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Development of immunoglobulin λ-chain–positive B cells, but not editing of immunoglobulin κ-chain, depends on NF-κB signals

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By genetically ablating IκB kinase (IKK)-mediated activation of the transcription factor NF-κB in the B cell lineage and by analyzing a mouse mutant in which immunoglobulin λ-chain–positive B cells are generated in the absence of rearrangements in the locus encoding immunoglobulin κ-chain, we define here two distinct, consecutive phases of early B cell development that differ in their dependence on IKK-mediated NF-κB signaling. During the first phase, in which NF-κB signaling is dispensable, predominantly κ-chain-positive B cells are generated, which undergo efficient receptor editing. In the second phase, predominantly λ-chain-positive B cells are generated whose development is ontogenetically timed to occur after rearrangements of the locus encoding κ-chain. This second phase of development is dependent on NF-κB signals, which can be substituted by transgenic expression of the prosurvival factor Bcl-2.

It is well established that the NF-κB family of transcription factors is critical to B cell physiology[1,2]. Activation of NF-κB by the alternative pathway, which is mediated by NF-κB-inducing kinase and the inhibitor of NF-κB kinase 1 (IKK1; A001170) downstream of interactions between B cell–activation factor of the tumor necrosis factor family (BAFF) and BAFF-receptor, is essential for mature B cell survival[3]. In addition, mature B cells depend on continuous signaling through the canonical NF-κB pathway, in which activation of the IKK complex, which consists of IKK1, IKK2 (A001172) and NF-κB essential modulator (NEMO; A001628), is central[1]. In contrast, the function of NF-κB signaling in B cell development remains unclear[1] and is indeed highly controversial.

Initial experiments addressed that issue in mice lacking one or two individual NF-κB transcription factors. Whereas the generation of mature B cells is generally impaired in most of these mutant mice, the effects are often mild in B cell progenitors and it has remained unresolved whether these defects are B cell autonomous[2]. Notably, genetic ablation of the BAFF-receptor or IKK1 seems not to affect B cell development in the bone marrow, at least in terms of proportions of cells at the various developmental stages[1,3]; the same is true for ablation of the canonical pathway by knockout of IKK2 or NEMO specifically in B cells[4,5]. However, the weak effect of these genetic manipulations on bone marrow B cell progenitors may have been due to redundancies and/or compensatory mechanisms among NF-κB proteins or IκB kinases[2,6]. Indeed, overexpression of a dominant negative form of the NF-κB inhibitor IκBα prevents efficient transition from the pro–B cell stage to the pre–B cell stage[7,8]. In addition, an earlier publication suggests that NF-κB signals regulate the expression of recombination-activating gene 1 (*Rag1*) and *Rag2* in developing B cells and are involved in the control of receptor editing[9]. The process of receptor editing, through which B cell progenitors change the immunoglobulin light chains in their B cell antigen receptor, is achieved initially by consecutive rearrangements of variable (V_κ) and joining (J_κ) gene segments at the locus encoding immunoglobulin κ-chain (*Igk*) and subsequently by V_λ-J_λ joining; the latter often occurs after rearrangement of the noncoding recombining sequence (RS) element with either a V_κ segment or a recombination signal sequence in the intronic region (IRS) of the *Igk* locus, leading to the inactivation of the *Igk* locus (RS recombination). Receptor editing is key to the generation of B cells bearing non-autoreactive and functionally intact B cell antigen receptors[10]. Published studies have emphasized the possible involvement of IKK-mediated NF-κB signals in early B cell development[11] by suggesting that a partially NF-κB-dependent transcriptional program is activated in B cell progenitors by the kinase ATM in response to DNA breaks that occur during variable-diversity-joining gene segments (V(D)J) recombination. The NF-κB

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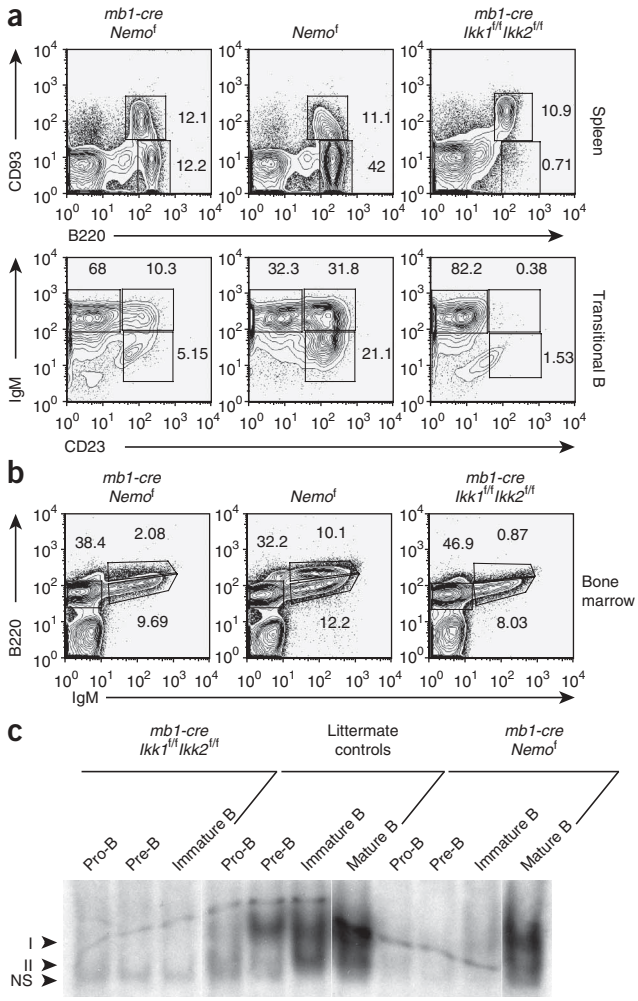

Figure 1 B cell development in the absence of NF-κB signaling. (**a**) Flow cytometry of transitional (B220$^+$CD93$^+$) and mature (B220$^+$CD93$^-$) B cells gated on lymphocytes (top), and T1 (IgM$^+$CD23$^-$), T2 (IgM$^+$CD23$^+$) and T3 (IgM^{lo}CD23$^+$) subsets gated on transitional B cells (bottom), among splenocytes from *mb1-cre Nemo*^{f}, *Nemo*^{f} and *mb1-cre Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ mice ($n = 7$). Numbers adjacent to outlined areas indicate percent cells in each gate. (**b**) Flow cytometry of *mb1-cre Nemo*^{f}, *Nemo*^{f} and *mb1-cre Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ bone marrow leukocytes stained for B220 and IgM to identify pro– and pre–B cells (B220^{lo}IgM$^-$), immature B cells (B220^{lo}IgM$^+$) and recirculating B cells (B220^{hi}IgM$^+$), gated on lymphocytes. Numbers adjacent to outlined areas indicate percent cells in each gate. (**c**) EMSA of NF-κB DNA-binding activity in total extracts of pro–B cells (B220^{lo}IgM$^-$c-Kit$^+$), pre–B cells (B220^{lo}IgM$^-$CD25$^+$), immature B cells (B220^{lo}IgM$^+$) and mature B cells (B220$^+$CD93$^-$) purified by flow cytometry from *mb1-cre Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ mice, littermate control mice and *mb1-cre Nemo*^{f} mice ($n = 6$–8 mice per group). I and II, NF-κB complexes; NS, nonspecific band. EMSA done twice.

signaling cascade thus has been linked to the control of B cell progenitor physiology at many stages through different mechanisms.

To directly address the function of canonical and alternative NF-κB signaling in early B cell development, we generated mice in which these pathways were ablated specifically in the B cell lineage; we induced conditional inactivation of NEMO or IKK1 and IKK2 with the *mb1-cre* transgene[12]. By combining that genetic system with various other mutant alleles, we obtained evidence that even when both NF-κB signaling pathways were ablated and the mutant B lineage cells lacked any biochemically detectable NF-κB DNA-binding activity, normal numbers of B cells were generated and receptor editing at the *Igk* locus was intact. However, in the mutant mice, the generation of B cells expressing immunoglobulin λ-chain was profoundly impaired; this defect was rescued by a transgene encoding the prosurvival protein Bcl-2 (A000367). Transgenic Bcl-2 also restored the development of NEMO-deficient λ-chain-positive B cells in a mouse model of induced *Igk* editing[13] and in mutant mice whose *Igk* loci do not undergo any gene rearrangements[14]. Thus, we conclude that NF-κB signals are dispensable for the development of κ-chain-positive B cells but are required for the efficient generation of λ-chain-positive B cells during a subsequent phase of B cell development.

RESULTS

Experimental design

To address the function of canonical and alternative NF-κB signaling pathways in early B cell development, we induced ablation of NEMO or IKK1 and IKK2 in the B cell lineage with the *mb1-cre* knock-in transgene[12]. Additional genetic modifications (a Bcl-2 transgene[15], the 'κ-macroself' transgene, which encodes a ubiquitously expressed single-chain chimeric antibody with specificity for κ-chains[13], and a mutant *Igk* allele in which the intronic κ enhancer is replaced with a neomycin-resistance gene (iEκT) in homozygous form[14]) were introduced into the compound-mutant mice during the course of the experiments. For efficient generation of the compound mutant mice, which harbored between four and six mutant alleles, we had to follow a breeding strategy yielding experimental and *mb1-cre* negative control mice, the latter collectively called littermate controls. We therefore obtained separate experimental evidence to ensure that the *mb1-cre* transgene did not affect our results because of toxicity of Cre recombinase or heterozygosity for *mb1* (ref. 16). In accordance with published work[12], we found that the *mb1-cre* transgene did not significantly affect early B cell development in the bone marrow in terms of cell numbers (**Supplementary Fig. 1** online) or receptor editing at the *Igk* locus when it was used to ablate NF-κB signaling in B cell progenitors (see below). Furthermore, in terms of the development of λ-chain-positive B cells, we found that *mb1-cre* alone or

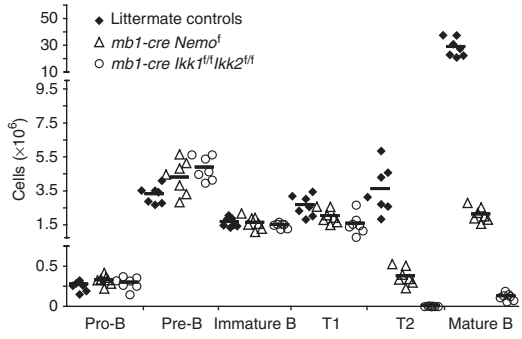

Figure 2 B cell numbers during B cell development in the presence and absence of NF-κB signaling. Absolute numbers of bone marrow pro–B cells (B220^{lo}IgM$^-$c-Kit$^+$), pre–B cells (B220^{lo}IgM$^-$CD25$^+$) and immature B cells (B220^{lo}IgM$^+$), as well as splenic T1 cells (B220$^+$CD93$^+$IgM$^+$CD23$^-$), T2 cells (B220$^+$CD93$^+$IgM$^+$CD23$^+$) and mature B cells (B220$^+$CD93$^-$), from littermate control mice ($n = 7$), *mb1-cre Nemo*^{f} mice ($n = 7$) and *mb1-cre Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ mice ($n = 7$). Each symbol represents an individual mouse; small horizontal lines indicate the mean. One control mouse was excluded from the analysis because its splenic B cell numbers were aberrantly higher than those in the other control mice.

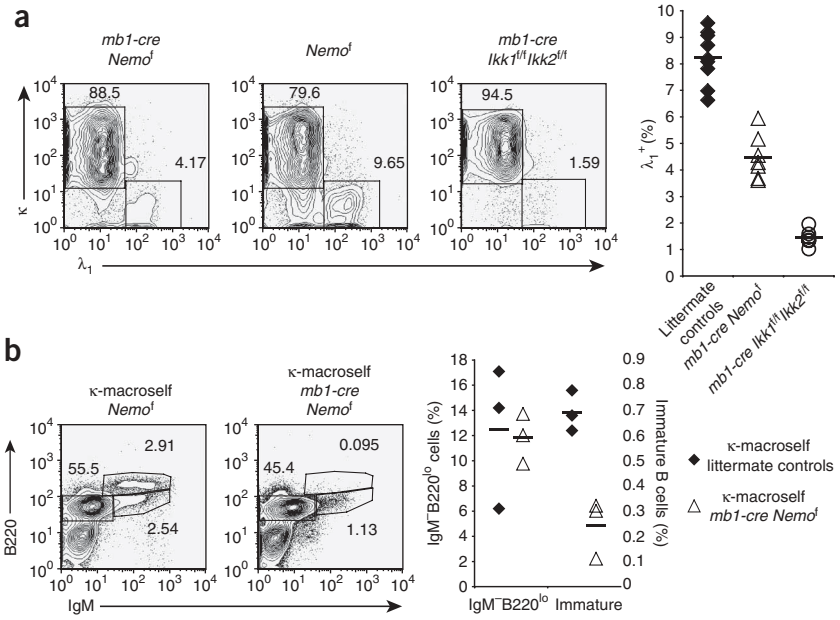

Figure 3 Generation of λ-chain-positive B cells in the presence and absence of IKK-mediated NF-κB activation. (**a**) Flow cytometry of bone marrow immature B cells ($B220^{lo}IgM^{+}$) from *mb1-cre Nemo*^{f} mice ($n = 7$), *mb1-cre Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ mice ($n = 7$) and littermate control mice ($n = 9$), stained for expression of κ-chain and λ_1-chain. Left, plots gated on immature B cells; numbers adjacent to outlined areas indicate percent cells in each gate. Right, proportion of λ_1-chain-positive immature B cells; each symbol represents one mouse and small horizontal bars indicate the mean. (**b**) Flow cytometry of pro– and pre–B cells ($IgM^{-}B220^{lo}$), immature B cells ($B220^{lo}IgM^{+}$) and recirculating B cells ($B220^{hi}IgM^{+}$) from the bone marrow of *mb1-cre Nemo*^{f} mice ($n = 3$) and littermate control mice ($n = 3$) expressing the κ-macroself transgene. Left, plots gated on lymphocytes; numbers adjacent to outlined areas indicate percent cells in each gate. Right, proportion of total bone marrow $IgM^{-}B220^{lo}$ B cells and immature B cells ($B220^{lo}IgM^{+}$); each symbol represents one mouse and small horizontal bars indicate the mean.

mb1-cre-mediated deficiency in IKK1 did not result in a smaller fraction of λ-chain-positive cells in the immature B cell compartment in the bone marrow (**Supplementary Fig. 1** and see below). On the basis of these results, we feel confident that our findings cannot be attributed to unwanted side effects of the *mb1-cre* transgene.

B cell development in the absence of IKKs

In agreement with published work[4,5], ablation of NEMO in the B cell lineage mostly abolished the generation of mature ($B220^{+}CD93^{-}$) peripheral B cells (**Figs. 1a** and **2**). This effect was even more pronounced after ablation of both canonical and alternative NF-κB signaling in *mb1-cre Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ mice, which are *mb1-cre* mice with *lox*P-flanked genes encoding IKK1 (*Chuk*; called '*Ikk1*' here) and IKK2 (*Ikbkb*; called '*Ikk2*' here). Indeed, the $B220^{+}CD93^{-}$ cells in these mice were presumably mostly non–B cells, as only a few of them expressed IgM or IgD (**Supplementary Fig. 2a** online). Ablation of IKK-mediated NF-κB signaling also affected the generation of transitional 1 (T1) and T2 B cells, identified according to a published classification[17]. Whereas some T2 cells, characterized by expression of CD93 and CD23, could still be identified in *mb1-cre Nemo*^{f} mice, which have one (male mice) or two (female mice) X-linked *lox*P-flanked allele(s) encoding NEMO (*Ikbkg*; called '*Nemo*' here), such cells were undetectable in mice lacking IKK1 and IKK2 in the B cell lineage (**Figs. 1a** and **2**). This could have resulted from CD23 regulation by NF-κB[18]. However, the impaired T2 cell generation in the latter mice was also evident from the bright staining of the entire population of transitional cells for CD93 (**Supplementary Fig. 2b**), which indicated that only the most immature (T1) subset of transitional B cells was present. T1 B cell numbers, on the other hand, were only slightly reduced in *mb1-cre Nemo*^{f} and *mb1-cre Ikk1*$^{f/f}$ *Ikk2*$^{f/f}$ mice.

B cell development in the bone marrow of the mutant mice seemed largely undisturbed in terms of subset distribution and cell numbers (**Figs. 1b** and **2**). Similar numbers of pro–B cells and immature B cells were present in mutant and control mice, except that the minor, more mature, $CD23^{+}$ subset of immature B cells was much smaller in the mutant mice[19] (**Supplementary Fig. 3a** online). Notably, mutant mice had approximately 25% more pre–B cells, although the fraction of cycling cells was not higher than that in control mice (**Fig. 2** and **Supplementary Fig. 3b**).

Electrophoretic mobility-shift assay (EMSA) showed that within the limits of the sensitivity of this assay, the efficient generation of B cells in the mutant mice was not due to incomplete ablation of NF-κB signaling (**Fig. 1c**). While traces of DNA-binding activity could still be detected in NEMO-deficient immature B cells, no binding activity was detectable in earlier progenitors, and in the IKK1- and IKK2-deficient cells, there was no detectable binding activity in any of the B lineage cells in the bone marrow. Supershift experiments with antibody to p50 (anti-p50) and anti-p52 showed that essentially all DNA-binding activity detected with this assay was due to NF-κB transcription factors (**Supplementary Fig. 4** online). Curiously, the few mature B cells present in *mb1-cre Nemo*^{f} mice showed strong NF-κB DNA-binding activity, suggesting that these cells, which presumably had not escaped NEMO deletion[5], had activated NF-κB by some other means. These results collectively suggest that abrogation of IKK-mediated NF-κB signaling results in a profound block of B cell development at the stage at which B cells begin to express CD23 but has little effect on earlier stages of B cell development in terms of cell numbers, except for a modest increase in the size of the pre–B cell compartment.

Fewer λ-chain-positive B cells in the absence of NF-κB signaling

Despite our finding of an immature B cell compartment of roughly normal size in the mutant mice (**Fig. 2**), we found that *mb1-cre Nemo*^{f} and mb1-cre *Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ mice had lower percentages of λ-chain-positive immature B cells, as exemplified for λ_1-chain expression (**Fig. 3a**). We confirmed that finding with a second antibody specific for λ-chains (**Supplementary Fig. 5** online). Thus, NF-κB signaling seems to be required for efficient generation of λ-chain-positive B cells. In contrast, ablation of either IKK1 or IKK2 alone in the B cell lineage did not appreciably alter the proportion of λ-chain-positive B cells (**Supplementary Fig. 6** online), which suggests redundant functions for these kinases in the generation of λ-chain-positive B cells.

A possible explanation for the lower number of λ-chain-positive B cells in *mb1-cre Nemo*^{f} and *mb1-cre Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ mice is a defect in

Table 1 J_κ usage in B cells

Genotype	J_κ	Unique sequences	%	J_κ1–2/J_κ4–5 %
Nemo^f iEκT/+	J_κ1	47	30.7	65.4
	J_κ2	53	34.6	
	J_κ4	21	13.7	34.6
	J_κ5	32	20.9	
mb1-cre Nemo^f iEκT/+	J_κ1	64	25.6	52.4
	J_κ2	67	26.8	
	J_κ4	40	16.0	47.6
	J_κ5	79	31.6	

V_κ-J_κ rearrangements involving J_κ1, J_κ2, J_κ4 and J_κ5 in splenic B cells from *mb1-cre Nemo*^{f} iEκT/+ mice (n = 3) and Nemo^f iEκT/+ mice (n = 2); V_κ-J_κ rearrangements were amplified by RT-PCR and percent of rearrangements for each J_κ element were calculated for unique sequences.

receptor editing, which is thought to be regulated by NF-κB[9]. We therefore assessed the effect of NEMO ablation in a mouse model of induced receptor editing. This mouse model is based on the κ-macroself transgene[13]. In κ-macroself mice, B cell progenitors are forced to edit their immunoglobulin light-chain loci away from κ-chain expression, so that only λ-chain-positive B cells can differentiate. We found that κ-macroself *mb1-cre Nemo*^{f} mice had a lower percentage of (λ-chain-positive) immature B cells in the bone marrow than did κ-macroself control mice (**Fig. 3b**). As this result is consistent with a role of NF-κB signaling in receptor editing, we proceeded to direct analysis of receptor editing at *Igk* in progenitor cells devoid of NF-κB signaling.

Receptor editing in the absence of NF-κB signaling

During receptor editing, downstream J_κ elements are progressively recombined; mouse models of B cell autoreactivity show greater fractions of $V_\kappa J_\kappa$ rearrangements using the downstream elements J_κ4 or J_κ5 than do wild-type mice[10,20]. On the basis of those observations, we compared J_κ usage in B cells from mice sufficient or deficient in NF-κB signaling and carrying the iEκT mutation in one of their *Igk* loci (iEκT/+). As noted above, this mutation replaces the intronic κ enhancer with a neomycin-resistance gene, thereby preventing V_κ-J_κ recombination in *cis*[14] and leading, in heterozygous mutant mice, to increased usage of downstream J_κ elements after receptor editing. We found that NEMO deficiency did not result in decreased usage of J_κ4-5 in these mice (**Table 1**). This finding lends no support for the idea that canonical NF-κB signals are involved in receptor editing at *Igk*.

To address that issue more directly, we measured RAG-generated DNA breaks in the *Igk* loci of pre–B cells from *mb1-cre Nemo*^{f} and *mb1-cre Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ mice by ligation-mediated PCR. This assay allows primary breaks to be distinguished from subsequent secondary breaks; the latter reflect sequential V_κ-J_κ rearrangements[21] (**Fig. 4a**). Both classes of DNA breaks were equally present in control, *mb1-cre Nemo*^{f} and *mb1-cre Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ pre–B cells, with the intensity of the bands being roughly proportional to the direct amplification of the control locus *Aprt* (which encodes adenine phosphorybosyltransferase) in the same samples. We also found equal RS recombination in control, *mb1-cre Nemo*^{f} and *mb1-cre Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ pre–B cells (**Fig. 4b**). In agreement with those data, expression of *Rag1* and *Rag2* transcripts was similar in pre–B cells from control, *mb1-cre Nemo*^{f} and *mb1-cre Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ mice, as determined by quantitative real-time PCR (**Fig. 4c**). These results collectively show that IKK-mediated NF-κB signals are dispensable for sequential gene rearrangements at *Igk*, including inactivation of the locus through RS recombination.

Rescue of λ-chain-positive B cell development by Bcl-2

In the absence for evidence of a defect in receptor editing in mice lacking NF-κB signaling, we hypothesized that λ-chain-positive B cells, which are generated with delayed kinetics relative to that of their κ-chain-positive counterparts in mice[22], may depend on NF-κB-mediated survival signals, given that regulation of cell survival is a prominent function of NF-κB transcription factors. We therefore

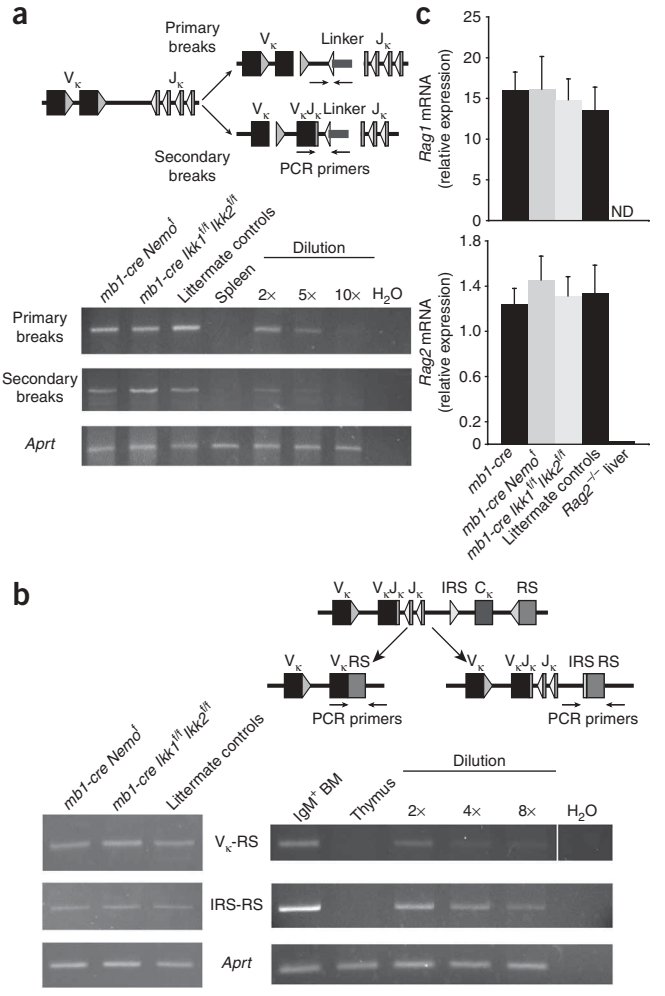

Figure 4 Effect of the presence or absence of IKK-mediated activation of NF-κB on receptor editing at the *Igk* locus. (**a**) Ligation-mediated PCR analysis of primary and secondary breaks at the *Igk* locus in pre–B cells purified from *mb1-cre Nemo*^{f}, *mb1-cre Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ and littermate control mice. Spleen, control DNA from spleen that should not contain breaks at the *Igk* locus. Dilution (2×, 5×, 10×), assessment of assay sensitivity by dilution of wild-type pre–B cell DNA with wild-type mature B cell DNA that should not contain DNA breaks at the *Igk* locus; APRT, confirmation of equal loading of template DNA by *Aprt* amplification. Top, strategy for the analysis of primary and secondary breaks at the *Igk* locus. (**b**) PCR amplification of endogenous V_κ-RS and IRS-RS rearrangements in pre–B cells (B220^{lo}IgM$^-$CD25$^+$) sorted from mice of various genotypes (above lanes; left). Right, assessment of assay sensitivity by dilution of wild-type bone marrow (BM) IgM$^+$ B cell DNA with thymus DNA that should not contain RS rearrangements. Top right, strategy for the analysis of rearrangements between the RS and either a V_κ segment or the IRS sequences. (**c**) Quantitative fluorescence real-time PCR analysis of the expression of *Rag1* and *Rag2* mRNA in pre–B cells (B220^{lo}CD93$^+$IgM$^-$CD25$^+$) from mice of various genotypes (horizontal axis) and from the liver of *Rag2*$^{-/-}$ mice (far right), normalized to expression of γ-actin mRNA. ND, not detected. Data are representative of two experiments (**a**,**b**) mean and s.d. of three mice per genotype, except for the liver control (**c**).

assessed whether transgenic overexpression of the prosurvival protein Bcl-2 in the B cell lineage was able to restore the generation of λ-chain-positive immature B cells in the absence of NF-κB signaling[15]. This was indeed the case, as a Bcl-2 transgene induced complete restoration of λ-chain-positive cells in *mb1-cre Nemo*^{f} mice and partial restoration in *mb1-cre Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ mice (**Fig. 5a** and **Supplementary Fig. 5**). Similar restoration was induced by the Bcl-2 transgene in the κ-macroself model (**Supplementary Fig. 7** online). In agreement with earlier work[23], we noted a greater proportion of λ-chain-positive B cells in Bcl-2-transgenic mice than in wild-type mice (**Figs. 3a** and **5a** and **Supplementary Fig. 5**). This result may have been due to the prolonged time window afforded by Bcl-2 overexpression during which cells can undergo sequential rearrangements in their immunoglobulin light-chain loci[23].

If the NF-κB dependence of the generation of λ-chain-positive B cells indeed reflects a dependence on NF-κB controlled prosurvival factors, these factors should be downregulated in NF-κB-signaling-deficient pre–B cells. One such protein is the Bcl-2 family member Bcl-x_L, which has been shown to play a role in early B cell development[24]. However, in contrast to *Bcl3* and *Nfkb2*, two other known NF-κB targets, expression of mRNA encoding Bcl-x_L was not substantially lower in pre–B cells lacking NEMO or IKK1 and IKK2 (**Supplementary Fig. 8** online), which suggests that expression of Bcl-x_L does not require NF-κB in these cells. A second candidate protein is the serine-threonine kinase Pim2, which phosphorylates the proapoptotic protein Bad, thereby interfering with its interaction with prosurvival Bcl-2 family members[25]. Pim2 has been shown to contribute to the survival of mature B cells, and its expression is controlled by NF-κB in pre–B cells and mature B cells in response to RAG-induced DNA double-stranded breaks and stimulation by BAFF, respectively[11,26]. We indeed found much lower expression of *Pim2* mRNA in pre–B cells lacking NEMO or IKK1 and IKK2 (**Fig. 5b**). Furthermore, the proportion of λ-chain-positive immature B cells was lower in *Pim2*-deficient mice than in control mice (**Fig. 5c**). These results indicate that NF-κB-mediated *Pim2* upregulation contributes to the generation of λ-chain-positive B cells.

λ-chain-positive B cells depend on NF-κB signals in iEκT/T mice

Although our results have established that gene rearrangements and receptor editing at *Igk* proceeds efficiently in B cell progenitors lacking NEMO or IKK1 and IKK2, our findings do not exclude some positive effect of NF-κB signals on these processes; this possibility would be consistent with the slightly enlarged pre–B cell compartment in the mutant mice. It therefore seemed possible that the impaired generation of λ-chain-positive B cells in the absence of NF-κB signals might have resulted from an extended time during which the mutant pre–B cells attempted to rearrange their *Igk* loci. To address that possibility, we investigated the effect of NEMO ablation on B cells of iEκT-homozygous (iEκT/T) mice, in which the *Igk* locus is developmentally 'frozen' so that neither $V_κ$-$J_κ$ rearrangements nor RS recombination takes place and the mice produce exclusively λ-chain-positive B cells[14]. Similar to the results we obtained with the κ-macroself model, the development of B cells was impaired in *mb1-cre Nemo*^{f} iEκT/T mice but was fully restored by a Bcl-2 transgene (**Fig. 6a**,**b** and **Supplementary Fig. 9** online). These experiments showed that λ-chain-positive B cells also depend on NF-κB signals in the absence of gene rearrangements in *Igk* and can be rescued by the prosurvival protein Bcl-2. The NF-κB dependence of the λ-chain-positive B cells correlated with the delayed appearance of these cells during B cell development, compared with that of κ-chain-positive cells, in wild-type mice as well as mice unable to rearrange their *Igk* loci[22] (**Fig. 6c**).

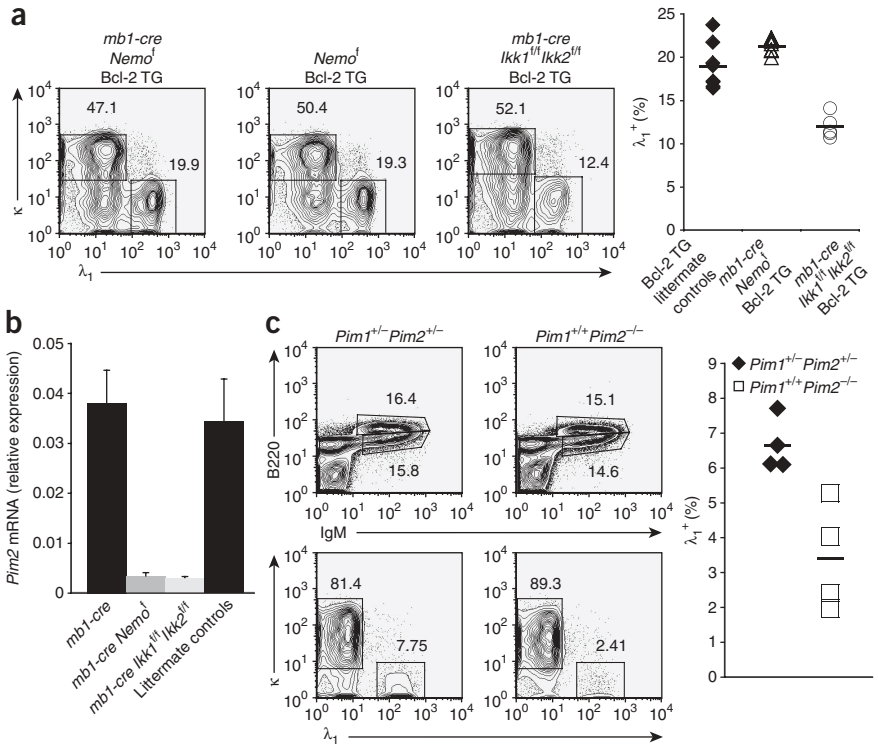

Figure 5 Effect of overexpression of Bcl-2 in the B cell lineage on the generation of λ-chain-positive B cells in the presence and absence of IKK-mediated NF-κB activation. (**a**) Flow cytometry of κ-chain-positive and $λ_1$-chain-positive bone marrow immature (B220^{lo}IgM^{lo}) B cells from *mb1-cre Nemo*^{f} mice ($n = 7$), *mb1-cre Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ mice ($n = 5$) and littermate control mice ($n = 8$) expressing a Bcl-2 transgene (Bcl-2 TG). Immature B cells are defined as B220^{lo}IgM^{lo} cells to exclude from the analysis an interfering B220^{+}IgM^{hi} B cell population present in Bcl-2-transgenic mice. Left, plots gated on immature B cells; numbers indicate proportion of cells in each gate. Right, proportion of $λ_1$-chain-positive immature B cells; each symbol represents one mouse and small horizontal bars indicate the mean. (**b**) Quantitative fluorescence real-time PCR of the expression of *Pim2* mRNA in B220^{lo}CD93^{+}IgM^{-}CD25^{+} pre–B cells sorted from *mb1-cre*, *mb1-cre Nemo*^{f}, *mb1-cre Ikk1*$^{f/f}$ *Ikk2*$^{f/f}$ and littermate control mice, normalized to the expression of γ-actin mRNA (mean and s.d. of three mice per genotype). (**c**) Flow cytometry of bone marrow immature B cells (B220^{lo}IgM^{+}) from *Pim1*$^{+/-}$*Pim2*$^{+/-}$ mice ($n = 4$) and *Pim1*$^{+/+}$*Pim2*$^{-/-}$ mice ($n = 4$), stained for κ-chain and $λ_1$-chain. Left, plots gated on immature B cells; numbers adjacent to outlined areas indicate percent cells in each gate. Right, proportion of $λ_1$-chain-positive immature B cells; each symbol represents one mouse and small horizontal bars indicate the mean.

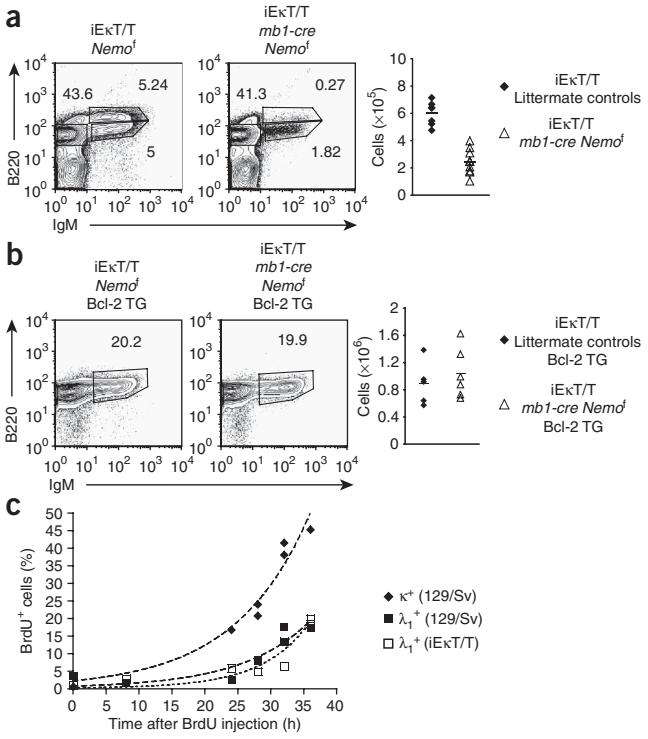

Figure 6 Generation of immature NEMO-deficient λ-chain-positive B cells in the presence and absence of rearrangements at *Igk* loci. (**a**) Flow cytometry of bone marrow cells from iEκT/T mice, which cannot undergo *Igk* rearrangements, on a wild-type ($n = 7$) or NEMO-deficient ($n = 9$) genetic background, stained with anti-B220 and anti-IgM to identify immature ($B220^{lo}IgM^{+}$) and recirculating ($B220^{hi}IgM^{+}$) B cells. Left, plots gated on lymphocytes; numbers adjacent to outlined areas indicate percent cells in each gate. Right, immature B cell numbers; each symbol represents one mouse and small horizontal bars indicate the mean. (**b**) Flow cytometry of immature B cells ($B220^{lo}IgM^{+}CD93^{+}CD23^{-}$) in the bone marrow of *mb1-cre Nemo*^{f} iEκT/T mice ($n = 6$) and *Nemo*^{f} iEκT/T mice ($n = 5$) overexpressing Bcl-2. The combination of fluorochromes in this analysis allows the inclusion of CD93 and CD23 as additional markers to separate immature B cells from an interfering $B220^{+}IgM^{hi}$ B cell population present in Bcl-2-transgenic mice. Left, plots gated on $CD93^{+}CD23^{-}$ cells; numbers adjacent to outlined areas indicate percent cells in each gate. Right, immature B cell numbers; each symbol represents one mouse and small horizontal bars indicate the mean. (**c**) Incorporation of the thymidine analog BrdU by κ-chain-positive and λ_1-chain-positive immature B cells ($B220^{lo}IgM^{+}$) from iEκT/T mice and littermate control mice (129/Sv) injected intraperitoneally with BrdU, assessed by flow cytometry of $BrdU^{+}$ immature B cells.

Development of λ-chain-positive B cells requires TRAF6

Candidate receptors involved in the activation of NF-κB in developing λ-chain-positive B cells include the B cell antigen receptor, Toll-like receptor 2, 4, 6 or 9, or CD40. All of these receptors are expressed in developing B cells and pre–B cell and immature mouse B cell lines[27,28], and all activate NF-κB and induce transcription of genes encoding prosurvival proteins. We therefore examined the proportion of λ-chain-positive cells in the immature B cell compartments of mice lacking Bcl10 and MyD88, which mediate NF-κB activation downstream of these receptors[1,29], or CD40. *Cd40*$^{-/-}$, *Bcl10*$^{-/-}$ and *Myd88*$^{-/-}$ mice had proportions of λ-chain-positive immature B cells similar to those of their wild-type counterparts (**Supplementary Fig. 10a** online). NF-κB activation in B cell progenitors can also be initiated by RAG-induced DNA double-stranded breaks, which activate NF-κB in pre–B cells through the kinase ATM, a regulator of the genotoxic stress response[11]. Thus, the generation of λ-chain-positive B cell could depend on NF-κB activation initiated by DNA double-stranded breaks. However, *Atm*$^{-/-}$ mice had an λ-chain-positive immature B cell population equal in size to that of control mice (**Supplementary Fig. 10b**).

Notably, B cell–specific ablation of TRAF6, an adaptor protein with ubiquitin ligase activity that is involved in NF-κB activation downstream of a variety of receptors such as Toll-like receptors and members of the tumor necrosis factor receptor superfamily[30], led to fewer λ-chain-positive immature B cells (**Supplementary Fig. 10c**). This could indicate that the development of λ-chain-positive immature B cells requires NF-κB activation induced by a receptor that is expressed on developing B cells and signals through TRAF6. The transmembrane activator and CAML-interactor (TACI) is one candidate receptor, as it is expressed on immature B cells, signals through TRAF6 and activates NF-κB[31,32]. However, the proportion of λ-chain-positive immature B cells was not altered in TACI-knockout mice compared with that in control mice (**Supplementary Fig. 10a** online).

DISCUSSION

To determine whether NF-κB is required for B cell development in the bone marrow, we interfered with IKK-mediated NF-κB activation by ablating either NEMO or IKK1 and IKK2 in combination specifically in the B cell lineage. The combined ablation of IKK1 and IKK2 would be expected to not only completely abolish canonical NF-κB activation, more profoundly perhaps than NEMO ablation due to the complete absence of IKK complex kinase activity but also shut off the alternative NF-κB pathway. Indeed, EMSA demonstrated the absence of detectable NF-κB DNA-binding activity in bone marrow B cells when both IKK1 and IKK2 were genetically ablated. NEMO ablation also resulted in a strong reduction of NF-κB DNA-binding activity during the early stages of B cell development, although trace binding activity was still detectable in immature B cells in the bone marrow. In contrast, in developing wild-type B cells, EMSA showed substantial NF-κB DNA-binding activity from the pre–B cell stage onward, in agreement with earlier work[8,33]. Although we cannot formally exclude the possibility that NF-κB transcription factors could be activated in B cell progenitors in ways other than through the canonical or alternative activation pathway, EMSA with progenitor cells lacking both IKK1 and IKK2 did not support this notion.

The results of our analysis of early B cell development in the absence of IKK-mediated NF-κB activation allow us to subdivide the developmental window during which gene rearrangements in the immunoglobulin light-chain loci take place in pre–B cells into two consecutive phases. During the first phase, neither canonical nor alternative NF-κB signals are critical, and pre–B cells undergo gene rearrangements in *Igk*. In agreement with previous work[34], these cells express *Rag1* and *Rag2* mRNA independently of NF-κB and, as shown in our experiments, efficiently undergo receptor editing in the absence of IKK-mediated NF-κB activation. Therefore, neither the initiation nor the progression of gene rearrangements in *Igk* loci require IKK-mediated activation of NF-κB. That conclusion is in agreement with earlier work documenting unimpaired *Igk* rearrangements after mutation of the NF-κB-binding site in the intronic *Igk* enhancer[35]. However, our results do not exclude the possibility that NF-κB signals may serve an auxiliary function in controlling *Igk* gene rearrangements, as earlier work in transformed pre–B cell lines has suggested[36]. Indeed, the slightly larger pre–B cell compartment in the mutant mice may indicate that the mutant cells need more time than do wild-type cells to rearrange their *Igk* loci. Along the same lines, other work has

suggested that NF-κB activity promotes receptor editing by regulating *Rag1* and *Rag2* transcription[9]. Our results presented here and other published work[34] indicate that such a control mechanism can serve only a minor function in B cell development.

During a subsequent phase of pre–B cell development, gene rearrangements at the *Igl* locus predominate, and this phase of development depends on NF-κB signals. It is possible that the requirement for NF-κB is characteristic of a special subset of B cell progenitors that are programmed to prospectively undergo gene rearrangements at *Igl* or that λ-chain-positive but not κ-chain-positive immature B cells depend on NF-κB. However, a simpler interpretation of our data connects the NF-κB dependence of the generation of λ-chain-positive B cells to a program of ontogenetic timing of gene rearrangements in *Igl* loci in the B cell lineage. Despite some controversy, most available evidence supports the idea that gene rearrangements in *Igl* usually follow those in *Igk* in developing B cells. Thus, most λ-chain-positive B cells in both mice and humans have undergone gene rearrangements in their *Igk* loci and often have inactivated *Igk* by RS recombination[10,37]. Although that observation has invited speculation about a possible interdependence of gene rearrangements in *Igk* and *Igl* loci, strong genetic evidence, including the work we have presented here, supports the view that the *Igl* locus is autonomously programmed to become accessible to RAG-mediated recombination at a later stage of B cell development than is the *Igk* locus[22,38].

While the mechanism that controls the differences in the timing of gene rearrangements in *Igk* and *Igl* loci remains obscure, our data indicate that B cell progenitors require NF-κB signals when gene rearrangements in the *Igl* locus occur. The rescue of λ-chain-positive B cells lacking NF-κB signaling by overexpression of Bcl-2 can be interpreted to mean that the differentiating cells become gradually dependent on NF-κB-mediated survival signals. We indeed found strong downregulation of *Pim2*, an NF-κB-dependent prosurvival protein, in the absence of NF-κB signaling in B cell progenitors. This serine-threonine kinase promotes the survival of mature B cells and is induced in B cell progenitors in an NF-κB dependent way[11,26]. In agreement with that result, *Pim2*-knockout mice had a smaller proportion of λ-chain-positive immature B cells than did wild-type control mice. However, it is still possible that NF-κB signals also promote the differentiation of progenitor B cells to λ-chain-positive B cells and that in the absence of NF-κB activation, the cells need extra time for maturation. In one such scenario, NF-κB signals would contribute to making the *Igl* locus accessible for V(D)J recombination. There is evidence in the literature that this may indeed be the case, although through an indirect rather than a direct mechanism[39]. Our results have demonstrated that IKK-mediated activation of NF-κB is dispensable for the development of immature κ-chain-positive B cells in the bone marrow and for receptor editing at *Igk* loci. In contrast, the differentiation of λ-chain-positive B cells strongly depends on an NF-κB-dependent gene expression program that includes upregulation of the NF-κB target gene *Pim2* and whose activation may involve an as-yet-unidentified receptor signaling through TRAF6.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A001170, A001172, A001628 and A000367.

Note: Supplementary information is available on the Nature Immunology website.

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ONLINE METHODS

Mice. *Nemo*^{f}, *Ikk1*^{f}, *Ikk2*^{f}, *Traf6*^{f}, *mb1-cre*, *Atm*$^{-/-}$, *Bcl10*$^{-/-}$, *Myd88*$^{-/-}$, *Pim2*$^{-/-}$, *Taci*$^{-/-}$, κ-macroself and iEκT mice have been described[12–14,40–48]. The strain 129/Sv was from Charles River Laboratories. *Cd40*$^{-/-}$ and Eμ-Bcl-2-22 (Bcl-2-transgenic) mice were from Jackson Laboratory[15,49]. The iEκT mice were on a 129/Sv background, *Atm*$^{-/-}$ mice were on either a 129/Sv or a mixed genetic background, and all other stains were generated on or backcrossed to a C57BL/6 genetic background. All mouse experiments were in compliance with guidelines of the Institutional Animal Care and Use Committee of Harvard University and the Immune Disease Institute.

Flow cytometry. Cells isolated from bone marrow and spleen were stained with the following antibodies conjugated to fluorescein isothiocyanate, phycoerythrin, peridin chlorophyll, allophycocyanin, indodicarbocyanine or biotin: anti-B220 (RA3-6B2; BD Biosciences), anti-CD19 (1D3; BD Biosciences), anti-CD23 (B3B4; eBioscience), anti-CD25 (PC61.5; eBioscience), anti-CD93 (AA4.1; eBioscience), anti-c-Kit (ACK2; eBioscience), anti-IgD (11-26; eBioscience), anti-κ-chain (187.1; BD Biosciences), anti-λ_1-chain (L22.18.2; a gift from S. Weiss), anti-$\lambda_{1,2,3}$-chain (R26-46; BD Biosciences) and anti-IgM (goat anti–mouse Fab; Jackson Immunoresearch). Data were acquired on a FACSCalibur (BD Biosciences) and were analyzed with FlowJo software (Tree Star).

B cell isolation. Samples were first enriched for bone marrow and splenic B cells by magnetic depletion with the MACS system (Miltenyi Biotec). Samples were depleted of bone marrow non–B cells with a 'cocktail' of biotinylated antibodies (anti-CD4 (GK1.5; eBioscience), anti-CD3ε (145-2C11; eBioscience), anti-Gr-1 (RB6-8C5; eBioscience), anti-CD11b (M1/70; eBioscience), anti-CD11c (N418; eBioscience), anti-NK1.1 (PK136; eBioscience) and anti-Ter119 (Ter119; eBioscience)), followed by incubation with streptavidin microbeads. Splenocyte samples were depleted of non–B cells with CD43 microbeads. B cell subsets were then sorted on a FACSAria (BD Biosciences) with various surface markers. Cells were allowed to 'rest' for 4 h in medium containing 0.5% (vol/vol) FBS before total protein extracts were prepared for EMSA with whole-cell lysis buffer (20 mM HEPES, pH 7.9, 350 mM NaCl, 20% (vol/vol) glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, pH 8.0, 0.1 mM EGTA, pH 8.0, 1%(vol/vol) Nonidet P-40 and protease inhibitors). Cell remnants were then treated with proteinase K and DNA was subsequently prepared for ligation-mediated PCR and RS-PCR analysis.

EMSA. Total protein extracts were incubated with poly(dI:dC) competitor DNA (GE Healthcare) and a ^{32}P-labeled κB consensus probe (Promega). Protein-DNA complexes and free probe were then separated by electrophoresis through a native polyacrylamide gel. For supershift experiments, protein extracts were preincubated for 30 min on ice with anti-p50 (NLS(X); Santa Cruz) or anti-p52 (1495; N. Rice).

Analysis of J_κ usage. Total RNA was extracted with TRIzol reagent (Invitrogen) from enriched splenic B cell preparations generated by CD43 magnetic bead depletion (Miltenyi Biotec); cDNA was synthesized according to the ThermoScript RT-PCR system protocol (Invitrogen). V_κ-J_κ rearrangements were amplified by PCR with high-fidelity Taq enzyme (Roche) and primers V_κ-FW3 (5′-AGCTTCAGTGGCAGTGGRTCWGGRAC-3′, where 'R' is G or A, and 'W' is A or T) and C_κ (5′-CTTCCACTTGACATTGATGTC-3′), as described[50]. PCR amplicons were then cloned with the TOPO-TA cloning kit (Invitrogen) and were sequenced (Dana Farber–Harvard Cancer Center DNA Resource Core facility). Unique sequences were analyzed for J_κ usage.

Ligation-mediated PCR. The BW linker oligonucleotides (BW-1, 5′-GCG GTGACCCGGGAGATCTGAATTC-3′ and BW-2, 5′-GAATTCAGATC-3′) were ligated to DNA overnight. PCR was then done with linker primer (5′-CC GGGAGATCTGAATTCCAC-3′), primer ko5 (5′-GCCCAAGCGCTTCCACGC ATGCTTGGAG-3′) and degenerated V_κ primer (5′-CCGAATTCGSTTCAG TGGCAGTGGRTCRGGRAC-3′, where 'S' is G or C, and 'R' is A or G) for amplification of primary breaks (BW and ko5) or secondary breaks (BW and V_κ)[21]. A 'touchdown' PCR program was used as follows: 94 °C for 1 min, 19 cycles of 92 °C for 30 s and 70 °C for 40 s; and a drop in temperature of 0.5 °C with each successive cycle. This was followed by 19 cycles of 92 °C for 30 s and 60 °C for 40 s with 1 s added at each successive cycle. PCR products were subsequently analyzed by electrophoresis on an agarose gel.

RS PCR. RS rearrangements were amplified from genomic DNA with RS reverse 1 primer (5′-GGACATCTACTGACAGGTTATCACAGGTC-3′) and IRS forward 1 primer (5′-ATGACTGCTTGCCATGTAGATACCATGG-3′) for the detection of RS-IRS recombination, or with V_κS primer (described above) for the detection of V_κ-RS rearrangements. PCR conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The *Aprt* loading control was amplified with primers APRT 747F (5′-TGC TAGACCAACCCGCACCCAGAAG-3′) and APRT 964R (5′-TCGTGACCG CACCTGAACAGCAC-3′) and the following cycling conditions: 95 °C for 5 min, followed by 21 cycles of 95 °C for 30 s, 63 °C for 30 s and 72 °C for 30 s.

BrdU labeling. A BrdU Flow kit was used for labeling of DNA with BrdU (5-bromo-2-deoxyuridine) according to the manufacturer's instructions (BD Biosciences). Both 129/Sv and iEκT/T mice were injected with 1 mg BrdU intraperitoneally and were analyzed at 8, 24, 28, 32 and 36 h thereafter. Bone marrow cells were stained with anti-B220, anti-IgM, anti-κ-chain or anti-λ_1-chain and anti-BrdU. The proportion of κ-chain-positive and λ_1-chain-positive immature B cells ($B220^{lo}IgM^{+}$) positive for BrdU were determined by flow cytometry on a FACSCalibur. Prism software (GraphPad Software) was used for best-fit regression curve analysis. The 0-hour time point shows anti-BrdU background staining in noninjected mice.

Quantitative real-time PCR. Total RNA was extracted from flow cytometry–purified pre–B cells with an RNeasy Micro kit (Qiagen) and cDNA was synthesized with the Thermoscript RT-PCR system according to the manufacturer's protocol (Invitrogen). Power SYBR Green was used for quantitative real-time PCR, followed by analysis with a StepOnePlus Real-Time PCR system (Applied Biosystems). Primers to detect transcripts were as follows: *Rag1* (5′-TTGCTATCTCTGTGGCATCG-3′ and 5′-AATTTCATCGGGTGCAGAAC-3′), *Rag2* (5′-AGTGACTCTTCCCCAAGTGC-3′ and 5′-CTTCCTGCTTGTG GATGTGA-3′), *Nfkb2* (5′-TTTCCTTCGAGCTAGCGATG-3′ and 5′-TTCGG GAGATCTTCAGGTTC-3′), Bcl-x_L (5′-GGTGAGTCGGATTGCAAGTT-3′ and 5′-GCTGCATTGTTCCCGTAGAG-3′) and γ-actin (5′-GGTGTCCGGAGGCA CTCTT-3′ and 5′-TGAAAGTGGTCTCATGGATACCA-3′). Primers for *Pim2* and *Bcl3* have been described[11]. Samples were analyzed in duplicate and messenger abundance was normalized to that of γ-actin.

Additional methods. Information on cell cycle analysis is available in the **Supplementary Methods** online.

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