International Immunology, Vol. 14, No. 9, pp. 983–991

Regulation of Ig class switch recombination by NF-κB: retroviral expression of RelB in activated B cells inhibits switching to IgG1, but not to IgE

Deepta Bhattacharya[1], Dong U. Lee[1] and **William C. Sha[1]**

[1]Division of Immunology, Cancer Research Laboratory, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720-3200, USA

Keywords: IL-4, isotype switching, lipopolysaccharide, retrovirus, splenic B cells

Abstract

Mutant NF-κB-deficient B cells from knockout mice lacking RelA, p105/p50 or the transactivation domain of c-Rel exhibit distinct and selective cell-intrinsic defects in their ability to undergo class switch recombination (CSR) to specific Ig isotypes. This isotype-specific requirement for particular NF-κB transcription factors in B cells activated to undergo CSR is intriguing because the NF-κB composition in B cells is also highly regulated and can vary significantly depending upon how B cells are activated. These studies prompted us to test by retroviral transduction of normal B cells whether changes in the NF-κB composition in activated B cells could modulate cytokine-driven CSR. RelB, RelA, c-Rel, p50 and p52 were first expressed in lipopolysaccharide-activated primary B cells and then induced by cytokine addition to undergo CSR to IgG1, IgE, IgG2a, IgG2b or IgA. Surprisingly, only retroviral expression of RelB altered CSR, resulting in a 3-fold decrease in CSR to IgG1 induced by IL-4. This effect was isotype specific as RelB expression did not affect CSR to IgE within the same culture or to other isotypes tested. The transactivation domain of RelB was required for inhibition of CSR to IgG1. Expression of p50–RelB or p52–RelB dimers joined covalently by a flexible peptide linker also specifically inhibited IgG1 CSR. RelB-mediated inhibition of IgG1 CSR was associated with a decrease in germline γ1 transcription, but not with changes in proliferation as assayed by CFSE labeling. Thus, RelB complexes can specifically inhibit CSR to IgG1, but not IgE, in activated, primary B cells.

Introduction

Naive mature B cells express surface Ig of the IgM and IgD isotypes. Following antigenic encounter B cells can undergo class switch recombination (CSR) and utilize a different antibody isotype (1). The choice of the new Ig isotype appears to be directed largely, if not exclusively, by the cytokines to which a particular B cell is exposed. IL-4 treatment, for example, has been shown to direct isotype switching to IgG1 and IgE (2), IFN-γ instructs murine B cells to switch to IgG2a or IgG3 (3,4), and transforming growth factor (TGF)-β effects class switching to IgA or IgG2b (5,6).

There is, however, growing evidence that B cell-intrinsic properties may cooperate with cytokine signaling to regulate class switching in an isotype-specific manner. For instance, studies have shown that the number of cell divisions a B cell undergoes in conjunction with CD40 ligand and IL-4 treatment can greatly influence the ability of a B cell to undergo cytokine-directed CSR to IgG1 or IgE (7). Similarly, the absence of certain B cell-intrinsic signaling molecules that are not directly regulated by cytokines involved in CSR, such as the NF-κB family of transcription factors, has been found to prevent CSR to certain isotypes (Table 1). Mutant NF-κB-deficient B cells from knockout mice lacking RelA, p105/p50 or the transactivation domain of c-Rel exhibit distinct and selective B cell-intrinsic defects in their ability to undergo CSR to specific Ig isotypes (8–10).

These studies examining the ability of NF-κB knockout B cells to undergo CSR clearly demonstrate that NF-κB transcription factors are part of the machinery required for cytokine-driven CSR to occur to certain isotypes. However, one important unanswered question raised by these studies is

Correspondence to: W. C. Sha; E-mail: bsha@uclink4.berkeley.edu

Transmitting editor: K. Murphy

Received 16 April 2002, *accepted* 28 May 2002

Table 1. NF-κB-mediated regulation of Ig isotype switching is both B cell extrinsic and intrinsic

Gene	*in vivo* serum Ig defects							B cell intrinsic isotype switching defects						
		IgG3	IgG1	IgE	IgG2a	IgG2b	IgA		IgG3	IgG1	IgE	IgG2a	IgG2b	IgA
RelB (30, 36, 37)	immune	↓	↓	NT	↓	NT	NT	knockout	=	=	=	=	=	=
	resting	↓	↑	↑↑	↓	=	=	retrovirus	=	↓	=	=	=	=
RelA (8, 25)	immune	NT	NT	NT	NT	NT	NT	knockout	↓	=	=	=	=	=
	resting	NT	↓	=	=	=	↓	retrovirus	=	=	=	=	=	=
c-Rel (26)	immune	↓	↓	NT	NT	NT	NT	knockout	NT	NT	NT	NT	NT	NT
	resting	↓	↓↓	NT	↓↓↓	↓	NT	retrovirus	=	=	=	=	=	=
c-RelΔC (9, 38)	immune	NT	NT	NT	NT	NT	NT	knockout	↓	↓↓	↓	NT	NT	=
	resting	↓↓	↓	NT	↑	=	↑	retrovirus	NT	=	=	NT	NT	NT
p50 (10, 27)	immune	↓	↓↓	NT	NT	↓↓	NT	knockout	↓	=	↓	NT	NT	↓↓
	resting	=	↓↓	↓↓↓	↓	↓	↓	retrovirus	=	=	=	=	=	=
p52 (39)	immune	NT	NT	NT	NT	NT	NT	knockout	NT	NT	NT	NT	NT	NT
	resting	=	=	=	=	=	=	retrovirus	=	=	=	=	=	=

NT, not tested.

whether natural differences in the cellular composition of NF-κB complexes within B cells can also modulate cytokine-driven CSR. A large body of research suggests that the cellular composition of NF-κB dimers is fluid, and reflects both the differentiation state of B cells (11,12) and the activation state of B cells induced by different innate and adaptive immune signaling pathways (13). Thus, if natural changes in the composition of NF-κB transcription factors within B cells participate in the regulation of CSR, the involvement of NF-κB factors in CSR may reflect not simply a hard-wired component activating the CSR machinery, but also a more complex regulatory mechanism that allows information on the state of the B cell to be integrated with cytokine-driven CSR.

In order to test this hypothesis that differences in the cellular composition of NF-κB complexes in activated B cells modulate cytokine-directed CSR, we chose to retrovirally infect activated splenic B cells with individual NF-κB family members. Because NF-κB transcription factors also regulate other aspects of B cell differentiation including proliferation and apoptosis, retroviral infection of wild-type B cells has several advantages over the analysis of mutant knockout B cells for specifically focusing on the regulation of cytokine-driven CSR. Mutant B cells from NF-κB-deficient mice may possess either subtle developmental differences that may accentuate defects in assays of CSR or long-term compensatory changes in function that may mask the particular roles of individual NF-κB family members in regulating CSR (14). For example, both p50- and RelB-deficient mice have been shown to have defects in marginal zone B cell development (15,16). Thus, the B cell-intrinsic defects in CSR reported in NF-κB-deficient B cells (see Table 1) may also reflect differences in the populations of splenic B cells used for these studies. These issues are more carefully controlled in the setting of transient expression of NF-κB members in wild-type B cells.

Although we hypothesized that the cellular NF-κB dimer composition might alter cytokine-driven CSR, we observed that RelA, c-Rel, p50 and p52 expression in lipopolysaccharide-activated primary B cells did not alter IL-4-driven CSR to IgG1 and IgE, IFN-γ-driven CSR to IgG2a, TGF-β-driven CSR to IgG2b, TGF-β-, IL-4- and IL-5-driven CSR to IgA or CSR to IgG3. Surprisingly, only retroviral expression of RelB altered CSR, resulting in a 3-fold decrease in CSR to IgG1 induced by IL-4. This effect was isotype specific as RelB expression did not affect CSR to IgE within the same culture or to other isotypes tested. Expression of p52–RelB or p50–RelB dimers joined covalently by a flexible peptide linker also specifically inhibited IgG1 CSR and the transactivation domain of RelB was required for inhibition of CSR to IgG1. RelB-mediated inhibition of IgG1 CSR was associated with a decrease in germline γ1 transcription, but not with changes in proliferation as assayed by CFSE labeling. Thus, although individual NF-κB family members may be required for CSR to specific isotypes, these results support only a limited role for natural differences in the cellular composition of NF-κB transcription factors within B cells as being a B cell-intrinsic regulatory mechanism for modulating cytokine-driven CSR.

Methods

Retroviral constructs and production

The generation of the murine stem cell virus 2.2 (MSCV) has been described elsewhere (17). The *pgk–neo* cassette in MSCV 2.2 was excised with *Bgl*II and *Cla*I, and replaced with a synthetic oligonucleotide polylinker containing *Bgl*II–*Xho*I–*Not*I–*Pme*I–*Sal*I–*Eco*RI–*Hin*dIII–*Cla*I. An IRES–green fluorescent protein (GFP) cassette was inserted into the *Eco*RI site of this vector to generate MSCV–IRES–GFP. The p50 cDNA was cloned into the *Xho*I–*Not*I sites, RelA and c-Rel cDNAs were cloned into the *Not*I–*Sal*I sites, the RelB cDNA was cloned as a *Bam*HI–*Not*I insert into the *Bgl*II–*Not*I sites, and the p52 cDNA was cloned into the *Not*I site of MSCV–IRES–GFP. Covalently linked dimers were generated by inserting an in-frame cassette encoding 10 copies of a $SerGly_4$ linker downstream of the p50 or p52 coding regions. RelB was cloned in-frame downstream of these linked cDNAs to generate MSCV–p50–L–RelB IRES–GFP or MSCV–p52–L–RelB IRES–GFP. The MSCV–IRES–Thy-1.1 and MSCV–RelB–IRES–Thy-1.1 constructs were provided by Dr Thomas Mitchell (University of Louisville). Then 10 μg of each retroviral construct was transfected into 4×10^6 Bosc23 packaging cells on 10-cm^2 plates using calcium phosphate co-precipitation. After 8 h,

media was replaced with 6 ml of fresh media and retroviral supernatants were harvested 48 h after transfection.

Reagents

Recombinant mouse IL-4 and mouse IL-5 were purchased from R & D Systems (Minneapolis, MN). TGF-β1 was purchased from PharMingen (San Diego, CA). IFN-γ was a gift of K. Murphy (Washington University, St Louis, MO). Biotinylated anti-mouse IgG1 (A85-1), IgA (R5-140), IgG2a (R19-15), IgG2b (R12-3), IgG3 (R40-82) and anti-mouse Thy-1.1 (OX-7 clone) were purchased from PharMingen. Purified anti-IgE antibody (84.1C) was a kind gift of Dr Z. Eshhar (Weizmann Institute, Rehovot, Israel) and was biotinylated using biotin sulfosuccinimidyl ester from Molecular Probes (Eugene, OR). Polyclonal anti-RelB rabbit serum and polyclonal anti-rabbit-horseradish peroxidase antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Western blots were developed with NEN (Boston, MA) Renaissance reagents. Streptavidin–TriColor was purchased from Caltag (Burlingame, CA). CFSE was purchased from Molecular Probes and used to label B cells as described in the manufacturer's protocol.

Isotype switching assays

Splenocytes were harvested from C57BL6/J mice, and red blood cells were lysed in 17 mM Tris–Cl and 144 mM NH_4Cl buffer and spun over Ficoll 1083 (Sigma, St Louis, MO). Cells at the interface were collected, washed, then stained with anti-Thy-1.2 ascites. Stained cells were then lysed in 10% rabbit complement (Accurate, Westbury, NY) in PBS for 45 min at 37°C. Cells were washed, spun over Ficoll 1119 (Sigma), stained using an anti-B220–phycoerythrin antibody (PharMingen; clone RA3-6B2) and analyzed using flow cytometry (XL; Coulter, Miami, FL) to assess purity (typically >90%). B cells were then incubated in 24-well plates at 10^6 cells/well in media [RPMI (Gibco, Carlsbad, CA) + 10% FBS (Hyclone, Logan, UT) + 50 μM β-mercaptoethanol] + 30 μg/ml LPS (Sigma) for 24 h. Cells were then spun over Ficoll 1119, resuspended in retroviral supernatant at 10^6 cells/ml and infected by spinning for 1.5 h in six-well plates at 32°C. These cells were resuspended at 10^6 cells/ml in media containing 30 μg/ml LPS in six-well plates and incubated for 2 days to allow expression of the retrovirus. Cells were then spun over Ficoll 1119 to remove dead cells, and diluted to 10^5 cells/ml in media containing 30 μg/ml LPS, 100 ng/ml IL-4 and 25 ng/ml IL-5; 2 ml of this suspension was added to each well of 24-well plates. For isotype switching to other isotypes, IL-4 was added along with or replaced by 10 U/ml IFN-γ (gift of K. Murphy and R. Schreiber, Washington University, St Louis) or 10 ng/ml TGF-β1 (PharMingen). After 4 days, these cells were spun over Ficoll 1119 and stained for flow cytometric analysis on a Coulter Epics XL. Data were plotted using the WinMDI program.

Immunoblots

Whole-cell extracts were obtained by resuspending cells in PBS + 0.5% NP-40, incubating on ice for 10 min, spinning at 30 000 *g* and harvesting the supernatant. The protein concentration from above was determined using BioRad (Hercules, CA) protein assay reagent. Aliquots of 5 μg from each sample were subjected to Western blotting.

Germline transcripts

Cells were treated as above in the isotype-switching assay, but were harvested after only 24 h in media containing IL-4. Cells were stained with biotinylated anti-Thy-1.1 antibody, streptavidin beads (Miltenyi, Auburn, CA) + streptavidin–phycoerythrin and sorted on an AUTOMacs machine (Miltenyi). Purity was assessed by flow cytometry and was typically >90%. Total RNA was isolated using TRIzol reagent (Gibco) and 1 μg was used as template for the RT-PCR reaction. Oligo$(dT)_{12–16}$ was used as the primer for the reverse transcription reaction using Superscript II (Gibco). Aliquots of 2 μl (1/10) of each reaction were used for the subsequent PCR reactions. The magnesium concentrations and oligonucleotides used for each PCR reaction are listed below. For all reactions, a single PCR cycle consisted of:

GAPDH (14 cycles): 1 mM $MgCl_2$ (150 bp amplicon)
5′ oligo: TACCCCCAATGTGTCCGTCGT
3′ oligo: GAAGTCGCAGGAGACAACCT
probe: GGTGAAGCAGGCATCTGAGGG

γ1 (18 cycles): 1 mM $MgCl_2$ (153 bp amplicon)
5′ oligo ($I_\gamma 1$): TGGCCCTTCCAGATCTTTGAGT
3′ oligo ($C_\gamma 1$): CAGGCTGTAGGCAAGCACCTTT
probe: CAGACCAGCCCAGGCAGA

Reactions were electrophoresed on a 2% agarose TBE gel and subjected to Southern blot analysis using the ^{32}P-labeled probes listed above. Band intensities were quantified using a Phosphorimager.

CFSE assays

Freshly purified splenic B cells were resuspended at 10^7 cells/ml in PBS + 0.1% BSA and CFSE was added to a final concentration of 10 nM. Reactions were incubated at 37°C for 15 min and quenched with media. Cells were plated at 2×10^6 cells/well of a 24-well plate with 30 μg/ml LPS for 12 h and analyzed by flow cytometry. At this point, cells were infected as above with MSCV–IRES–Thy-1.1 or MSCV–RelB–IRES–Thy-1.1. Cells were plated at 10^5 cells/ml, 2 ml/well of a 24-well plate in media containing 30 μg/ml LPS and 100 ng/ml IL-4, and analyzed each day after infection by flow cytometry.

Luciferase assays

293T cells (10^6) were transfected with 10 ng each of HIV–firefly luciferase (18) and pEF1α–renilla luciferase (gift of H. Kasler and A. Winoto, University of California, Berkeley) and 100 ng of control or NF-κB-encoding retroviral vectors. After 24 h, cells were lysed in 0.5 ml Passive Lysis Buffer (Promega, Madison, WI). An aliquot of 20 μl of each lysate was used per reaction. Firefly and renilla luciferase activity was assayed according to manufacturer's instructions (Promega) on a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA).

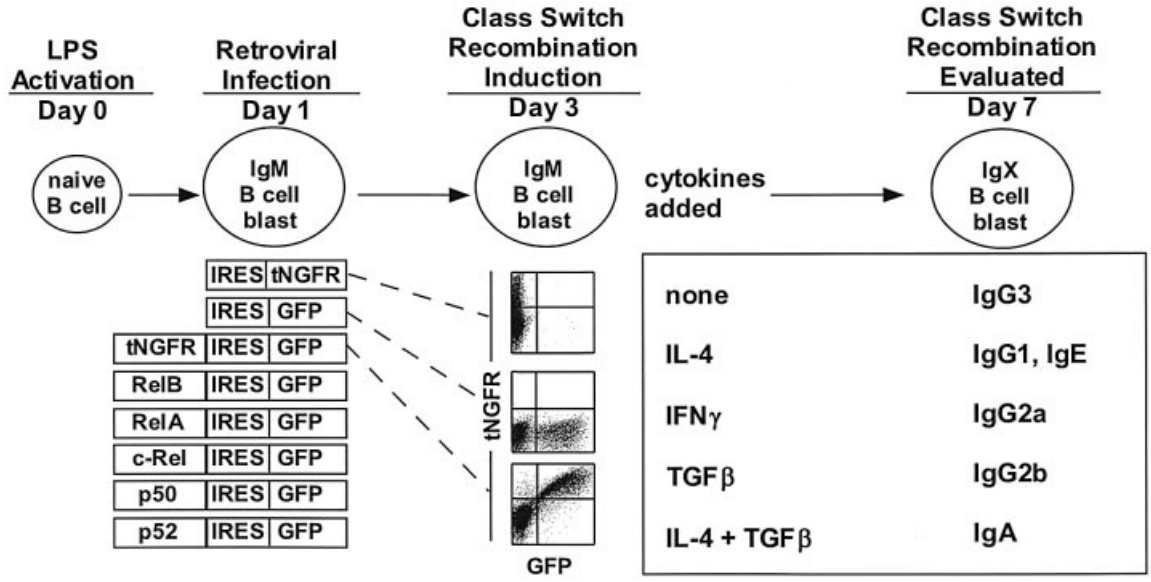


Fig. 1. Analysis of cytokine-directed CSR in LPS-activated B cells retrovirally expressing different NF-κB family members. Purified splenic B cells were activated with LPS and then infected on day 1 with NF-κB-encoding retroviruses tagged with an IRES–GFP marker. On day 3 when GFP-expressing cells were expressing NF-κB genes, cytokines were added to induce CSR to distinct isotypes. Modulation of CSR by retroviral expression of different NF-κB family members was assessed on day 7 by flow cytometric analysis, using the IRES–GFP marker to compare differences between CSR in retrovirally infected and non-infected populations within the same cultures.

Results

Recombinant retroviruses were generated expressing individual NF-κB family member genes for RelB, RelA, c-Rel, p50 and p52 cloned upstream of an IRES–GFP cassette in a MSCV–IRES–GFP retroviral vector (19). Each of these constructs was tested functionally by an NF-κB-dependent luciferase reporter assay (Fig. 3C and D). Proper expression of p50 and p52 was further confirmed by immunoblots and electrophoretic mobility shift assays (data not shown). Recently, retroviral expression of RelB, RelA and c-Rel using these constructs was shown to modulate activated T cell survival upon antigen and adjuvant challenge *in vivo* (20). We utilized these NF-κB retroviruses to test whether changes in the composition of NF-κB complexes in activated primary B cells modulated cytokine-directed CSR to different Ig isotypes.

The GFP expressed from the IRES–GFP cassette provided an accurate fluorescent marker of retrovirally infected cells expressing different NF-κB family members as shown by the expression pattern of a dual marker MSCV–truncated nerve growth factor receptor (tNGFR)–IRES–GFP retrovirus in primary B cells (Fig. 1). Using an antibody specific for tNGFR, we monitored expression of both tNGFR and GFP simultaneously by flow cytometric analysis of retrovirally transduced B cells that were activated by LPS. Virtually all B cells that expressed tNGFR also expressed GFP and, conversely, very few B cells expressed GFP without the concomitant expression of tNGFR. These results demonstrate that GFP expression served as a stringent marker that distinguishes B cell populations expressing and not expressing genes cloned upstream of the IRES sequence within cultures of retrovirally transduced cells. Further, the relative expression levels of GFP correlated with those of tNGFR, indicating that high GFP-expressing B cells tended to also express high levels of tNGFR. These results indicate that the expression level of GFP reflected the relative expression level of the gene cloned upstream.

Although retroviral markers were detected as early as 24 h after infection, maximal expression of retroviral markers and the genes cloned upstream of the IRES sequence was clearly reached by 48 h after infection (data not shown). Thus, day 3, when LPS-stimulated B cells were maximally expressing transduced NF-κB subunits, was chosen as the timepoint for addition of cytokines to initiate CSR to specific isotypes and assess the ability of retroviral expression of individual NF-κB family members to modulate cytokine-directed CSR. B cells were activated to isotype switch to IgG1 and IgE via addition of IL-4, to IgG2a by adding IFN-γ, to IgG2b with addition of TGF-β, to IgA with addition of TGF-β, IL-4 and IL-5 or to IgG3 via culturing in the absence of cytokines. On day 7, B cells were stained with isotype-specific antibodies to determine percentages of cells that had undergone CSR. As small differences in cytokine addition can cause significant changes in the percentage of cells that undergo CSR, we chose to compare the effects of NF-κB-transduced cells to internally controlled non-transduced cells within the same cultures rather than to separate control samples using GFP marker expression. Since CSR induced in *ex vivo* splenic B cell cultures typically occurs 3–4 days after cytokine addition, retroviral expression of NF-κB subunits preceded CSR in our assays. This allowed us to study how the cellular NF-κB composition of a B cell prior to cytokine exposure can affect CSR to specific Ig isotypes.

In examining IL-4 cytokine-mediated CSR, we observed that expression of RelB resulted in a 3-fold inhibition of isotype switching to IgG1, but not to IgE, relative to non-transduced

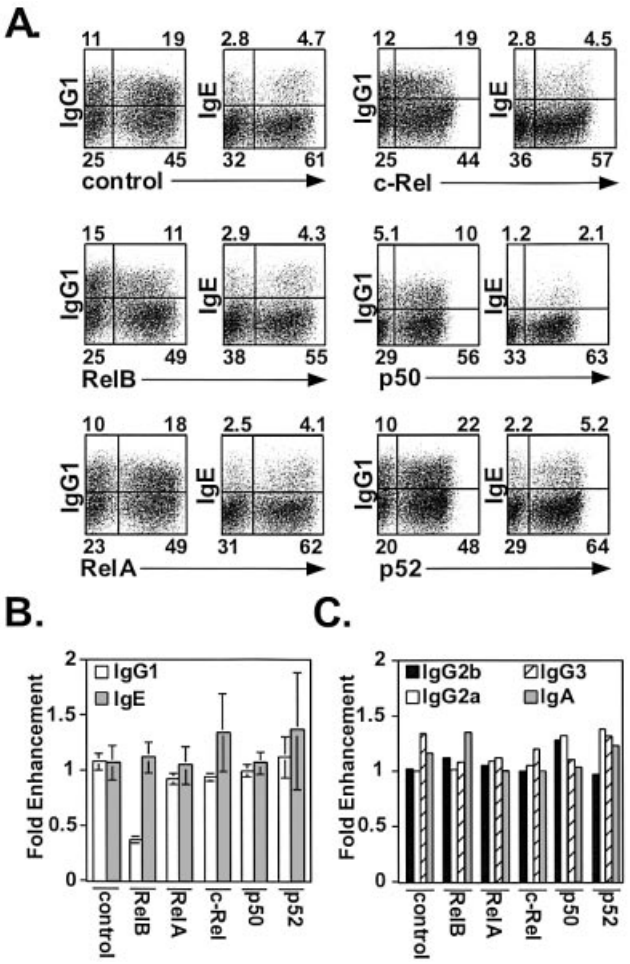


Fig. 2. Retroviral expression of RelB inhibits IL-4-directed CSR to IgG1, but not to IgE. (A) Expression of RelB, but not other NF-κB family members, impairs isotype switching to IgG1, but not to IgE, within the same cultures. Splenic B cells were transduced with control or NF-κB family member encoding retroviruses and induced to switch to IgG1 or IgE with LPS + IL-4 addition. Cells from the same culture were split and stained for either IgG1 or IgE expression. (B) RelB expression leads to a 3-fold decrease in isotype CSR to IgG1 relative to non-transduced cells within the same culture. Percentages of $IgG1^+$ or IgE^+ isotype-switched cells were compared between retrovirally transduced and non-transduced cells within the same cultures using the following equation: % of transduced cells that have isotype switched/% of non-transduced cells that have isotype switched. Values shown are means ± SD from six independent experiments. (C) Expression of NF-κB family members does not modulate isotype switching to IgG2a, IgG2b, IgA or IgG3. After retroviral transduction, B cells were induced to isotype switch to IgG2b, IgG2a, IgG3 or IgA by addition of the appropriate cytokines (see Methods). Values shown compare the percentage of isotype-switched cells in retrovirally transduced cells with non-transduced cells within the same cultures.

cells within the same culture (Fig. 2A). This inhibitory effect of isotype switching to IgG1 was reproducible and specific for RelB, as expression of RelA, c-Rel, p50 or p52 NF-κB family members did not alter isotype switching to either IgG1 or IgE (Fig. 2B). Further, in examining IFN-γ and TGF-β cytokine-mediated CSR, neither RelB nor any of the other individual NF-κB family members affected isotype switching to IgG2a, IgG2b, IgA or IgG3 (Fig. 2C). These results indicate that alterations in NF-κB complexes in activated B cells had only a modest effect on cytokine-driven CSR and were limited to the ability of RelB expression to specifically inhibit IL-4-mediated CSR to IgG1, but not IgE.

Because NF-κB transcription factors function as dimers, we next sought to address whether RelB expression led to inhibition of CSR to IgG1 by functioning as dimers corresponding to p52–RelB dimers normally found in B cells or by forming unnatural dimers not normally found in B cells. To test whether expression of p52–RelB dimers could also lead to specific inhibition of CSR to IgG1, we created forced dimers corresponding to endogenous RelB complexes by creating a fusion protein of RelB covalently joined to either p52 or p50 by a flexible $(Gly_4Ser)_{10}$ linker (Fig. 3A). These p52–L–RelB and p50–L–RelB forced dimer proteins were expressed as full-length proteins within cells with minimal release of free RelB from degradation of the protein linker (Fig. 3B). Further, in transfection experiments, these p52–L–RelB and p50–L–RelB forced dimers were capable of activating transcription of an HIV-κB-dependent luciferase reporter at levels that were comparable to co-transfection of individual p50, p52 and RelB subunits (Fig. 3D). These results suggest that that the retroviral expression of p52–L–RelB and p50–L–RelB forced dimers would correspond to overexpression of endogenous p52–RelB complexes.

When retrovirally transduced into activated B cells, expression of both p52–L–RelB and p50–L–RelB dimers resulted in similar modulation of IL-4-driven CSR as expression of RelB alone (Fig. 4). These forced dimers specifically inhibited isotype switching to IgG1. These results indicate that retroviral expression of RelB functioned to inhibit IL-4-mediated CSR to IgG1 by dimers corresponding to endogenous p52–RelB complexes rather than by generation of aberrant dimers not normally found within B cells.

We next sought to address whether the increases in RelB complexes led to inhibition of CSR to IgG1 by transactivation of transcription or by competitive inhibition through binding to DNA sites used by other transcription factors. If RelB was functioning to inhibit CSR to IgG1 by competitive inhibition, expression of a dominant-negative form of RelB should also lead to inhibition of CSR to IgG1. We utilized a dominant-negative RelB mutant (amino acids 103–392) that retains its ability to dimerize and bind DNA, but can no longer activate transcription (21). Retroviral expression of this dominant-negative RelB did not lead to a decrease in isotype switching to IgG1, demonstrating that the transcriptional activation domain of RelB was required for inhibition of CSR to IgG1 (Fig. 4). These results were consistent with a model whereby increases in RelB complexes led to inhibition of IL-4-driven CSR to IgG1 by transactivation of RelB-regulated genes.

Because germline transcription through the switch region and the constant region of a particular isotype is always observed prior to CSR, we next sought to determine whether retroviral expression of RelB led to altered germline γ1 transcription (Fig. 5). While no definitive causal relationship has been established between germline transcription and CSR, several reports have suggested that the spliced germline transcripts themselves may participate in the CSR event (22). B cells were infected with a RelB-expressing retrovirus

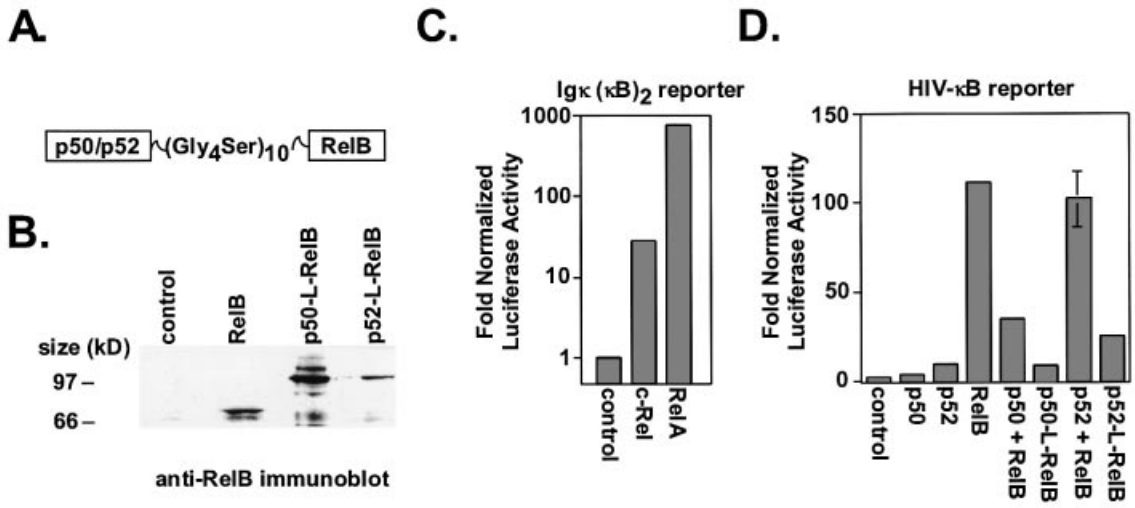


Fig. 3. Forced NF-κB dimers of RelB covalently linked to either p52 or p50 are functional. (A) Generation of covalently linked p52–RelB and p50–RelB heterodimers using a flexible $(Gly_4Ser)_{10}$ peptide linker. The cDNAs encoding these forced dimer proteins were cloned into a retroviral vector. (B) Appropriate full-length covalently linked heterodimers are expressed in cells with minimal degradation. 293T cells were transfected with control or retroviral plasmids encoding RelB, p50–L–RelB or p52–L–RelB heterodimers. Cell lysates were subjected to an anti-RelB immunoblot. (C) Retroviral plasmids encoding RelA and c-Rel activate transcription of a minimal NF-κB-dependent luciferase reporter gene. Retroviral constructs were co-transfected with a luciferase reporter controlled by two copies of the NF-κB-binding site from the Igκ intronic enhancer. (D) Covalently linked heterodimers activate transcription of a κB reporter gene with similar efficiency to co-transfected monomeric RelB. 293T cells were co-transfected with an HIV-κB-dependent firefly luciferase construct and control or NF-κB-encoding retroviral constructs. A control renilla luciferase reporter gene was included in each sample and was used to normalize all values. Expression of p50 and p52 were confirmed by immunoblotting and electrophoretic mobility shift assays.

containing an IRES–Thy-1.1 marker after 1 day of activation with LPS. On day 3 when RelB was expressed in activated B cells, the cytokine IL-4 was added to induce germline transcription. After 24 h, retrovirally infected cells were isolated from non-infected cells by magnetic bead selection using the Thy-1.1 marker. RNA was purified from both cell populations and RT-PCR was used to semi-quantitatively compare spliced γ1 germline transcripts between B cell populations retrovirally expressing or not expressing RelB.

We repeatedly observed that B cells retrovirally expressing RelB showed decreased germline γ1 transcription in response to IL-4 treatment in comparison to their non-transduced counterparts from the same cultures (Fig. 5). In five separate experiments, RelB-expressing cells consistently showed lower levels of spliced γ1 germline transcripts in comparison to their non-transduced counterparts. This decrease was specific as controls for GAPDH transcripts were equivalent between RelB-expressing and non-expressing populations. The extent of the decrease in germline γ1 transcripts was variable, ranging from a 2-fold decrease to an absence of detectable transcripts. To confirm that this reduction in germline γ1 transcripts was caused by RelB expression and did not arise as an artifact of retroviral infection or magnetic bead selection, we repeated the experiments using B cells infected with a control MSCV–IRES–Thy-1.1 vector. In contrast to infection with the MSCV–RelB–IRES–Thy-1.1 vector, no differences in germline γ1 transcripts were observed between retrovirally transduced and non-transduced cell populations with the control vector within the same IL-4-transduced cultures. These data indicate that RelB expression results in decreased germline γ1 transcription in LPS-activated B cells treated with IL-4.

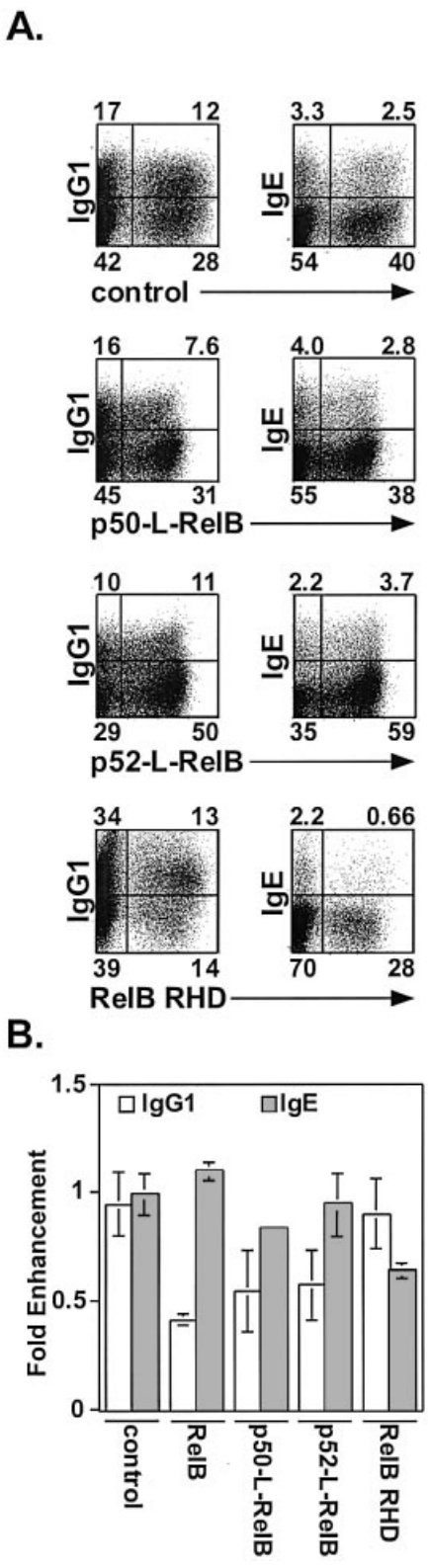
We next sought to address whether decreases in germline γ1 transcription might be related changes in the proliferation of activated B cells expressing RelB (Fig. 6). Earlier studies have suggested that B cells must undergo at least three divisions prior to isotype switching to IgG1, while five cell divisions are required prior to IgE class switching (7). These division numbers are apparently linked to the onset of germline transcription for these isotypes (23). To assess whether RelB affected proliferation of B cells in our CSR assays, we CFSE-labeled B cells, and then activated them in the presence of LPS and IL-4. Activated splenic B cells were then infected with either a control or RelB-encoding retroviruses and CFSE profiles of control or RelB-infected B cells were followed over 4 days. No differences were observed in CSFE profiles, suggesting that RelB expression did not substantially alter the proliferative behavior of activated B cells within our assays.

Discussion

The NF-κB family of transcription factors has been implicated in a broad range of processes involving innate and adaptive immunity. The phenotypes of knockout mice lacking individual and multiple genes corresponding to the five mammalian family members, RelA, RelB, c-Rel, NFKB-1 (p50 and its precursor p105) and NFKB-2 (p52 and its precursor p100), are complex, with defects in multiple hematopoietic cell lineages, including B cells where NF-κB was originally identified (24). Analyses of different knockout B cells reveal that NF-κB factors regulate B cell activation and proliferation by a variety of general B cell activators, including LPS, CD40 ligand and antigen receptor cross-linking (25–28).

Studies with B cell lines and primary B cells indicate that the composition of NF-κB dimers found within B cells is variable and reflects both the activation and differentiation state of a B cell, presumably due in part to transcriptional cross-regulation of different family member genes (11,12,29). These studies showed that in pre-B cells, the major NF-κB complex consists of p50 and RelA, while the cellular NF-κB composition shifts to p50 and c-Rel in mature B cells. Intriguingly, high levels of p52–RelB complexes are observed in plasma cells, suggesting a role for RelB-containing complexes in regulating terminal B cell maturation events such as plasma cell differentiation or CSR. Thus, activation of the same extracellular signaling pathways may result in the translocation of different NF-κB dimers depending upon the state of the B cell when activated. Since the pool of NF-κB dimers available for translocation likely reflects information from multiple signaling pathways, signaling by NF-κB transcription factors in B cells has the complex capacity to evoke differential transcriptional programs of gene activation that are specific to the activation state of the B cell.

Studies with knockout B cells have also revealed that NF-κB activation by general B-cell activators is critical for CSR to occur to specific isotypes (see Table 1). B cells from Rel and p50 knockout mice, for example, have distinct intrinsic defects in their ability to class switch to IgE, IgA and IgG1 (9,10,30). The involvement of NF-κB factors in signaling CSR is consistent with the identification of κB-binding sites within



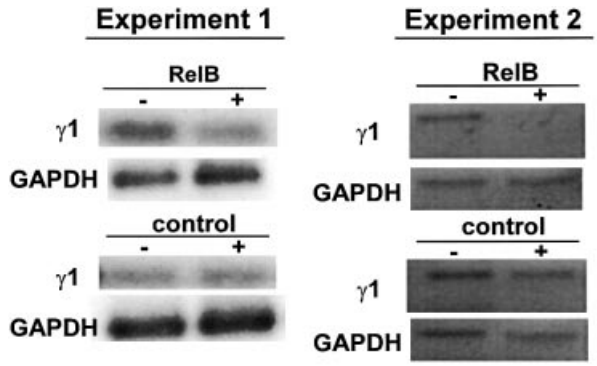


Fig. 5. RelB expression inhibits germline γ1 transcription in LPS and IL-4 activated B cells. MSCV–IRES–Thy-1.1-infected or MSCV–RelB–IRES–Thy-1.1-infected B cells were treated with LPS + IL-4. Non-transduced and transduced cells were separated to >90% purity using magnetic bead-based sorting and total RNA from these populations was isolated and subjected to RT-PCR for GAPDH and germline γ1 transcripts. Two experiments showing the range of inhibition observed in five separate experiments [ranging from a 2-fold (experiment 1) to a complete decrease (experiment 2)] of germline γ1 transcripts observed in RelB-expressing over non-expressing B cells are shown.

Fig. 4. Expression of RelB covalently linked to either p52 or p50 also inhibits IL-4-induced CSR to IgG1, but expression of a RelB mutant lacking the transcriptional activation domain does not. (A) Forced RelB dimer expression inhibits isotype switching to IgG1, but not to IgE in LPS + IL-4-activated B cells. Splenic B cells were transduced with control or p50–L–RelB or p52–L–RelB forced dimers or a transactivation domain-deleted mutant of RelB (RelB RHD) and treated with LPS + IL-4. Cells from each infection were split and stained for IgG1 or IgE expression. (B) Values in the graph compare percentages of $IgG1^+$ and IgE^+ cells between the transduced and non-transduced populations within each sample. Means ± SD from three independent experiments are shown.

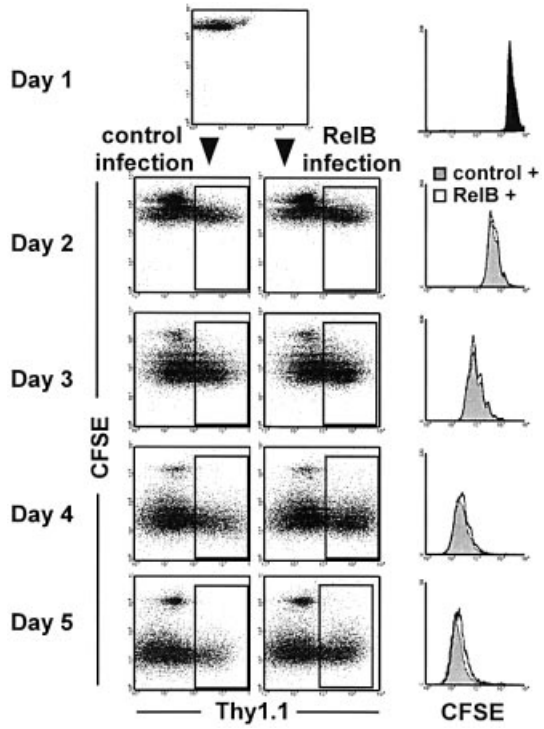


Fig. 6. RelB expression does not alter proliferation of LPS + IL-4-activated B cells. CFSE-labeled purified B cells were transduced with control or RelB-encoding retroviruses and treated with LPS + IL-4. Samples were analyzed each day after infection by flow cytometry. The CFSE profiles to the right compares control-transduced (gray shaded) with RelB-transduced cells (no shading).

the germline C_H promoters regulating class switching to IgG1 and IgE and within the 3′ IgH enhancers that have been implicated in isotype switch regulation (31–35). Taken together, these studies demonstrate that activation of NF-κB in B cells is required along with activation of cytokine signaling pathways to direct CSR to specific isotypes.

Although the precise mechanisms by which NF-κB factors regulate CSR remain to be elucidated, we sought to test whether potential natural differences that can arise in the cellular composition of NF-κB complexes in activated B cells could potentially modulate cytokine-driven CSR. If so, the involvement of NF-κB factors in CSR would reflect not simply a hard-wired signaling response activating CSR, but also a more complex regulatory mechanism that allows information on the state of the B cell to be integrated with cytokine-driven CSR.

In order to test this hypothesis that differences in the cellular composition of NF-κB complexes in activated B cells modulate cytokine-directed CSR, we chose to retrovirally infect activated splenic B cells with individual NF-κB family members. Retroviral expression of RelA, c-Rel, p50 and p52 expression in LPS-activated primary B cells, however, did not alter IL-4-driven CSR to IgG1 and IgE, IFN-γ-driven CSR to IgG2a, TGF-β-driven CSR to IgG2b, TGF-β-, IL-4- and IL-5-driven CSR to IgA or CSR to IgG3. Surprisingly, only retroviral expression of RelB altered CSR, resulting in a 3-fold decrease in CSR to IgG1 induced by IL-4. While our data suggest that RelB may play a role in modulating IL-4-driven IgG1 isotype switching, we have not observed significant differences in RelB expression levels upon cytokine treatment or between B cell subpopulations (data not shown). Thus, although individual NF-κB family members are required for CSR to specific isotypes, these results suggest that natural alterations in the cellular composition of NF-κB transcription factors may have only a limited capacity to modulate cytokine-driven CSR in activated B cells.

The ability of RelB to inhibit IL-4-driven CSR to IgG1 in LPS-activated B cells was isotype specific as RelB expression did not affect CSR to IgE within the same culture or to other isotypes tested. Expression of p52–RelB or p50–RelB dimers joined covalently by a flexible peptide linker also specifically inhibited IgG1 CSR and the transactivation domain of RelB was required for inhibition of CSR to IgG1. These results are consistent with a model where increases in RelB complexes corresponding to endogenous RelB complexes are required for transcription of genes that lead to inhibition of IgG1 CSR. Although others have reported that RelB can actually increase transcription of the γ1 promoter in reporter assays (32), we found that retroviral RelB expression was associated in activated primary B cells with a decrease in germline γ1 transcription, but not with changes in proliferation as assayed by CFSE labeling. Since the C-terminal transactivation domain of RelB was required for inhibition of IgG1 isotype switching, it seems likely that downstream genes regulated by RelB in activated primary B cells are responsible for the inhibition of γ1 germline transcripts and that this effect takes precedence over any direct transcriptional effects of RelB on the γ1 germline promoter in B cells. Our results utilizing retroviral transduction highlight the need to study CSR in primary B cells where NF-κB factors regulate multiple aspects of B cell differentiation.

Acknowledgements

This work was supported by NIH grant AI42252 and the Pew Charitable Trusts. We would like to thank Dr Z. Eshhar (Weizmann Institute, Rehovot, Israel) for generously providing the 84.1C anti-IgE antibody.

Abbreviations

CSR	class switch recombination
GFP	green fluorescent protein
LPS	lipopolysaccharide
MSCV	murine stem cell virus
tNGFR	truncated nerve growth factor receptor
TGF	transforming growth factor

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