Synergism between NF-κB1/p50 and Notch2 during the Development of Marginal Zone B Lymphocytes

Stewart T. Moran, Annaiah Cariappa, Haoyuan Liu, Beth Muir, Dennis Sgroi, Cristian Boboila and Shiv Pillai

*J Immunol* 2007; 179:195-200; doi: 10.4049/jimmunol.179.1.195

http://www.jimmunol.org/content/179/1/195

**Why *The JI***?

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**  This article **cites 40 articles**, 19 of which you can access for free at: http://www.jimmunol.org/content/179/1/195.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Synergism between NF-κB1/p50 and Notch2 during the Development of Marginal Zone B Lymphocytes

Stewart T. Moran,2 Annaiah Cariappa,2 Haoyuan Liu, Beth Muir, Dennis Sgroi, Cristian Boboila, and Shiv Pillai3

NF-κB1 and Notch2 are both required for the development of marginal zone (MZ) B cells. Analysis of B lymphocyte development in mice that are doubly heterozygous at the Notch2 and NF-κB1 loci revealed synergism between Notch2 and NF-κB1 during MZ B cell development. Two known transcriptional targets of the Notch pathway, Hes-5 and Deltex-1, were found to be preferentially expressed in MZ B cells and regulated by NF-κB1. These studies provide in vivo evidence for a genetic interaction between the Notch and NF-κB pathways. The Journal of Immunology, 2007, 179: 195–200.

The Notch and NF-κB protein families represent two major evolutionarily conserved pathways that affect cell fate and cell survival in all known metazoans, but genetic interactions between these pathways have not been hitherto described. Notch proteins are cell surface molecules that influence binary cell fate decisions in all multicellular organisms. The activation of a Notch receptor results in the proteolytic liberation of its intracellular domain, which is then translocated to the nucleus. The cleaved intracellular domain of Notch forms ternary complexes with recombination signal binding protein-Jκ (RBP-Jκ) and mastermind-like proteins, resulting in the conversion of RBP-Jκ from a transcriptional repressor to an activator of transcription that ultimately mediates the induction of a set of target genes (reviewed in Ref. 1).

The NF-κB pathway, like the Notch pathway, also influences cell fate decisions in metazoans. It is also relevant in terms of the induction of gene expression in a number of other biological contexts, including inflammation and cancer. There are five distinct NF-κB proteins in vertebrates and these exist as homodimers or heterodimers that are retained in the cytosol by IκB proteins before activation. Following the ubiquitination and proteasomal degradation of IκB, NF-κB dimers are released and enter the nucleus. IκB is marked for ubiquitination following its phosphorylation by the IκB kinase (IκK) complex, which is comprised of two catalytic subunits, IKKα/IKK-1 and IKKβ/IKK-2, and a regulatory subunit, IKKγ/NEMO (NF-κB essential modulator). Two of the five members of the NF-κB family, NF-κB1/p50 and NF-κB2/p52, possess DNA-binding Rel-homology domains, but lack a transactivation domain. The other three members, p65/RelA, RelB, and c-Rel, all possess DNA binding as well as transactivation domains (2). As a result, NF-κB activates the transcription of target genes as a heterodimer, generally with p65 or c-Rel, and less frequently with RelB. NF-κB p50 homodimers lack the ability to activate transcription and may recruit histone deacetylases resulting in transcriptional repression (3).

Both Notch and NF-κB are key players in lymphocyte development. Although Notch1 is a crucial mediator of the T vs B lymphoid cell fate decision (4, 5), Notch2 is required at the mature follicular (FO) vs marginal zone (MZ) B lymphoid cell fate transition. MZ B cells are lost but FO B cells are preserved in mice in which Notch2 or RBP-Jκ are conditionally deleted in the B lineage (6, 7). In addition, the deletion of the gene that encodes Delta-like-1, a ligand for Notch proteins, or the overexpression of a dominant-negative mutant of mastermind-like-1, also results in the absence of MZ B cells (8, 9), while the loss of MINT (Mx2 interacting nuclear target protein), a negative regulator of the Notch pathway, contributes to an increase in MZ B cells (10). Signaling via Notch2 is therefore required for the development of MZ B cells. The role of Notch2 during peripheral B cell development may not be restricted to the generation or maintenance of MZ B cells. Although conditional Notch2−/− mice exhibited no defect in peritoneal B-1 B cells (6), examination of Notch2−/− B cells in another study implicated Notch2 in peritoneal B-1 B cell development (11).

Mice that lack NF-κB1 have a marked reduction of MZ B cells (12). A less prominent reduction in MZ B cell numbers was noted in Rag-2−/− mice that were reconstituted with p65−/− hemopoietic stem cells, or in mice lacking c-Rel (12). These data suggest that while NF-κB1/p65 and NF-κB1/c-Rel both contribute to MZ B cell development, the requirement for NF-κB1 as a component of these heterodimers is stringent. Alternatively, it remains formally possible that NF-κB1 homodimers are required to repress crucial targets to permit MZ B cell differentiation. Regardless of whether NF-κB1 functions as a transcription activator or a repressor during MZ B cell development, it is also possible that it is required for MZ B cell generation because it functions as a negative regulator of the BCR/Bruton’s tyrosine kinase (Btk) pathway. The loss of negative regulators of BCR signaling, such as Aiolos and CD22, can contribute to a block in MZ B cell development (13, 14).

Cancer Center, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02129

Received for publication January 2, 2007. Accepted for publication April 30, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Institutes of Health Grants AI 064930 and CA102793.

2 S.T.M. and A.C. were equal contributors.

3 Address correspondence and reprint requests to Dr. Shiv Pillai, Massachusetts General Hospital Cancer Center, Building 149, 13th Street, Charlestown, MA 02129. E-mail address: pillai@helix.mgh.harvard.edu

4 Abbreviations used in this paper: IκK, IκB kinase; FO, follicular; MZ, marginal zone; Btk, Bruton’s tyrosine kinase; BAFF, B cell-activating factor of the TNF family; BAF-F-R, BAFF receptor; FO-I, FO type I; FO-II, FO type II; MZP, MZ precursor; NF, newly formed.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00

www.jimmunol.org

Downloaded from http://www.jimmunol.org/ by guest on October 30, 2017
Many other genes contribute to the generation or maintenance of MZ B cells, some at the level of cell survival and others in maintaining MZ B cells in their niche (14). Signaling via the B cell-activating factor of the TNF family (BAFF) receptor (BAFF-R) may drive MZ development as suggested by the expansion of MZ B cells in BAFF-transgenic mice (15); BAFF may contribute to the activation of the canonical NF-κB pathway and thus contribute not just to MZ B cell development but possibly to maintenance as well (16). Other proteins that are believed to influence MZ B cell survival include CD19 (17) and PI3Kp110 (18, 19). The LFA-1 (16). Other proteins that are believed to influence MZ B cell just to MZ B cell development but possibly to maintenance as well (16).

Some biochemical interactions between Notch and NF-κB have been described in studies in cell lines, but no in vivo or genetic links between these pathways have been established in any species studied. One such biochemical interaction is the association of Notch with NF-κB1 in overexpression studies in human cell lines and this interaction has been linked to the inhibition of NF-κB translocation activity by Notch (25). In other studies, IκBα and p65 have been shown to influence the regulation of Notch target gene expression (26, 27). c-Rel has also been shown to be capable of regulating the expression of Jagged 1, a Notch ligand, and to thus be capable of regulating Notch signaling (28).

In this study, we have used a genetic approach to demonstrate that NF-κB1 is not a negative regulator of Btk. NF-κB1 is required for the development of B-1 B cells as well as of MZ B cells. NF-κB1 and Notch2 function synergistically during MZ B cell development as revealed by the analysis of splenic B cell development in mice haploinsufficient for both these genes, but no evidence was found for a genetic interaction between Notch2 and NF-κB1 during B-1 B cell development. These studies further our understanding of an important cell fate decision in lymphocytes and provide the first in vivo evidence that the Notch and NF-κB pathways interact.

Materials and Methods

Mice

p50-null mice were obtained from The Jackson Laboratory and have been described previously (12). Notch2+/− mice were provided by Dr. Y Hamada (National Institute for Basic Biology, Okazaki Japan). C57BL/6 mice and xid (CBA/N) mice were purchased from The Jackson Laboratory. Xid/p50-null double mutants were generated in a manner similar to that described for Aiolos+/−/xid mice (13). p50−/− mice were mated with N2+/− mice to generate double heterozygous mice that were then intercrossed to obtain N2+/−/p50−/− or N2+/−/p50−/− mice. All mice were housed in a pathogen-free facility. Animal procedures were cleared by the Subcommittee on Research Animal Care at Massachusetts General Hospital.

Flow cytometric analysis and FACS

Single-cell suspensions from the spleen were obtained using standard methods. Peritoneal B cells were harvested by injecting 10 ml of PBS containing 0.2% BSA into the peritoneal cavity followed by aspiration with an 18-gauge needle. Flow cytometry and flow sorting were performed as previously described (29). The following murine mAb conjugates were used: r-PE (R-PE)-1B4B1 (anti-IgM, rat IgG2a, κ; both obtained from Southern Biotechnology Associates), and FITC-7G6 (anti-CD21/CD35, rat IgG2b, BD Pharmingen). Biotinylated Abs were revealed using streptavidin-allophycocyanin (BD Pharmingen). Flow cytometric analysis was performed on a dual-laser FC500 (Beckman Coulter) and sorting was performed on a MoFlo sorter (DakoCytomation). The purity of sorted samples exceeded 96%. Gates in the spleen were set according to Hardy et al. (30) and Cariappa et al. (13, 31). Processed sample data were analyzed using FloJo version 8.2 software (Tree Star).

TagMan RT-PCR analysis

Splenocytes from C57BL/6 and p50 mutant mice were stained as described above with anti-IgM, IgD, and CD21 Abs; IgMhighIgDlow/CD21high MZ B cells, IgM+/−IgD+/−CD21+/− FO type 1 (FO-I), and IgM+/−IgD+/−CD21+/− FO type II (FO-II) were sorted. Total RNA was isolated from each sorted fraction by the Absolutely RNA Microprep kit (Stratagene) and converted into cDNA. TaqMan quantitative RT-PCR and primer design was performed as previously described (32). Primers used were: Deltex 1 forward CTGATGCGCGCTCACAGT, reverse CATGCACCCACCCATAG; TaqMan probe CTGAGCAGGCAGCAAAGCGTTAACTTC. Hes5 formed as previously described (32). Primers used were: Xid 1 forward TGTTCCAGGTATACAGCCATCAA, reverse CCACCGCCACCTTCA AG; TaqMan probe CAGGAGGGAAGCAATACGTTCTT. Hex5 forward CTAAGGAGGCCGTCAG; reverse CACGGCCACGTCAGC ACAA; TaqMan probe TCTCCACGATGATCCTTAAAGGATT.
Results

The defect in MZ B cell development in NF-κB1-null mice is not linked to the activation of Btk. Aiolos-null mice present with an enhancement in BCR signal strength and a significant defect in MZ B cell development. In double mutant mice that lack Aiolos and also carry the xid mutation (an inactivating point substitution within the pleckstrin homology domain of Btk), the MZ B cell population is restored, presumably because Btk-dependent BCR signal strength is no longer enhanced (13). Like Aiolos-deficient mice, NF-κB1 mutant mice present with decreased numbers of MZ B cells (12). We used a similar genetic approach to the one used with Aiolos-null mice to determine whether the decreased numbers of MZ B cells in NF-κB1 mutant mice may be attributed to enhanced signaling via Btk.

We crossed the NF-κB1-null mutation onto the xid (CBA/N) background and, in contrast to the “reappearance” of the IgMhighIgDlowCD21int MZ B cell fraction observed in xid/Aiolos−/− mice (13), xid/NF-κB1−/− mice present with a virtually complete absence of MZ B cells (Fig. 1). Although there appears to be an increase in the newly formed (NF/T) B cell population in the double mutant mice (right side panel of Fig. 1), this is an apparent increase that reflects the paucity of MZ B cells. We conclude that the loss of MZ B cells in NF-κB1 mutant mice cannot be attributed to increased Btk-derived signals.

NF-κB1 is required for B-1 B cell development

NF-κB1 is activated downstream of the BAFF-R in developing and mature B cells (16). Although Notch2 may be required for...
B-1 B cell development (11), BAFF knockout mice exhibit normal B-1 B cell development (33). It was of some interest to therefore examine whether NF-κB1-null mice have a defect in B-1 B cell development. As can be seen from Fig. 2, peritoneal IgM<sup>high</sup>IgD<sup>high</sup>CD5<sup>+</sup> B-1a B cell development is markedly compromised in the absence of NF-κB1. A less striking reduction in peritoneal IgM<sup>high</sup>IgD<sup>high</sup>Mac-1<sup>+</sup> B-1b B cells was also noted in these mice.

NF-κB1 and Notch2 synergize during the development of MZ B cells

NF-κB1-null mice, Notch2 conditionally null mice, as well as Notch2+/−/− mice all present with a significant reduction in MZ B cells (6, 11, 12). We therefore entertained the possibility that Notch2 and NF-κB1 might cooperate to influence MZ B cell development. To test this hypothesis, we analyzed FO and MZ B cell development in NF-κB1+/−, Notch2+/−, and NF-κB1−/−/Notch2−/− doubly heterozygous mice.

We have previously suggested that IgM<sup>high</sup>IgD<sup>high</sup>CD21<sup>int</sup> B cells may represent the last common precursor of MZ and IgD<sup>high</sup>IgM<sup>low</sup> mature FO B cells (14, 34, 35). The majority of these cells are long-lived posttransitional B cells (A. Cariappa, H. Liu, C. Boboila, S. T. Moran, and S. Pillai, submitted for publication). These cells are present in mutants, such as the Aiolos-null and Notch2+/−/− mice, that lack MZ B cells, but are also preserved in xid mice and other mutants in the Btk pathway in which IgD<sup>high</sup>IgM<sup>low</sup> mature FO B cells fail to develop or are lost. We currently refer to IgM<sup>high</sup>IgD<sup>high</sup>CD21<sup>int</sup> B cells as FO-II cells. We distinguish them from IgD<sup>high</sup>IgM<sup>low</sup> mature FO B cells, which we refer to as FO-I B cells. Consistent with previous reports, NF-κB1−/− mice have no obvious defect in splenic B cell development and Notch2−/−/− mice have a reduction in a presumed MZ precursor (MZP; IgM<sup>high</sup>IgD<sup>high</sup>CD21<sup>high</sup>) population and in MZ (IgM<sup>high</sup>IgD<sup>low</sup>CD21<sup>high</sup>) B cells, while other B cell populations (NF/T1, IgM<sup>low</sup>IgD<sup>high</sup>CD21<sup>low</sup>; FO-II, IgM<sup>high</sup>IgD<sup>high</sup>CD21<sup>int</sup>; and IgM<sup>low</sup>IgD<sup>low</sup>CD21<sup>int</sup> FO-I B cells) are not reduced (6, 11, 12) (Fig. 3). Strikingly, in NF-κB1−/−/Notch2−/− doubly heterozygous mice, there is an almost complete absence of MZP and MZ B cells (Fig. 3; see Table I for absolute numbers). These data establish that the NF-κB1 and Notch2 genes interact genetically and function synergistically to promote MZ B cell development.
However, in contrast, examination of peritoneal B-1 B cell development in NF-κB1+/−/Notch2+/− and NF-κB1−/−/Notch2+/− mice revealed no evidence of synergism between Notch2 and NF-κB1 during B-1B cell development (Fig. 4).

**Hes5 and Deltex1 are regulated by NF-κB1**

There are a number of potential ways in which the Notch2 and NF-κB1 transcriptional regulators could potentially synergize during MZ B cell development. One possible mechanism is the cooperative transcriptional regulation of target genes. The Deltex1 (Dtx1) gene is known to be positively regulated by Notch2 and to be expressed at higher levels in MZ B cells than in other B cell populations (6). The hairy/enhancer of split homolog 5 (Hes5) is also expressed most strongly in MZ B cells (6). We tested the hypothesis that these known Notch targets might represent NF-κB1-regulated genes in B cells. We performed quantitative real-time RT-PCR on RNA obtained from wild-type and NF-κB1 mutant FO-II cells (the cell type that represents the presumed last common precursor for both MZ B cells and FO-I B cells) as well as on RNA extracted from wild-type MZ and FO-I B cells. Fig. 5A shows that the IgMhighIgDhighCD21int FO-II B cell population is preserved in NF-κB1−/− mice even though the MZP B cell population is reduced. As shown in Fig. 5B, Hes5 and Dtx1 are more highly expressed in wild-type FO-II cells than in NF-κB1-null FO-II cells, suggesting that NF-κB1 is a positive regulator of these genes. In addition, quantitative real-time RT-PCR analysis confirmed that both Hes5 and Dtx1 are more highly expressed in wild-type MZ B cells relative to FO-I B cells. These data suggest that in differentiating B cells, some genes, such as Deltex1, may be dually regulated by both Notch2 and NF-κB1.

**Discussion**

We have observed a strong genetic interaction between two hemizygous loci. Such collaboration between two distinct heterozygous null alleles has very rarely been observed in studies of mouse development. Although Notch2+/− mice do have a clearly discernible reduction in MZ B cells, Notch2+/−/NF-κB1+/− mice present with a far more striking phenotype. Our genetic analyses show that the IgMhighIgDhighCD21int FO-II B cell population is a common precursor for both MZ B cells and FO-I B cells (the cell type that represents the presumed last common precursor for both MZ B cells and FO-I B cells) as well as MZ B cell development or survival.

We have shown that both Deltex1 and Hes5 are expressed at relatively high levels in MZ B cells and that both these genes are expressed in the B lineage in an NF-κB1-dependent manner. The dependence of Deltex1 expression on Notch2 is clear but the evidence for Notch2-dependent regulation of Hes5 is weaker (6). Our data are consistent with the possibility that a set of genes may be coordinately regulated by Notch2 and NF-κB to orchestrate a program that potentially commits developing B cells to a MZ B cell fate. Clearly a large set of genes, and not merely Dtx1 (and possibly Hes5), may be dual Notch and NF-κB targets. It will be necessary to perform chromatin immunoprecipitation assays at specific stages of B cell development to examine the in vivo occupancy by transcription factors of regulatory sites in putative target genes to fully reveal the transcriptional targets of Notch2 and NF-κB that contribute to MZ B cell development.

What signaling receptor is responsible for the induction of NF-κB1-containing heterodimers during MZ B cell development? The actual signaling pathway that contributes to the activation of NF-κB during MZ B cell development remains unclear. Although basal or low-level BCR signaling may be the source of NF-κB activation during MZ B cell development, the participation of NF-κB1 does not appear to depend on the activation of the BCR/Blk phospholipase Cγ pathway, because this latter pathway is not required for MZ B cell development. A likely source of NF-κB activation in the context of MZ B cell development is the BAFF-R.

BAFF-null mice present with a severe reduction of both MZ and FO B cell populations (33) and this defect is phenocopied by conditional IKKγ-null mice defective in the canonical NF-κB-signaling pathway (36). Constitutively active IKKβ can rescue the BAFF-R defect, indicating that canonical NF-κB signaling during B cell development is largely mediated by the BAFF-R (16). BAFF signaling may mediate the survival of peripheral B cells by posttranslationally down-regulating the expression of Bim (37). However, it might separately contribute to the transcriptional induction of target genes in collaboration with Notch2 to mediate MZ B cell development or survival.

It is interesting that there appears to be an absolute requirement for NF-κB1, an NF-κB protein that lacks an activation domain, during MZ B cell development. We assume that the activation in vivo of a subset of genes required for MZ B cell development depends on NF-κB1-containing heterodimers, and that c-Rel homodimers for instance, cannot functionally replace NF-κB1 containing heterodimers in this context, although they may suffice for FO B cell survival. These stringent in vivo requirements are consistent with the growing appreciation that the composition and posttranslational modifications of NF-κB dimers critically influence target gene activation (2, 38). We have previously demonstrated that the absence of c-Rel or of p65/RelA only partly reduces MZ B cell numbers (12), suggesting that both NF-κB1/c-Rel and NF-κB1/p65 heterodimers may contribute to MZ B cell development. The requirement for NF-κB1 is particularly intriguing when one considers that BAFF signaling via the alternative pathway is likely to contribute to the processing of p100 to yield NF-κB2 in all B cells in lymphoid follicles (39), suggesting indirectly that NF-κB2 cannot substitute for NF-κB1 during MZ B cell development. Nevertheless, there appears to be an independent B cell-intrinsic as well as a B cell-extrinsic requirement for RelB during MZ B cell development (40), suggesting a requirement for both the canonical and alternative NF-κB pathways during MZ B cell development and/or survival.

Our studies suggest that Notch2 and NF-κB1 function in a different way during B-1 B cell development, distinct from their synergistic roles in MZ B cells. The role of Notch2 in B1 B cell
development remains unclear—a defect was not seen in conditional Notch2−/− mice but was observed in a separate study examining Notch2−/− heterozygotes. How exactly NF-κB1 functions in B-1 B cell development is also unclear, and given that limited role, if any, for Btk during MZ B cell development, it may well be that the BCR/Btk pathway induces the activity of NF-κB1-containing heterodimers during B-1 B cell development.

In conclusion, our results indicate that Notch2 and NF-κB1 function synergistically in vivo during the development of MZ B cells. The identification of the specific Notch and NF-κB1 targets that are required during MZ B cell development will be essential to obtain a detailed molecular elucidation of the programs that drive the development of cells of this lymphoid lineage.

Acknowledgments
We thank Joanne Yetze-Aldape, John Duley, and Susan Lazo-Kallian for their contributions to flow cytometric sorting.

Disclosures
The authors have no financial conflict of interest.

References
20. Espinosa, L., J. Ingles-Esteve, E.