

# Differential Requirement for Rel/Nuclear Factor $\kappa$ B Family Members in Natural Killer T Cell Development

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## Abstract

Natural killer T (NKT) cells have been implicated in diverse immune responses ranging from suppression of autoimmunity to tumor rejection. Thymus-dependent NKT cells are positively selected by the major histocompatibility complex class I-like molecule CD1d, but the molecular events downstream of CD1d are still poorly understood. Here, we show that distinct members of the Rel/nuclear factor (NF)- $\kappa$ B family of transcription factors were required in both hematopoietic and nonhematopoietic cells for normal development of thymic NKT cells. Activation of NF- $\kappa$ B via the classical I $\kappa$ B $\alpha$ -regulated pathway was required in a cell autonomous manner for the transition of NK-1.1-negative precursors that express the TCR V $\alpha$ 14-J $\alpha$ 18 chain to mature NK-1.1-positive NKT cells. The Rel/NF- $\kappa$ B family member RelB, on the other hand, had to be expressed in radiation resistant thymic stromal cells for the generation of early NK-1.1-negative NKT precursors. Moreover, NF- $\kappa$ B-inducing kinase (NIK) was required for both constitutive thymic DNA binding of RelB and the specific induction of RelB complexes in vitro. Thus, distinct Rel/NF- $\kappa$ B family members in hematopoietic and nonhematopoietic cells regulate NKT cell development with a unique requirement for NIK-mediated activation of RelB in thymic stroma.

Key words: RelB • NKT cells • thymus • aly • NIK

## Introduction

NKT cells represent a subset of mature T lymphocytes, which are characterized by the coexpression of NK cell surface markers and the TCR as well as high levels of cytokine production, in particular IL-4 and IFN- $\gamma$ . NKT cells are most frequent in thymus, bone marrow (BM),\* spleen, and liver and represent a smaller proportion of T cells in lymph nodes, blood, and lung. In mouse thymus, NKT cells are either CD4<sup>-</sup>CD8<sup>-</sup> or CD4<sup>+</sup>CD8<sup>-</sup> and represent 0.3–0.8% of total thymocytes and up to 20% of the mature heat stable antigen (HSA)<sup>neg-low</sup> thymocyte fraction (for reviews, see references 1–3). Thymus-dependent NKT cells

are positively selected by CD1d, a nonclassical MHC class I-like,  $\beta_2$ -microglobulin-associated molecule, and they predominantly express the invariant V $\alpha$ 14-J $\alpha$ 18 TCR (4–7). These so-called V $\alpha$ 14i NKT cells respond to the marine sponge-derived glycolipid  $\alpha$ -galactosyl ceramide ( $\alpha$ -GalCer) and can be identified by staining with  $\alpha$ -GalCer-loaded CD1d tetramers (8–10). Other populations of NKT cells, defined by NK-1.1 and TCR expression, do not express the V $\alpha$ 14-J $\alpha$ 18 TCR and thus are unresponsive to  $\alpha$ -GalCer (11, 12).

Several independent observations indicate that the requirements for NKT cell and conventional TCR $\alpha\beta$  T lymphocyte development are fundamentally different. First, whereas thymic epithelial cells are the predominant cell type for positive selection of conventional T cells, BM-derived immature CD4<sup>+</sup>CD8<sup>+</sup> double positive (DP) thymocytes are required for the positive selection of NKT cells (6, 13). Second, many mutations/gene deletions that impair development of conventional T cells do not have a severe effect on the development of NKT cells and in a few cases the opposite is true (for a review, see reference 14).

The online version of this article contains supplemental material.

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\*Abbreviations used in this paper:  $\alpha$ -GalCer,  $\alpha$ -galactosylceramide; BM, bone marrow; DP, double positive; EMSA, electrophoretic mobility shift assay; HSA, heat-stable Ag; LT, lymphotoxin; MEF, mouse embryonic fibroblast; NF, nuclear factor; NIK, NF- $\kappa$ B-inducing kinase.

These observations suggest that mouse NKT cells belong to a special lineage of T lymphocytes and that NKT precursors follow the conventional developmental pathway to the CD4<sup>+</sup>CD8<sup>+</sup> DP thymocyte to branch off into the NKT lineage as a result of CD1d mediated instructions given to the V $\alpha$ 14-J $\alpha$ 18 TCR (1). The recent finding that NKT cells are indeed derived from DP thymocytes (15) supports this model, but the molecular events downstream of CD1d selection are still poorly understood.

Gene knockout studies demonstrate that lymphotoxin (LT) plays an important role in organogenesis and maintenance of secondary lymphoid tissue microarchitecture (for a review, see reference 16). Membrane LT signaling is defective in the absence of functional nuclear factor (NF)- $\kappa$ B-inducing kinase (NIK) and NIK-deficient as well as alymphoplasia (*aly/aly*) mice, which carry an amino acid substitution in the COOH-terminal domain of NIK, have similar defects in lymphoid organ development (16–19). Interestingly, both LT signaling and NIK are required for normal mouse NKT cell development, but dispensable for the development of conventional T cells (20–22).

The Rel/NF- $\kappa$ B transcription factors, consisting of the family members NF- $\kappa$ B1 (encoding p50 and its precursor p105), NF- $\kappa$ B2 (encoding p52 and its precursor p100), RelA (p65), RelB, and c-Rel, play an important role in immune responses, inflammation, cell survival, and cancer (23–25). In resting cells, NF- $\kappa$ B proteins are retained in the cytoplasm as inactive forms via the association with inhibitory I $\kappa$ B molecules. The classical induction of p50-RelA NF- $\kappa$ B heterodimers involves activation of the I $\kappa$ B kinase (IKK) complex by a wide range of stimuli, resulting in the phosphorylation and ubiquitin-dependent degradation of I $\kappa$ B $\alpha$ . As a consequence, RelA NF- $\kappa$ B complexes translocate to the nucleus and regulate the expression of  $\kappa$ B target genes (26, 27). Recently, an alternative pathway has been described that involves NIK/IKK $\alpha$ -mediated phosphorylation of the inhibitory p100 precursor, its processing to p52, and nuclear translocation of p52-RelB heterodimers (28).

Rel/NF- $\kappa$ B proteins have essential and distinct roles in development and function of the immune system. Similar to LT signaling, Rel/NF- $\kappa$ B family members are required for proper organogenesis and maintenance of secondary lymphoid tissues (29–31). Importantly, while NF- $\kappa$ B regulates specific T cell functions, such as antigen/mitogen-induced proliferation and cytokine production, thymic development of conventional T cells is not affected by the targeted deletion of individual Rel/NF- $\kappa$ B family members or the blockage of the classical NF- $\kappa$ B pathway with a mutant nondegradable mI $\kappa$ B $\alpha$  super-inhibitor (32).

We show here that, similar to *aly/aly* mice, RelB-deficient animals lacked thymus-dependent V $\alpha$ 14i NKT cells. Whereas RelB had to be expressed in radiation-resistant thymic stromal cells, classical NF- $\kappa$ B was required in NKT precursors for their maturation and expansion. We further show that NIK regulates RelB DNA binding in vitro and in vivo, suggesting a unique requirement for NIK-mediated activation of RelB in NKT cell development.

## Materials and Methods

**Mice.** Generation of *nfkb1*<sup>-/-</sup> (33), *nfkb2*<sup>-/-</sup> (34), *relB*<sup>-/-</sup> (35), *lbr*<sup>-/-</sup> (36), *tnfr1*<sup>-/-</sup> (37), and mI $\kappa$ B $\alpha$ <sup>tg</sup> mice (38) has been described previously. Alymphoplasia (*aly/aly*) (17, 19) mice were kindly provided by Dr. Thomas Böhm, Max-Planck-Institute of Immunobiology, Freiburg, Germany. C57BL/6-Ly-5.1 mice were obtained from Charles River Laboratories. Adoptive BM transfers were performed as previously described (30) and chimeric mice were analyzed 12–14 wk after reconstitution. All animals were housed and bred under standardized conditions with water and food *ad libitum* in the SPF mouse facility of the Forschungszentrum Karlsruhe.

**Flow Cytometric Analyses.** Flow cytometry was performed using a Becton Dickinson LSR flow cytometer. Single cell suspensions were washed twice with staining buffer (PBS, 2% FCS) and incubated with Fc Block<sup>TM</sup> (clone 2.4G2). The following conjugated mAbs were used: anti-NK-1.1-PE (clone PK136, diluted 1:200), anti-TCR $\beta$ -FITC (clone H57–597, diluted 1:100), anti-CD24/HSA-Biotin (clone M1/69, diluted 1:100), anti-CD4-FITC/Biotin (clone RM4–5, diluted 1:100), anti-CD8 $\alpha$ -PE/Biotin (clone 53–6.7, diluted 1:200), anti-CD1d-FITC (clone 1B1, diluted 1:100), anti-CD44-Biotin (clone IM7, diluted 1:100), and anti-Ly-5.2-Biotin (clone 104, diluted 1:100). All mAbs were from BD Biosciences. Biotinylated mAbs were detected with Streptavidin-PerCP (BD Biosciences, diluted 1:200). Apoptotic cells were detected by Annexin V-FITC staining according to the manufacturer's recommendations (Roche). Incubations were for 30 min on ice followed by two washes with staining buffer. Staining with  $\alpha$ -GalCer-loaded CD1d tetramers was performed as described previously (39). Data were analyzed using CELLQuest Pro<sup>TM</sup> software (BD Biosciences).

**Cell Culture.** Primary mouse embryonic fibroblasts (MEFs) were prepared from E15.5 embryos according to standard procedures (40), cultured in DMEM supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), L-glutamine (2 mM), and treated with agonistic anti-LT $\beta$ R mAb (1  $\mu$ g/ml clone AC.H6; gift from Dr. Jeffrey Browning and Dr. Paul Rennert, Biogen, Cambridge, MA).

**RNA Analysis.** RNA extraction and quantitative RT-PCR were performed as reported previously (30). Briefly, amplification conditions using an MJ Research PTC-225 thermal cycler were 94°C for 1 min, 55°C for 1 min, 72°C for 1 min in the presence of 1  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]dCTP. The linear range of amplification was determined for each primer pair: V $\alpha$ 14/J $\alpha$ 18, 26 cycles; C $\beta$ , 18 cycles; IL-15, 29 cycles. Amplified products were separated in 6% polyacrylamide gels. Quantifications were done with a Fujix BAS 1000 Phosphorimager. PCR primers were: V $\alpha$ 14 (GTT GTC CGT CAG GGA GAG AA) and J $\alpha$ 18 (CAA TCA GCT GAG TCC CAG CT), IL-15 (TCA GCA GAT AAC CAG CCT AC and TTT CTC CTC CAG CTC CTC AC), and C $\beta$  (CAC TGA TGT TCT GTG TGA CA and GAG GAT CTG AGA AAT GTG ACT CCA C).

**Electrophoretic Mobility Shift Assays.** Whole thymus tissue extracts and nuclear extracts from fibroblasts were prepared and electrophoretic mobility shift assays (EMSA) were performed as described previously (38, 41). Integrity of extracts was checked using an Oct oligodeoxynucleotide.

**Online Supplemental Material.** Fig. S1 shows reduced numbers of V $\alpha$ 14i/NKT cells in *relB*<sup>-/-</sup> and mI $\kappa$ B $\alpha$ <sup>tg</sup> mice. Thymocytes from 3-mo-old mice were analyzed for tetramer binding and TCR $\beta$  expression by flow cytometry (lymphocyte gate). Numbers indicate mean values  $\pm$  SD from three mice. Online supple-

## Results

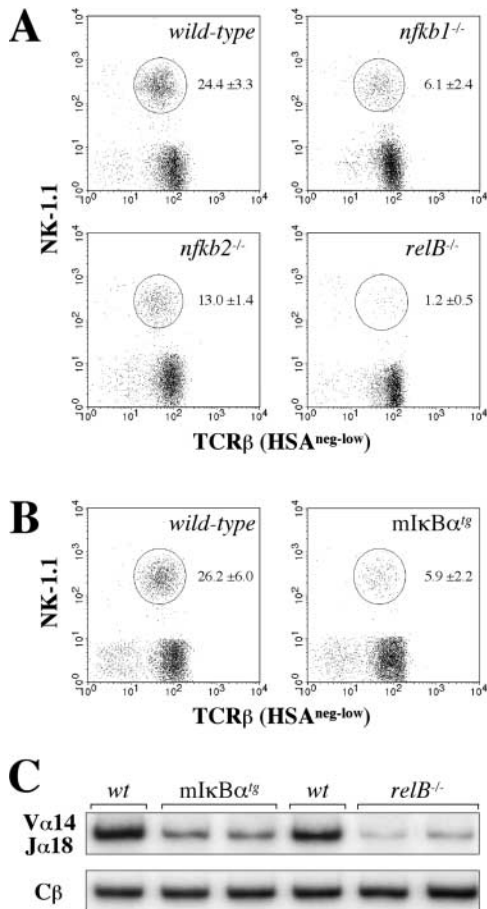
**NF- $\kappa$ B Is Essential for Normal NKT Cell Development.** To examine the role of individual Rel/NF- $\kappa$ B subunits in NKT cell development we analyzed mice with targeted disruptions of RelB (*relB*<sup>-/-</sup>), p50/NF- $\kappa$ B1 (*nfkb1*<sup>-/-</sup>), and p52/NF- $\kappa$ B2 (*nfkb2*<sup>-/-</sup>) as well as transgenic mice, in which NF- $\kappa$ B function is inhibited in T cells by the expression of a nondegradable mutant form of the I $\kappa$ B $\alpha$  molecule under the control of the T cell-specific *lck* proximal promoter (mI $\kappa$ B $\alpha$ <sup>tg</sup>). The percentage of NK-1.1<sup>+</sup>TCR $\beta$ <sup>+</sup> cells in thymus of *relB*<sup>-/-</sup> mice was dramatically reduced (~20-fold) compared with wild-type mice (Fig. 1 A). Thymic NK-1.1<sup>+</sup>TCR $\beta$ <sup>+</sup> cells were also markedly reduced in *nfkb1*<sup>-/-</sup> mice (~4-fold), whereas *nfkb2*<sup>-/-</sup> mice showed a milder phenotype with two- to threefold reduced NK-

1.1<sup>+</sup>TCR $\beta$ <sup>+</sup> cell numbers (Fig. 1 A). The observed defects in NKT cell development in *relB*<sup>-/-</sup>, *nfkb1*<sup>-/-</sup>, and *nfkb2*<sup>-/-</sup> mice could be due to the lack of these proteins in all cells of the thymus (including stroma) or to impaired NF- $\kappa$ B function in hematopoietic thymocytes that develop into mature T cells. We therefore analyzed mI $\kappa$ B $\alpha$ <sup>tg</sup> mice, in which NF- $\kappa$ B function is blocked in *Lck*-expressing thymocytes (38). Interestingly, the specific inhibition of NF- $\kappa$ B in these cells also resulted in a ~5-fold reduced NKT cell population compared with nontransgenic littermates (Fig. 1 B).

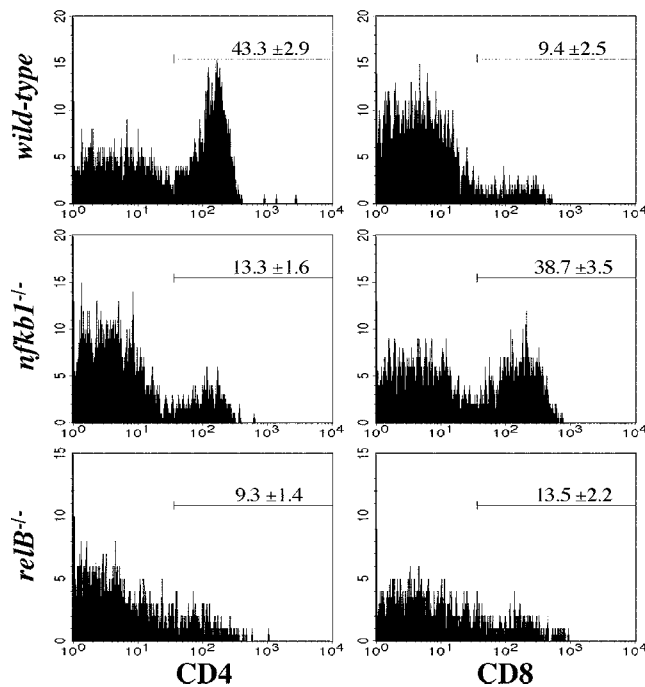
Since NKT cell development can still occur in the absence of the NK-1.1 marker (42), the observed reduction of NK-1.1<sup>+</sup>TCR $\beta$ <sup>+</sup> cells in *relB*<sup>-/-</sup>, *nfkb1*<sup>-/-</sup>, *nfkb2*<sup>-/-</sup>, and mI $\kappa$ B $\alpha$ <sup>tg</sup> mice could be due to the lack of NK-1.1 expression. Expression of the V $\alpha$ 14-J $\alpha$ 18 chain is considered a more stringent measure for the major population of NKT cells in thymus (43). Semiquantitative RT-PCR analysis revealed reduced V $\alpha$ 14-J $\alpha$ 18 mRNA levels in *relB*<sup>-/-</sup>, *nfkb1*<sup>-/-</sup>, *nfkb2*<sup>-/-</sup>, and mI $\kappa$ B $\alpha$ <sup>tg</sup> thymocytes (Fig. 1 C, and unpublished data). Staining with  $\alpha$ -GalCer/CD1d tetramers confirmed the reduced V $\alpha$ 14i NKT cell numbers, indicating that these mice have a true defect in NKT cell development (online supplemental Fig. S1).

**Selective Loss of CD4<sup>+</sup> NKT Cells in Spleen and Bone Marrow of *relB*<sup>-/-</sup> and *nfkb1*<sup>-/-</sup> Mice.** Spleen and BM are other major lymphoid organs where NKT cells are found. Despite the significant reduction in thymic NKT cells, only a moderate reduction in total NKT cell numbers was observed in spleen and BM of *relB*<sup>-/-</sup>, *nfkb1*<sup>-/-</sup>, and mI $\kappa$ B $\alpha$ <sup>tg</sup> mice (unpublished data). When different peripheral NKT cell subpopulations were analyzed thymus-dependent CD4<sup>+</sup> NKT cells were clearly reduced in spleens from *relB*<sup>-/-</sup> (4.5-fold) and *nfkb1*<sup>-/-</sup> mice (threefold). Interestingly, *nfkb1*<sup>-/-</sup> spleens showed a selective fourfold increase in the percentage of CD8<sup>+</sup> NK-1.1<sup>+</sup> T cells, whereas this subpopulation was only minimally increased in *relB*<sup>-/-</sup> mice (Fig. 2). As the CD8<sup>+</sup> NK-1.1<sup>+</sup> subset is unrelated to thymus-dependent CD4<sup>+</sup> NKT cells (12), this result indicates a specific requirement of NF- $\kappa$ B for the development of V $\alpha$ 14i NKT cells rather than for the total NK-1.1<sup>+</sup> T cell population.

**Differential Requirement of Distinct NF- $\kappa$ B Complexes in Hematopoietic and Radiation-resistant Stromal Cells for NKT Cell Development.** Thymic NKT cells are positively selected via the interaction of their V $\alpha$ 14-J $\alpha$ 18 TCR with CD1d on DP cortical thymocytes (6) but stromal components are also required for normal NKT cell development (22). Flow cytometric analysis revealed normal CD1d levels on thymocytes from *relB*<sup>-/-</sup>, *nfkb1*<sup>-/-</sup>, *nfkb2*<sup>-/-</sup>, and mI $\kappa$ B $\alpha$ <sup>tg</sup> mice, indicating that NF- $\kappa$ B is not required for the expression of CD1d (unpublished data). Next, we examined the requirement of individual NF- $\kappa$ B members in both hematopoietic and nonhematopoietic cells by radiation chimera analysis. Reciprocal BM transplantations between wild-type and *relB*<sup>-/-</sup> mice revealed that RelB in radiation resistant stromal components was required for NKT cell development, as wild-type BM transferred into



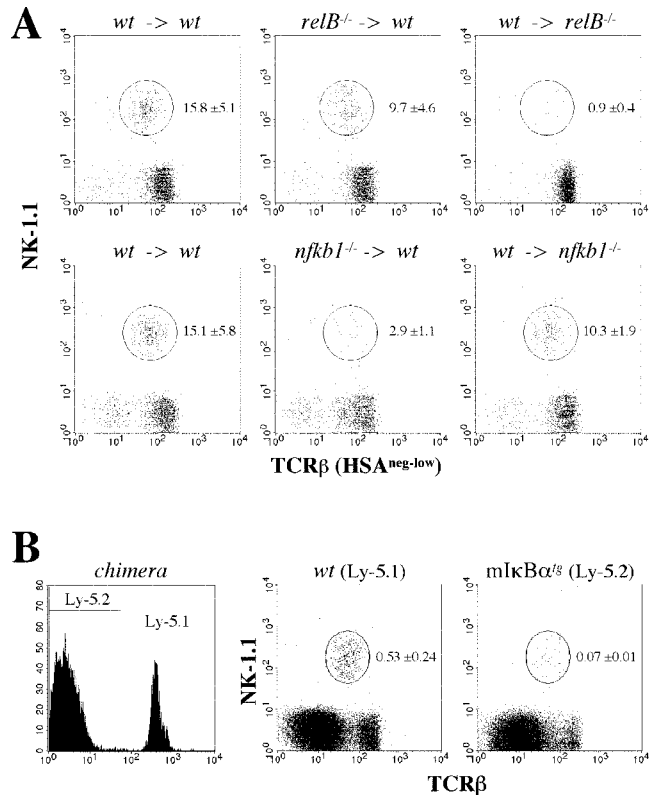
**Figure 1.** Reduced numbers of thymic NKT cells in NF- $\kappa$ B-deficient mice. Thymocytes from adult wild-type, *nfkb1*<sup>-/-</sup>, *nfkb2*<sup>-/-</sup>, and *relB*<sup>-/-</sup> (A) as well as from control and mI $\kappa$ B $\alpha$ <sup>tg</sup> mice (B) were analyzed for NK-1.1 and TCR $\beta$  expression by flow cytometry. Percentages of NKT cells (circles) were increased by gating on HSA<sup>neg-low</sup> cells. Numbers indicate mean values  $\pm$  SD from 5–7 mice. (C) RT-PCR analysis of V $\alpha$ 14-J $\alpha$ 18 expression in wild-type, mI $\kappa$ B $\alpha$ <sup>tg</sup>, and *relB*<sup>-/-</sup> mice. Expression of the C $\beta$  chain is shown as an amplification control.



**Figure 2.** Selective loss of peripheral CD4<sup>+</sup> NKT cells in *relB*<sup>-/-</sup> and *nfkb1*<sup>-/-</sup> mice. NKT cells from adult wild-type, *nfkb1*<sup>-/-</sup>, and *relB*<sup>-/-</sup> spleens were analyzed for CD4 and CD8 expression by flow cytometry. Histograms show CD4 (left) and CD8 (right) expression levels on NK-1.1<sup>+</sup>TCRβ<sup>+</sup> cells. Note the reduction of CD4<sup>+</sup> NKT cells in *nfkb1*<sup>-/-</sup> and *relB*<sup>-/-</sup> mice and the increase of CD8<sup>+</sup> NK-1.1<sup>+</sup> T cells in *nfkb1*<sup>-/-</sup> but not in *relB*<sup>-/-</sup> animals. Numbers indicate mean values ± SD from 3–7 mice.

lethally irradiated *relB*<sup>-/-</sup> recipients resulted in severely impaired development of NKT cells (Fig. 3 A). RelB appeared to play a minor role in hematopoietic cells, as adoptive transfers of *relB*<sup>-/-</sup> BM into wild-type recipients resulted only in a 1.5-fold reduction of NKT cell numbers compared with controls. Interestingly, reciprocal BM transfers between wild-type and *nfkb1*<sup>-/-</sup> mice revealed that p50/NF-κB1 was required in hematopoietic cells rather than in the stromal compartment (Fig. 3 A). In contrast to p50/NF-κB1 but similar to RelB, p52/NF-κB2 was required in radiation-resistant nonhematopoietic cells for proper NKT cell development (unpublished data).

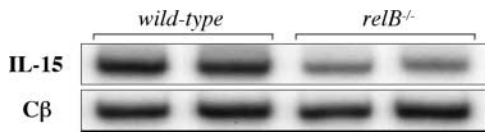
To determine whether the reduced numbers of NKT cells in *mIkBα*<sup>tg</sup> mice was a direct effect of blocking the NF-κB pathway in NKT precursors or due to the lack of NF-κB in accessory thymocytes, mixed chimeric mice were generated. BM cells from Ly-5.1 wild-type and Ly-5.2 *mIkBα*<sup>tg</sup> mice were mixed and injected into lethally irradiated Ly-5.2 wild-type recipients. Flow cytometric analysis showed that ~70% of the thymocytes were derived from Ly-5.2 *mIkBα*<sup>tg</sup> BM whereas the Ly-5.1 wild-type BM contributed the remaining 30% (Fig. 3 B). While only Ly-5.1 wild-type BM resulted in the generation of NKT cells, BM from Ly-5.2 *mIkBα*<sup>tg</sup> mice was unable to efficiently generate NKT cells despite the coexistence of



**Figure 3.** Analysis of NKT cell development in BM chimeras. (A) Thymocytes were isolated from lethally irradiated mice that were reconstituted with BM as indicated (donor BM → irradiated recipient). HSA<sup>neg-low</sup> cells were analyzed for NK-1.1 and TCRβ expression by flow cytometry. Percentages of NKT cells (mean values from 3–7 mice ± SD) are indicated. (B) Competitive BM chimeras were generated by mixing wild-type Ly-5.1 with *mIkBα*<sup>tg</sup> Ly-5.2 BM to reconstitute lethally irradiated wild-type Ly-5.2 mice. Chimerism was checked by flow cytometry (left). Thymocytes derived from wild-type (Ly-5.1<sup>+</sup>) and from *mIkBα*<sup>tg</sup> (Ly-5.2<sup>+</sup>) BM were stained for NK-1.1 and TCRβ expression and analyzed by flow cytometry (right). Percentages of NKT cells among total thymocytes (mean values from 2–3 mice ± SD) are indicated.

wild-type thymocytes (Fig. 3 B). Together, these data indicate that thymic NKT cell development is dependent on both RelB in the nonhematopoietic compartment and, in a cell-autonomous manner, on classical NF-κB activity in NKT precursors.

*RelB Is Required for Normal Thymic IL-15 mRNA Expression.* As NF-κB regulates gene expression we analyzed total thymus steady-state mRNA levels of several genes that have been shown to be crucial for NKT cell development (14). Focusing on *relB*<sup>-/-</sup> and *mIkBα*<sup>tg</sup> mice, we did not observe significant defects in the expression of cathepsin S, cathepsin L, Ets-1, Fyn, GM-CSFRβ, IL-15Rα, IRF-1, LTα, LTβ, and Vav-1 compared with wild-type controls (unpublished data). In contrast, steady-state levels of IL-15 mRNA were reduced ~3-fold in *relB*<sup>-/-</sup> but not in *mIkBα*<sup>tg</sup> thymus (Fig. 4). IL-15 expression was not detected in thymocyte single cell suspensions (unpublished data), indicating that RelB in stromal cells is required for the maintenance of normal IL-15 levels.

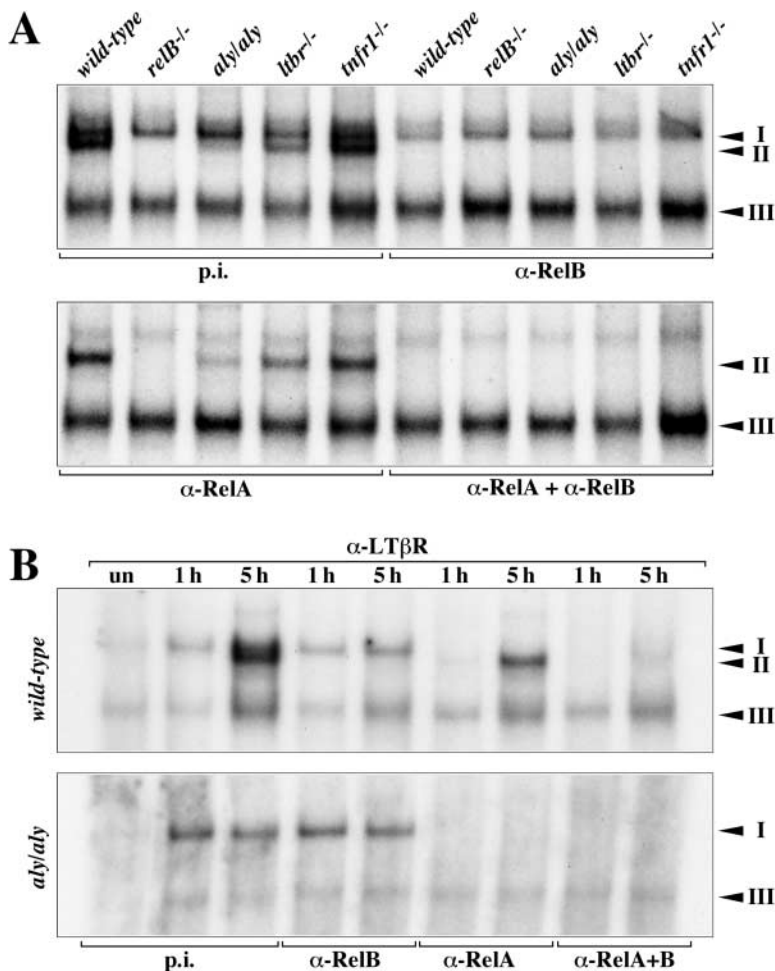


**Figure 4.** Analysis of IL-15 mRNA expression in thymus from NF- $\kappa$ B-deficient mice. RT-PCR analysis of whole thymus RNA revealed reduced IL-15 steady-state mRNA levels in *relB*<sup>-/-</sup> mice. Expression of IL-15 in thymus from m $\kappa$ B $\alpha^{\text{g}}$  mice was similar to controls (unpublished data). Expression of the C $\beta$  chain is shown as an amplification control.

*DNA Binding of RelB Complexes Is Defective in Thymus of aly/aly Mice.* NIK is a key player in LT signaling and it has previously been shown that NIK is required in nonhematopoietic cells for normal NKT cell development (22). As thymic NKT cell development was also dependent on stromal RelB expression we analyzed whether the lack of functional NIK affects constitutive DNA-binding of RelB complexes in thymus. When whole thymus extracts were analyzed in EMSAs a marked reduction of RelB  $\kappa$ B-binding (complex II) was observed in *aly/aly* extracts and to a lesser extent also in *ltbr*<sup>-/-</sup> mice, whereas binding of RelA (complex I) was not affected (Fig. 5 A). Extracts from *relB*<sup>-/-</sup> and *tnfr1*<sup>-/-</sup> mice were included as a specificity control.

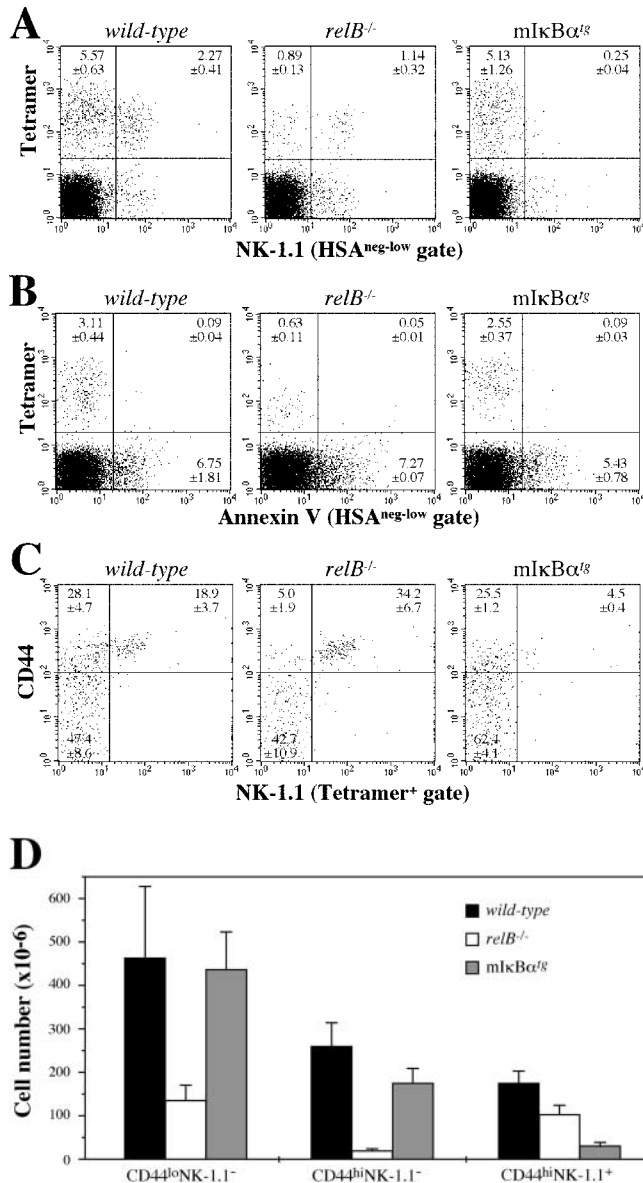
To address whether NIK is also required for the induced binding of RelB complexes we stimulated fibroblasts with agonistic anti-LT $\beta$ R mAb. LT $\beta$ R activation in wild-type MEFs resulted in the rapid induction of RelA (complex I) and at later time points also RelB (complex II), while no binding of RelB complexes could be detected in *aly/aly* MEFs despite normal induction of RelA (Fig. 5 B). Thus, both constitutive and surface receptor-induced RelB  $\kappa$ B-binding requires functional NIK, whereas binding of classical RelA NF- $\kappa$ B complexes is independent of this kinase.

*Lack of RelB and NF- $\kappa$ B Blocks Early NKT Cell Development at Different Steps.* Recent evidence indicates that NK-1.1<sup>-</sup> NKT precursors that bind to CD1d tetramers give rise to mature NK-1.1<sup>+</sup> tetramer<sup>+</sup> cells in both thymus and peripheral lymphoid tissues (15, 44, 45). As the frequency of NK-1.1<sup>-</sup> precursors is low in adult compared with young animals, we analyzed 2-wk-old mice by flow cytometry. Staining of wild-type thymocytes with  $\alpha$ -GalCer-loaded CD1d tetramer and anti-NK-1.1 mAb revealed that tetramer<sup>+</sup> cells at this age consisted predominantly of NK-1.1<sup>-</sup> precursors and a smaller portion of mature NK-1.1<sup>+</sup> NKT cells. In contrast, the NK-1.1<sup>-</sup> precursor population was markedly reduced in *relB*<sup>-/-</sup> mice whereas mature NKT cells were less severely affected. On



**Figure 5.** LT $\beta$ R and NIK regulate RelB DNA-binding in thymus. (A) Whole thymus extracts from wild-type, *relB*<sup>-/-</sup>, *aly/aly*, *ltbr*<sup>-/-</sup>, and *tnfr1*<sup>-/-</sup> mice were prepared and analyzed for  $\kappa$ B-binding activity in EMSAs. Complexes are indicated by arrowheads and their identity was determined with Abs specific for individual Rel/NF- $\kappa$ B family members. p.i., preimmune serum; complex I, RelA heterodimers; complex II, RelB heterodimers. Complex III consists of p50 homodimers (unpublished data). (B) LT $\beta$ R-mediated induction of RelB is dependent on NIK. Primary MEFs from wild-type and *aly/aly* mice were either left untreated (un) or treated for 1 and 5 h with anti-LT $\beta$ R mAb and nuclear extracts were analyzed for  $\kappa$ B-binding. Complexes were identified with specific Abs.

the other hand,  $mIkB\alpha^{tg}$  mice had a normal NK-1.1<sup>-</sup> precursor frequency but a ninefold reduction in the percentage of mature NK-1.1<sup>+</sup> tetramer<sup>+</sup> NKT cells (Fig. 6 A). Annexin V staining of thymocytes from 2-wk-old mice did not reveal increased apoptosis of tetramer<sup>+</sup> cells in  $relB^{-/-}$  or  $mIkB\alpha^{tg}$  mice (Fig. 6 B).



**Figure 6.** Analysis of developmental intermediates of V $\alpha$ 14i NKT cells in NF- $\kappa$ B-deficient mice. (A) Staining of thymocytes from 2-wk-old C57BL/6 wild-type,  $relB^{-/-}$ , and  $mIkB\alpha^{tg}$  mice with anti-NK-1.1 and  $\alpha$ -GalCer CD1d tetramer. (B) Analysis of apoptotic cells in thymus from 2-wk-old C57BL/6 wild-type,  $relB^{-/-}$ , and  $mIkB\alpha^{tg}$  mice. Thymocytes were stained with Annexin V and  $\alpha$ -GalCer CD1d tetramer. HSA<sup>neg-low</sup> cells in panels A and B were gated as in Fig. 1. (C) Expression of CD44 and NK-1.1 on tetramer<sup>+</sup> thymocytes from 2-wk-old wild-type,  $relB^{-/-}$ , and  $mIkB\alpha^{tg}$  mice was determined by flow cytometric analysis. Numbers in quadrants indicate mean values of percentages  $\pm$  SD from three mice. (D) Numbers of tetramer<sup>+</sup> cells in 10<sup>6</sup> thymocytes from 2-wk-old wild-type,  $relB^{-/-}$ , and  $mIkB\alpha^{tg}$  mice based on their CD44 and NK-1.1 expression patterns.

After positive selection, NKT precursors progress through CD44<sup>lo</sup> and CD44<sup>hi</sup> stages before they become positive for the NK-1.1 marker (for a review, see reference 14). We therefore compared the CD44/NK-1.1 profiles of tetramer<sup>+</sup> thymocytes in 2-wk-old wild-type,  $relB^{-/-}$ , and  $mIkB\alpha^{tg}$  mice. Whereas the percentage of mature CD44<sup>hi</sup>NK-1.1<sup>+</sup> NKT cells was not reduced, the frequency of the intermediate CD44<sup>hi</sup>NK-1.1<sup>-</sup> NKT population was strongly reduced in  $relB^{-/-}$  mice. In contrast, intermediate CD44<sup>hi</sup>NK-1.1<sup>-</sup> NKT precursors were only mildly affected in  $mIkB\alpha^{tg}$  mice, while further differentiation into mature CD44<sup>hi</sup>NK-1.1<sup>+</sup> NKT cells was clearly impaired (Fig. 6 C). This difference between wild-type,  $relB^{-/-}$ , and  $mIkB\alpha^{tg}$  mice became even more obvious when numbers of the different subpopulations were calculated (Fig. 6 D). Fig. 6 D also shows that RelB is required for the generation of normal numbers of early CD44<sup>lo</sup>NK-1.1<sup>-</sup> NKT precursors, whereas this population is not reduced in  $mIkB\alpha^{tg}$  mice. Our data indicate that these early but distinct blocks caused by the lack of RelB in stromal and classical NF- $\kappa$ B in hematopoietic cells, respectively, result in severely reduced numbers of mature V $\alpha$ 14i NKT cells in adult mice.

## Discussion

This study reveals distinct nonredundant roles for the NF- $\kappa$ B family members p50/NF- $\kappa$ B1, p52/NF- $\kappa$ B2, and RelB in both hematopoietic and nonhematopoietic cells for the development of thymic NKT cells. While p50/NF- $\kappa$ B1 is required in hematopoietic cells, RelB is required in the nonhematopoietic compartment. Interestingly, the lack of RelB in radiation-resistant stromal cells results in a more severe decrease in NKT cell numbers than blocking total NF- $\kappa$ B function via the nondegradable  $mIkB\alpha$  inhibitor in NKT precursors. CD4<sup>+</sup> NKT cells are thymus-dependent (11, 12) and because this subpopulation is also reduced in  $nfkb1^{-/-}$ ,  $relB^{-/-}$ , and  $mIkB\alpha^{tg}$  spleen it is unlikely that the reduced numbers of thymic NKT cells is due to increased emigration from thymus. The selective loss of these cells is accompanied by an increase in CD8<sup>+</sup> NK-1.1<sup>+</sup> T cells in the spleen of  $nfkb1^{-/-}$  but not in  $relB^{-/-}$  mice. One possible explanation for this observation is that p50/NF- $\kappa$ B1 prevents the expansion of CD8<sup>+</sup> NK-1.1<sup>+</sup> T cells. Alternatively, the lack of p50/NF- $\kappa$ B1 may result in the up-regulation of the NK-1.1 marker on conventional CD8<sup>+</sup> T cells (46). Due to the very low frequency, it is difficult to analyze CD8<sup>+</sup> NKT cells and the specific increase of this subpopulation in  $nfkb1^{-/-}$  mice may help to study the function and origin of CD8<sup>+</sup> NKT cells in more detail.

NKT cells are derived from a CD4<sup>+</sup>CD8<sup>+</sup> DP thymic subset (15), suggesting that NKT cell development is similar to conventional T cells until the DP stage and that at this stage the interaction of CD1d with the V $\alpha$ 14-J $\alpha$ 18 TCR triggers both positive selection and NKT lineage commitment. As a CD1d/V $\alpha$ 14-J $\alpha$ 18 TCR-specific signal regulating positive selection and/or commitment to the

NKT lineage has not been identified yet (14) it is important to identify key factors within signaling pathways, which regulate the development of NKT independent from conventional T cells.

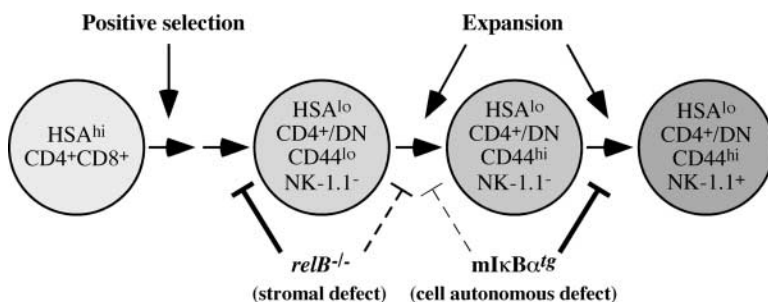
Functional NIK in nonhematopoietic cells is essential for NKT cell development (22), which is consistent with the lack of constitutive RelB DNA binding in thymus of *aly/aly* mice and the stromal requirement of RelB for NKT cell development. Both *relB*<sup>-/-</sup> and *aly/aly* mice show similar structural disorganization of the thymus (17, 35, 47) and the marked defect in RelB DNA binding in *aly/aly* thymus offers an explanation for the similarity of this phenotype. In contrast to *aly/aly* mice, thymic DNA binding of RelB is only mildly affected in *ltbr*<sup>-/-</sup> and normal in *tnfr1*<sup>-/-</sup> mice, indicating that another receptor associated with NIK may be involved in RelB activation, NKT cell development, and the establishment of proper thymic architecture. NIK is required for the processing of the NF- $\kappa$ B2 p100 precursor to p52 (48, 49) and since the COOH-terminal domain of p100 specifically inhibits RelB function (50–52), lack of RelB DNA binding in the absence of functional NIK most likely is due to defective processing of the inhibitory p100 precursor. Similar to RelB, p52/NF- $\kappa$ B2 is also required in stromal cells for normal NKT cell development. This observation correlates with a stromal requirement of both RelB and p52/NF- $\kappa$ B2 for proper spleen (29, 30) and Peyer's patch development (52, 53), emphasizing that p52-RelB rather than p50-RelB heterodimers are involved in the regulation of NKT cell development, although we cannot rule out that p50-RelB complexes also contribute.

Thymic steady-state mRNA levels of many genes that have been demonstrated in knockout mouse models to be important for NKT cell development are not affected in *relB*<sup>-/-</sup> and *mIkB $\alpha$* <sup>tg</sup> mice. However, we observed a reproducible reduction of IL-15 mRNA levels in thymus from *relB*<sup>-/-</sup> mice. This finding is consistent with a stromal function of RelB since IL-15 produced by thymic stromal cells has been shown to be essential for early NKT cell development and homeostasis (54, 55). An NF- $\kappa$ B binding site has been reported in the IL-15 promoter (56) but it remains to be shown whether RelB directly or indirectly regulates expression of IL-15.

Recent reports indicate that committed NK-1.1<sup>-</sup> NKT precursors are derived from a CD4<sup>+</sup>CD8<sup>+</sup> DP thymocyte subpopulation. They proliferate and differentiate through

CD44<sup>lo</sup>NK-1.1<sup>-</sup> and CD44<sup>hi</sup>NK-1.1<sup>-</sup> stages into mature CD44<sup>hi</sup>CD4<sup>+</sup>/DN NK-1.1<sup>+</sup> NKT cells (15, 44, 45). We did not observe a significant reduction in the frequency of tetramer<sup>+</sup> DP<sup>hi</sup> NKT precursors in 12-d-old *relB*<sup>-/-</sup> and *mIkB $\alpha$* <sup>tg</sup> mice (unpublished data), indicating that NF- $\kappa$ B is not required for the rearrangement of the V $\alpha$ 14-J $\alpha$ 18 TCR. As expression of CD1d is normal in *relB*<sup>-/-</sup> and *mIkB $\alpha$* <sup>tg</sup> mice, it is unlikely that initial positive selection is impaired in these mice. The analysis of 2-wk-old mice revealed, however, that RelB function is required for the generation of normal numbers of early CD44<sup>lo</sup>NK-1.1<sup>-</sup> NKT precursors, indicating that RelB in stromal cells regulates maturation/expansion of positively selected V $\alpha$ 14i NKT precursors. We also observed markedly reduced percentages and numbers of CD44<sup>hi</sup>NK-1.1<sup>-</sup> NKT precursors in thymus from 2-wk-old *relB*<sup>-/-</sup> mice. This can be explained by an impaired transition from the CD44<sup>lo</sup> to the CD44<sup>hi</sup> stage, which is supported by the analysis of 6-wk-old mice (unpublished data), but normal progression from the CD44<sup>hi</sup>NK-1.1<sup>-</sup> to the mature CD44<sup>hi</sup>NK-1.1<sup>+</sup> stage (see also Fig. 7). Interestingly, IL-15-deficient mice also show an early block in NKT cell development (55) and the reduced IL-15 expression in *relB*<sup>-/-</sup> thymus may contribute to the reduced NKT cell numbers in RelB-deficient mice. Analysis of *mIkB $\alpha$* <sup>tg</sup> mice, on the other hand, revealed that classical NF- $\kappa$ B function within NKT precursors is dispensable for the early generation of tetramer<sup>+</sup> NK-1.1<sup>-</sup> cells, whereas it is required at a later step of NKT cell development. In particular, the progression from CD44<sup>hi</sup>NK-1.1<sup>-</sup> to the mature CD44<sup>hi</sup>NK-1.1<sup>+</sup> stage is impaired with a milder block at the CD44<sup>lo</sup> to CD44<sup>hi</sup> transition (Fig. 7). It is unlikely that the blocks in NKT cell development are due to increased apoptosis since we did not observe increased Annexin V staining of tetramer<sup>+</sup> cells in *relB*<sup>-/-</sup> and *mIkB $\alpha$* <sup>tg</sup> mice compared with wild-type controls.

Our results demonstrate a distinct involvement of different NF- $\kappa$ B subunits in the development of NKT cells with an important role of the classical NF- $\kappa$ B pathway within NKT precursor cells, while the alternative NIK/NF- $\kappa$ B2/RelB pathway operates in the thymic stroma. The identification of stromal cell-derived factor(s) regulated by RelB and hematopoietic cell-derived factor(s) regulated by classical NF- $\kappa$ B would help to better define the role of NF- $\kappa$ B in the regulation of different stages of NKT cell development.



**Figure 7.** Model of NF- $\kappa$ B-regulated V $\alpha$ 14i NKT cell development in thymus. Lack of RelB in stromal cells results in impaired generation of CD44<sup>lo</sup>NK-1.1<sup>-</sup> precursors. Blocking NF- $\kappa$ B in precursors results in impaired expansion of NKT cells predominantly at the NK-1.1<sup>-</sup> to NK-1.1<sup>+</sup> transition. For more details see Discussion.

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## References

- Bendelac, A., M.N. Rivera, S.H. Park, and J.H. Roark. 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu. Rev. Immunol.* 15:535–562.
- Godfrey, D.I., K.J. Hammond, L.D. Poulton, M.J. Smyth, and A.G. Baxter. 2000. NKT cells: facts, functions and fallacies. *Immunol. Today.* 21:573–583.
- MacDonald, H.R. 2002. Development and selection of NKT cells. *Curr. Opin. Immunol.* 14:250–254.
- Lantz, O., and A. Bendelac. 1994. An invariant T cell receptor  $\alpha$  chain is used by a unique subset of major histocompatibility complex class I-specific CD4<sup>+</sup> and CD4<sup>-</sup>8<sup>-</sup> T cells in mice and humans. *J. Exp. Med.* 180:1097–1106.
- Bendelac, A., O. Lantz, M.E. Quimby, J.W. Yewdell, J.R. Bennink, and R.R. Brutkiewicz. 1995. CD1 recognition by mouse NK1<sup>+</sup> T lymphocytes. *Science.* 268:863–865.
- Bendelac, A. 1995. Positive selection of mouse NK1<sup>+</sup> T cells by CD1-expressing cortical thymocytes. *J. Exp. Med.* 182:2091–2096.
- Mendiratta, S.K., W.D. Martin, S. Hong, A. Boesteanu, S. Joyce, and L. Van Kaer. 1997. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity.* 6:469–477.
- Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, et al. 1997. CD1d-restricted and TCR-mediated activation of V $\alpha$ 14 NKT cells by glycosylceramides. *Science.* 278:1626–1629.
- Burdin, N., L. Brossay, Y. Koezuka, S.T. Smiley, M.J. Grusby, M. Gui, M. Taniguchi, K. Hayakawa, and M. Kronenberg. 1998. Selective ability of mouse CD1 to present glycolipids:  $\alpha$ -galactosylceramide specifically stimulates V $\alpha$ 14<sup>+</sup> NK T lymphocytes. *J. Immunol.* 161:3271–3281.
- MacDonald, H.R. 2000. CD1d-glycolipid tetramers: a new tool to monitor natural killer T cells in health and disease. *J. Exp. Med.* 192:F15–F20.
- Eberl, G., R. Lees, S.T. Smiley, M. Taniguchi, M.J. Grusby, and H.R. MacDonald. 1999. Tissue-specific segregation of CD1d-dependent and CD1d-independent NK T cells. *J. Immunol.* 162:6410–6419.
- Hammond, K.J., S.B. Pelikan, N.Y. Crowe, E. Randle-Barrett, T. Nakayama, M. Taniguchi, M.J. Smyth, I.R. van Driel, R. Scollay, A.G. Baxter, and D.I. Godfrey. 1999. NKT cells are phenotypically and functionally diverse. *Eur. J. Immunol.* 29:3768–3781.
- Robey, E., and B.J. Fowlkes. 1994. Selective events in T cell development. *Annu. Rev. Immunol.* 12:675–705.
- Kronenberg, M., and L. Gapin. 2002. The unconventional lifestyle of NKT cells. *Nat. Rev. Immunol.* 2:557–568.
- Gapin, L., J.L. Matsuda, C.D. Surh, and M. Kronenberg. 2001. NKT cells derive from double-positive thymocytes that are positively selected by CD1d. *Nat. Immunol.* 2:971–978.
- Fu, Y.-X., and D.D. Chaplin. 1999. Development and maturation of secondary lymphoid tissues. *Annu. Rev. Immunol.* 17:399–433.
- Miyawaki, S., Y. Nakamura, H. Suzuka, M. Koba, R. Yasumizu, S. Ikehara, and Y. Shibata. 1994. A new mutation, *aly*, that induces a generalized lack of lymph nodes accompanied by immunodeficiency in mice. *Eur. J. Immunol.* 24:429–434.
- Matsumoto, M., K. Iwamasa, P.D. Rennert, T. Yamada, R. Suzuki, A. Matsushima, M. Okabe, S. Fujita, and M. Yokoyama. 1999. Involvement of distinct cellular compartments in the abnormal lymphoid organogenesis in lymphotoxin- $\alpha$ -deficient mice and alymphoplasia (*aly*) mice defined by the chimeric analysis. *J. Immunol.* 163:1584–1591.
- Shinkura, R., K. Kitada, F. Matsuda, K. Tashiro, K. Ikuta, M. Suzuki, K. Kogishi, T. Serikawa, and T. Honjo. 1999. Alymphoplasia is caused by a point mutation in the mouse gene encoding NF- $\kappa$ B-inducing kinase. *Nat. Genet.* 22:74–77.
- Iizuka, K., D.D. Chaplin, Y. Wang, Q. Wu, L.E. Pegg, W.M. Yokoyama, and Y.-X. Fu. 1999. Requirement for membrane lymphotoxin in natural killer cell development. *Proc. Natl. Acad. Sci. USA.* 96:6336–6340.
- Elewaut, D., L. Brossay, S.M. Santee, O.V. Naidenko, N. Burdin, H. De Winter, J. Matsuda, C.F. Ware, H. Cheroutre, and M. Kronenberg. 2000. Membrane lymphotoxin is required for the development of different subpopulations of NK T cells. *J. Immunol.* 165:671–679.
- Nakagawa, K., K. Iwabuchi, K. Ogasawara, M. Ato, M. Kajiwara, H. Nishihori, C. Iwabuchi, H. Ishikura, R.A. Good, and K. Onoe. 1997. Generation of NK1.1<sup>+</sup> T cell antigen receptor  $\alpha/\beta$ <sup>+</sup> thymocytes associated with intact thymic structure. *Proc. Natl. Acad. Sci. USA.* 94:2472–2477.
- Karin, M., and A. Lin. 2002. NF- $\kappa$ B at the crossroads of life and death. *Nat. Immunol.* 3:221–227.
- Karin, M., Y. Cao, F.R. Greten, and Z.W. Li. 2002. NF- $\kappa$ B in cancer: from innocent bystander to major culprit. *Nat. Rev. Cancer.* 2:301–310.
- Li, Q., and I.M. Verma. 2002. NF- $\kappa$ B regulation in the immune system. *Nat. Rev. Immunol.* 2:725–734.
- Pahl, H.L. 1999. Activators and target genes of Rel/NF- $\kappa$ B transcription factors. *Oncogene.* 18:6853–6866.
- Karin, M., and Y. Ben-Neriah. 2000. Phosphorylation meets ubiquitination: the control of NF- $\kappa$ B activity. *Annu. Rev. Immunol.* 18:621–663.
- Pomerantz, J.L., and D. Baltimore. 2002. Two pathways to NF- $\kappa$ B. *Mol. Cell.* 10:693–695.
- Poljak, L., L. Carlson, K. Cunningham, M.H. Kosco-Vilbois, and U. Siebenlist. 1999. Distinct activities of p52/NF- $\kappa$ B required for proper secondary lymphoid organ microarchitecture: functions enhanced by Bcl-3. *J. Immunol.* 163:6581–6588.
- Weih, D.S., Z.B. Yilmaz, and F. Weih. 2001. Essential role of RelB in germinal center and marginal zone formation and proper expression of homing chemokines. *J. Immunol.* 167:1909–1919.



31. Alcamo, E., N. Hacohen, L.C. Schulte, P.D. Rennert, R.O. Hynes, and D. Baltimore. 2002. Requirement for the NF- $\kappa$ B family member RelA in the development of secondary lymphoid organs. *J. Exp. Med.* 195:233–244.
32. Gerondakis, S., M. Grossmann, Y. Nakamura, T. Pohl, and R. Grumont. 1999. Genetic approaches in mice to understand Rel/NF- $\kappa$ B and I $\kappa$ B function: transgenics and knock-outs. *Oncogene*. 18:6888–6895.
33. Sha, W.C., H.-C. Liou, E.I. Tuomanen, and D. Baltimore. 1995. Targeted disruption of the p50 subunit of NF- $\kappa$ B leads to multifocal defects in immune responses. *Cell*. 80:321–330.
34. Caamaño, J.H., C.A. Rizzo, S.K. Durham, D.S. Barton, C. Raventos-Suarez, C.M. Snapper, and R. Bravo. 1998. Nuclear factor (NF)- $\kappa$ B2 (p100/p52) is required for normal splenic microarchitecture and B cell-mediated immune responses. *J. Exp. Med.* 187:185–196.
35. Weih, F., D. Carrasco, S.K. Durham, D.S. Barton, C.A. Rizzo, R.-P. Ryseck, S.A. Lira, and R. Bravo. 1995. Multi-organ inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF- $\kappa$ B/Rel family. *Cell*. 80:331–340.
36. Fütterer, A., K. Mink, A. Luz, M.H. Kosco-Vilbois, and K. Pfeffer. 1998. The lymphotoxin  $\beta$  receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity*. 9:59–70.
37. Pfeffer, K., T. Matsuyama, T.M. Kündig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P.S. Ohashi, M. Krönke, and T.W. Mak. 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. *Cell*. 73:457–467.
38. Vallabhapurapu, S., R.-P. Ryseck, M. Malewicz, D.S. Weih, and F. Weih. 2001. Inhibition of NF- $\kappa$ B in T cells blocks lymphoproliferation and partially rescues autoimmune disease in *gld/gld* mice. *Eur. J. Immunol.* 31:2612–2622.
39. Matsuda, J.L., O.V. Naidenko, L. Gapin, T. Nakayama, M. Taniguchi, C.R. Wang, Y. Koezuka, and M. Kronenberg. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J. Exp. Med.* 192:741–754.
40. Hogan, B., R. Beddington, F. Costantini, and E. Lacy. 1994. Preparing mouse embryo fibroblasts. In *Manipulating the Mouse Embryo*. Cold Spring Harbor Laboratory Press, New York. 260–261.
41. Weih, F., D. Carrasco, and R. Bravo. 1994. Constitutive and inducible Rel/NF- $\kappa$ B activities in mouse thymus and spleen. *Oncogene*. 9:3289–3297.
42. Lantz, O., L.I. Sharara, F. Tilloy, A. Andersson, and J.P. DiSanto. 1997. Lineage relationships and differentiation of natural killer (NK) T cells: intrathymic selection and interleukin (IL)-4 production in the absence of NKR-P1 and Ly49 molecules. *J. Exp. Med.* 185:1395–1401.
43. Tilloy, F., J.P. Di Santo, A. Bendelac, and O. Lantz. 1999. Thymic dependence of invariant V $\alpha$ 14<sup>+</sup> natural killer-T cell development. *Eur. J. Immunol.* 29:3313–3318.
44. Benlagha, K., T. Kyin, A. Beavis, L. Teyton, and A. Bendelac. 2002. A thymic precursor to the NK T cell lineage. *Science*. 296:553–555.
45. Pellicci, D.G., K.J. Hammond, A.P. Uldrich, A.G. Baxter, M.J. Smyth, and D.I. Godfrey. 2002. A natural killer T (NKT) cell developmental pathway involving a thymus-dependent NK1.1-CD4<sup>+</sup> CD1d-dependent precursor stage. *J. Exp. Med.* 195:835–844.
46. Slifka, M.K., R.R. Pagarigan, and J.L. Whitton. 2000. NK markers are expressed on a high percentage of virus-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells. *J. Immunol.* 164:2009–2015.
47. Burkly, L., C. Hession, L. Ogata, C. Reilly, L.A. Marconi, D. Olson, R. Tizard, R. Cate, and D. Lo. 1995. Expression of *relB* is required for the development of thymic medulla and dendritic cells. *Nature*. 373:531–536.
48. Xiao, G., E.W. Harhaj, and S.-C. Sun. 2001. NF- $\kappa$ B-inducing kinase regulates the processing of NF- $\kappa$ B2 p100. *Mol. Cell*. 7:401–409.
49. Yamada, T., T. Mitani, K. Yorita, D. Uchida, A. Matsushima, K. Iwamasa, S. Fujita, and M. Matsumoto. 2000. Abnormal immune function of hemopoietic cells from alymphoplasia (*aly*) mice, a natural strain with mutant NF- $\kappa$ B-inducing kinase. *J. Immunol.* 165:804–812.
50. Dobrzanski, P., R.-P. Ryseck, and R. Bravo. 1995. Specific inhibition of RelB/p52 transcriptional activity by the C-terminal domain of p100. *Oncogene*. 10:1003–1007.
51. Solan, N.J., H. Miyoshi, E.M. Carmona, G.D. Bren, and C.V. Paya. 2002. RelB cellular regulation and transcriptional activity are regulated by p100. *J. Biol. Chem.* 277:1405–1418.
52. Yilmaz, Z.B., D.S. Weih, V. Sivakumar, and F. Weih. 2003. RelB is required for Peyer's patch development: differential regulation of p52-RelB heterodimers by lymphotoxin and TNF. *EMBO J.* 22:121–130.
53. Paxian, S., H. Merkle, M. Riemann, M. Wilda, G. Adler, H. Hameister, S. Liptay, K. Pfeffer, and R.M. Schmid. 2002. Abnormal organogenesis of Peyer's patches in mice deficient for NF- $\kappa$ B1, NF- $\kappa$ B2, and Bcl-3. *Gastroenterology*. 122:1853–1868.
54. Ohteki, T., S. Ho, H. Suzuki, T.W. Mak, and P.S. Ohashi. 1997. Role for IL-15/IL-15 receptor  $\beta$ -chain in natural killer 1.1<sup>+</sup> T cell receptor- $\alpha\beta$ <sup>+</sup> cell development. *J. Immunol.* 159:5931–5935.
55. Matsuda, J.L., L. Gapin, S. Sidobre, W.C. Kieper, J.T. Tan, R. Ceredig, C.D. Surh, and M. Kronenberg. 2002. Homeostasis of V $\alpha$ 14i NKT cells. *Nat. Immunol.* 3:966–974.
56. Azimi, N., K. Brown, R.N. Bamford, Y. Tagaya, U. Siebenlist, and T.A. Waldmann. 1998. Human T cell lymphotropic virus type I Tax protein trans-activates interleukin 15 gene transcription through an NF- $\kappa$ B site. *Proc. Natl. Acad. Sci. USA*. 95:2452–2457.