HHS Public Access

Author manuscript

Nat Immunol. Author manuscript; available in PMC 2009 December 01.

Published in final edited form as:

Nat Immunol. 2009 June ; 10(6): 647–654. doi:10.1038/ni.1732.

Igλ+ B cell development but not Igκ editing depends on NF-κB signals

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Abstract

By genetically ablating IκB kinase (IKK)-mediated NF-κB activation in the B cell lineage, and by analyzing a mouse mutant in which Igλ$^+$ B cells are generated in the absence of rearrangements in *Igk*, we define 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 Igκ$^+$ B cells are generated and undergo efficient receptor editing. In the second phase, predominantly Igλ$^+$ B cells are generated, whose development is ontogenetically timed to occur after *Igk* rearrangements. This second phase of development is dependent on NF-κB signals, which can be substituted by transgenic expression of the pro-survival factor Bcl2.

INTRODUCTION

It is well established that the NF-κB family of transcription factors plays a critical role in B cell physiology1,2. Activation of NF-κB via the alternative pathway, which is mediated by

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NF-κB inducing kinase (NIK) and IKK1 (http://www.signaling-gateway.org/molecule/query?afcsid=A001170) downstream of B cell activation factor of the TNF family (BAFF)-BAFF receptor (BAFF-R) interactions, is essential for mature B cell survival3. 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 (http://www.signaling-gateway.org/molecule/query?afcsid=A001172) and NF-κB essential modulator (NEMO) (http://www.signaling-gateway.org/molecule/query?afcsid=A001628), plays a central role1. In contrast, the role of NF-κB signaling in B cell development remains unclear1, and is indeed highly controversial.

Initial experiments addressed this issue in mice lacking one or two individual NF-κB transcription factors. Whereas the generation of mature B cells was generally impaired in most of these mutant mice, the effects were often mild in B cell progenitors, and it remained unresolved whether these defects were B cell autonomous2. Notably, genetic ablation of BAFF-R or IKK1 appeared not to affect B cell development in the bone marrow (BM), at least in terms of proportions of cells at the various developmental stages1,3; the same was true for ablation of the canonical pathway by knocking out IKK2 or NEMO specifically in B cells4,5. However, the weak impact of these genetic manipulations on BM B cell progenitors may have been due to redundancies and/or compensatory mechanisms among NF-κB proteins or IκB kinases2,6. Indeed, the over-expression of a dominant negative form of the NF-κB inhibitor IκBα prevented efficient transition from the pro- to the pre-B cell stage7,8. In addition, the work of Verkoczy et al.9 suggested 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. The process of receptor editing, through which B cell progenitors change the immunoglobulin (Ig) light (L) chains in their B cell antigen receptor (BCR), is achieved initially by consecutive V_κ-J_κ rearrangements, and subsequently by V_λ-J_λ joining; the latter often occurs after rearrangement of the non-coding recombining sequence (RS) element with either a V_κ segment or a recombination signal sequence within the *Igk* intronic region (IRS), leading to the inactivation of the *Igk* locus (RS recombination). Receptor editing plays a key role in the generation of B cells bearing non-autoreactive and functionally intact BCRs10. The recent work of Bredemeyer et al. emphasizes a possible involvement of IKK-mediated NF-κB signals in early B cell development11, by suggesting that a partially NF-κB-dependent transcriptional program is activated in B cell progenitors via the ataxia telangiectasia mutated (ATM) kinase in response to DNA breaks that occur during V(D)J recombination. The NF-κB signaling cascade thus has been implicated in the control of B cell progenitor physiology at multiple stages through different mechanisms.

To directly address the role of canonical and alternative NF-κB signaling in early B cell development, we generated mice in which these pathways are ablated specifically in the B cell lineage; we induced conditional inactivation of NEMO or IKK1 and IKK2 using the *mb1-cre* transgene12. By combining this genetic system with various other mutant alleles, we obtained evidence that even when both NF-κB signaling pathways are ablated and the mutant B lineage cells lack any biochemically detectable NF-κB DNA binding activity, normal numbers of B cells are generated and receptor editing at the *Igk* locus is intact.

However, in the mutant mice the generation of Igλ expressing B cells is profoundly impaired; this defect can be rescued by a transgene encoding the pro-survival protein Bcl2 (http://www.signaling-gateway.org/molecule/query?afcsid=A000367). Transgenic Bcl2 also rescued the development of NEMO-deficient Igλ$^+$ B cells in a mouse model of induced *Igk* editing13, and in mutant mice whose *Igk* loci do not undergo any gene rearrangements14. Thus we conclude that NF-κB signals are dispensable for the development of Igκ$^+$ B cells, but are required for the efficient generation of Igλ$^+$ B cells during a subsequent phase of B cell development.

RESULTS

Experimental design

To address the role 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 using the *mb1-cre* knock-in transgene12. Additional genetic modifications, namely a Bcl2 transgene15, the κ-macroself transgene and the iEκT allele in homozygous form13,14, were introduced into the compound mutant mice during the course of the experiments. Our strategy for the generation of the various compound mutant mice did not always yield appropriate *mb1-cre* controls that were heterozygous for, or lacking loxP-flanked target genes. We therefore obtained separate experimental evidence to ensure that the *mb1-cre* transgene did not impact our results because of Cre toxicity or heterozygosity for *mb1*16. In accordance with previous work12, we found that the *mb1-cre* transgene did not significantly affect early B cell development in the BM in terms of cell numbers (Supplementary Figure 1, online) or receptor editing at the *Ig*κ locus, when it was used to ablate NF-κB signaling in B cell progenitors (Fig. 4). Furthermore, with respect to the development of Igλ$^+$ B cells, we have shown that *mb1-cre* alone or *mb1-cre* mediated deficiency in IKK1 did not result in a reduction of the fraction of Igλ$^+$ cells in the immature B cell compartment in the BM in absence of IKK1 and IKK2 or NEMO (Fig. 3a; Supplementary Figure 1, Supplementary Figure 6 online). On the basis of these results, we feel confident that our results cannot be attributed to unwanted side effects of the *mb1-cre* transgene.

B cell development in the absence of IKKs

In accord with previous work4,5, ablation of NEMO in the B cell lineage largely abolished the generation of mature (B220$^+$CD93$^-$) peripheral B cells (Fig. 1a, Fig. 2). This effect was even more pronounced upon ablation of both canonical and alternative NF-κB signaling, in *mb1-cre Ikk1$^{f/f}$Ikk2$^{f/f}$* mice. Indeed, the B220$^+$CD93$^-$ cells in these animals 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 (T) 1 and T2 B cells, identified according to the classification of Allman et al.17. Whereas some T2 cells, characterized by CD93 and CD23 expression, could still be identified in *mb1-cre Nemo^f* mice (carrying a single (X-linked) *Nemo^f* allele in males, and two such alleles in females), such cells were undetectable in mice lacking IKK1 and IKK2 in the B cell lineage (Fig. 1a, Fig. 2). This could have resulted from CD23 regulation by NF-κB18. 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, indicating that only the

most immature (T1) subset of transitional B cells was present (Supplementary Fig. 2b, online). T1 B cells 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 BM of the mutant mice appeared largely undisturbed in terms of subset distribution and cell numbers (Fig. 1b, Fig. 2). Similar numbers of pro-B and immature B cells were present in mutant and control mice, except that the minor, more mature, CD23^{+} subset of immature B cells was substantially reduced in the mutants (Supplementary Fig. 3a, online)19. Interestingly, the numbers of pre-B cells were increased in the mutants by approximately 25%, although the fraction of cycling cells not higher than in the controls (Fig. 2; Supplementary Fig. 3b, online).

Electromobility shift assays demonstrated that within the limits of the sensitivity of this assay the efficient generation of B cells in the mutant animals was not due to an 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 BM. Supershift experiments using anti-p50 and anti-p52 demonstrated that essentially all DNA binding activity detected in this assay was due to NF-κB transcription factors (Supplementary Fig. 4, online). Curiously, strong NF-κB DNA binding activity was seen in the few mature B cells present in *mb1-cre Nemo^{f}* mice, suggesting that these cells, which presumably have not escaped NEMO deletion5, have activated NF-κB by some other means.

Together, these results suggest that abrogation of IKK-mediated NF-κB signaling results in a profound block of B cell development at the stage when 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 Igλ^{+} B cells in the absence of NF-κB signaling

Despite a roughly normal sized immature B cell compartment (Fig. 2), we detected a 1.8 and 5.7 fold reduction in the percent Igλ^{+} immature B cells in *mb1-cre Nemo^{f}* and mb1-cre *Ikk1$^{f/f}$Ikk2$^{f/f}$* mice, respectively (Fig. 3a). This finding was confirmed using a second antibody specific for Igλ chains (Supplementary Fig. 5, online). Thus, NF-κB signaling appears to be required for efficient generation of Igλ^{+} B cells. In contrast, ablation of either IKK1 or IKK2 alone in the B cell lineage did not appreciably alter the proportion of Igλ^{+} B cells (Supplementary Fig. 6, online), suggesting redundant functions for these kinases in the generation of Igλ^{+} B cells.

A possible explanation for the reduction of Igλ^{+} B cells in *mb1-cre Nemo^{f}* and *mb1-cre Ikk1$^{f/f}$Ikk2$^{f/f}$* mice is a defect in receptor editing, which is thought to be regulated by NF-κB9. 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, which encodes a ubiquitously expressed single chain chimeric antibody with specificity for Igκ chains13. In κ-macroself mice, B cell progenitors are forced to edit their IgL chain loci away from Igκ expression, so that only Igλ^{+} B cells can differentiate. κ-macroself *mb1-cre Nemo^{f}* mice

displayed a 2.7 fold reduction in the percent (Igλ$^+$) immature B cells in the BM, compared to κ-macroself control mice (Fig. 3b). As this result is consistent with a role for NF-κB signaling in receptor editing, we proceeded to a 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; compared to wild-type animals, mouse models of B cell autoreactivity show increased fractions of $V_\kappa J_\kappa$ rearrangements using the downstream elements $J_{\kappa 4}$ or $J_{\kappa 5}$10,20. Based on these observations, we compared J_κ usage in B cells from mice proficient or deficient in NF-κB signaling and carrying the iEκT mutation in one of their *Igk* loci. 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 the heterozygous mutant mice, to an increased usage of downstream J_κ elements upon receptor editing. We found that NEMO deficiency did not decrease $J_{\kappa 4-5}$ usage in these mice (Table 1). This finding lends no support to a role for canonical NF-κB signals in receptor editing at *Igk*.

To address this 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, using ligation-mediated PCR (LM-PCR). This assay allows one to distinguish between primary breaks and subsequent secondary breaks, the latter reflecting sequential V_κ-J_κ rearrangements21 (Fig. 4a). Both classes of DNA breaks were equally observed 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 a control locus (adenine phosphorybosyltransferase; APRT) 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 accord with these data, the 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$^{f/f}$* animals, as determined by quantitative real time PCR (Fig. 4c). Together, these results show that IKK-mediated NF-κB signals are dispensable for sequential gene rearrangements at *Igk*, including the inactivation of the locus through RS recombination.

Rescue of Igλ$^+$ B cell development by Bcl2

In the absence of evidence for a defect in receptor editing in mice lacking NF-κB signaling, we hypothesized that Igλ$^+$ B cells, which are generated in mice with delayed kinetics compared to their Igκ$^+$ counterparts22, 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 assessed whether transgenic over-expression of the pro-survival protein Bcl2 in the B cell lineage was able to rescue the generation of Igλ$^+$ immature B cells in the absence of NF-κB signaling15. This was indeed the case, as a Bcl2 transgene induced a complete rescue of Igλ$^+$ cells in *mb1-cre Nemo^f* mice, and a partial rescue in *mb1-cre Ikk1$^{f/f}$Ikk2$^{f/f}$* mice (Fig. 5a and Supplementary Fig. 5, online). A similar rescue was induced by the Bcl2 transgene in the κ-macroself model (Supplementary Fig. 7, online). In accord with earlier work23, we observed an increased proportion of Igλ$^+$ B cells in Bcl2 transgenic compared to wild-type mice (Fig. 3a, Fig. 5a; Supplementary Fig. 5, online). This result may

be due to a prolonged time window afforded by Bcl2 overexpression, during which cells can undergo sequential rearrangements in their Ig light chain loci23.

If the NF-κB dependence of the generation of Igλ$^+$ B cells indeed reflects a dependence on NF-κB controlled pro-survival factors, these factors should be downregulated in NF-κB-deficient pre-B cells. One such protein is the Bcl2 family member Bcl-x_L which has been shown to play a role in early B cell development24. However, in contrast to *Bcl3* and *Nfkb2*, two other known NF-κB targets, *Bcl-x_L* mRNA expression was not significantly reduced in pre-B cells lacking NEMO or IKK1 and IKK2, suggesting that expression of *Bcl-x_L* does not require NF-κB in these cells (Supplementary Fig. 8, online). A second candidate protein is the serine threonine kinase Pim2, which phophorylates the pro-apoptotic protein Bad, thereby interfering with its interaction with pro-survival Bcl2 family members25. Pim2 has been shown to contribute to the survival of mature B cells, and its expression is controlled by NF-κB in pre-B and mature B cells in response to RAG-induced DNA double strand breaks and BAFF stimulation, respectively11,26. We indeed observed markedly reduced *Pim2* mRNA expression in pre-B cells lacking NEMO or IKK1 and IKK2 (Fig. 5b). Furthermore, the proportions of Igλ$^+$ immature B cells were reduced in *Pim2*-deficient mice compared to control mice (Fig. 5c). These results indicate that NF-κB-mediated *Pim2* up-regulation contributes to the generation of Igλ$^+$ B cells.

Igλ$^+$ B cells depend on NF-κB signals in iEκT/T mice

While our results have established that gene rearrangements and receptor editing at *Igk* proceeded efficiently in B cell progenitors lacking NEMO or IKK1 and IKK2, our findings do not exclude some positive impact 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 appeared possible that the impaired generation of Igλ$^+$ B cells in the absence of NF-κB signals might result from an extended time window in which the mutant pre-B cells attempt to rearrange their *Igk* loci. To address this possibility, we investigated the impact 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 Igλ$^+$ B cells14. Similar to what we had observed in the κ-macroself model, the development of B cells was impaired in *mb1-cre Nemo^f* iEκT/T mice, but was fully rescued by a Bcl2 transgene (Fig. 6a,b and Supplementary Fig. 9, online).

These experiments demonstrated that the dependence of Igλ$^+$ B cells on NF-κB signals is also seen in the absence of gene rearrangements in *Igk*, and can be rescued by the pro-survival protein Bcl2. The NF-κB dependence of the Igλ$^+$ B cells correlated with the delayed appearance of these cells in B cell development, compared to that of Igκ$^+$ cells, in wild-type mice as well as mice unable to rearrange their *Igk* loci22 (Fig. 6c).

Igλ$^+$ B cell development requires TRAF6

Candidate receptors involved in the activation of NF-κB in developing Igλ$^+$ B cells include the BCR, Toll-like receptor 2 (TLR2), TLR4, TLR6, TLR9, or CD40. All of these receptors are expressed in developing B cells and pre- and immature mouse B cell lines27,28, and all

activate NF-κB and induce transcription of genes encoding pro-survival proteins. We therefore examined the proportions of Igλ$^+$ cells in the immature B cell compartments of mice lacking Bcl10 and MyD88, which mediate NF-κB activation downstream of these receptors1,29, or CD40. *Cd40*$^{-/-}$; *Bcl10*$^{-/-}$ and *Myd88*$^{-/-}$ mice had similar proportions of Igλ$^+$ immature B cells as wild-type counterparts (Supplementary Fig. 10a, online). NF-κB activation in B cell progenitors can also be initiated by RAG-induced DNA double strand breaks, which activate NF-κB in pre-B cells through the kinase ATM, a regulator of the genotoxic stress response11. Thus, the generation of Igλ$^+$ B cell could depend on NF-κB activation initiated by DNA double strand breaks. However, *Atm*$^{-/-}$ mice displayed an Igλ$^+$ immature B cell population equal in size to control mice (Supplementary Fig. 10b, online).

Interestingly, B cell-specific ablation of TNF-receptor associated factor (TRAF) 6, an adaptor protein with ubiquitin ligase activity that is involved in NF-κB activation downstream of a variety of receptors such as TLRs and members of the TNF receptor superfamily30, led to a reduction of Igλ$^+$ immature B cells (Supplementary Fig. 10c, online). This could indicate that Igλ$^+$ immature B cell development requires NF-κB activation induced by a receptor that is expressed on developing B cells and signals via TRAF6. Transmembrane activator CAML-interactor (TACI) is one candidate receptor, as it is expressed on immature B cells, signals through TRAF6 and activates NF-κB31,32. However, the proportions of Igλ$^+$ immature B cells were not altered in *Taci*$^{-/-}$ mice compared to control mice (Supplementary Fig. 10a, online).

DISCUSSION

To determine whether NF-κB is required for B cell development in the BM, 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 to shut off the alternative NF-κB pathway. Indeed, electromobility shift assays demonstrated the absence of detectable NF-κB DNA binding activity in BM 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 early stages of B cell development, although trace binding activity was still detectable in immature B cells in the BM. In contrast, in developing wild-type B cells the electromobility shift assay revealed substantial NF-κB DNA binding activity from the pre-B cell stage onward, in accord with earlier work8,33. While 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, electromobility shift assays performed using progenitor cells lacking both IKK1 and IKK2 do 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 IgL 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 accord with the work of Amin and Schlissel34,

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 NF-κB activation. This conclusion is in agreement with previous work that documented unimpaired *Igk* rearrangements after mutation of the NF-κB binding site in the intronic Igκ enhancer35. However, our results do not exclude the possibility that NF-κB signals may play an auxiliary role in controlling *Igk* gene rearrangements, as earlier work in transformed pre-B cell lines had suggested36. Indeed, the slight increase of the size of the pre-B cell compartment in the mutant animals may indicate that the mutant cells need more time than wild-type cells to rearrange their *Igk* loci. Along the same lines, other work suggested that NF-κB activity promotes receptor editing by regulating *Rag1* and *Rag2* transcription9. The present results and those of Amin and Schlissel34 indicate that such a control mechanism can only play a minor role 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 $Ig\lambda^+$ but not $Ig\kappa^+$ immature B cells depend on NF-κB. However, a simpler interpretation of our data connects the NF-κB dependence of the generation of $Ig\lambda^+$ B cells to a program of ontogenetic timing of gene rearrangements in IgL chain loci in the B cell lineage. Despite some controversy, most of the available evidence supports the notion that gene rearrangements in *Igl* usually follow those in *Igk* in developing B cells. Thus, most $Ig\lambda^+$ B cells in both mouse and human have undergone gene rearrangements in their *Igk* loci, and often have inactivated the latter by RS recombination10,37. While this observation has invited speculations about a possible interdependence of gene rearrangements in *Igk* and *Igl* loci, strong genetic evidence, including the present work, 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 the *Igk* locus22,38.

While the mechanism controlling the differential 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 $Ig\lambda^+$ B cells lacking NF-κB signaling by the over-expression of Bcl2 can be interpreted to mean that the differentiating cells become gradually dependent on NF-κB mediated survival signals. We indeed noted strong downregulation of *Pim2*, an NF-κB dependent pro-survival protein, in 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 manner11,26. In accord with this result, *Pim2* knockout mice exhibited a decrease in the proportion of $Ig\lambda^+$ immature B cells compared to wild-type controls. However, it is still possible that NF-κB signals also promote the differentiation of progenitor B cells to $Ig\lambda^+$ B cells, and that in the absence of signals of the latter kind the cells need extra time for maturation. In one such scenario NF-κB signals would contribute to make 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 mechanism39.

Our results demonstrate that IKK-mediated NF-κB activation is dispensable for the development of immature Igκ$^+$ B cells in the BM, and for receptor editing at *Igk* loci. In contrast, the differentiation of Igλ$^+$ B cells strongly depends on a NF-κB-dependent gene expression program that includes up-regulation of the NF-κB target gene *Pim2*, and whose activation may involve an as yet unidentified receptor signaling through TRAF6.

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 previously12-14,40-48. The strain 129/Sv was purchased from Charles River Laboratories. *Cd40$^{-/-}$* and Eμ-Bcl2−22 (Bcl2 Tg) mice were obtained from Jackson Laboratory15,49. 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 were backcrossed to a C57BL/6 genetic background. All mouse experiments were performed in compliance with guidelines of the Institutional Animal Care and Use Committee of Harvard University and the Immune Disease Institute.

Flow cytometry

Cells were isolated from BM and spleen and stained with the following antibodies conjugated to fluorescein isothiocyanate, phycoerythrin, peridin chlorophyll, allophycocyanin, cyanine 5 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-Igκ (187.1, BD Biosciences), anti-Igλ$_1$ (L22.18.2, gift from S. Weiss, Helmholtz Centre for Infection Research, Braunschweig, Germany), anti-Igλ$_{1,2,3}$ (R26−46, BD Biosciences) and anti-IgM (goat anti-mouse Fab, Jackson Immunoresearch). Data were acquired on a FACSCalibur (BD Biosciences) and analyzed with the FlowJo software (Tree Star).

B cell isolation

BM and splenic B cells were first enriched by magnetic depletion using the MACS system (Miltenyi Biotec). BM non-B cells were depleted using a cocktail of biotinylated antibodies (anti-CD4 (GK1.5, eBioscience), anti-CD3ε (145−2C11, eBioscience), anti-Gr1 (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. Splenocytes were depleted of non-B cells with CD43 microbeads. B cell subsets were then sorted on a FACSAria (BD Biosciences) using the surface markers described in figure legends. Cells were rested for 4 h in medium containing 0.5% fetal bovine serum before total protein extracts were prepared for EMSA using whole cell lysis buffer (20 mM Hepes pH 7.9, 350 mM NaCl, 20% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA pH 8.0, 0.1 mM EGTA pH 8.0, 1% NP-40 and protease inhibitors). Cell remnants were then treated with proteinase K, and DNA was subsequently prepared for LM-PCR and RS-PCR analyses.

EMSA

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

Analysis of J_κ usage

Total RNA was extracted using TRIzol (Invitrogen) from enriched splenic B cell preparations generated by CD43 magnetic bead depletion (Miltenyi Biotec). cDNA was synthesized following the ThermoScript RT-PCR system protocol (Invitrogen). V_κ-J_κ rearrangements were amplified by PCR using high fidelity Taq enzyme (Roche) and the following primers: V_κ-FW3 5'-AGCTTCAGTGGCAGTGGRTCWGGRAC; C_κ 5'-CTTCCACTTGACATTGATGTC (R = G ro A, W = A or T), as previously described50. PCR amplicons were then cloned using the TOPO-TA cloning kit (Invitrogen) and sequenced (DF-HCC DNA resource core facility). Unique sequences were analyzed for J_κ usage.

LM-PCR

The BW linker oligonucleotides (BW-1 5'-GCGGTGACCCGGGAGATCTGAATTC and BW-2 5'-GAATTCAGATC) were ligated to DNA overnight. A PCR reaction was then performed using linker (5'-CCGGGAGATCTGAATTCCAC), ko5 (5'-GCCCAAGCGCTTCCACGCATGCTTGGAG) and degenerated V_κ (5'-CCGAATTCGSTTCAGTGGCAGTGGRTCRGGRAC; S = G or C and R = A or G) primers to amplify primary (BW and ko5) or secondary (BW and V_κ) breaks21. A touchdown PCR program was used: 94°C for 1min, 19 cycles of 92°C for 30 s then 70°C for 40 s; and a 0.5°C drop in temperature with each successive cycle. This was followed by 19 cycles of 92°C for 30 s then 60°C for 40 s with 1 s added at each successive cycle. PCR products were separated by electrophoresis on an agarose gel and were analyzed by Southern blot with ko6 (5'-AGCCAGACAGTGGAGTACTACCAC) or J_κ rev (5'-GAGTAAGATTTTATACATCATTTTTAGACA) probes to detect primary and secondary breaks, respectively.

RS PCR

RS rearrangements were amplified from genomic DNA using RS reverse #1 primer (5'-GGACATCTACTGACAGGTTATCACAGGTC) and IRS forward #1 primer (5'-ATGACTGCTTGCCATGTAGATACCATGG) to detect RS-IRS recombination, or V_κS primer (see above) to detect V_κ-RS rearrangements. PCR conditions were as follows: 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The Adenine Phosphoribosyltransferase (APRT) loading control was amplified using APRT 747F (5'-TGCTAGACCAACCCGCACCCAGAAG) and APRT 964R (5'-TCGTGACCGCACCTGAACAGCAC) primers, and the following cycling conditions: 95°C for 5 minutes, followed by 21 cycles of 95°C for 30 seconds, 63°C for 30 seconds, and 72°C for 30 seconds.

BrdU labeling

5-bromo-2-deoxyuridine (BrdU) labeling of DNA was performed using BrdU Flow Kit (BD Biosciences) according to manufacturer's instructions. 129/Sv and iEκT/T mice were injected with 1 mg BrdU intraperitoneally and analyzed at 8, 24, 28, 32 and 36 h thereafter. BM cells were stained with anti-B220, anti-IgM, anti-Igκ or anti-Igλ$_1$ and anti-BrdU. The proportions of Igκ$^+$ and Igλ$_1$$^+$ immature B cells (B220^{lo}IgM$^+$) positive for BrdU were determined by flow cytometry on a FACSCalibur. Best-fit regression curve analysis was determined using Prism software (GraphPad Software). The 0 h time point of the graph shows anti-BrdU background staining in non injected mice.

Quantitative real-time PCR

total RNA was extracted with RNeasy Micro kit (Qiagen) from FACS-purified pre-B cells and cDNA synthesis was performed using the Thermoscript RT-PCR system plus (Invitrogen) according to the manufacturer's protocol. Quantitative real-time PCR was done using Power SYBR green and analyzed with a StepOne plus real-time PCR system (Applied Biosystems). Primers for *Rag1* (5'-TTGCTATCTCTGTGGCATCG and 5'-AATTTCATCGGGTGCAGAAC), *Rag2* (5'-AGTGACTCTTCCCCAAGTGC and 5'-CTTCCTGCTTGTGGATGTGA), *Nfkb2* (5'-TTTCCTTCGAGCTAGCGATG and 5'-TTCGGGAGATCTTCAGGTTC), *Bcl-x$_L$* (5'-GGTGAGTCGGATTGCAAGTT and 5'-GCTGCATTGTTCCCGTAGAG), and *γ-actin* (5'-GGTGTCCGGAGGCACTCTT and 5'-TGAAAGTGGTCTCATGGATACCA) were used to detect the transcripts. *Pim2* and *Bcl3* primers have been described elsewhere11. Samples were analyzed in duplicates and messenger abundance was normalized to *γ-actin*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We regret that for space reasons we often had to quote reviews instead of the original papers. We thank S. Akira (Osaka University), F. Alt (Children's Hospital, Boston), R. Bram (Mayo Medical School, Rochester), R. Geha (Children's Hospital, Boston), T. Mak (Campbell Family Institute for Cancer Research, Toronto), D. Nemazee (The Scripps Research Institute, La Jolla) and M. Reth (University of Freiburg) for the *Myd88*$^{-/-}$, *Atm*$^{-/-}$, *Taci*$^{-/-}$, *Bcl10*$^{-/-}$, κ-macroself and *mb1-cre* cells and mouse strains; T. Chakraborty, G. Galler and S. Koralov for help with the quantitative real-time PCR, EMSA and cell cycle analysis; M. Bamberg for antibody preparation and N. Barteneva for cell sorting; D. Ghitza, A. Pellerin, J. Grundy and J. Xia for technical assistance with mouse work. M.S-S. is a recipient of an Emmy Noether grant from the DFG. Supported by the US National Institutes of Health (M.S.S. and K.R.).

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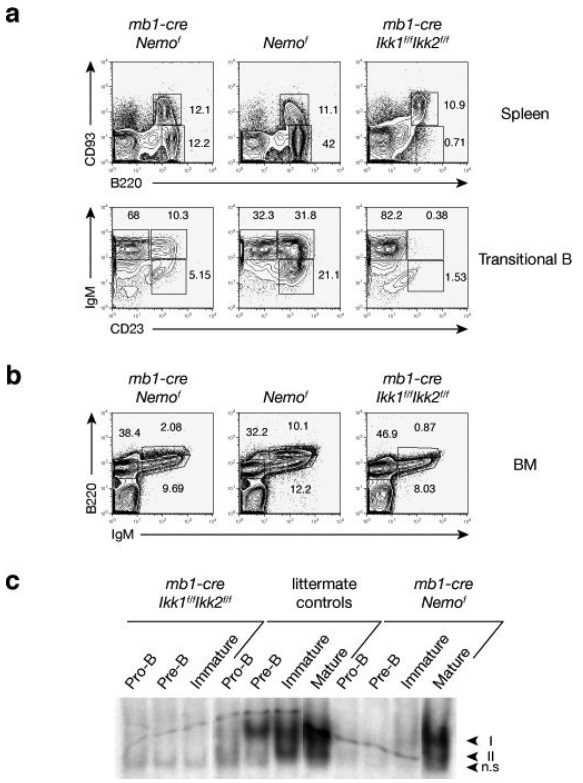

Figure 1.

B cell development in the absence of NF-κB signaling. (**a**) Transitional ($B220^{+}CD93^{+}$) and mature ($B220^{+}CD93^{-}$) B cells (top) and T1 ($IgM^{+}CD23^{-}$), T2 ($IgM^{+}CD23^{+}$) and T3 ($IgM^{lo}CD23^{+}$) transitional subsets (bottom) in splenocytes of the indicated mice (*n*=7) were examined by flow cytometry. Numbers indicate percentages of cells within each gate. Gated on lymphocytes (top) or transitional B cells (bottom). (**b**) BM leukocytes from the indicated mice were stained for B220 and IgM to identify proand pre-B cells ($B220^{lo}IgM^{-}$), immature B cells ($B220^{lo}IgM^{+}$) and recirculating ($B220^{hi}IgM^{+}$) B cells. Numbers indicate percent cells within each gate. Gated on lymphocytes. (**c**) Electrophoretic mobility shift assay (EMSA) of NF-κB DNA-binding activity in total extracts of B cell subsets from indicated mice. Pro-B ($B220^{lo}IgM^{-}$c-Kit^{+}), pre-B ($B220^{lo}IgM^{-}CD25^{+}$), immature ($B220^{+}IgM^{+}$) and mature ($B220^{+}CD93^{-}$) B cells were purified by FACS from 6−8 mice per experimental group. EMSA has been performed twice. I and II, NF-κB complexes; n.s, non-specific.

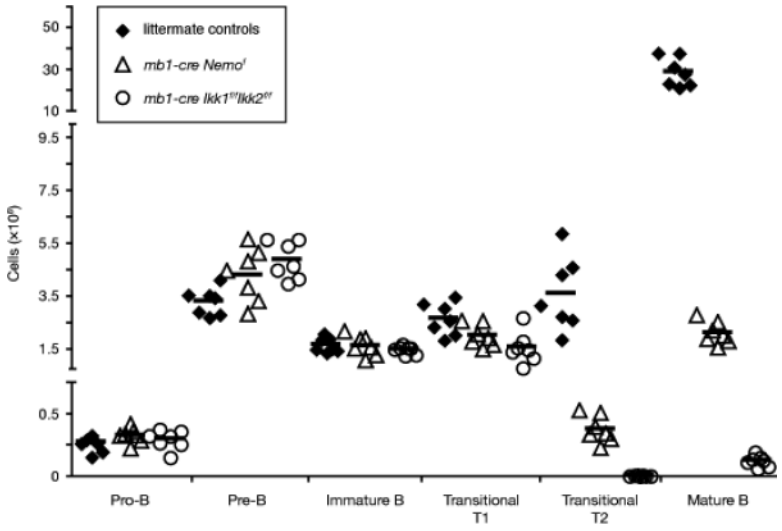

Figure 2.

B cell numbers are reduced from the T2 stage of B cell development onwards in the absence of NF-κB signaling. Absolute numbers of BM pro-B ($B220^{lo}IgM^{-}c\text{-}Kit^{+}$), pre-B ($B220^{lo}IgM^{-}CD25^{+}$), and immature B ($B220^{lo}IgM^{+}$) cells, as well as splenic T1 ($B220^{+}CD93^{+}IgM^{+}CD23^{-}$), T2 ($B220^{+}CD93^{+}IgM^{+}CD23^{+}$) and mature B ($B220^{+}CD93^{-}$) cells from littermate control (*n*=7), *mb1-cre Nemo*^{f} (*n*=7) and *mb1-cre Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ (*n*=7) mice. One control mouse was excluded from the analysis because splenic B cell numbers were aberrantly higher than in the other control mice.

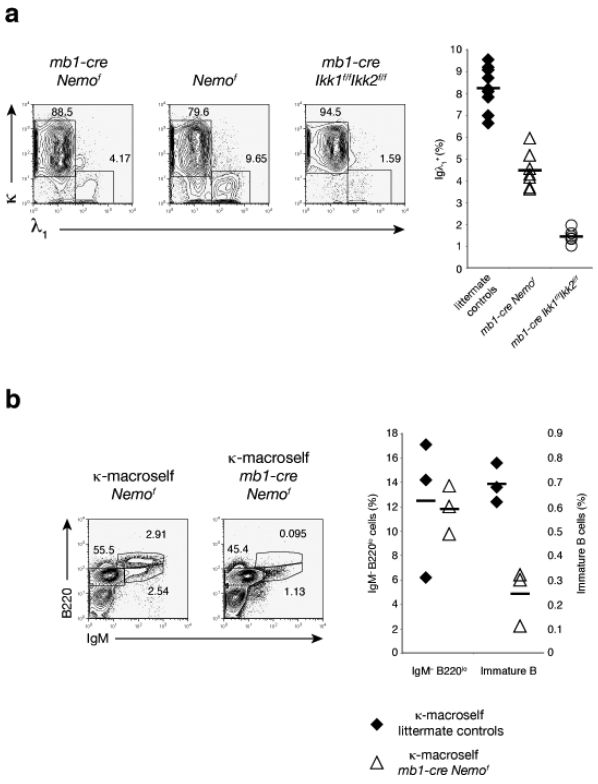

Figure 3.

Impaired generation of Igλ^+ B cells in the absence of IKK-mediated NF-κB activation. (**a**) BM immature B cells (B220^{lo}IgM$^+$) from *mb1-cre Nemo^f* (*n*=7), *mb1-cre Ikk1$^{f/f}$Ikk2$^{f/f}$* (*n*=7) and littermate control (*n*=9) mice were stained for Igκ and Igλ_1 expression. Left, representative contour plots. Numbers indicate percent cells in each gate. Gated on immature B cells. Right, proportion of Igλ_1^+ immature B cells. Each symbol represents one mouse, and black bars depict arithmetic mean. (**b**) Pro- and pre-B (IgM$^-$B220^{lo}), immature (B220^{lo}IgM$^+$) and recirculating (B220^{hi}IgM$^+$) B cells in the BM of *mb1-cre Nemo^f* (*n*=3) and littermate control (*n*=3) mice expressing the κ-macroself transgene were determined by flow cytometry. Left, representative contour plots. Numbers indicate percent cells within each gate. Gated on lymphocytes. Right, percent total BM IgM$^-$B220^{lo} and immature B cells (B220^{lo}IgM$^+$) from indicated mice. Each symbol represents one mouse, and black bars depict mean.

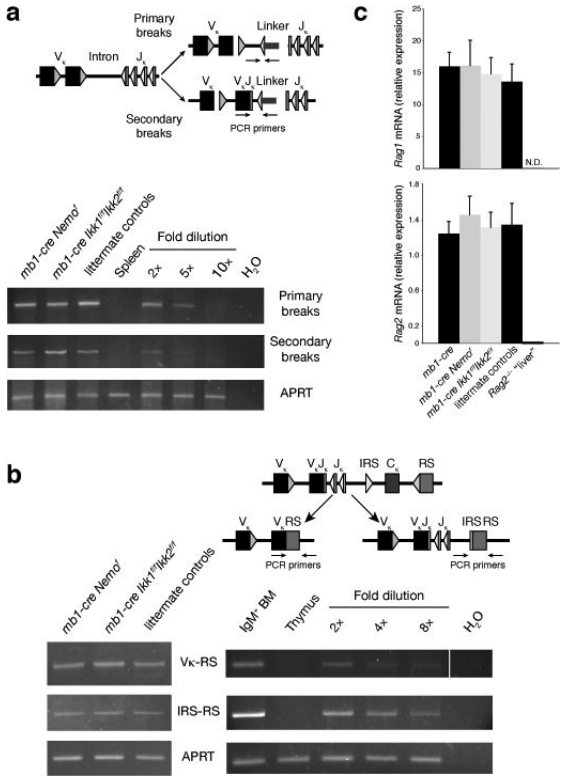

Figure 4.

Receptor editing at the *Igk* locus is not affected in the absence of IKK-mediated NF-κB activation. (**a**) LM-PCR analysis of primary and secondary breaks at the *Igk* locus of pre-B cells purified from *mb1-cre Nemo*^{f}, *mb1-cre Ikk1*$^{f/f}$*Ikk2*$^{f/f}$ and littermate control mice. The sensitivity of the assay was tested by diluting wild-type pre B cell DNA with wild-type mature B cell DNA that should not contain DNA breaks at the *Igk* locus. Genomic DNA quantity was determined by optical density and equal loading of template DNA was confirmed by APRT amplification. The cartoon details the strategy used to analyze 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 the indicated mice. The sensitivity of the assay was tested by diluting wild-type BM IgM^{+} B cell DNA with thymus DNA that should not contain RS rearrangement. PCR amplification of APRT served as a genomic DNA template control. The cartoon depicts rearrangements between the RS and either a V_{κ} segment or the IRS sequences. (**a**) and (**b**) were repeated, using two different sets of samples. (**c**) *Rag1* and *Rag2* mRNA expression in pre-B cells ($B220^{lo}CD93^{+}IgM^{-}CD25^{+}$) from the indicated mice and from the liver of *Rag2*$^{-/-}$ mice was determined by quantitative fluorescence real-time PCR. mRNA abundance was normalized to *γ-actin* mRNA expression. Mean and s.d. was calculated from mRNA isolated from 3 mice per genotype except for the liver control. N.D., not detected.

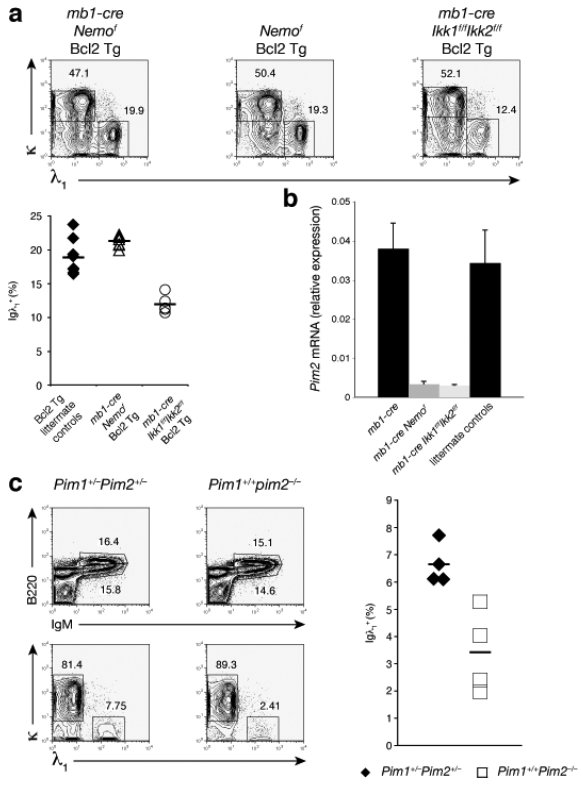

Figure 5.

Over-expression of Bcl2 in the B cell lineage rescues Igλ$^+$ B cell generation in absence of IKK-mediated NF-κB activation. (**a**) Igκ$^+$ and Igλ$_1^+$ BM immature B (B220^{lo}IgM^{lo}) cells from *mb1-cre Nemo^f* (*n*=7) *mb1-cre Ikk1$^{f/f}$Ikk2$^{f/f}$* (*n*=5) and littermate controls (*n*=8) expressing a Bcl2 transgene (Bcl2 Tg) were examined by flow cytometry. Immature B cells were defined as B220^{lo}IgM^{lo} cells to exclude from the analysis an interfering B220$^+$IgM^{hi} B cell population present in Bcl2 Tg mice. Top, representative contour plots. Numbers indicate proportions of cells in each gate. Gated on immature B cells. Bottom, percent Igλ$_1^+$ immature B cells in indicated mice. Each symbol represents one mouse and black bars indicate mean. (**b**) *Pim2* mRNA expression in B220^{lo}CD93$^+$IgM$^-$CD25$^+$ pre-B cells sorted from the indicated mice was determined by quantitative fluorescence real-time PCR. mRNA abundance was normalized to *γ-actin* mRNA. Mean and s.d. was calculated for 3 mice per genotype. (**c**) BM immature B cells (B220^{lo}IgM$^+$) from *Pim1$^{+/-}$Pim2$^{-/-}$* (*n*=4) and *Pim1$^{+/-}$Pim2$^{+/-}$* (*n*=4) mice were stained for Igκ and Igλ$_1$ expression. Left, representative contour plots. Numbers indicate percent cells within each gate. Gated on immature B cells. Right, proportions of Igλ$_1^+$ immature B cells in indicated mice. Each symbol represents one mouse and black bars depict mean.

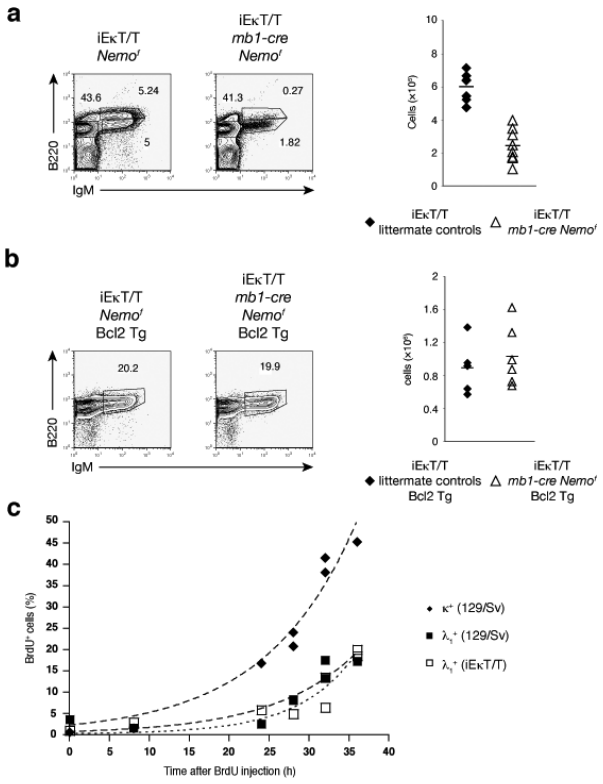

Figure 6.

Impaired generation of immature NEMO-deficient Igλ$^+$ B cells in the absence of rearrangements at *Igk* loci. (**a**) BM cells from iEκT homozygous mice (iEκT/T)— which cannot undergo *Igk* rearrangements— on wild-type (*n*=7) or NEMO-deficient (*n*=9) genetic backgrounds were stained with anti-B220 and anti-IgM to identify immature (B220^{lo}IgM$^+$) and recirculating (B220^{hi}IgM$^+$) B cells. Left, representative contour plots. Numbers indicate percent cells within each gate. Gated on lymphocytes. Right, immature B cell numbers in indicated mice. Each symbol represents one mouse and black bars depict mean. (**b**) Immature B cells (B220^{lo}IgM$^+$CD93$^+$CD23$^-$) in the BM of *mb1-cre Nemo^f* iEκT/T (*n*=6) and *Nemo^f* iEκT/T (*n*=5) mice over-expressing Bcl2 were examined by flow cytometry. (The combination of fluorochromes in this analysis allowed inclusion of CD93 and CD23 as additional markers to separate immature B cells from an interfering B220$^+$IgM^{hi} B cell population present in Bcl2 Tg mice) Left, representative contour plots. Numbers indicate percent cells within each gate. Gated on CD93$^+$CD23$^-$ cells. Right, immature B cell numbers in indicated mice. Each symbol represents one mouse and black bars depict mean. (**c**) BrdU incorporation in Igκ$^+$ and Igλ$_1{}^+$ immature B cells (B220^{lo}IgM$^+$). Mice were injected intra-peritoneally with BrdU and analyzed at the indicated time points thereafter. The percentage of BrdU$^+$Igκ$^+$ and BrdU$^+$Igλ$_1{}^+$ immature B cells in iEκT/T and littermate control mice (129/Sv) was determined by flow cytometry.

Table 1

$J_{\kappa 4-5}$ usage is not decreased in NEMO-deficient B cells. Splenocytes from Mb1-Cre*NEMO*$^{f/y}$ iEκT/+ (*n*=3) and *NEMO*$^{f/y}$ iEκT/+ (*n*=2) mice were subjected to CD43^{+} cell depletion and total RNA was prepared. Functional endogenous V_{κ}-J_{κ} rearrangements were amplified by RT-PCR and percent of rearrangements involving $J_{\kappa 1}$, $J_{\kappa 2}$, $J_{\kappa 4}$ and $J_{\kappa 5}$ were calculated among the unique sequences.

	J_{κ}	Unique sequences	%	$J_{\kappa 1-2}/J_{\kappa 4-5}$ %
Mb1-Cre^{-} *NEMO*$^{f/y}$ iEκT/+	$J_{\kappa 1}$	47	30.7	65.4
	$J_{\kappa 2}$	53	34.6	
	$J_{\kappa 4}$	21	13.7	34.6
	$J_{\kappa 5}$	32	20.9	
Mb1-Cre^{+} *NEMO*$^{f/y}$ iEκT/+	$J_{\kappa 1}$	64	25.6	52.4
	$J_{\kappa 2}$	67	26.8	
	$J_{\kappa 4}$	40	16.0	47.6
	$J_{\kappa 5}$	79	31.6	