytokines.

IL-4, IFN-γ, and TGF-β each induce class switching in appropriately activated mouse B cells; IL-4 to IgG1 and IgE; IFN-γ to IgG3 and IgG2a; and TGF-β to IgG2b and IgA (for review see reference 28). This is accomplished, at least in large part, by their apparent ability to selectively

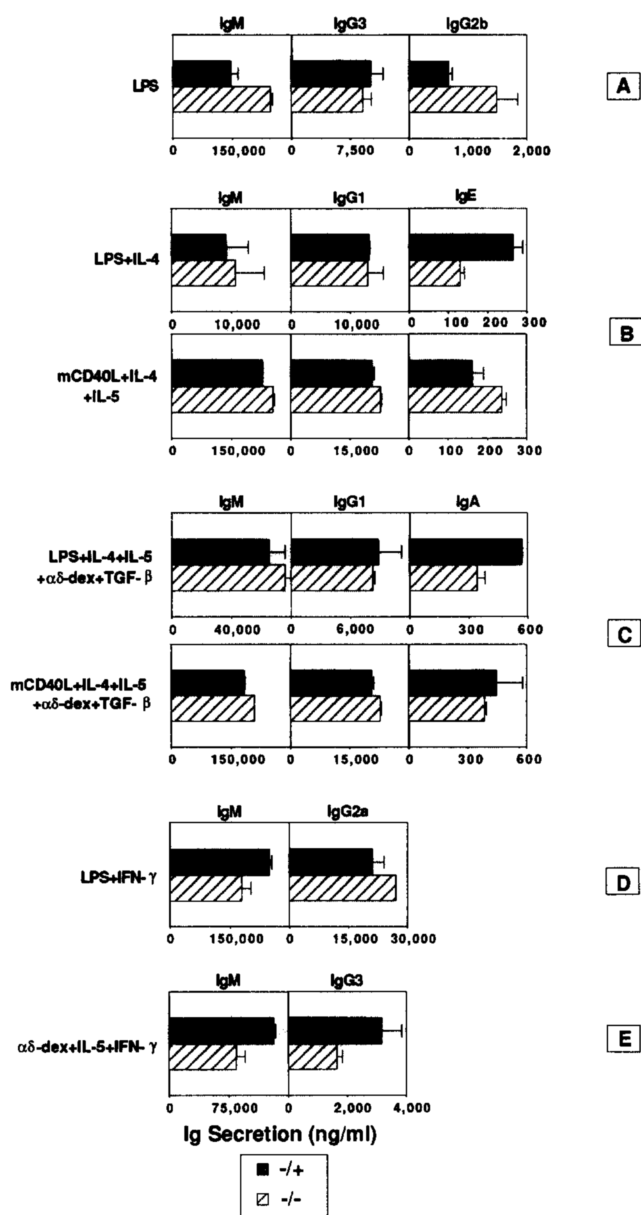

Figure 2. B cells lacking RelB show normal levels of maturation to IgM secretion and expression of downstream Ig isotypes relative to controls. Sort-purified resting B cells from *relB*$^{-/-}$ and control mice were cultured for 6 d at 1 × 10^5 cells/ml in the presence of (*A*) LPS (20 μg/ml), (*B*) LPS + IL-4 (3,000 U/ml), mCD40L (1/2,000 vol/vol) + IL-4 + IL-5 (150 U/ml), (*C*) LPS + IL-4 + IL-5 + αδ-dex (3 ng/ml) + TGF-β (3 ng/ml) or mCD40L + IL-4 + IL-5 + αδ-dex (0.3 ng/ml) + TGF-β (29), (*D*) LPS + IFN-γ (10 U/ml), (*E*) αδ-dex + IL-5 + IFN-γ. After 6 d of culture, SN were harvested for determination of Ig isotype concentrations by ELISA. The means of duplicate cultures ± SEM are indicated. Similar results were obtained in a second set of experiments.

stimulate transcriptional activation of germline C_H genes, thus targeting them for switch recombination. Combinations of stimuli optimal for induction of class switching to different sets of isotypes were used to assess switch recombination in *RelB*$^{-/-}$ B cells. Maturation to IgM secretion was essentially comparable between B cells lacking RelB and similarly-activated control B cells, in response to a variety of distinct stimuli (Fig. 2, *A–E*). This finding contrasted sharply with observations made using *p50*$^{-/-}$ B cells in which IgM secretion was markedly defective in response to LPS, or in the presence of cytokines to either αδ-dex or CD40 activation (18). Restoration of cytokine-dependent IgM secretion in *p50*$^{-/-}$ B cells was accomplished only with the combined actions of CD40L and αδ-dex. Thus, despite its appearance late during B cell maturation, RelB does not appear to be critical for maturation of B cells into IgM-secretors.

Likewise, induction of all 6 non-IgM, non-IgD isotypes in B cells lacking RelB was comparable to that observed for control B cells. The essentially normal levels of IgG1 and IgG3 secretion by B cells lacking RelB correlated with comparable percentages of membrane (m)IgG1$^+$ and mIgG3$^+$ cells by flow cytometry, relative to similarly activated control B cells (data not shown). This contrasted sharply with *p50*$^{-/-}$ B cells in which class switching to IgG3, IgE, and IgA, was markedly reduced compared to control B cells, although induction of IgG1 was significant (18). This was associated with normal steady-state levels of germline $C_H\gamma1$ and $C_H\alpha$ RNA, but markedly reduced germline $C_H\gamma3$ and $C_H\epsilon$ RNA in *p50*$^{-/-}$ B cells relative to controls. Studies on IgM secretion and class switching by B cells from *c-rel* knockout mice have not been reported (16). However, preliminary data from our laboratories indicates selective defects in germline C_H RNA expression and Ig class switching in *c-rel*$^{-/-}$ B cells, disinct from that observed in B cells from *p50*$^{-/-}$ mice (P. Zelazowski et al., in preparation). Thus, although RelB binding to the 3′αE (hs 1,2), as well as 3′α-hs4 was shown to be functionally relevant in a B cell tumor line (15), and 3′αE (hs1,2) has been implicated in regulating germline C_H transcription (26), RelB by itself does not appear to play a critical role in expression of any of the Ig isotypes.

A more comprehensive understanding of the role of the NF-κB/Rel family members in normal B cell function, will require additional analyses of B cells from p52 and RelA knockout mice plus further analyses of B cells from double, and perhaps triple knockout mice. Through multiparameter functional analyses of these model systems, a clear view of the role of the NF-κB/Rel family in the B cell functional program should emerge.

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