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IκB Kinase Complex α Kinase Activity Controls Chemokine and High Endothelial Venule Gene Expression in Lymph Nodes and Nasal-Associated Lymphoid Tissue

Danielle L. Drayton,* Giuseppina Bonizzi,† Xiaoyan Ying,* Shan Liao,* Michael Karin,† and Nancy H. Ruddle2*

The lymphotactic (LT) β receptor plays a critical role in secondary lymphoid organogenesis and the classical and alternative NF-κB pathways have been implicated in this process. IKKα is a key molecule for the activation of the alternative NF-κB pathway. However, its precise role and target genes in secondary lymphoid organogenesis remain unknown, particularly with regard to high endothelial venules (HEV). In this study, we show that IKKα–/– mutant mice, who lack inducible kinase activity, have hypocellular lymph nodes (LN) and nasal-associated lymphoid (NALT) tissue characterized by marked defects in microarchitecture and HEV. In addition, IKKα–/– LNs showed reduced lymphoid chemokine CCL19, CCL21, and CXCL13 expression. IKKα–/– LN- and NALT-HEV were abnormal in appearance with reduced expression of peripheral node addressin (PNAd) explained by a severe reduction in the HEV-associated proteins, glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1), and high endothelial cell sulfotransferase, a PNAd-generating enzyme that is a target of LTαβ. In this study, analysis of LTβR+/− mice identifies GlyCAM-1 as another LTβ-dependent gene. In contrast, TNFRI−/− mice, which lose classical NF-κB pathway activity but retain alternative NF-κB pathway activity, showed relatively normal GlyCAM-1 and HEC-6ST expression in LN-HEV. In addition, in this communication, it is demonstrated that LTβR is prominently expressed on LN- and NALT-HEV. Thus, these data reveal a critical role for IKKα in LN and NALT development, identify GlyCAM-1 and high endothelial cell sulfotransferase as new IKKα-dependent target genes, and suggest that LTβR signaling on HEV can regulate HEV-specific gene expression. 

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2 Address correspondence and reprint requests to Dr. Nancy H. Ruddle, Yale University School of Medicine, Department of Epidemiology and Public Health, 60 College Street, P.O. Box 208034, New Haven, CT 06520-8034. E-mail address: nancy.ruddle@yale.edu

Abbreviations used in this paper: LT, lymphotactic; PP, Peyer’s patches; NALT, nasal-associated lymphoid tissue; LN, lymph node; CD62L, L-selectin; BAFF, B cell-activating factor; IKK, IκB kinase complex; IKKα, IKKβ and IKKγ inactive; NIK, NF-κB inducing kinase; MLN, mesenteric lymph node; HEV, high endothelial venules; HEC-6ST, high endothelial cell sulfotransferase; PNAd, peripheral node addressin; GlyCAM-1, glycosylation-dependent cell adhesion molecule 1; DIG, digoxigenin; WT, wild type; FDC, follicular dendritic cell; PLN, peripheral lymph node.

Given the relatively mild defects in TNF-α−/− mice, the phenotype of TNFRI−/− mice does not reflect signaling from TNF-α only but suggests an important role for LTα:TNFRI interaction in secondary lymphoid organogenesis. LTβR−/− mice lack PP and peripheral LNs (PLNs) but retain mesenteric LNs (MLN) and cervical LNs, and exhibit splenic defects that are less severe than those of LTα−/− mice (7).

Two NF-κB signaling pathways, the canonical and alternative pathways that can be activated in response to TNFRI or LTβR engagement, have been implicated in differential regulation of secondary lymphoid organogenesis (8). Additional TNF family receptors, B cell-activating factor (BAFF)-R and CD40, also appear to participate in the alternative pathway (9). In mammals, the NF-κB family of transcription factors includes five members that form various homo- and heterodimeric complexes: NF-κB1 (p105 processed to p50), NF-κB2 (p100 processed to p52), RelA (p65), RelB, and c-Rel (9). Activation of the canonical NF-κB pathway can be induced by a wide variety of stimuli including proinflammatory cytokines, such as IL-1 and TNF-α, bacterial endotoxins, and viral proteins (10). In most cell types, NF-κB is sequestered in the cytoplasm by inhibitory IκB proteins. Activation of NF-κB depends on the IκB kinase complex (IKK), which contains the IκB regulatory subunit and two catalytic subunits, IKKα and IKKβ (9). IKKβ and IκBγ are critical mediators of the canonical NF-κB pathway required for phosphorylation of IκB proteins, whereas IKKα is dispensable for activation of this pathway in response to most stimuli (10–12). Following IKK activation, the IκB proteins are phosphorylated and degraded through a ubiquitin-dependent process, thereby allowing nuclear translocation of NF-κB dimers that activate expression of proinflammatory genes, including VCAM, MIP-1β, and MIP-2 (8, 9).
LTαβ binding to LTβR results in activation of the canonical NF-κB pathway as well as the alternative NF-κB pathway. The latter is based on phosphorylation-dependent proteolytic processing of cytosolic NF-κB2 (p100) to p52 and nuclear translocation of p52:RelB heterodimers (8, 9, 13–15). Activation of the alternative pathway requires the NF-κB-inducing kinase (NIK) and IKKα, but is independent of IKKβ and IKKγ (8, 13, 16). Early studies in embryonic fibroblasts from alymphoplasia mice (aly/aly), which lack functional NIK (17), and from IKKα−/− mice revealed that NIK and IKKα are dispensable for TNFR1-induced NF-κB activation but are required for activation of the alternative NF-κB pathway in response to LTβR engagement (14). Other studies have demonstrated that LTβR-induced NF-κB2 processing and RelB nuclear translocation are NIK- and IKKα-dependent (8, 16). Early data implicated both the canonical and alternative NF-κB pathways in control of lymphoid chemokine expression—CCL19 (EBV-induced molecule 1 ligand chemokine), CCL21 (secondary lymphoid chemokine), and CXCL13 (B lymphocyte chemoattractant; Ref. 18). More recent results derived from the activation of the LTβR in vivo by treatment of mice with an LTβR agonistic reagent suggests that the LTβR→NIK→IKKα alternative pathway induces expression of these lymphoid chemokine genes (8). Furthermore, studies in LTβ-deficient and rat insulin promoter LTαβ transgenic mice indicate an involvement of LTβR signaling in regulation of high endothelial venule (HEV)-specific gene expression (19). Though the classical NF-κB pathway has been analyzed in endothelial cell lines in vitro, the roles of the classical and alternative NF-κB pathways in HEV in vivo is completely unknown. This is the major focus of the present communication.

HEV are specialized postcapillary venules that facilitate L-selectin (CD62L)+ lymphocyte extravasation into LN and NALT through expression of adhesion molecules, peripheral node addressin (PNAd), and lymphoid chemokines. PNAd, a CD62L ligand detected by the MECA 79 Ab, is generated by high endothelial cell transferrin receptor (HEC-6ST)-mediated sulfaLT of various glycoproteins including CD34 and glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1; Ref. 19). In the absence of HEC-6ST, the normal luminal MECA 79 staining pattern is lost, though abluminal staining remains (20). Although the LTα:TNFRI system can regulate mucosal addressin cell adhesion molecule 1 expression (21–23), presumably through the canonical NF-κB pathway, our previous data suggest that the LTαβLTβR system is a key regulator of PNAd through induction of HEC-6ST (19). One hypothesis to be tested in this study is that this occurs through the alternative NF-κB pathway.

Analysis of aly/aly mice or mice with targeted disruptions of alternative NF-κB pathway genes, including NIK, NF-κB2, and RelB have revealed important roles for all of these molecules in the generation of normal splenic microarchitecture and PP development (24). aly/aly and NIK−/− mice lack all LNs, while mice deficient in the downstream molecules, RelB and NF-κB2, have a less profound defect in that they lack some, but not all LNs (24, 25). A role for IKKα in LN organogenesis has not been established. IKKα−/− mice exhibit perinatal death associated with defects in skeletal morphogenesis and epidermal differentiation (26, 27). Given the complete absence of LNs in NIK−/− mice, the relative paucity of LNs in RelB−/− and NF-κB2−/− mice, and IKKα perinatal lethality, studies addressing the role of these molecules in secondary lymphoid organogenesis have focused on PP and spleen development and have revealed the absence of PP and multiple splenic defects (24).

The NALT is an organized lymphoid tissue found in the rodent nasal tract that has structural similarities to PPs as well as the tonsils and adenoids in humans due to its common origin from the Waldeyer’s ring (28). Previous studies have demonstrated that initiation of NALT organogenesis is independent of LTα, LTβ, LTβR, and NIK, NF-κB2, and RelB. However, the NALT of mice deficient in these molecules exhibit reduced cellularity and diminished expression of lymphoid chemokines and vascular addressins (24, 29). To address the contribution of the alternative NF-κB pathway to LN and NALT organogenesis, particularly the role of IKKα in these processes, we have studied IKKα−/− mutant mice, in which two amino acid substitutions were introduced into the activating phosphorylation sites of IKKα, resulting in prevention of its activation by upstream stimuli (30). IKKα−/− mice are viable and display only minor developmental defects (30). In regard to lymphoid organ development, IKKα−/− mice were found to lack PP and exhibit defects in splenic organization similar to IKKα−/− mice (13).

In this study, we provide in vivo evidence that IKKα kinase activity is required for normal LN and NALT organogenesis through regulation of tissue microarchitecture, cellular composition, lymphoid chemokine expression, and HEV-specific gene expression. In addition to highlighting a role for IKKα kinase activity in LN and NALT organogenesis, these studies identify GlyCAM-1 and HEC-6ST as new IKKα-dependent target genes.

Materials and Methods

Mice

TNFRI−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). LTβ−/− mice have been previously described (31). TNFRI−/− and LTβ−/− mice, on a C57BL/6 background, were studied under a protocol approved by the Yale University Institutional Animal Care and Use Committee. IKKα−/− mice, on a 129/C57BL/6 genetic background, were previously described (30) and studied under a standard protocol approved by the University of California, San Diego, Office of Animal Care (La Jolla, CA) according to National Institutes of Health guidelines. All mice were studied between 6 and 10 wk of age.

LN visualization

Adult mice were injected in the hind footpads with 10 μl of 1% Evans blue in PBS (Eastman Kodak, Rochester, NY) 24 h before sacrifice. The dye becomes concentrated within lymphoid tissue and facilitates macroscopic detection of even very small LNs.

Histologic analysis

For immunofluorescence, LN and NALT tissues were dissected and frozen in OCT compound (Tissue-Tek; Sakura Finetek, Torrance, CA) on dry ice. NALT tissue was isolated as previously described (32). Seven-micrometer tissue sections were cut (longitudinal sections of NALT tissue) onto poly-L-lysine-coated glass slides (Sigma-Aldrich, St. Louis, MO), fixed in 100% cold acetone for 10 min, and stored at −70°C. For staining, slides were air-dried at room temperature. Sections were double-stained with anti-HEC-6ST generated by our laboratory (19) and MECA 79 (anti-PNAd; BD Pharmingen, San Diego, CA) as previously described (19), or with MECA 79 and anti-LTβR (BD Pharmingen) Abs. For MECA 79 and LTβR double-staining, MECA 79 was detected using Cy3-conjugated goat anti-rat IgM Ab (Jackson ImmunoResearch Laboratories, West Grove, PA), and LTβR was detected using biotinylated anti-hamster IgG (Jackson ImmunoResearch Laboratories). Slides were analyzed by fluorescence microscopy using a Zeiss Axioskop microscope (Carl Zeiss Microimaging, Thornwood, NY).

For immunohistochemistry, frozen sections of LN tissue were prepared and stained as previously described (19). Primary Abs used were as follows: anti-CD35 (CR1) from BD Pharmingen, and rabbit-anti-mouse GlyCAM-1 (CAMA 02) was a generous gift from Dr. S. Rosen (University of California, San Francisco, CA).

In situ hybridization

The technique previously described by Drayton et al. (19) was used. Briefly, fixed LNs were cut onto poly-L-lysine-coated slides and analyzed with the following digoxigenin (DIG)-labeled riboprobes: CCL21, CCL19, and CXCL13 sense and antisense probes. Signal was detected by overnight...
incubation of sections with alkaline-phosphatase-conjugated sheep anti-DIG Ab (Roche Diagnostic Systems, Mannheim, Germany) and was developed with NBT/5-bromo-4-chloro-3-indolyl phosphate (Invitrogen Life Technologies). CCL21 and CXCL13 oligonucleotide primers have been previously described (8).

**Flow cytometric analysis**

LN and thymic tissues were harvested from mice, homogenized in PBS, and passed over a 40-μm cell strainer (BD Biosciences, Bedford MA). Isolated cells were resuspended in FACS buffer (PBS supplemented with 1% FBS and 0.1% sodium azide). FACS staining was performed by conventional procedures using either FITC- or PE-conjugated anti-CD3, -CD4, -CD8, -CD19, -CD11c, -CD11b, and -CD62L Abs. The percentage of positive cells was determined by flow cytometry.

**Quantitative RT-PCR**

Total RNA was prepared from WT and IKKαAA LNs with an RNeasy kit (Qiagen, Valencia, CA) and treated with RNase-Free DNase set to remove residual genomic DNA (Qiagen). First strand cDNA was prepared using oligo(dT) primers and SuperScript II reverse transcriptase (Invitrogen Life Technologies, Gaithersburg, MD). CCL21 and CXCL13 oligonucleotide primers have been previously described (8).

**Results**

**Rudimentary LNs from IKKαAA mice exhibit structural and cellular defects**

Previous studies have established a role for IKKα kinase activity in PP and splenic development through analysis of IKKαAA mutant mice (30). However, LN development has not been studied in detail. To further determine the contribution of IKKα to lymphoid organs, we investigated the presence of LNs in IKKαAA mice by injection of Evans blue in the hind footpad. Upon initial macroscopic examination of untreated IKKαAA mice, inguinal LNs appeared to be absent while the presence of other LNs, including the MLNs, was apparent. However, closer examination of Evans blue-injected animals revealed the presence of several other LNs, including inguinal LNs (Fig. 1A). Although all LNs analyzed (axillary, brachial, iliac, lumbar, inguinal, popliteal, cervical, and mesenteric) could be detected in IKKαAA mice, they were smaller than those of wild-type (WT) mice (Fig. 1A) and exhibited reduced cellularity (Table I). Of the LNs examined, inguinal, axillary, and popliteal LNs exhibited the most striking reduction in size (Fig. 1A, and data not shown). Given the reduction in the size of IKKαAA LNs, we next examined the cellular composition of these tissues by flow cytometric analysis (FACS). Quantitation of absolute cell number and FACS analysis of IKKαAA LN cells revealed a reduction in total cellularity. All populations were affected (Fig. 1 and Table I) and, in fact, there was a large proportion of cells that did not react with standard phenotypic markers. Most notably, the proportion and number of mature B220+CD19+ B cells was markedly reduced (Fig. 1B and Table I). Although B220+CD19+ B cells comprised 40% of the total LN cells in WT controls, this population was reduced to 4% in IKKαAA LNs. No significant differences in the proportion of CD4+ and CD8+ T cells were observed in WT and IKKαAA LNs (Fig. 1B) or the thymus (data not shown). In addition to B cell defects, we also observed a reduction in the proportion and number of CD11c+ dendritic cells and CD11b+ macrophages in IKKαAA LNs (Fig. 1B and Table I). This impaired LN cellular composition prompted us to investigate the contribution of IKKα kinase activity to other hallmarks of normal LNs. Two-color immunofluorescence analysis of WT and IKKαAA LNs revealed numerous defects in IKKαAA LN microarchitecture. WT LNs exhibited T and B cell compartmentalization and prominent B cell follicles (Fig. 2). Consistent with the paucity of B cells revealed by flow cytometric analysis, IKKαAA LNs were characterized by only a thin rim of B cells located in the LN cortex (Fig. 2). Further immunohistochemical analysis of the B cell compartment in these LNs with anti-CD35 revealed fewer and smaller follicular dendritic cell (FDC) networks than in WT controls (Fig. 2).

**Table I. Cellularity of WT and IKKαAA PLNs**

|          | Total Cell Number (× 10⁶) | CD4⁺ | CD8⁺ | CD19⁺B220⁺ | CD11c⁺ | CD11b⁺ |
|----------|--------------------------|------|------|------------|--------|--------|
| WT PLN  | 12.83 ± 0.97             | 4.68 ± 0.58 | 3.17 ± 0.52 | 4.04 ± 1.10 | 0.72 ± 0.23 | 0.49 ± 0.26 |
| IKKαAA PLN | 9.87 ± 2.11             | 2.11 ± 0.95 | 0.95 ± 0.19 | 0.33 ± 0.01 | 0.35 ± 0.21 | 0.15 ± 0.11 |

α Cells were isolated from WT and IKKαAA PLNs. PLNs included pooled brachial and axillary LNs. Cells were first counted by trypan blue exclusion on a hemacytometer, the proportion of the indicated cell populations was determined by flow cytometry, and the absolute cell number was determined; (n = 3).
IKKα kinase activity contributes to HEV gene expression and the generation of functional HEV ligands

Given a previously hypothesized role for LTαβ and the alternative NF-κB pathway in HEV genesis (19), the hypocellularity of IKKα AA LNs, and diminished CCL21 expression in IKKα AA LN-HEV, we investigated the HEV phenotype in IKKα AA PLNs. First, WT, TNFRI−/−, LTβ−/−, and IKKα AA LNs were analyzed by immunohistochemistry for GlyCAM-1 expression. In WT LN, GlyCAM-1 exhibited high HEV-specific expression (Fig. 3A). TNFRI−/− mice exhibited slightly reduced expression of Gly-CAM-1 on LN-HEV (Fig. 4B). In contrast, both IKKα AA and LTβ−/− LN-HEV exhibited marked reductions in GlyCAM-1 expression (Fig. 4A). Whereas low levels of GlyCAM-1 could be detected in IKKα AA LNs, it was undetectable on LTβ−/− MLN-HEV. Consistent with the marked reduction in the Gly-CAM-1 protein, GlyCAM-1 mRNA transcripts were not detectable by in situ hybridization analysis in LTβ−/− MLN-HEV, and in five of six (∼83%) IKKα AA mice, while one IKKα AA mouse exhibited severely reduced GlyCAM-1 expression (data not shown).

To further examine the contribution of IKKα kinase activity to the HEV phenotype, we investigated PNAd, the HEV-borne CD62L ligand, and HEC-6ST expression by two-color immunofluorescence on WT, TNFRI−/−, LTβ−/−, and IKKα AA LNs. WT LN-HEV exhibited concomitant pericellular (i.e., luminal and abluminal) PNAd expression and high levels of HEC-6ST (Fig. 4, E and I). Analysis of TNFRI−/− LNs revealed a pattern of PNAd and HEC-6ST expression similar to that seen in WT controls, although a few small vessels displayed only abluminal PNAd expression.

with fewer B cells in IKKα AA LNs, CXCL13 transcripts were significantly reduced and in some cases, undetectable by in situ hybridization analysis (Fig. 3A). Stromal expression of CCL21 and CCL19 was also reduced in IKKα AA LNs (Fig. 3A). Moreover, HEV-specific CCL21 expression was greatly diminished (Fig. 3A, inset). Quantitative RT-PCR analysis revealed a respective 10- and 27-fold reduction in CCL21 and CXCL13 expression compared with WT controls (Fig. 3B).

FIGURE 3. Lymphoid chemokine expression is diminished in IKKα AA LNs. A. In situ hybridization analysis of WT littermate and IKKα AA LNs with DIG-labeled anti-sense riboprobes to CCL21, CCL19, or CXCL13. Stromal expression of CCL21 and CCL19 and follicular expression of CXCL13 (open arrowhead) are dramatically reduced in IKKα AA LNs. A high magnification inset (filled arrowhead) also reveals a marked reduction in CCL21 expression on IKKα AA LN-HEV. Positive signal is seen as dark purple staining. Original objective, ×20. CCL21 inset, ×100. B. Real-time PCR of total RNA was extracted from WT and IKKα AA LNs, reverse transcribed, and CCL21 and CXCL13 expression levels were monitored. CCL21 and CXCL13 mRNA expression is reported relative to GAPDH mRNA expression.
with no detectable HEC-6ST (Fig. 4, F and J). The HEV phenotype in TNFRI−/− mice is the reciprocal of that seen in LTβ−/− mice. Whereas TNFRI−/− mice exhibited predominantly pericylular PNAd expression on HEV, we have previously shown that LTβ−/− mice exhibit a higher proportion of HEV displaying only abluminal PNAd and a corresponding absence of HEC-6ST on these vessels (Fig. 4, H and L; Ref. 19). In contrast to WT and TNFRI−/− LN-HEV, IKKαAA LN-HEV displayed an abnormal pattern of PNAd staining characterized by a lack of distinct pericylular PNAd expression (Fig. 4G) presumably due to the modification of other glycoproteins, such as CD34, by a sulfotransferase other than HEC-6ST as previously discussed (19, 20). Furthermore, HEC-6ST expression was markedly reduced compared with WT controls and TNFRI−/− mice (Fig. 4K). Consistent with diminished HEC-6ST expression on IKKαAA LN-HEV, quantitative RT-PCR revealed a 4-fold reduction in this transcript compared with WT (data not shown).

Given diminished HEC-6ST expression and nearly undetectable GlyCAM-1 expression in IKKαAA LN-HEV, we next examined CD62Lhigh cell recruitment to these LNs as an indicator of HEV function. We have previously reported that CD62Lhigh cell accumulation to these LNs is an indicator of HEV function. We have previously reported that CD62Lhigh cell accumulation is reduced in LTβ−/− MLN (19). Although the proportion of CD62Lhigh cells was similar in both WT, TNFRI−/−, and IKKαAA MLNs, the intensity of CD62L staining was reduced on only those cells isolated from IKKαAA MLNs pointing to a reduction in functional CD62L on HEV (Fig. 5A, and data not shown). Whereas nearly 70% of WT MLN cells exhibited high CD62L expression, this population was dramatically reduced to 24% in IKKαAA MLNs (Fig. 5B).

**FIGURE 4.** IKKα kinase activity is crucial for HEV-specific gene expression. WT, TNFRI−/−, IKKαAA, and LTβ−/− LN sections were analyzed with Abs directed against GlyCAM-1, PNAd, and HEC-6ST. WT and TNFRI−/− LNs exhibited high HEV-specific expression of all markers, while IKKαAA LN-HEV displayed reduced levels of all markers. LTβ−/− LN-HEV exhibited undetectable GlyCAM-1 expression and a large proportion (40–50%) of the HEV exhibited only abluminal PNAd expression and a corresponding absence of HEC-6ST as previously described (19). Original objective, ×40.

IKKα kinase activity is important for normal NALT organogenesis

We also investigated the involvement of IKKα in NALT development. Gross histology revealed the presence of this organ in both WT and IKKαAA mice (Fig. 6). However, when compared with WT controls, IKKαAA NALTs were reduced in size (Fig. 6, A and E). Whereas the WT NALT is characterized by a large proportion of B cells surrounded by a smaller ring of T cells (Fig. 6B), IKKαAA NALTs exhibited marked structural disruptions including comingling of T and B cells (Fig. 6F). As was seen in the LN, both the number of T and B cells in the NALT appeared reduced but the effect was more pronounced with regard to B cells.

**FIGURE 5.** Reduced levels of CD62L expression on IKKαAA LN cells. A, Flow cytometric analysis of WT and IKKαAA MLN cells stained with anti-CD62L revealed a reduction in CD62Lhigh cell accumulation in IKKαAA MLN. B, The percentage of CD62Lhigh and CD62Llow cells was determined from the total CD62L+ cells.
FIGURE 6. Impaired NALT organogenesis in IKKα^AA^ mice. A and E. Comparison of WT littermate and IKKα^AA^ NALT tissue stained with hematoxylin reveals reduction in the size of IKKα^AA^ NALT. B and F. Cellular compartmentalization of NALT tissue was analyzed by two-color immunofluorescence with anti-CD3 PE (red) and anti-B220 FITC (green). In WT NALT, a central area of B cells (green) is surrounded by a small ring of T cells (T, red) whereas the IKKα^AA^ NALT was characterized by an absence of distinct T and B cell compartments. C–H. HEV-specific gene expression is markedly reduced on IKKα^AA^ NALT-HEV. Fixed WT NALT-HEV exhibited high coexpression of PNAd and HEC-6ST while expression of these markers were severely reduced on IKKα^AA^ NALT-HEV. Original objective, x20.

We next examined the NALT-HEV phenotype by two-color immunofluorescence. Similar to WT LN-HEV, WT NALT-HEV exhibited intense PNAd staining with coincidental HEC-6ST expression (Fig. 6, C and D). However, IKKα^AA^ NALT had few PNAd^+^ HEV (Fig. 6G). These vessels were small with primarily abluminal PNAd staining compared with predominantly pericellular PNAd expression found in WT NALT. Similarly, HEC-6ST expression was dramatically reduced in IKKα^AA^ NALT-HEV (Fig. 6H).

LTβR is expressed on LN- and NALT-HEV

We have previously shown that LTβ, likely signaling through the LTβR, is important for the optimal expression of HEC-6ST and PNAd on HEV (19), and shown here with analysis of LTβ/−/− mice that GlyCAM-1 is also an LTβ target. Given that IKKα is a crucial component of LTβR signaling and that there were HEV defects in IKKα^AA^ LN and NALT tissue, we sought to determine whether LTβR was expressed on HEV to determine whether LTβR activity could contribute directly to HEV-specific gene expression. C57BL/6 LN and NALT tissue were analyzed by two-color immunofluorescence with Meca 79 (anti-PNAd) and anti-LTβR Abs (Fig. 7). Examination of LN and NALT tissue revealed prominent coexpression of both PNAd and LTβR on HEV. Interestingly, while PNAd^LTβR^+^ HEV were readily detected in both LN and NALT tissue, there are also some HEV that expressed only the LTβR, particularly in the NALT.

Discussion

In this paper, we have studied the contribution of IKKα, an essential component of the alternative NF-κB pathway, to LN and NALT organogenesis. By using IKKα^AA^ knockin mice, we provide evidence that IKKα activation and kinase activity contribute in a crucial manner to both LN and NALT organogenesis through regulation of tissue cellularity, organization, chemokine expression, and HEV development. IKKα^AA^ mice were characterized by the presence of small, hypocellular LN and NALT tissue exhibiting multiple structural defects. Although IKKα^AA^ kinase activity is not critical for T and B cell compartmentalization in the LN, it plays an important role in B cell follicle development and is also crucial for compartmentalization of the NALT. Furthermore, consistent with the hypothesis that signaling via the alternative NF-κB pathway regulates lymphoid chemokine expression (8), we find that IKKα^AA^ mice have severe defects in LN expression of CCL21, CCL19, and CXCL13. The reduced expression of these chemokines involved in the recruitment of lymphoid and myeloid cells to secondary lymphoid organs (33) may account, in part, for the reduced numbers of cells. Further analysis also revealed a previously unidentified role for IKKα in HEV development through regulation of HEV-specific gene expression. It is likely that impaired expression of HEV genes also contributes to the significant reduction in cell recruitment to IKKα^AA^ LN and NALT organs. It is of interest that CD62L expression on B cells is only half that of T cells, and that when CD62L expression is limited, reduced rolling is seen on HEV where CD62L ligand expression is also reduced (34). In previous reports, it has been noted that additional CD62L ligands probably exist (35, 36) and these ligands could bind cells expressing different levels of CD62L and could account for the entry of CD62L^low^ cells into IKKα^AA^ LNs. It is also possible that a different ligand/receptor pair could be used by these cells.

Numerous reports addressing the cellular and molecular interac-
suggesting that signaling via the alternative NF-κB pathway is not crucial for initiation of NALT development (29). In this study, we report that IKKα kinase activity is also dispensable for NALT formation but plays an important role in NALT maturation. Unlike LNs, which complete development during embryogenesis or shortly thereafter, the NALT continues to develop up to 6 wks after birth. Similar to LNs, IKKα kinase activity plays a crucial role in NALT architecture, cellularity, and HEV development.

We have previously described a role for LTαβ in HEV development through the regulation of PNAd and HEC-6ST expression and proposed that this regulation occurs through the LTαβ→NIK→IKKα alternative NF-κB pathway (19). In support of this hypothesis, in this study, we show that IKKα is necessary for optimal PNAd and HEC-6ST expression on LN- and NALT-HEV. In addition, we demonstrate that a mechanism by which LTαβ regulates the HEV phenotype is through regulation of GlyCAM-1 expression. We established that LTαβ expressed on endothelial cells, engages the alternative NF-κB pathway, triggers IKKα activation and subsequent transcription of HEC-6ST, GlyCAM-1, and CCL21. Although LTαβ expression has been detected on HUVEC cells (42), its expression on HEV had not been described previously. In this paper, we establish that LTβR is highly expressed on HEV in both the LN and NALT; a finding in support of the hypothesis that LTαβ can exert its effects directly on high endothelial cells. Future studies will address whether direct signaling via the LTβR on high endothelial cells in vivo regulates gene expression.

Targeted disruptions of alternative NF-κB pathway components including LTβ, LTβR, NIK, NF-κB2, and RelB, result in extensive defects in secondary lymphoid organogenesis (7, 24). The defects in IKKαAA mice are not as severe as those seen in mice deficient in the upstream signaling molecule, NIK, in that IKKαAA mice retain LN and NALT albeit these organs are defective. One possibility for the retention of small, rudimentary LNs and NALT tissue in IKKαAA mice is that the IKKαAA mutation only prevents inducible IKKα kinase activity but permits basal activity (13). However, as the phenotype of IKKαAA mice is similar to that of mice deficient in downstream molecules of the alternative NF-κB pathway including NF-κB2 and RelB, the basal activity of the IKKαAA variant must be very low. Most notably, NF-κB2AA mice have strikingly similar LN defects—small, undeveloped LNs, defective follicle formation, and a marked reduction in B cell accumulation (24). Therefore, while IKKα kinase activity is not essential for initiation of LN and NALT organogenesis, it is crucial for later stages of development including HEV formation, lymphoid chemokine expression, and proper tissue microarchitecture.

Given the gross similarities between IKKαAA, NF-κB2−/−, and RelB−/− mice, a previously identified role for IKKα in LTβR activation of the alternative NF-κB pathway, and the normal activation of the canonical NF-κB pathway by TNF-α in IKKαAA mice (8, 30), it is likely that the secondary lymphoid organ defects in IKKαAA mice are attributable to abrogated activation of the alternative NF-κB pathway. However, the fact that LNs are not completely eliminated in IKKαAA, NF-κB2−/−, and RelB−/− mice suggests that components of the canonical pathway are also involved in this process. For example, molecules such as VCAM-1 and mucosal addressin cell adhesion molecule 1, induced by both TNFRI and LTβR through the canonical pathway, are also crucial for normal LN development (7). In addition, both the LTα:TNFRI and LTαβ:LTβR ligand:receptor pairs regulate lymphoid chemokine expression (8, 18). Therefore, while both NF-κB pathways differentially regulate gene expression, their activities likely cooperate in secondary lymphoid organogenesis and HEV development.

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