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Activation of IKKα target genes depends on recognition of specific κB binding sites by RelB:p52 dimers

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IκB Kinase (IKK)α is required for activation of an alternative NF-κB signaling pathway based on processing of the NF-κB2/p100 precursor protein, which associates with RelB in the cytoplasm. This pathway, which activates RelB:p52 dimers, is required for induction of several chemokine genes needed for organization of secondary lymphoid organs. We investigated the basis for the IKKα dependence of the induction of these genes in response to engagement of the lymphotoxin β receptor (LTβR). Using chromatin immunoprecipitation, we found that the promoters of organogenic chemokine genes are recognized by RelB:p52 dimers and not by RelA:p50 dimers, the ubiquitous target for the classical NF-κB signaling pathway. We identified in the IKKα-dependent promoters a novel type of NF-κB-binding site that is preferentially recognized by RelB:p52 dimers. This site links induction of organogenic chemokines and other important regulatory molecules to activation of the alternative pathway.

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Introduction

The classical NF-κB signaling pathway, which is activated by proinflammatory cytokines and pathogen-associated molecular patterns (PAMPs), depends on inducible degradation of specific inhibitors, IκBs, which retain different NF-κB dimers in the cytoplasm (Ghosh and Karin, 2002). This pathway is largely dependent on IKKβ, a component of a complex that also contains the IKKα catalytic subunit and the IKKγ/NEMO regulatory subunit (Rothwarf and Karin, 1999). In this pathway, IKKβ phosphorylates IκBs at N-terminal sites to trigger their ubiquitin-dependent degradation and induce nuclear entry of NF-κB dimers (Karin and Ben-Neriah, 2000). Recently, a second NF-κB activation pathway based on regulated processing of the NF-κB2/p100 precursor protein was identified (Senftleben *et al*, 2001; Xiao *et al*, 2001). NF-κB2/p100 consists of an N-terminal Rel homology domain (RHD), common to all NF-κB proteins, and an inhibitory IκB-like C-terminal domain (Ghosh *et al*, 1998). The presence of the latter prevents nuclear translocation of p100 and its partners.

IKKα and IKKβ activate at least a dozen NF-κB dimers, composed of five subunits (Ghosh and Karin, 2002). While the mechanisms of NF-κB activation are well understood (Ghosh and Karin, 2002), the generation of biological specificity by this complex system is more enigmatic (Pomerantz and Baltimore, 2002). Mouse mutagenesis experiments indicate that IKKβ activates the classical NF-κB pathway, represented by RelA:p50 dimers, in response to stimuli such as tumor necrosis factor (TNF)α (Li *et al*, 1999; Chen *et al*, 2003). The mechanisms by which IKKα regulates cytokine-induced gene expression are more obscure and controversial (Israel, 2003). *In vivo* analysis revealed that IKKα activates an alternative NF-κB pathway based on processing of NF-κB2/p100 and release of RelB:p52 dimers in response to LTα/β trimers (Dejardin *et al*, 2002) and other TNF family members (Claudio *et al*, 2002; Kayagaki *et al*, 2002). This pathway is required for secondary lymphoid organogenesis and induction of genes involved in this process, but has no apparent role in TNFα-induced functions (Senftleben *et al*, 2001; Dejardin *et al*, 2002). We have used mice in which IKKα was rendered inactivateable (Cao *et al*, 2001) to study the mechanism responsible for selective gene induction by the alternative NF-κB signaling pathway. Using primary cultures of splenic stromal cells and bone marrow-derived myeloid dendritic cells (BMDCs) as experimental systems, we found that generation of gene induction specificity by IKKα depends on selective activation of RelB:p52 dimers, which recognize a unique type of the NF-κB-binding site.

Results

Reconstitution of lethally irradiated mice with *Ikkα*$^{-/-}$ fetal liver hematopoietic progenitors revealed a role for IKKα in late B-cell maturation, splenic organization and germinal center (GC) formation (Kaisho *et al*, 2001; Senftleben *et al*, 2001). However, embryonic lethality precludes the use of *Ikkα*$^{-/-}$ mice to identify functions for IKKα in other cell

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types involved in spleen development and organization. Homozygous knockin mice expressing an IKKα variant that cannot be activated ($Ikk\alpha^{AA/AA}$ mice) are viable, yet show defects in lymphoid organogenesis and GC formation (Senftleben *et al*, 2001). Using an antibody against FDC-M2, a follicular dendritic cell (FDC) marker, we found that $Ikk\alpha^{AA/AA}$ mice lack mature FDCs (Figure 1A). To identify the cells in which IKKα acts, reciprocal bone marrow chimeras were generated using $Ikk\alpha^{AA/AA}$ and WT mice. At 6 weeks after adoptive transfer, mice were challenged with a T-cell-dependent antigen, sheep red blood cells (SRBC), and killed 7 days later. Using an antibody against CD35, another FDC marker, we examined the formation of mature FDCs. FDC maturation was impaired in $Ikk\alpha^{AA/AA}$ recipients reconstituted with WT bone marrow, whereas a mature FDC network formed in WT recipients reconstituted with $Ikk\alpha^{AA/AA}$ bone marrow (Figure 1B). These results suggest that IKKα acts in stromal cells of the spleen to induce maturation of FDCs, which are thought to be derived from mesenchymal stromal cells (Fu and Chaplin, 1999).

Another aspect of spleen development is segregation of B and T lymphocytes to the follicles and the periarterial lymphatic sheath (PALS), respectively. WT chimeras reconstituted with $Ikk\alpha^{AA/AA}$ bone marrow, but not $Ikk\alpha^{AA/AA}$ mice reconstituted with WT bone marrow, exhibited normal B- and T-cell segregation detected by staining with anti-B220 and anti-CD5 antibodies, respectively (Figure 1C). These results also point to a critical action of IKKα in stromal cells, which, in addition to giving rise to FDCs, control splenic microarchitecture through production of organogenic chemokines that dictate cell migration and localization (Ansel and Cyster, 2001). Critical organogenic chemokines for spleen development include: ELC and SLC, ligands for the chemokine receptor CCR7; BLC, which binds CXCR5 (Forster *et al*, 1999; Ansel *et al*, 2000) and SDF-1, which promotes trafficking of both immature and naïve lymphocytes to lymphoid tissues (Kim and Broxmeyer, 1999). Previous work revealed that induction of these chemokines in response to engagement of LTβR is defective in $Ikk\alpha^{AA/AA}$ mice (Dejardin *et al*, 2002). We extended these observations to SRBC immunized mice (Figure 1D). Based on previous experiments, we examined the expression of the different genes 48 h post-immunization. While induction of the mRNAs for BLC, ELC, SLC and SDF-1 was readily detected in WT spleens, these genes were barely induced in the mutant.

The defects shown above are very similar to those exhibited by mice lacking LTβR (Fu and Chaplin, 1999). The majority of LTβR expression is restricted to stromal cells of the spleen; however, BMDCs have also been shown to express LTβR (Browning and French, 2002). We therefore isolated and cultured both of these cell types from WT and

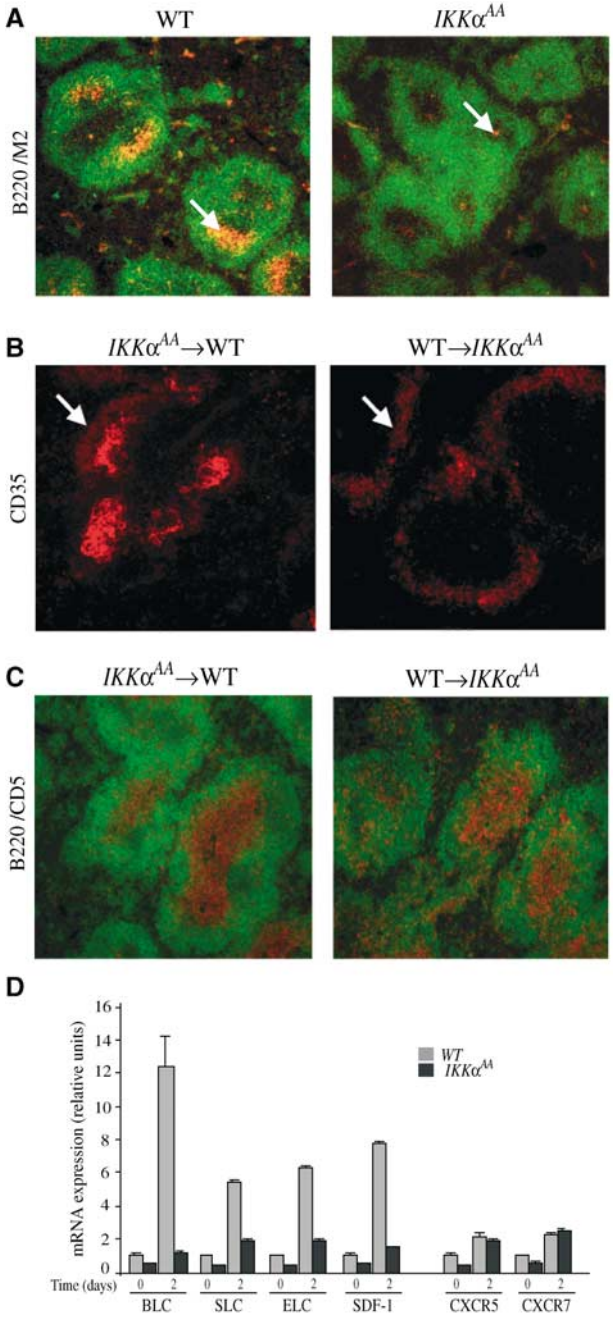


Figure 1 Impaired FDC maturation and chemokine production in stromal cell-derived FDC requires IKKα. (**A**) Absence of mature FDC network in $Ikk\alpha^{AA/AA}$ mice. Cryosections of spleen from WT ($n=6$) and $Ikk\alpha^{AA/AA}$ ($n=6$) mice, isolated 7 days post-immunization with SRBC, were stained for FDCs (arrows) with FDC-M2 (orange) and anti-B220 (green). (**B**) Impaired FDC maturation is inherent to the $Ikk\alpha^{AA/AA}$ stroma. Lethally irradiated WT ($n=6$) or $Ikk\alpha^{AA/AA}$ ($n=6$) mice were reconstituted with $Ikk\alpha^{AA/AA}$ or WT bone marrow, respectively. Spleens were isolated 7 days after immunization with SRBC, cryosectioned and stained with anti-CD35. An FDC network is present in WT mice reconstituted with $Ikk\alpha^{AA/AA}$ bone marrow, while only perifollicular rings of $CD35^+$ immature FDCs are present in $Ikk\alpha^{AA/AA}$ mice reconstituted with WT bone marrow. (**C**) Impaired B/T cell segregation in $Ikk\alpha^{AA/AA}$ spleens. Lethally irradiated WT ($n=6$) or $Ikk\alpha^{AA/AA}$ ($n=6$) mice reconstituted with $Ikk\alpha^{AA/AA}$ or WT bone marrow cells were immunized and analyzed as above, using anti-CD5 (to recognize T cells) and anti-B220 (to recognize B cells). Impaired B/T cell segregation is intrinsic to the $Ikk\alpha^{AA/AA}$ stroma. (**D**) Defective chemokine gene expression in $Ikk\alpha^{AA/AA}$ spleens. Total splenocytes from naïve and SRBC-immunized (day 2) WT ($n=6$) and $Ikk\alpha^{AA/AA}$ ($n=6$) mice were isolated. RNA was extracted and analyzed by RT–PCR for expression of mRNAs encoding BLC, SLC, ELC and SDF-1 and two of their receptors (CXCR5, CCR7). The results are averages ± s.d. of three independent experiments normalized to the level of cyclophilin mRNA.

Ikkα$^{AA/AA}$ mice. Stimulation of WT stromal cells with agonistic anti-LTβR antibody (Dejardin *et al*, 2002) resulted in four- to six-fold induction of BLC, SDF-1, TNFα, VCAM-1 and IκBα mRNA (Figure 2A). Modest induction of ELC and SLC mRNAs was also observed. Both basal expression and induction of BLC, SDF-1, ELC and SLC mRNAs were defective in *Ikkα*$^{AA/AA}$ stromal cells. Similar defects in expression of these chemokines have been described in *RelB*$^{-/-}$ and *Nfkb2*$^{-/-}$ mice (Poljak *et al*, 1999; Weih *et al*, 2001). Induction of TNFα, IκBα and VCAM-1 in *Ikkα*$^{AA/AA}$ stromal cells remained intact or was even elevated. The increased expression of VCAM-1 could be related to the defective nuclear entry of RelB in *Ikkα*$^{AA/AA}$ cells (see below), as RelB deficiency was previously found to increase the expression of certain inflammatory genes (Xia *et al*, 1999). By contrast, very few differences in expression of TNFα-inducible genes were found between WT and *Ikkα*$^{AA/AA}$ stromal cells (Figure 2A). Unlike anti-LTβR, TNFα was a poor inducer of the organogenic chemokines, but was a potent inducer of TNFα, IκBα and VCAM-1.

TNFα induced both rapid and delayed nuclear translocation of RelA in WT and *Ikkα*$^{AA/AA}$ stromal cells (Figure 2B). This response was not considerably different in *Ikkα*$^{AA/AA}$ cells (Figure 2B, right panel). Neither TNFα nor anti-LTβR had a significant effect on the subcellular distribution of p50, as this NF-κB subunit was constitutively nuclear (Figure 2B). Both TNFα and anti-LTβR induced nuclear translocation of RelB in WT cells, but only TNFα was capable of sending RelB to the nucleus of *Ikkα*$^{AA/AA}$ cells (Figure 2B). In either case, the nuclear translocation of RelB is delayed relative to that of RelA. As expected, only anti-LTβR, but not TNFα, stimulated nuclear entry of p52, and this effect was seen only in WT cells (Figure 2B). Similar results with regard to both gene expression and nuclear translocation of NF-κB subunits were observed in BMDCs. In WT BMDCs, LTβR engagement led to induction of SLC, ELC and IκBα mRNA (Figure 2C). However, SLC and ELC were not induced in BMDC from *Ikkα*$^{AA/AA}$ mice. Again, we found that at least one gene, in this case CXCR5, was elevated in mutant cells. Whereas engagement of LTβR resulted in nuclear entry of RelB and p52 in WT BMDCs, this response was defective in *Ikkα*$^{AA/AA}$ cells (Figure 2D). Nuclear translocation of RelA was not affected in *Ikkα*$^{AA/AA}$ cells. These results and the previous genetic analysis of NF-κB2- (Poljak *et al*, 1999) and RelB- (Weih *et al*, 2001) deficient mice strongly suggest that *Blc*, *Sdf-1*, *Elc* and *Slc* gene induction requires RelB:p52 nuclear translocation. Curiously, the induction of RelB and p52 nuclear entry following LTβR engagement was considerably faster in BMDCs than in splenic stromal cells. This is likely to be related to the different origins of these cell types and/or the expression of different levels of LTβR molecules on their surface.

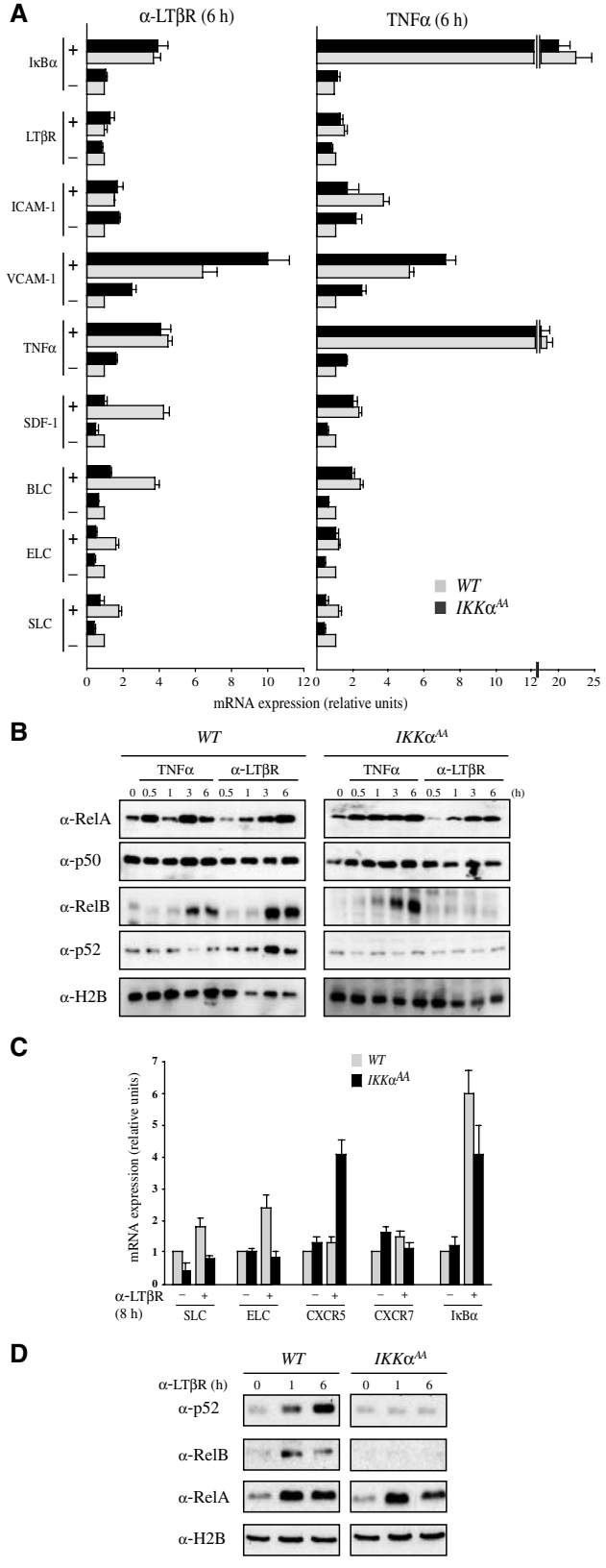


Figure 2 IKKα is required for LTβR-induced RelB:p52 nuclear translocation and chemokine expression in splenic stromal cells and myeloid dendritic cells. (**A**) *Ikkα*$^{AA/AA}$ stromal cells and (**C**) BMDC exhibit specific defects in LTβR-induced gene expression. Total RNA was extracted from either WT or *Ikkα*$^{AA/AA}$ stromal cells or BMDC before and after stimulation with 2 μg/ml agonistic anti-LTβR antibody or 20 ng/ml TNFα. Gene expression was analyzed by real-time PCR. Results are averages ± s.d. of three independent experiments normalized to the level of cyclophilin mRNA. (B, D) Nuclear translocation of NF-κB proteins. Stromal cells (**B**) and BMDC (**D**) were stimulated with either anti-LTβR antibody or TNFα as indicated. At the indicated time points (h), nuclear extracts were prepared and analyzed by immunoblotting for the presence of the indicated NF-κB subunits. The levels of histone H2B were examined to control for loading and proper cell fractionation. Contamination with cytoplasmic proteins was monitored by blotting with anti-actin antibody (not shown).

To address whether the IKKα-dependent genes are in fact direct targets for RelB-containing dimers and whether they are also recognized by RelA-containing dimers, we performed chromatin immunoprecipitation (ChIP) experiments (Saccani and Natoli, 2002). In splenic stromal cells, anti-LTβR induced efficient recruitment of RelB, but not RelA, to the *Blc* and *Sdf-1* promoters (Figure 3A), which encode the two organogenic chemokines that are most efficiently expressed by these cells (Cyster, 2003). As previously shown, recruitment of NF-κB subunits to promoter DNA may be detected at earlier time

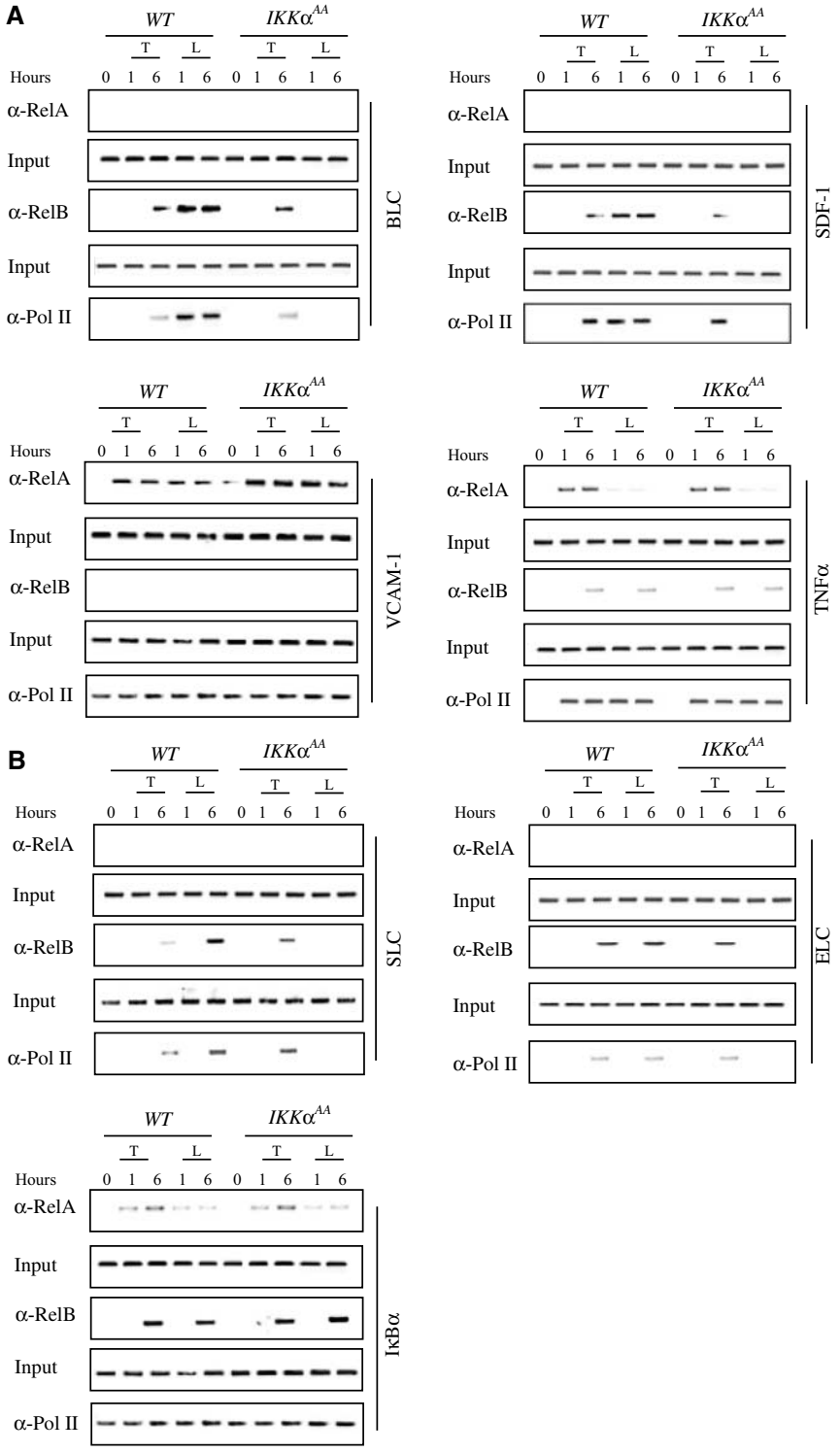


Figure 3 IKKα is required for recruitment of RelB to the *Blc*, *Sdf-1*, *Elc* and *Slc* promoters. Primary cultures of stromal cells (**A**) and BMDC (**B**) from WT and $Ikk\alpha^{AA/AA}$ mice were left unstimulated or stimulated with TNFα (T) or anti-LTβR (L). At the indicated time points (h), the cells were collected and recruitment of RelA, RelB and the large subunit of RNA polymerase (Pol II) to the indicated promoter regions was examined by ChIP experiments.

points than the ones revealed by immunoblot analysis of nuclear translocation, due to the increased sensitivity of the ChIP assay (Saccani *et al*, 2001). Anti-LTβR-induced recruitment of RelB to target gene promoters was abolished in $Ikk\alpha^{AA/AA}$ cells. However, TNFα-induced RelB promoter recruitment, which was slower and weaker than the response elicited by anti-LTβR, was not affected by the $Ikk\alpha^{AA}$ mutation (Figure 3A). The response to TNFα may depend on the formation of RelB:p50 dimers. As a control, we analyzed the same immunoprecipitates for the presence of the *Tnfα* and *Vcam1* promoter regions. We found efficient precipitation of both promoter fragments by anti-RelA antibodies, but a weak signal was obtained with anti-RelB (Figure 3A). Recruitment of either Rel protein to these promoters was not IKKα-dependent. Importantly, recruitment of Pol II to the *Blc* and *Sdf-1* promoters correlated with recruitment of RelB and was seen only in anti-LTβR stimulated WT cells (Figure 3A).

As mentioned above, splenic stromal cells are the major source of production of BLC and SDF, while BMDCs are a major source of ELC and SLC (Cyster, 2003). Therefore, in BMDCs, we examined recruitment of the different NF-κB subunits in response to LTβ signaling to the *Elc* and *Slc* promoters. Treatment with anti-LTβR induced efficient recruitment of RelB, but not RelA, to the *Elc* and *Slc* promoters (Figure 3B). No recruitment of RelA was observed. By contrast, both RelB and RelA were recruited to the *IκBα* promoter in response to either TNFα or anti-LTβR, but neither response was IKKα-dependent (Figure 3B). As observed for RelB, the LTβR-induced recruitment of Pol II to the *Slc* and *Elc* promoters was IKKα-dependent (Figure 3B).

Selective recruitment of RelB-containing NF-κB dimers to the *Blc*, *Sdf-1*, *Elc* and *Slc* promoters could reflect, previously unknown, intrinsic differences in sequence selectivity between RelB- and RelA-containing dimers. To examine this possibility, we analyzed binding of NF-κB proteins to the *Blc* and *Elc* promoters. In this experiment, we used truncated recombinant NF-κB proteins to generate NF-κB dimers of known composition. All of the proteins used in these experiments were fully characterized and even crystallized (Ghosh *et al*, 1995; Chen *et al*, 1999; G Ghosh, unpublished data). Several ^{32}P-labeled probes were derived from the 700 base pair (bp) proximal region (−688 to +12) of the *Blc* promoter, contained within the ChIP primer set (Figure 4A). One of the probes, spanning positions −191 to −20, exhibited strong binding to recombinant RelB:p52 and weak

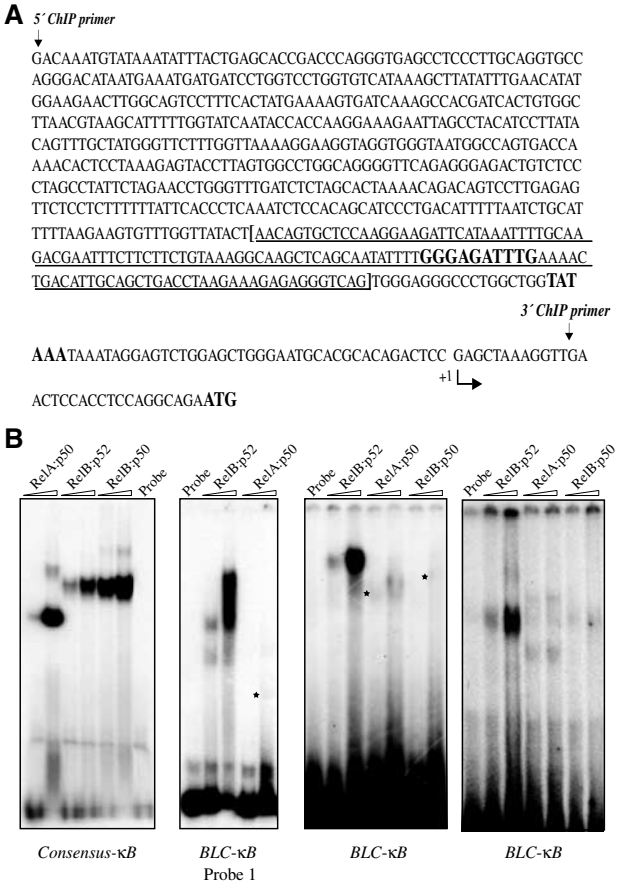
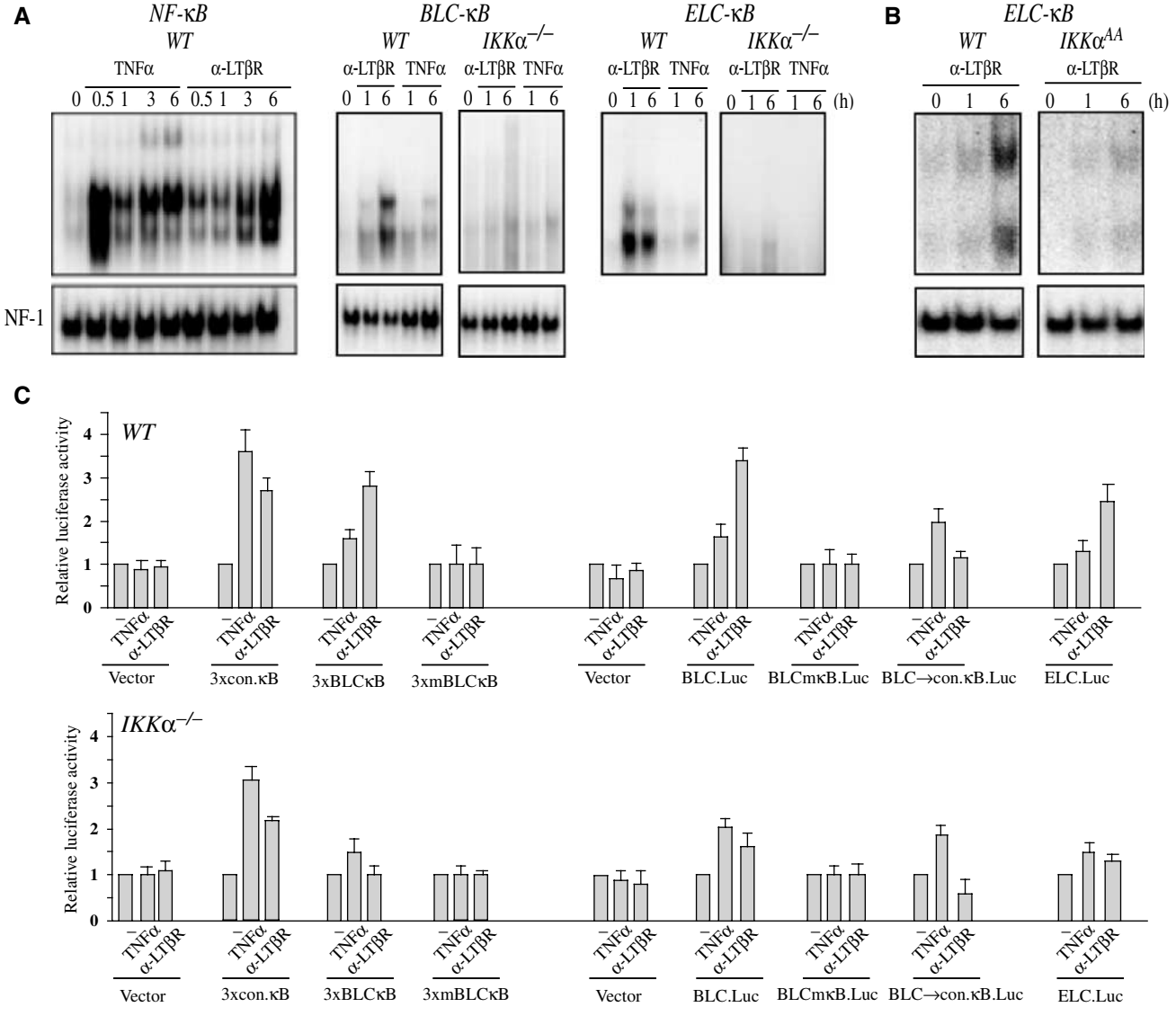


Figure 4 The *Blc* and *Elc* promoters contain a unique κB site that is selectively recognized by RelB:p52 dimers. (**A**) The sequence of the 700 bp region, covering the proximal *Blc* promoter, contained within the ChIP primer set. The RelB-selective κB site and the TATA box are highlighted. The sequence contained within Probe 1 is indicated by the brackets and is underlined. (**B**) DNA-binding analysis. The different probes were incubated with two different amounts (250 and 500 ng) of the indicated NF-κB dimers and DNA binding was analyzed by EMSA. Note that the NF-κB subunits are not the full-length proteins, thus giving rise to complexes with different electrophoretic mobilities. (**C**) The sequences of the different κB sites used in these experiments.

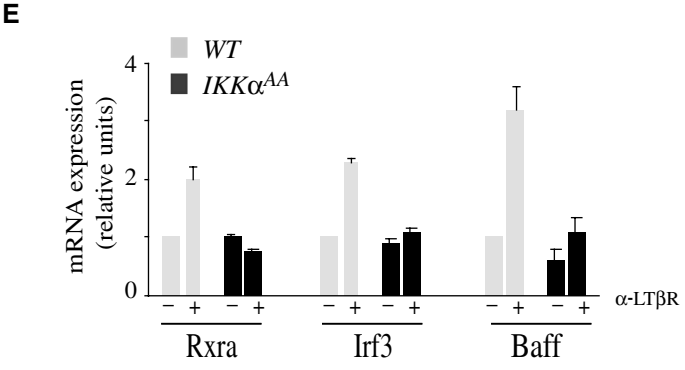
Figure 5 Selective, IKKα-dependent activation of the *Blc* and *Elc* promoters by LTβR engagement and IKKα-dependent induction of *Rxra*, *Irf3* and *Baff* mRNAs. (**A, B**) Engagement of LTβR selectively induces *Blc*-κB- and *Elc*-κB-binding activities. WT and IKKα-defective MEFs (A) and BMDC (B) were left unstimulated or stimulated with either TNFα or anti-LTβR for the indicated times. Nuclear extracts were prepared and incubated with ^{32}P-labeled probes corresponding to the consensus κB site (NF-κB) or the *Blc*-κB and *Elc*-κB sites. DNA-binding activity was analyzed by EMSA. NF-1 DNA-binding activity was measured as an internal control. (**C**) Functional analysis of the different κB sites in the *Blc* and *Elc* promoters. Triple repeats of the consensus κB (conκB), *Blc*-κB and a mutant *Blc*-κB (m*Blc*-κB) sites were cloned upstream to a minimal SV40 promoter (pGL3-Promoter vector, Promega). In addition, the *Blc* (+12 to −688) and *Elc* (+530 to −320) promoter regions were cloned upstream to a luciferase reporter (pGL3-Basic vector, Promega). To determine the importance of the *Blc*-κB site, it was converted by site-directed mutagenesis either to an inactive mutant version (mκB) or the consensus κB (conκB) site. The different plasmids were transfected into WT and $Ikk\alpha^{-/-}$ MEFs. After 6 h with TNFα or anti-LTβR, luciferase activity was determined. The results are averages ± s.d. of three independent experiments normalized to β-galactosidase activity, produced by a cotransfected β-galactosidase expression vector. (**D**) Alignment of novel κB sites from the control regions of IKKα-dependent genes. The novel κB sites from the *Blc*, *Elc* and *Sdf-1* 5′ regulatory region were aligned with those identified by computer analysis in the regulatory regions of three other IKKα-dependent genes. These sites form a consensus sequence (Alt. consensus) that, although similar, is distinct from the one associated with the classical NF-κB pathway (Class. consensus). (**E**) Induction of *Baff*, *Rxra* and *Irf3* is IKKα-dependent. Expression of the indicated mRNAs was analyzed by real-time PCR as described above, using RNA isolated from nonstimulated and anti-LTβR-stimulated stromal cells (*Rxra* and *Irf3*) and BMDCs (*Baff*) of the indicated genotypes.

binding to RelA:p50 dimers (data not shown). Several other probes (from −770 to −460, −460 to −380 and −380 to −150, as well as from −770 to −980) did not detectably bind either dimer (data not shown). To narrow down the sequence responsible for RelB:p52 binding, we generated a shorter probe (Probe 1) covering the region from −191 to −64. This probe exhibited very strong binding to recombinant RelB:p52 and only weak binding to RelA:p50 (Figure 4B). On the other hand, the RelA:p50 and RelB:p52 dimers exhibited little differences in their ability to bind a consensus κB probe, whereas a 200 bp probe (Probe 2) derived from the far 5′ upstream region (−1900 to −1700) of the *Blc* gene was preferentially recognized by RelA:p50 (data not shown). Probe 1 (−191 to −64) contained only one potential NF-κB-binding site. We synthesized two overlapping smaller probes containing this site



D

BLC-κB	(−115)	**GGGAGATTTG**
ELC-κB	(−64)	**AGGAGATTTG**
SDF-κB	(−675)	**GGGAGACCTG**
Baff #1	(−14)	**AGGAGACTTG**
Baff #2	(−71)	**GTGAGATTTG**
Rxra	(−4764)	**AGGAAATTTA**
Irf3	(−450)	**GGGAGATGTA**
Alt. consensus		**PuGGAGAPyTTPu**
Class. consensus		**GGGACTTTCC**



(Figure 4C) and used them to examine binding of RelA:p50, RelB:p52, as well as RelB:p50. Both probes, which contained the sequence 5′-GGGAGATTTG-3′, were efficiently recognized by RelB:p52 and only weakly by RelA:p50 (Figure 4B; data not shown). Binding of RelB:p50 to these probes was barely detectable. In all cases, the detected protein–DNA complexes were specific, as indicated by competition experiments (data not shown).

To identify whether nother IKKα-dependent chemokine genes contain a similar sequence, we used the Trafac server (Jegga *et al*, 2002), which identifies ortholog conserved transcription factor binding sites, to examine the human and rodent *Elc* genes. The putative binding sites were first identified using the MatInspector program (Professional Version 4.3, 2000) that utilizes a database of eukaryotic transcription factor binding sites (Jegga *et al*, 2002). This procedure identified a sequence very similar to the *Blc*-κB site at positions −64 to −50 of the *Elc* genes (Figure 4C). This site, termed the *Elc*-κB site, was also preferentially recognized by RelB:p52 dimers (Figure 4B).

We next used MEFs, which, unlike the related stromal cells, are amenable to transfection (Bebien M, unpublished results), to examine the function of the RelB:p52-specific sites. Stimulation of WT MEFs with either TNFα or α-LTβR induced DNA-binding activities recognized by the consensus κB site (Figure 5A). Using the *Blc*-κB and *Elc*-κB sites as probes, we detected induced DNA-binding activity only in WT MEFs stimulated with anti-LTβR (Figure 5A). This activity was not induced in *Ikkα*$^{-/-}$ MEFs. Similar results were obtained in BMDCs analyzed with the *Elc*-κB probe (Figure 5B). Next, we cloned three copies of either the consensus κB site, the *Blc*-κB site or an inactive version of the latter (m*Blc*-κB), upstream to a minimal SV40 promoter driving a luciferase reporter, and transfected the constructs into WT and *Ikkα*$^{-/-}$ MEFs. The consensus κB site conferred inducibility by either TNFα or anti-LTβR, whereas the *Blc*-κB site conferred an efficient response to anti-LTβR but only a weak response to TNFα, and the mutated *Blc*-κB site was inactive (Figure 5C). While the consensus κB site was equally active in WT and *Ikkα*$^{-/-}$ MEFs, the *Blc*-κB site did not confer anti-LTβR responsiveness in *Ikkα*$^{-/-}$ MEFs (Figure 5C). Using the intact *Blc* promoter fused to a luciferase reporter, we found efficient induction by anti-LTβR in WT but not in *Ikkα*$^{-/-}$ MEFs. This response was dependent on the integrity of the *Blc*-κB site and even its conversion to a consensus κB site attenuated the response to anti-LTβR (Figure 5C). The *Elc* promoter also exhibited preferential activation by anti-LTβR that was IKKα-dependent.

To further examine the relevance of the RelB:p52 selective binding site, we conducted a pattern search with two strings, namely AGGAGATTTG (*Elc*-κB) and GGGAGATTTG (*Blc*-κB), using the Trafac server and the BlastZ algorithm (http://bio.cse.psu.edu). Closely similar (at least 8/10 identity) sites were detected within 5 kb upstream to the start sites of the *Sdf-1* and *Baff* genes, whose expression is known to be *Ikkα*-dependent (Dejardin *et al*, 2002) (Figures 1D, 2A and D; data not shown). We also detected similar and evolutionary conserved sites with the same region of several other genes, whose IKKα dependence was previously unknown (Figure 5D). RT–PCR analysis revealed that two of these genes, *Rxra* and *Irf3*, coding for important transcription factors, were induced in stromal cells in response to anti-LTβR in a manner dependent on IKKα (Figure 5E).

Discussion

Two distinct pathways leading to selective activation of RelA:p50 and RelB:p52 dimers, dependent on IKKβ or IKKα, respectively, were identified (Ghosh and Karin, 2002). Each pathway has distinct biological functions (Li *et al*, 1999; Senftleben *et al*, 2001; Chen *et al*, 2003), that could be mediated in part through selective gene activation (Dejardin *et al*, 2002). How this occurs was previously unknown. We now show in two different cell types, splenic stromal cells and BMDC, that IKKα is required for induction of four genes encoding chemokines critical for spleen organogenesis and maintenance of tissue microarchitecture, because these genes are selectively recognized by RelB-containing dimers, most likely RelB:p52. These genes are preferentially activated by engagement of LTβR and are only weakly induced by TNFα. Whereas the TNFα response is IKKα-independent, the response to LTβR engagement is strictly IKKα-dependent. The latter requires two events. First, RelB:p52 dimers have to enter the nucleus, a process dependent on IKKα-mediated p100 processing (Dejardin *et al*, 2002; Yilmaz *et al*, 2003). Second, RelB:p52 dimers are selectively recruited to the IKKα-dependent gene promoter. The selective recruitment of RelB to the *Blc* and the *Elc* promoters is likely to depend on a novel κB site, whose consensus sequence (Figure 5D) is distinct from that of the classical κB site. Unlike the classical site, the novel site is preferentially recognized by RelB:p52 dimers. This unique sequence specificity is entirely consistent with sequence differences between the DNA-binding loops of RelA and RelB, but was previously unknown (Ghosh *et al*, 1995). It is entirely possible, however, that additional factors may contribute to selective IKKα-dependent gene activation and that IKKα may also be responsible in certain cell types for activation of the canonical NF-κB pathway (Cao *et al*, 2001) or for potentiating its ability to activate transcription (Anest *et al*, 2003; Israel, 2003; Yamamoto *et al*, 2003). Nonetheless, an important mechanism responsible for selective gene activation through the IKKα-dependent alternative NF-κB signaling pathway is based on specific recruitment of RelB:p52 dimers to target gene promoters. Sites similar to the RelB:p52 selective κB site were detected in the 5′ regulatory region of three other genes, whose expression was found to be IKKα-dependent (Figure 5D).

What is the purpose of the functional separation between the two NF-κB signaling pathways? The IKKα-dependent organogenic chemokines optimize adaptive immunity through proper organization of secondary lymphoid organs. By contrast, IKKβ is mostly involved in inflammatory and innate immune responses. Thus, IKKβ-mediated NF-κB signaling is responsible for rapid responses to infection and injury, which require recruitment of immune cells out of lymphoid organs to sites of infection. This response depends on pro-inflammatory chemokines, such as MIP-1, MCP-1 and RANTES, which are induced by the canonical NF-κB signaling pathway (Alcamo *et al*, 2001). The arrival of antigens to secondary lymphoid tissues from distal sites of infection and

their processing, presentation and recognition require coordinated activity of DC, macrophages, T cells and B cells, whose recruitment to secondary lymphoid organs depends on IKKα-regulated organogenic chemokines. Premature expression of such chemokines would compromise the immediate antimicrobial response as it may abort the emigration of immune cells to the periphery. It is, therefore, logical that expression of organogenic chemokines would not be induced through the canonical NF-κB signaling pathway. Consistent with its delayed function in adaptive immunity, activation of the alternative NF-κB signaling pathway is slower than the canonical NF-κB signaling pathway and seems to depend on prior activation of the latter (Dejardin *et al*, 2002). The dependence of the two pathways on distinct but related protein kinases and transcription factors allows for both functional integration and kinetic separation.

Materials and methods

Primary cell cultures

Stromal cell cultures were established from spleens of WT and $Ikk\alpha^{AA/AA}$ mice as described (Skibinski *et al*, 1998). Spleens were gently ground and released cells cultured in DMEM supplemented with heat-inactivated FCS (Invitrogen, Carlsbad, CA). After 1 week, nonadherent cells were removed, adherent cells were washed twice with PBS and cultured for one more week in DMEM/FCS. The absence of contaminating myeloid and lymphoid cells was verified by flow cytometry (FACSCalibur, Becton Dickinson). Stromal cells are uniformly positive for ICAM-1 (data not shown). BMDCs were cultured as described (Wu and Hwang, 2002).

Adoptive transfers

Bone marrow cells ($3–4 \times 10^6$ cells per mouse) were isolated from femurs of WT or $Ikk\alpha^{AA/AA}$ mice and injected intravenously into lethally irradiated recipients. Mice were H-2 matched and, in the case of $Ikk\alpha^{AA/AA}$, were from the F3–F5 backcross to C57Bl/6. Mice were provided antibiotics in drinking water and killed 6–8 weeks post reconstitution. When indicated, mice were immunized i.p. with SRBC (Colorado Serum Company, Denver, CO) 7 days prior to killing (Poljak *et al*, 1999).

Immunohistochemical analysis

Cryosections (8–10 μM) of spleen were prepared, dried and fixed with acetone before immunohistochemical analysis (Poljak *et al*, 1999; Weih *et al*, 2001). Staining reagents were: FDC-M2 (ImmunoKontact, UK), ICAM-1 (Santa-Cruz Biologicals, CA), B220, and CD35-bio (clone 8C12) (all from BD Pharmingen). Immune-complexes were detected using species-specific secondary reagents. Sections were viewed by immunofluorescence microscopy (HM505E Microm Inc, Walldorf, Germany) and images captured with a digital camera (Nikon E800 Scope with Spot Diagnostics Digital Camera, AG Heinze Inc., Lake Forest, CA).

Electrophoretic mobility shift assay and immunoblots

Nuclear and cytoplasmic extracts were prepared and analyzed for levels of NF-κB subunits and DNA-binding activity (Bonizzi *et al*, 1999; Senftleben *et al*, 2001). Recombinant NF-κB subunits (not full-length proteins) were produced in *Escherichia coli* and purified as described (Chen *et al*, 1999). All antibodies and immunoblotting procedures were described (Senftleben *et al*, 2001).

Real-time PCR analysis and ChIP assay

Real-time PCR was performed using a PE Biosystems 5700 thermocycler following the SyBr Green™ protocol. Briefly, 12 ng of total cDNA, 50 nM of each primer and 1 × SyBr Green™ mix were used in a total volume of 25 μl. All values were standardized to that of cyclophilin mRNA. Primer sequences are available upon request. ChIP assays were as described (Saccani and Natoli, 2002). Polyclonal antibodies to p65 (C-20), RelB (C-19) and Pol II (N-19) were from Santa Cruz. The sequences of the promoter-specific primers (*Blc* +12 to −688, *Sdf-1* +22 to −678, *Vcam-1* +30 to −640, *Iκbα* +20 to −340, *Tnfα* +20 to −545) and a detailed experimental protocol are available upon request.

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