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Role of CXC Chemokine Ligand 13, CC Chemokine Ligand (CCL) 19, and CCL21 in the Organization and Function of Nasal-Associated Lymphoid Tissue

Javier Rangel-Moreno,* Juan Moyron-Quiroz,* Kim Kusser,* Louise Hartson,* Hideki Nakano,† and Troy D. Randall2*

Nasal-associated lymphoid tissue (NALT),3 is specialized to provide immunity to Ags that contact the mucosal surface (1). Thus, unlike lymph nodes, NALT is not encapsulated and is not supplied by afferent lymphatics (1). Instead, NALT has a specialized dome epithelium, with membranous cells (M cells) that transport Ag from the luminal surface of the epithelium to the APCs directly underneath (2). Membranous cells also allow the transport of bacteria or viruses into NALT (2, 3), while immune responses to these pathogens are initiated (4). In response to stimulation by pathogens or Ags, NALT facilitates cell differentiation and iso- type switching to IgA (5), which is efficiently transported across the epithelium to the mucosal surface (6). CD4 responses to group A streptococcus are induced in NALT (4) as are CD8 responses to respiratory viruses, such as influenza (7). Thus, the NALT of mice serves as an important mucosal inductive site, similar in function to the palatine tonsils in humans.

Despite the structural and functional differences between encapsulated lymph nodes and NALT, all secondary lymphoid organs share a related architecture, with separated B and T cell areas and specialized populations of stromal cells (8). The architecture of lymphoid organs is maintained by homeostatic chemokines, which are constitutively expressed and direct the recruitment and placement of lymphocytes and dendritic cells (DCs) (9). CXCL13 (B lymphocyte chemotactic) is expressed in B cell folicies and attracts CXC5+B cells (10) and activated CX5+ T cells (11). CXCL13 is also expressed by some high endothelial venules (HEVs) and helps to recruit CXCR5-expressing cells into lymphoid organs (12). In contrast, CCL21 (secondary lymphoid organ chemokine) and CCL19 (EB1 ligand chemokine) are expressed in the T cell areas of secondary lymphoid organs and are primarily responsible for the recruitment of naive CCR7+ T cells and activated CCR7+ APCs to these areas (13, 14). CCL21 is also strongly expressed on HEVs and is instrumental for the recruitment of T cells from blood into lymph nodes (15). Together, the homeostatic chemokines organize the B and T cell areas of secondary lymphoid organs and help to orchestrate the recruitment and interaction of lymphocytes and APCs.

The constitutive expression of homeostatic chemokines in spleen is dependent on the lymphotxin-α (LTα) signaling pathway (16, 17). As a result, the production of CXCL13, CCL21, and CCL19 is reduced in the spleens of Ltα−/− mice (16), which prevents the formation of B cell folicies, gerinmal centers, marginal zones, and follicular DCs (FDCs) and disrupts the proper segregation of B and T cell areas (18, 19). In addition, Ltα−/− mice lack lymph nodes and Peyer’s patches due to an inability of lymphoid tissue inducer cells to trigger the differentiation of mesenchymal cells into mature stromal cells during embryogenesis (20, 21). Homoeostatic chemokines are also required to recruit lymphoid tissue

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2 Address correspondence and reprint requests to Dr. Troy D. Randall, Trudeau Institute, 154 Algonquin Avenue, Saranac Lake, NY 12983. E-mail address: trandal@trudeauinstitute.org
3 Abbreviations used in this paper: NALT, nasal-associated lymphoid tissue; AID, activation-induced cytokine deaminase; DC, dendritic cell; FDC, follicular DC; HEV, high endothelial venule; LTα, lymphotxin-α; NP, macroprotein; PA, acidic polymers; PNA, peanut agglutinin; PNAd, peripheral node addressin.
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inducer cells to sites of lymph node development (22, 23). Thus, the interplay between LT signaling and the expression of homeostatic chemokines controls the development of secondary lymphoid organs as well as their organization and function.

A notable exception to this model is the development of NALT (7, 24). Surprisingly, LTα does not develop in the absence of the cytokines, LTα, LTβ, and TNF-α; the receptors TNP1 and LTβR; and the signaling molecule NF-κB-inducing kinase (7, 24), demonstrating that neither TNF nor LT signaling pathways are required for NALT development. Despite the fact that LTα development is initiated in the absence of LT signaling, the structure of LTα is severely compromised in the absence of LTα (7), suggesting that the loss of this signaling pathway leads to impaired chemokine expression in NALT (25). The function of LTα is also impaired in Lta−/− mice, because it is unable to support germinal centers or promote isotype switching to IgA, even though IgA terminal center formation or promote isotype switching to IgA, even

Council. conducted according to the principles outlined by the National Research Trudeau Institute Institutional Animal Care and Use Committee and were of Trudeau Institute. All procedures using animals were approved by the California, San Francisco, CA). All gene-targeted mice were on the region from behind the incisors to the posterior end of the soft palette, was removed from the incisors forward, as was any muscle. Finally the

expression of CXCL13 and CCL19, but not that of CCL21, is highly suggesting that the loss of this signaling pathway leads to impaired chemokine expression in NALT (25). The function of LTα is also impaired in Lta−/− mice, because it is unable to support germinal centers or promote isotype switching to IgA, even though IgA terminal center formation or promote isotype switching to IgA, even

In this study we tested whether the structural and functional defects in the NALT of Lta−/− mice are related to impaired chemokine expression or to the loss of LTα. We found that the expression of CXCL13 and CCL19, but not that of CCL21, is highly impaired in the NALT of Lta−/− mice. We also found that stromal cell defects in the NALT of Lta−/− mice are related to the lack of LTα-induced CXCL13 expression, whereas defects in the development of peripheral node addressin (PNAd)-expressing HEVs are related primarily to the loss of LTα. Furthermore, we found that the NALT of Cxcl13−/− and Lta−/− mice does not support germinal center formation or promote isotype switching to IgA, even though IgA + T cells accumulate in the nasal mucosa of these mice. In contrast, although CD8 T cell responses are impaired in the NALT of Lta−/− mice, they are normal in the NALT of Cxcl13−/− mice. Thus, many of the structural and functional defects in the NALT of Lta−/− mice are due to the impaired expression of homeostatic chemokines, whereas impaired HEV development is directly due to the loss of LTα.

Materials and Methods

Mice

C57BL/6 and Lta−/− mice were obtained from The Jackson Laboratory. Cxcl13−/− and pit/pit mice were obtained from Dr. J. Cyster (University of California, San Francisco, CA). All gene-targeted mice were on the C57BL/6 genetic background and were bred at the animal breeding facility of Trudeau Institute. All procedures using animals were approved by the Trudeau Institute Institutional Animal Care and Use Committee and were conducted according to the principles outlined by the National Research Council.

Preparation of NALT and nasal mucosa

For histochemical staining of NALT, heads of euthanized mice were fixed in neutral buffered formalin for 24 h. The skull was decalcified in 7% EDTA in PBS for several days, then embedded in paraffin, sectioned, and stained with H&E. For immunofluorescence analysis, cells from the nasal mucosa were prepared for ELISPOT analysis. Cells from the nasal mucosa were prepared for ELISPOT analysis by first removing the skin, lower jaw, and brain from the head. The nose was removed from the incisors forward, as was any muscle. Finally the region from behind the incisors to the posterior end of the soft palate, including a large area of nasal mucosa and NALT, was mechanically disrupted through a metal screen. Viable cells were isolated by density centrifugation.

Immunofluorescence

Biotinylated anti-B220, anti-CD3, anti-CD11c, and anti-CD21/CD35 Abs were purchased from BD Pharmingen. Biotinylated Abs were detected with strepavidin conjugated to either Alexa 488 or Alexa 594. Purified anti-PNAa was a gift from Dr. R. Muebs (Vrije Universiteit Medical Center, Amsterdam, The Netherlands). Anti-PNAa was detected with rabbit anti-rat IgM. Polyclonal anti-CXCL13, anti-CCL21, and anti-CCL20 Abs (R&D Systems) were detected using donkey anti-rabbit IgG. Anti-BP3 (Research Diagnostics) was biotinylated using NHS-LC-biotin (Pierce). Anti-ERTR7 Ab was detected using Alexa 488-conjugated donkey anti-rat IgG. All slides were viewed with a Zeiss Axioplan 2 microscope. Images were recorded with a Zeiss AxioCam digital camera. Images were cropped and rotated in Adobe Photoshop 7.0 and saved as TIFF files.

Polymerase chain reaction

Mice were killed at 8 wk of age, and NALT was dissected with the help of a stereoscopic microscope. Total RNA was extracted using the RNeasy kit (Qiagen). DNase-treated RNA was reverse transcribed with oligo(dt) and SuperScript II (Invitrogen Life Technologies). Quantitative PCR was performed using TaqMan Universal PCR Master Mix, following the Applied Biosystems protocol. Primers and probes for GAPDH, CXCL12, CXCL13, CCL19, CCL20, LTβ, and activation-induced cytidine deaminase (AID) were obtained from Applied Biosystems. Primers for CCL21 (5′-AGACT CAGGAGCCCAAAGCA-3′ and 5′-GTGAAAAGCCGAGGAAGGGT-3′) were synthesized by IDT, and the probe for CCL21 (5′-FAM-CACCT CATGTCGCTCTCGT-BHQ-3′) was synthesized by Biosearch Technology. Quantitative PCRs were performed using a PRISM 7700 instrument from Applied Biosystems available through the Molecular Biology Core Facility at Trudeau Institute. Standard PCRs were performed with 50 ng of cDNA using the primers 5′-CTCTGGCCGTTATTGTTG-3′ and 5′-GACGTCGTTGAGTTCAGTG-3′ to amplify IκBα transcripts and the primers 5′-GGCCGAAAACCGAGGATTGACA-3′ and 5′- GTAGCCTGGCCGTGGATTGAGTC-3′ to amplify RPL32. Conditions for the amplification included a denaturing step of 95°C for 5 min, followed by 35 PCR cycles (94°C for 30 s, 58°C for 1 min, 72°C for 1 min). At the end, an extension step of 5 min at 72°C was added to the amplification reaction. PCR products were resolved on 1% agarose gels.

Influenza infection and analysis

Mice were infected intranasally with 100 egg infectious units of A/PR/38 influenza and were killed 10 days after infection. Cells from the NALT were isolated and analyzed by flow cytometry. Viral titers in the nasal mucosa were determined by homogenizing the excised tissue in 2.5 ml of PBS and inoculating embyonated chicken eggs with 100 μl of 10-fold serial dilutions of the homogenate. Allantoic fluid was harvested from inoculated eggs 4 days later, and infected eggs were scored by hemagglutination of chicken RBC. The viral end-point titer was defined as the highest dilution in which two or more eggs scored positively in the hemagglutination assay.

Flow cytometry

Cells from NALT were incubated in 3% FCS in PBS containing 10 μg/ml 2,4-DMA to block FcR binding, followed by the addition of fluorochrome-conjugated Abs or MHC class I tetramers. Fluorochrome-conjugated Abs were obtained from BD Biosciences. Peanut agglutinin (PNA) was obtained from Vector Laboratories. The H-2Dβ class I tetramers containing either the nucleoprotein366–374 peptide (NP) or the acidic polymerase224–236 peptide (PA) were generated by the Trudeau Institute Molecular Biology Core Facility. Flow cytometry was performed on a dual-laser FACSCalibur available through the Flow Cytometry Core Facility at the Trudeau Institute.

ELISPOT assays

Multiscreen 96-well plates (Millipore) were coated overnight with 1 μg/ml protein from purified, disrupted influenza virus in 50 mM NaHCO3 pH 9.5. Plates were blocked with complete RPMI 1640 medium containing 10% FCS. Cells from NALT were cultured in 3-fold serial dilutions for 6 h. The plates were washed in PBS with 0.1% Tween 20 and incubated overnight with alkaline phosphatase-conjugated goat anti-mouse IgG or anti-IgA (Southern Biotechnology Associates). The plates were washed again and developed with 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) and NBT (Sigma-Aldrich). Spots were counted under a dissecting microscope.
Results

LTα and homeostatic chemokines control the structure of NALT

Because LTα controls the splenic expression of the chemokines, CXCL13, CCL19, and CCL21 (16), we hypothesized that the organizational and functional defects in the NALT of Lta<sup>-/-</sup> mice were due to impaired expression of these molecules. To test this hypothesis, we first used histology to examine the structure of NALT in normal C57BL/6 mice as well as in mice lacking LTα (Lta<sup>-/-</sup> mice), mice lacking CCL19 and CCL21 (plt/plt mice), and mice lacking CXCL13 (Cxcl13<sup>-/-</sup> mice). As shown in Fig. 1A, the NALT of C57BL/6 mice formed large lymphoid structures on either side of the nasal passages. In contrast, the NALT of Lta<sup>-/-</sup> mice was underdeveloped and lymphopenic (Fig. 1B), consistent with previous reports (7, 24). The NALT of plt/plt mice was larger than that of Lta<sup>-/-</sup> mice and had more lymphocytes (Fig. 1C), whereas the NALT of Cxcl13<sup>-/-</sup> mice was similar to that of Lta<sup>-/-</sup> mice (Fig. 1D). We also found that the NALT of plt/plt × Cxcl13<sup>-/-</sup> mice was structurally similar to that of Lta<sup>-/-</sup> mice (Fig. 1E). Thus, the loss of CCL21/CCL19 and CXCL13 did not prevent NALT development, but did appear to impact the structure of NALT.

LTα promotes the expression of homeostatic chemokines in NALT

We next used immunofluorescence to examine whether the loss of LTα impaired chemokine expression in NALT. As shown in Fig. 2, A–C, the NALT of C57BL/6 mice expressed CXCL13 in a reticular pattern in the center of the B cell follicle (Fig. 2A), whereas CCL21 was expressed on reticular cells in the corners of NALT surrounding the follicle and was also expressed on vascular endothelial cells (Fig. 2B). The chemokine CCL20 was expressed in the dome epithelium of NALT (Fig. 2C, arrows), similar to its placement in the dome epithelium of Peyer’s patches (26). In contrast, the NALT of Lta<sup>-/-</sup> mice (Fig. 2, D–F) did not express CXCL13 at levels detectable by immunofluorescence (Fig. 2D) and CCL21 expression was reduced relative to that in normal mice, particularly on reticular cells around the edge of the follicle (Fig. 2E). The expression of CCL20 in the dome epithelium was also reduced to near-background levels (Fig. 2F). These data demonstrate that LTα is important for the proper expression of chemokines in NALT.

We next tested whether the plt/plt mutation and the loss of CXCL13 lead to similar defects in chemokine expression in NALT. As shown in Fig. 2G, CXCL13 was expressed at nearly normal levels in the NALT of plt/plt mice, whereas CCL21 was not detected (Fig. 2H). In addition, the expression of CCL20 in the dome epithelium appeared nearly normal (Fig. 2I). As expected, CXCL13 was not detected in Cxcl13<sup>-/-</sup> mice (Fig. 2J). In contrast, CCL21 was highly expressed in Cxcl13<sup>-/-</sup> mice and was found at high levels in vascular endothelium and at lower levels in reticular cells (Fig. 2K). Finally, the levels of CCL20 appeared nearly normal in the NALT of Cxcl13<sup>-/-</sup> mice (Fig. 2L). Thus, the loss of CXCL13 alone and the mutation of the CCL21/CCL19 locus appeared to recapitulate some (but not all) of the defects in chemokine expression observed in the NALT of Lta<sup>-/-</sup> mice.

Because immunofluorescence was not sensitive enough to consistently detect CCL19 or CXCL12 (not shown), particularly after the decalcification of NALT, we also examined the expression of homeostatic chemokines using quantitative PCR. As shown in Fig. 2M, the expression of CCL19, CXCL13, and CCL20 was substantially reduced in the NALT of Lta<sup>-/-</sup> mice, whereas the expression of CCL21 and CXCL12 was not impaired. As expected, the expression of CCL19 and CCL21 was impaired in the NALT of plt/plt mice, whereas the expressions of CXCL13, CXCL12, and CCL20 was essentially normal or slightly reduced (CCL20). Unexpectedly, the expression of CCL19 was considerably reduced in the NALT of Cxcl13<sup>-/-</sup> mice, even though CCL21 expression was normal. As expected, CXCL13 was not expressed in Cxcl13<sup>-/-</sup> mice. Interestingly, the expression of CXCL13 correlated with the expression of LTβ and TNF-α, which were reduced in Lta<sup>-/-</sup> mice and in Cxcl13<sup>-/-</sup> mice. In contrast, another TNF family member, LIGHT, was expressed at similar levels in the NALT of all groups (27). Thus, the relative production of chemokine mRNAs correlated with their staining patterns detected by immunofluorescence. In addition, the expression of CXCL13 appears to be codependent on the expression of LTβ and TNF-α.

LTα and CXCL13 are required for the differentiation of stromal cells and HEVs in NALT

To determine whether CXCL13 or CCL21/CCL19 played a role in the differentiation of stromal cells in NALT, we examined the expression of BP3, CD21, and ERTR7. As shown in Fig. 3, A–D,
we observed an extensive network of BP3-expressing stromal cells in the NALT of C57BL/6 and plt/plt mice (Fig. 3, A and C), but only a few BP3-expressing fibroblast-like cells were observed in the NALT of Ltα−/− mice (Fig. 3, B and D). Similarly, the NALT of C57BL/6 and plt/plt mice contained a central B cell follicle with CD21-expressing FDCs (Fig. 3, E and G),

FIGURE 3. LTα and CXCL13 are required for the differentiation of stromal cells in NALT. Frozen sections of NALT from C57BL/6 mice (A–C), Ltα−/− mice (D–F), plt/plt mice (G–I), and Cxcl13−/− mice (J–L) were probed with Abs to CXCL13 (A, D, G, and J), CCL21 (B, E, H, and K), and CCL20 (C, F, I, and L). Sections were counterstained with 4′,6-diamidino-2-phenylindole hydrochloride (DAPI). Arrows in A, I, and L indicate epithelial expression of CCL20. All images were obtained using a 10× objective, except for the image in F, which was originally obtained using a 20× objective. The data are representative of images obtained from at least three individual mice.
whereas CD21-expressing FDCs were never observed in the NALT of Lta<sup>-/-</sup> or Cxcl13<sup>-/-</sup> mice (Fig. 3, F and H). Finally, although fibroblast-like cells expressing ERTR7 were observed surrounding the edge of NALT in all mice (Fig. 3, I–L), reticular networks of ERTR7<sup>+</sup> cells (yellow) were only observed in the lymphoid areas of NALT from C57BL/6 and plt/plt mice (Fig. 3, I and K). Thus, the development of stromal-type cells in NALT was impaired in the absence of LTA and CXCL13, but not in the absence of CCR7 ligands.

We also examined the role of LTA and chemokines in the differentiation of PNAd-expressing HEVs. As shown in Fig. 4, A and B, well-developed HEVs that express PNAd were easily detected in the NALT of C57BL/6 mice. In contrast, PNAd-expressing vascular endothelial cells were rarely detected in the NALT of Lta<sup>-/-</sup> mice (Fig. 4, C and D), and Fig. 4D is the only example in which PNAd expression was observed. Although PNAd-expressing HEVs were observed in both plt/plt mice (Fig. 4, E and F) and in Cxcl13<sup>-/-</sup> mice (Fig. 4, G and H), the HEVs appeared to be less well developed. However, PNAd expression on HEVs was still observed in the NALT of plt/plt × Cxcl13<sup>-/-</sup> mice (Fig. 4F). Thus, although the loss of CCL21/CCL19 and CXCL13 minimally impaired HEV development, the loss of LTA had a substantial impact and almost entirely prevented the development of HEVs that expressed PNAd, consistent with previous results (25).

**CXCL13 and LTA are required for germinal center formation and isotype switching to IgA in NALT**

The loss of FDCs and BP3-expressing stromal cells in the NALT of Lta<sup>-/-</sup> and Cxcl13<sup>-/-</sup> mice suggested that the formation of B cell follicles and the ability of B cells to respond to Ag would be impaired in the NALT of these mice. As shown in Fig. 5A, the frequency of CD19<sup>+</sup> B cells was reduced in the NALT of Lta<sup>-/-</sup> and Cxcl13<sup>-/-</sup> mice compared with that in the NALT of C57BL/6 mice, but was normal in the NALT of plt/plt mice. The reduced frequency of B cells in Lta<sup>-/-</sup> and Cxcl13<sup>-/-</sup> mice was observed in all B cell populations, but was most pronounced in CD19<sup>+</sup>IgD<sup>-</sup>CXCR5<sup>+</sup> mature resting B cells (Fig. 5B). These differences were reflected in the absolute numbers of CD19<sup>+</sup> B cells and CD19<sup>+</sup>IgD<sup>-</sup>CXCR5<sup>+</sup> B cells (Fig. 5, C and D) and suggested that CXCL13 is important for the recruitment or retention of mature follicular B cells in NALT.

To test whether CXCL13 was also required for B cell responses in NALT, we intranasally infected mice with influenza and assayed the frequency of germinal center B cells on day 10 after infection. As shown in Fig. 5E, germinal center B cells were not observed in the NALT of influenza-infected Lta<sup>-/-</sup> or Cxcl13<sup>-/-</sup> mice, but were observed at normal frequency in the NALT of plt/plt mice after influenza infection. Similar results were observed on days 14 and 21 after infection. The presence of germinal centers was confirmed by immunofluorescence (not shown). We also tested whether CXCL13 expression in NALT was required for B cells to switch to IgA and differentiate into IgA-secreting cells. As shown in Fig. 5F, IgA-expressing B cells were observed in the NALT of all groups by day 14 after infection. Surprisingly, the frequency of B cells that expressed IgA was highest in the NALT of Lta<sup>-/-</sup> mice (Fig. 5F), although this was not reflected in the total number of IgA<sup>+</sup> B cells (Fig. 5H) due to the reduced frequency of B cells and the smaller size of NALT in Lta<sup>-/-</sup> mice. However, many of these IgA<sup>+</sup> B cells were specific for influenza, because we observed the highest number of IgA-secreting, influenza-specific B cells in the nasal mucosa of Lta<sup>-/-</sup> mice on days 10 and 14 after infection (Fig. 5, I and J). Interestingly, we observed that the mRNA levels of AID were profoundly reduced in the NALT of Lta<sup>-/-</sup> and Cxcl13<sup>-/-</sup> mice and were also reduced in the NALT of plt/plt mice (Fig. 5K). Similarly, the levels of Igα-Cα mRNA transcripts were reduced in the NALT of Lta<sup>-/-</sup>, plt/plt, and Cxcl13<sup>-/-</sup> mice (Fig. 5L). Because AID is required for isotype switching and because the presence of Igα-Cα transcripts is a hallmark of recently switched B cells, it appears that isotype switching to IgA is impaired in the NALT of mutant mice and that switching must occur in other locations. Thus, both germinal center formation and switching to IgA in NALT are dependent on LTA and CXCL13.

**Impaired T cell responses in Lta<sup>-/-</sup> mice are due to loss of LTα and CCL19/CCL21**

The poor CCL19 expression in Lta<sup>-/-</sup> mice, plt/plt mice, and Cxcl13<sup>-/-</sup> mice suggested that T cell recruitment and T cell responses may be impaired in these mice. To test this possibility, we first determined the number of T cells in the NALT of the various mouse strains. As shown in Fig. 6A, CD3<sup>+</sup> T cells make up only a minority of cells in the NALT in all mice tested. Surprisingly, the number of T cells in the NALT of Lta<sup>-/-</sup> and plt/plt mice was similar to that in C57BL/6 mice, whereas the number of T cells in the NALT of Cxcl13<sup>-/-</sup> mice was substantially reduced (Fig. 6C). These observations were consistent between multiple experiments.
and suggested that CCL21/CCL19 were not instrumental for the maintenance of normal steady-state levels of T cells in NALT. These results were also surprising, because the plt mutation (pau-

city of lymph node T cells) leads to extremely poor T cell recruit-

ment to the lymph nodes due to lack of CCL21 expression on HEVs of lymph nodes (15). Interestingly, although reductions in

the number of T cells in the NALT of Cxcl13$$^{-/-}$$ mice were ob-

served in both CD4 and CD8 populations, the CD4 population was

more severely affected. For example, CD4 cells made up 76 ± 6.2% of T cells in C57BL/6 NALT, 83 ± 4.1% of T cells in

Lta$$^{-/-}$$ NALT, and 85 ± 1.6% of T cells in plt/plt NALT, but only

60 ± 9.1% of T cells in Cxcl13$$^{-/-}$$ NALT. The selective loss of

CD4 cells in the NALT of Cxcl13$$^{-/-}$$ mice suggested that CD4 T

cells in NALT were primarily CXCR5$$^+$$ follicular helper cells (11).

However, as shown in Fig. 6, B and D, only a minority of the

cells in NALT expressed CXCR5, and this population was main-

tained in the NALT at a similar frequency in all groups of mice.

Thus, although the number of CXCR5$$^+$$ T cells was reduced in

the NALT of Cxcl13$$^{-/-}$$ mice (Fig. 6D), the reduced numbers of total

cells in the NALT of Cxcl13$$^{-/-}$$ mice was not due to the selective

loss of CXCR5$$^+$$ T cells.

We also wanted to know whether homeostatic chemokines

played a role in the development of influenza-specific CD8 re-

sponses in the NALT. To test this possibility, we intranasally in-

fected mice with influenza and assayed the number of CD8 cells in

NALT that were responding to influenza NP$$^{366-374}$$ and influenza

PA$$^{224-233}$$ presented in H2D$$^d$$. As shown in Fig. 6, E and F, both

NP-specific and PA-specific CD8 cells were observed in the

NALT of wild-type and Cxcl13$$^{-/-}$$ mice, but were not observed in

the NALT of Lta$$^{-/-}$$ or plt/plt mice at this time. The reduced

frequencies of influenza-specific CD8 cells were reflected in the

reduced numbers of influenza-specific CD8 cells in the NALT of

Lta$$^{-/-}$$ and plt/plt mice (Fig. 6F). This was not due to a general

defect in the recruitment of CD8 cells to NALT, because similar

numbers of CD8 cells were found in the NALT of all groups of mice

at this time (Fig. 6G). We also assessed the CTL activity of

NP-specific CD8 cells in NALT in an in vitro CTL assay. Given

the limited number of cells obtained from NALT, particularly in

the mutant mice, we pooled cells from NALT of 18–20 mice/

group (shown in Fig. 6H). However, CTL activity was minimal in

FIGURE 5. B cell responses are impaired in Lta$$^{-/-}$$ and Cxcl13$$^{-/-}$$

mice. Naive mice (four or five mice per group) were killed, and the NALT

was removed and analyzed by flow cytometry. A. B cells were identified

using Abs to CD19. The numbers in each panel refer to the percentage

and SD of CD19$$^+$$ cells in the total cell population. B. Follicular B cells were

identified using Abs to CD19, CXCR5, and IgD. The cells shown were

gated on CD19$$^+$$ expression. The numbers in each panel refer to the per-

centage and SD of CXCR5$$^{+}$$IgD$$^+$$ cells within the B cell population. C. The

total number of CD19$$^+$$ B cells gated in A is shown. Significance was
determined by unpaired t test (ns, not significant). D. The total number of

CXCR5$$^+$$IgD$$^+$$ follicular B cells gated as described in B is shown. Signif-

cance was determined by unpaired t test. The data are representative of

five independent experiments. E. Mice were infected with influenza, and

germinal center B cells in NALT were identified by the expression of Fas

and the binding of PNA on day 10 after infection. The plots shown were

gated on CD19$$^+$$ cells. The numbers in each panel refer to the percentage

and SD of PNA$$^{+}$$Fas$$^+$$ cells in the B cell population. F. Mice were infected

with influenza, and IgA-expressing B cells in NALT were evaluated on day

14 after infection. The numbers in each panel refer to the percentage of

B cells that express IgA. Cells from five NALTs were pooled for this analy-

sis. G. The total number of PNA$$^{+}$$Fas$$^+$$CD19$$^+$$ germinal center B cells in

NALT. Differences were not significant by unpaired t test. H. The total

number of IgA$$^+$$ B cells in NALT. I and J. Mice were infected with influenza,

and influenza-specific IgA-secreting cells from the nasal mucosa were

analyzed by ELISPOT on day 10 (I) and day 14 (J) after infection. Cells from

five NALTs were pooled for this assay. The data are representa-

tive of three independent experiments. K. Mice were infected with influ-

enza, and mRNA levels of AID on day 14 after infection were evaluated by

quantitative PCR. The expression of AID was first normalized to the ex-

pression of GAPDH and was then normalized to the average expression of

AID mRNA in C57BL/6 mice. RNA was extracted from five mice per

group. Significance was determined by unpaired t test. L. Mice were in-

fected with influenza, and mRNA levels for Ig-CO and the housekeeping

gene RPL32 were evaluated by PCR in NALT on day 14 after infection.
The numbers in each panel refer to the percentage and SD of CD3 and CXCR5. The numbers in each panel refer to the percentage and SD of CD8 cells. The numbers in each panel refer to the percentage of tetramer-binding cells within the CD8 T cell population. The total number of CXCR5+ CD3+ T cells is shown. Significance was evaluated by unpaired t test. The data are representative of five independent experiments. E–G, Mice were infected with influenza, and virus-specific CD8 T cells in NALT were enumerated by flow cytometry on day 10 after infection. E, Influenza-specific CD8 T cells in NALT were identified by tetramer binding. The numbers in each panel refer to the percentage of tetramer-binding cells within the CD8 T cell population. F, The total number of H2-DNP366–374 and H2-DPA224–233 CD8 T cells in NALT is shown. Significance was evaluated by an unpaired t test. G, The total number of CD8 T cells is shown. No significant differences were found by unpaired t test. The data are representative of five independent experiments using four or five mice per group. H, Mice were infected with influenza, and cells from the NALT and spleen were pooled from 18–20 mice/group on day 10 after infection. Influenza-specific CD8 T cells were identified by tetramer binding. The plots shown were gated on CD8+ cells. The numbers in each panel refer to the percentage of tetramer-binding cells within the CD8 T cell population.

Viral clearance is impaired in Ltα−/− mice

The activities of influenza-specific B and T cells are both responsible for clearing influenza from the airways. To test whether clearance of influenza was impaired in the upper airways of mice lacking LTα or the homeostatic chemokines, we intranasally infected mice with influenza and determined the viral titers in nasal mucosa on day 10 after infection. As shown in Fig. 7, viral titers were significantly higher in the nasal mucosa of Ltα−/− mice, but were similar in all other groups at this time. In fact, virus was cleared from the nasal mucosa of two of four C57BL/6 mice, two of five plt/plt mice, and two of five Cxcl13−/− mice at this time, whereas virus was still detectable in five of five Ltα−/− mice. Thus, viral clearance is delayed in Ltα−/− mice, but occurs with normal kinetics in mice lacking homeostatic chemokines.

Discussion

The data presented in this report are consistent with a model in which LTα is important for the normal expression of homeostatic chemokines in NALT. In turn, homeostatic chemokines, particularly CXCL13, play an essential role in maintaining the lymphoid architecture of NALT. For example, the overall structure of NALT is similarly compromised in the absence of either LTα or CXCL13 (Fig. 1). In addition, both LTα and CXCL13 are necessary for the differentiation of FDCs as well as BP3+ and ERTR7+ stromal cells in NALT (Fig. 3). This is consistent with previous data showing that CXCL13 is necessary for primary follicle formation and all groups, even at an E:T cell ratio of 50:1 (C57BL/6, 8.7%; Ltα−/−, 6.1%; plt/plt, 6.8%; Cxcl13−/−, 9.3%). The nonspecific killing of target cells in the absence of peptide averaged 6.8%. The poor killing activity of cells from NALT is explained by the low frequency of NP-specific CD8 cells in NALT. For example, the NALT of C57BL/6 mice contains 13% T cells, 20% of which are CD8 cells, and only 11% of those are NP specific. Thus, NP-specific CD8 T cell make up only 0.28% of the NALT, resulting in an adjusted E:T cell ratio of 0.14:1. Regardless of the poor CTL activity in NALT, however, it appears that the spleen and NALT of both Ltα−/− and plt/plt mice are impaired in their ability to rapidly generate influenza-specific CD8 T cells (Fig. 6H). However, in data not shown we found that all groups of mice eventually make CD8 responses, but that influenza-specific CD8 responses are delayed by 1–2 days in the NALT of plt/plt mice and are delayed by an additional day in the NALT of Ltα−/− mice. Thus, the rapid generation of influenza-specific CD8 cells in NALT is dependent on CCL21/CCL19 and LTα, but is independent of CXCL13.

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T cells gated as described in A is shown. Significance was evaluated by an unpaired t test (ns, not significant). D, The total number of CXCR5+ CD3+ T cells is shown. Significance was evaluated by unpaired t test. The data are representative of five independent experiments. E–G, Mice were infected with influenza, and virus-specific CD8 T cells in NALT were enumerated by flow cytometry on day 10 after infection. E, Influenza-specific CD8 T cells in NALT were identified by tetramer binding. The numbers in each panel refer to the percentage of tetramer-binding cells within the CD8 T cell population. F, The total number of H2-DNP366–374 and H2-DPA224–233 CD8 T cells in NALT is shown. Significance was evaluated by an unpaired t test. G, The total number of CD8 T cells is shown. No significant differences were found by unpaired t test. The data are representative of two independent experiments using four or five mice per group. H, Mice were infected with influenza, and cells from the NALT and spleen were pooled from 18–20 mice/group on day 10 after infection. Influenza-specific CD8 T cells were identified by tetramer binding. The plots shown were gated on CD8+ cells. The numbers in each panel refer to the percentage of tetramer-binding cells within the CD8 T cell population.

FIGURE 6. T cell responses are impaired in the NALT of Ltα−/− mice.

A, T cell responses are impaired in the NALT of Ltα−/− mice. The numbers in each panel refer to the percentage and SD of CD3+ cells in the total population. B, Follicular Th cells were identified using Abs to CD3 and CXCR5. The numbers in each panel refer to the percentage and SD of CXCR5+ cells within the T cell population. C, The total number of
The differentiation of HEVs is also impaired in the NALT of \( \text{Lta}^{-/-} \) mice. However, PNA\(d \)-expressing HEVs appear nearly normal in the NALT of \( \text{Cxc13}^{-/-} \), \( \text{plt}/\text{plt} \), and \( \text{plt}/\text{plt} \times \text{Cxc13}^{-/-} \) mice. These results are similar to those published by Ying et al. (25), who showed that LT\(\alpha \) and LT\(\beta \) are required for the expression of PNA\(d \) on the luminal side of HEVs in NALT. This study also showed that LT\(\alpha \beta \) is required for the expression of the sialotransferase HEC-6ST and the protein GlyCAM-1, which is modified on sialyl Lewis rats by HEC-6ST to generate PNA\(d \) (25). Our results extend this observation and show that the effect of LT\(\alpha \) signaling is probably mediated directly rather than indirectly via the LT\(\alpha \)-dependent expression of chemokines. However, the HEVs in \( \text{Cxc13}^{-/-} \) and \( \text{plt}/\text{plt} \) mice appear somewhat smaller than those in normal mice, possibly due to reduced trafficking of cells through the HEVs.

Chemokines are also important for the function of NALT. For example, our results show that LT\(\alpha \) and CXCL13 are both required for the formation of germinal centers in NALT and for the expression of AID and \( \text{Ig} \text{-} \text{Ca} \) mRNAs. Because AID is required for isotype switching (31), and the expression of \( \text{Ig} \text{-} \text{Ca} \) transcripts is indicative of B cells that recently switched to IgA (32), these data suggest that isotype switching to IgA should be highly impaired in the NALT of \( \text{Lta}^{-/-} \), \( \text{Cxc13}^{-/-} \), and even \( \text{plt}/\text{plt} \) mice. However, despite these local defects, IgA\(^+ \) B cells accumulate in the NALT of all groups after influenza infection, albeit at lower levels in the NALT of \( \text{Lta}^{-/-} \) and \( \text{Cxc13}^{-/-} \) mice (Fig. 5H). Similarly, IgA-secreting influenza-specific plasma cells are found in the nasal mucosa of all groups and are found at highest numbers in \( \text{Lta}^{-/-} \) mice. These data contrast with those recently reported by Ying et al. (25), who showed that OVA-specific serum IgG is reduced \(~250\)-fold in \( \text{Lta}^{-/-} \) mice and that OVA-specific vaginal IgA is undetectable after intranasal immunization with OVA and cholera toxin. Other studies also demonstrate that the production of IgA in the intestine is highly impaired in \( \text{Lta}^{-/-} \) mice (33). However, our published data showed that influenza-specific IgA in serum is normal in \( \text{Lta}^{-/-} \) mice after infection (34). One possible explanation for these results is that isotype switching occurs in other locations, such as the inducible bronchus-associated lymphoid tissue (35), and that IgA-secreting cells subsequently home to the NALT and nasal mucosa after switching. However, switching to IgA is thought to occur only in lymphoid tissues, such as NALT, Peyer’s patches, and isolated lymphoid follicles (5, 36), despite previous studies suggesting that switching to IgA can occur during the development of B cells in bone marrow (37), outside of lymphoid organs (33, 38), and independently of cognate interactions with T cells (39, 40). Thus, switching to IgA may occur via unusual mechanisms in \( \text{Lta}^{-/-} \) mice, possibly due to delayed viral clearance and prolonged inflammation in the respiratory tract. Thus, despite apparently inefficient isotype switching in the NALT of \( \text{Lta}^{-/-} \), \( \text{Cxc13}^{-/-} \), and \( \text{plt}/\text{plt} \) mice, IgA\(^+ \) B cells eventually accumulate in the nasal mucosa of these mice.

Our previous results show that influenza-specific CD8 T cell responses are impaired in the NALT of \( \text{Lta}^{-/-} \) mice (7, 34). We now show that these defects correlate with impaired expression of CXCL21/CCL19 in the NALT of \( \text{Lta}^{-/-} \) and \( \text{plt}/\text{plt} \) mice. This is consistent with previous results showing that T cell responses to contact Ags or s.c. Ags are delayed in \( \text{plt}/\text{plt} \) mice (41). However, T cell responses to other viruses are not significantly delayed in

![FIGURE 7. Viral clearance is impaired in \( \text{Lta}^{-/-} \) mice. Mice were intranasally infected with influenza, and viral titers in the nasal mucosa were determined on day 10 after infection. There were four mice in the C57BL/6 group and five mice in all other groups. Significance was evaluated using an unpaired t test.](http://www.jimmunol.org/)
partly responsible for the structural and functional defects in the NALT organogenesis is independent of the IL-7R, LTα/β, R on the lamina propria stromal cells of the gut is required for homeostasis of lymphoid tissues. Nature 98: 12620–12623.


