Growth Factors, Cytokines, Cell Cycle Molecules

Lymphotoxin Plays a Crucial Role in the Development and Function of Nasal-Associated Lymphoid Tissue through Regulation of Chemokines and Peripheral Node Addressin

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The mechanism of nasal-associated lymphoid tissue (NALT) development is incompletely understood with regard to the roles of cytokines, chemokines, and vascular addressins. Development of the wild-type NALT continued in the immediate postnatal period with gradual increases in cellularity, compartmentalization into T- and B-cell zones, and expression of lymphotoxin (LT)- α , LT- β , and lymphoid chemokines (CCL21, CCL19, CXCL13). High endothelial venules (HEVs) developed that expressed GlyCAM-1, HEC-6ST [an enzyme crucial for expression of luminal peripheral node addressin (PNAd)], and PNAd itself. LT- $\beta^{-/-}$ and LT- $\alpha^{-/-}$ NALTs had fewer cells than those of wild-type mice, reduced (LT- $\beta^{-/-}$) or absent (LT- $\alpha^{-/-}$) lymphoid chemokines, and no T- and B-cell compartmentalization. LT- $\beta^{-/-}$ HEVs expressed only abluminal PNAd and no HEC-6ST or GlyCAM-1. LT- $\alpha^{-/-}$ HEVs had no PNAd, HEC-6ST, or GlyCAM-1. Because intranasal immunization gives rise to vaginal IgA, immunization of LT- $\beta^{-/-}$ mice, which retain cervical lymph nodes, might generate such a response. Intranasal immunization with ovalbumin and cholera toxin revealed lower cytokine levels in the LT- $\alpha^{-/-}$ and LT- $\beta^{-/-}$ NALTs, and undetectable vaginal IgA. In contrast, splenic cytokines and serum IgG titers, although reduced, were detectable. These data indicate that LT- α_3 and LT- $\alpha_1\beta_2$ cooperatively contribute to NALT development and function through regulation of lymphoid chemokines and adhesion molecules; they are the first to implicate LT- $\alpha_1\beta_2$ in GlyCAM-1 regulation in NALT HEV development. (*Am J Pathol* 2005, 166:135–146)

The nasal-associated lymphoid tissue (NALT), a pair of lymphoid organs above the soft palate in mice and rats, is considered analogous to human tonsils and adenoids because of its common embryonic origin from Waldeyer's ring.¹ Its biological importance lies not only in its immediate physical proximity to the external environment, but more importantly its demonstrated crucial role with regards to generation of responses in the genitourinary tract.² Systemic immunization with the major capsid protein of human papillomavirus type 16 results in specific IgG in serum, but no secretory IgA. Only intranasal immunization results in high titers of IgG and IgA in vaginal washes in addition to serum immunoglobulins.³ Similar generation of vaginal IgA was found after intranasal immunization with bovine papillomavirus.⁴ The NALT drains into the cervical lymph node (CLN) and that node has been demonstrated to play a role in its activity.⁵ Although it was known that cells continued to accumulate in the NALT in the postnatal period,^{6,7} scant information was available concerning the kinetics of NALT development, particularly with regard to lymphoid chemokines and vascular adhesion molecules, and the mechanism of the lymphotoxin (LT) family's contribution to postnatal development was unexplored.^{6–10}

The LT family members are critical for lymphoid organ development. LT- α_3 expression is sufficient for mucosal LNs that include the mesenteric, cervical, and sacral LNs,^{11–13} whereas the LT- $\alpha\beta$ complex is required for development of peripheral LNs.^{11,14} Both LT- α_3 and LT- $\alpha\beta$ contribute to Peyer's patches and splenic development and organization. The embryonic initiation of NALT appears to be independent of LT- α or LT- β in that the organ is present in mice that lack expression of these

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cytokines.⁷ However, even though the NALT is detectable, it is sparsely populated, and in the tumor necrosis factor (TNF)/LT- $\alpha^{-/-}$ mouse a loss of T- and B-cell compartmentalization is apparent, reminiscent of the defects seen in the spleens of LT- $\alpha^{-/-}$ mice.^{7,15} Members of the LT/TNF family play crucial, but not completely redundant roles in the development of LNs, Peyer's patches, and spleen,⁸ in part through regulation of chemokines⁹ and adhesion molecules.¹⁰ Therefore, we wished to identify the mechanism by which the individual family members contribute to the development and function of the NALT to determine whether a similar pattern would emerge even in an organ in which the cytokines were not essential for its initiation. Furthermore, we hypothesized that LT- $\beta^{-/-}$ mice, which retain CLNs could still exhibit some NALT function with regard to induction of vaginal IgA, but that LT- $\alpha^{-/-}$ mice might be less likely to mount a normal immune response to intranasal inoculation. In this study, we address the postnatal development of the NALT and the role the members of the LT family play in this process.

We place particular emphasis on the development and regulation of high endothelial venules (HEVs). Because the NALT had been considered to be a mucosal-associated lymphoid tissue similar to Peyer's patches because of its embryonic origin, location, and presence of M cells, it was expected that the NALT HEVs would express the mucosal cell adhesion molecule-1 (MAdCAM-1). However, the NALT expresses only low levels of that molecule but high levels of peripheral node addressin (PNAd).^{7,16} Furthermore, Csencsits and colleagues¹⁶ showed that the initial naïve lymphocyte binding to NALT HEVs is mediated primarily by PNAd-L-selectin interaction. Thus, we have concentrated on PNAd as a marker of HEVs.

PNAd, a sulfated L-selectin ligand detected by MECA-79 antibody, is dependent on several modifications of a variety of core proteins including CD34, GlyCAM-1, podocalyxin, and Sgp200.¹⁷ Key to PNAd's biological activity is the sulfation of sialyl Lewis x, a posttranslational modification mediated by a HEV sulfotransferase (HEC-6ST), originally called HEC-GlcNAc6ST¹⁸ or L-selectin ligand sulfotransferase (gene name CHST4).¹⁹ This sulfation is necessary for the epitope recognized by the monoclonal antibody MECA-79 and also for the high affinity binding of L-selectin. The LN HEVs of mice that are genetically defective in HEC-6ST lose luminal reactivity with MECA-79 antibody, although abluminal expression is retained.²⁰ LT- α expression is sufficient for abluminal PNAd but not HEC-6ST in ectopic lymphoid accumulations in transgenic mice and in the mesenteric LN in LT- $\beta^{-/-}$ mice; however the LT- $\alpha\beta$ complex plays a crucial role in luminal PNAd and HEC-6ST.¹⁰ Here in the NALT we investigated whether the LT- $\alpha\beta$ complex controls PNAd in the NALT through regulation of GlyCAM-1 and HEC-6ST. Although it was known that the NALT is present in LT- $\alpha^{-/-}$ mice, its function with regard to induction of vaginal IgA had not been determined nor had it been compared with that of LT- $\beta^{-/-}$ mice, with their retained CLNs. The data presented here confirm that the NALT continues its development in the immediate postnatal period with increased expression of lymphoid organ chemokines and HEV mRNAs and proteins. Although LT is not required for the initiation of the organ, it does play key roles in its postnatal

development, organization, and function through induction of chemokines and adhesion molecules. LT- α 's role is quantitatively and qualitatively different from that of the LT- $\alpha\beta$ complex with regard to chemokines and especially HEV development.

Materials and Methods

Mice

C57BL/6 [wild type (WT)] mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and the National Institutes of Health (Bethesda, MD). LT- $\alpha^{-/-21}$ and LT- $\beta^{-/-11}$ mice on a C57BL/6 background were maintained in a specific pathogen-free colony at Yale University. Mice were used between birth and 3 months of age as indicated in individual experiments. All animal work was done under the approval of the Yale University Institutional Animal Care and Use Committee.

NALT Isolation and Cell Preparation

NALT tissue, still tightly attached to the palate, was isolated from mice according to the technique of Heritage and colleagues.²² To obtain lymphoid cells without bone debris, NALTs were gently pushed off the palates and teased in medium with syringe needles and then the cells were passed through a 40- μ m Falcon cell strainer (BD Biosciences, Bedford, MA), isolated by Percoll (Amersham Biosciences, Piscataway, NJ), and washed with phosphate-buffered saline (PBS).

Histological Analysis

Standard hematoxylin and eosin-staining procedures were used on formalin-fixed, paraffin-embedded tissue for histological analysis. The Dermatopathology Laboratory at the Yale University School of Medicine performed slide preparation and staining. For immunohistochemistry, palates were dissected and immediately frozen with the NALT facing downward in a mold filled with Tissue-Tek O.C.T. compound medium (Sakura Finetek, Torrance, CA) on dry ice. Longitudinal sections of 7 μ m were cut onto poly-L-lysine-coated glass slides (Sigma Diagnostics, St. Louis, MO), fixed in cold acetone for 10 minutes, and stored at -70°C until use. For staining, slides were air-dried at room temperature and blocked in 0.5% TNB (New England Nuclear, Boston, MA) for 45 minutes, followed by incubation in 3% goat serum in 0.1 mol/L Tris-HCl, pH 8.2, for 30 minutes. Sections were stained in a humidified tray with 2 μ g/ml of primary antibodies anti-CD3 (Caltag Laboratories, Burlingame, CA), anti-B220, or anti-PNAd (MECA-79) (BD Pharmingen, San Diego, CA), diluted in blocking solution. Sections were incubated for 30 minutes with species-specific biotinylated secondary antibodies diluted in blocking solution. Slides were developed by an initial incubation with streptavidin-conjugated alkaline phosphatase VectaStain ABC reagent (Vector Laboratories, Burlingame, CA), followed by Vector Red (alkaline phosphatase) substrate

solution (Vector Laboratories) containing 100 mmol/L levamisole. In all experiments, species-specific isotype-matched irrelevant antibody was used as a negative control. Sections were counterstained with 3% methyl green (Sigma Aldrich, St. Louis, MO), and mounted in Crystal/Mount (Biomed, Foster City, CA). For immunofluorescence, NALT tissues were isolated and slides prepared as above. Sections were stained with anti-B220, anti-CD4, anti-PNAd (MECA-79) (BD PharMingen) and anti-HEC-6ST, a rabbit anti-peptide polyclonal antibody developed in our laboratory, as previously described.^{10,23} All above experiments were performed at least three times.

In Situ Hybridization

Digoxigenin-labeled sense and anti-sense RNA probes were generated by *in vitro* transcription from I.M.A.G.E Consortium expressed DNA sequence tags 596050 (SLC or CCL21), 389013 (BLC or CXCL13), and AA286397 (GlyCAM-1), obtained from Genome Systems (St. Louis, MO), and ELC (CCL19) DNA, as previously described,²⁴ obtained from Jason Cyster (University of California at San Francisco).²⁵ Palates were fixed in 4% paraformaldehyde in 0.14 mol/L Sorenson's Buffer at 4°C for 2 to 4 hours, soaked in 30% sucrose in diethylpyrocarbonate-treated PBS at 4°C overnight, then embedded in O.C.T. and frozen as above. Seven- μ m sections were cut onto diethylpyrocarbonate-poly-L-lysine-coated slides, and *in situ* hybridization was performed according to the methods described by Hjelmström and colleagues.²⁶ All experiments were performed at least three times.

Flow Cytometry

Approximately 1×10^5 to 10^6 cells per sample were incubated with either fluorescein isothiocyanate- or phycoerythrin-conjugated antibodies against B220, CD4, CD8 α , CD3 ϵ , CD11b, GR-1, and L-selectin (BD PharMingen). Data were acquired on a Becton Dickinson FACSCalibur flow cytometer and analyzed with FlowJo software (Tree Star Inc., Ashland, OR).

Quantitative Real-Time Polymerase Chain Reaction (PCR)

Upper palates containing NALTs from 0-, 4-, 7-, 10-, 14-, 21-, 28-, 42-, and 75-day-old WT mice were isolated for analysis of CXCL13, LT- α , LT- β , HEC-6ST, and GlyCAM-1 mRNA expression during development. NALTs from 6-week-old WT, LT- $\alpha^{-/-}$, and LT- $\beta^{-/-}$ mice were analyzed for GlyCAM-1 and HEC-6ST mRNA expression, and NALTs from 2- to 3-month-old WT, LT- $\alpha^{-/-}$, and LT- $\beta^{-/-}$ mice immunized intranasally with ovalbumin (OVA) (Sigma Aldrich) were analyzed for cytokine mRNAs on day 30. To optimize RNA recovery from NALT, and because there is no other lymphoid-like tissue present, RNA was extracted from the isolated NALTs and upper palates (three per sample in developing group,

two per sample in adult group) using an RNeasy mini kit (Qiagen Inc., Valencia, CA), and reverse-transcribed with oligo primers and Superscript II (Invitrogen, Carlsbad, CA). cDNA was then analyzed by real-time PCR (SYBR Green PCR kit, Qiagen) to investigate gene expression with GAPDH as reference, using the following primer sequences: GAPDH: forward: 5'-CTG,CAC,CAC,-CAA,CTG,CTT,AG-3'; reverse: 5'-GAT,GGC,-ATG,GAC,TGT,GGT,CAT-3'; CXCL13: forward: 5'-CAT,AGA,TCG,GAT,TCA,AGT,TAC,GCC-3'; reverse: 5'-TCT,TGG,TCC,AGA,TCA,CAA,CTT,CA-3'; LT- α : forward: 5'-GCT,TGG,CAC,-CCC,TCC,TGT,C-3'; reverse: 5'-GAT,GCC,ATG,GGT,CAA,GTG,CT-3'; LT- β : forward: 5'-CCA,GCT,GCG,GAT,TCT,ACA,CCA-3'; reverse: 5'-AGC,CCT,TGC,CCA,CTC,ATC,C-3'; HEC-6ST: forward: 5'-ACC,CAG,GCC,CCC,GGA,AAC,AGT,C-3'; reverse: 5'-CTC,GTG,GGC,AGG,GAA,GAA,GTC,A-3'; GlyCAM-1: forward: 5'-CCT,GCC,TGG,GTC,CAA,AGA,TGA,AC-3'; reverse: 5'-CTG,GTG,-TAG,CTG,-GTG,GGA,GTG,GAC-3'; interferon (IFN)- γ : forward: 5'-GTC,CAG,CGC,CAA,GCA,TTC,AA-3'; reverse: 5'-ACC,CCG,AAT,CAG,CGA,CTC-3'; interleukin (IL)-4: forward: 5'-CCT,CAC,AGC,-AAC,GAA,GAA,CAC,C-3'; reverse: 5'-TCA,AGC,ATG,GAG,TTT,TCC,CAT,G-3'. Each experiment was performed at least three times.

Intranasal Immunization

Female WT, LT- $\alpha^{-/-}$, and LT- $\beta^{-/-}$ mice (2 to 3 months old; five per group) were lightly anesthetized for the duration of the inoculation with 10 μ l per gram of 10% ketamine, 5% xylazine in sterile water. Mice were immunized intranasally on days 0, 10, and 20 with 8 μ g of OVA and 2 μ g of cholera toxin (List Biological Laboratories Inc., Campbell, CA) in a total volume of 6 μ l per mouse (3 μ l per nostril). Five control mice were given PBS in the same volume on days 0, 10, and 20.

Antibody Detection

Serum and vaginal samples were collected on days 0, 10, 20, and 30. Blood was collected by retro-orbital sinus puncture while mice were under light anesthesia. All samples were microcentrifuged for 10 minutes and serum collected and stored at -20°C. Vaginal samples were obtained by injecting 100 μ l of PBS into the vaginal cavity and washing five times. For detection of OVA-specific vaginal IgA and serum IgG, high protein-binding 96-well plates (Fisher Scientific, Pittsburgh, PA) were incubated overnight at 4°C with a capture layer of OVA (50 μ g/ml in carbonate buffer, pH 9.6). After three washes with PBS-0.05% Tween-20, plates were blocked with 3% bovine serum albumin in PBS for 2 to 4 hours at room temperature, then samples were applied in duplicate at various dilutions in 3% bovine serum albumin-PBS as indicated in individual experiments, and incubated at 4°C overnight. Plates were washed five times, incubated with peroxidase-conjugated goat anti-mouse IgA (Zymed Laboratories, Inc., South San Francisco, CA), diluted 1:5000 in 3% bovine serum albumin-PBS or anti-mouse IgG (Zymed Laboratories, Inc.), diluted 1:2500, at room temperature

Figure 1. Development and analysis of B- and T-cell compartmentalization in NALTs of WT mice. NALTs were isolated at different times in the postnatal period (A–D) and double stained with anti-B220 antibody (green) to detect B cells and anti-CD4 antibody (red) to detect T cells. To determine the localization of HEVs, serial sections (D and E) were stained to detect B and T cells (D) or B cells (green) and MECA-79 (red) to detect PNA-d (E). Scale bar, 100 μ m.

for 2 to 3 hours. After seven washes, 100 μ l of TMB (Sigma Aldrich) were added to each well. Colorimetric reactions were performed at room temperature in the dark, stopped by adding 1 N H_2SO_4 , and ODs were measured at 450 nm using a 96-well spectrophotometer (PowerWave XS; Bio-Tek Instruments, Inc., Winooski, VT). The average OD for controls was 0.08, the endpoint was defined as 0.2 OD.

Enzyme-Linked Immunospot (ELISPOT) Assays

Ninety-six-well Multiscreen-HA filtration plates (Millipore, Billerica, MA) were coated overnight at 4°C with 50 μ l/well of either anti-IFN- γ , anti-IL-4, or anti-IL-5 (all antibodies from BD PharMingen) diluted at 4 μ g/ml in carbonate buffer (pH 9.6). After three washes with filtered PBS, wells were blocked with 200 μ l of 10% fetal calf serum in RPMI (Mediatech, Inc., Herndon, VA) for 2 hours at 37°C. NALT cells (5×10^5) or 1×10^6 CLN, mesenteric LN, or spleen cells from WT, LT- $\alpha^{-/-}$, and LT- $\beta^{-/-}$ mice 20 days after intranasal immunization were then applied to the wells and incubated at 37°C in 10% CO_2 in the presence or absence of 100 μ g/ml OVA for 48 hours for IFN- γ analysis, or for 72 hours for IL-4 and IL-5. After three washes with PBS and three more with PBS/0.05% Tween 20, 100 μ l of biotinylated anti-IFN- γ , anti-IL-4, or anti-IL-5 (BD PharMingen), diluted at 1 μ g/ml in filtered PBS/Tween 20 were added to each well for 2 hours. Plates were washed five times with filtered PBS/Tween 20, and 100 μ l of Streptavidin-AP (Zymed Laboratories, Inc.) diluted at 1:500 were added to each well for 1 hour at room temperature. After three washes with PBS/Tween 20 and three more with PBS, a solution of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma Aldrich) was added as substrate. Spots, representing single IFN- γ , IL-4-, or IL-5-producing cells, were counted using Immunospot analyzer and software (Cellular Technology Ltd. Analyzers, LLC, Cleveland, OH). The number of antigen-specific spot-forming cells was determined by subtracting the number of spots obtained with cells exposed to medium alone from those stimulated with OVA.

Results

Compartmentalization and Chemokine Expression during NALT Postnatal Development

The postnatal development of the NALT was followed to evaluate the timing of T- and B-cell population, compartmentalization, and expression of lymphoid chemokines and endothelial adhesion molecules. The NALT was present at birth, but contained few cells. By 4 days of age, a small number of T and B cells were randomly dispersed through the organ; these were even more apparent by 7 days. By 14 days, the number of cells increased. Distinct T- and B-cell zones apparent by 21 days were even more striking by 6 weeks (Figure 1; A to D). The adult NALT contained a higher proportion of B cells than T cells (Figure 1D). Flow cytometry analysis confirmed this qualitative observation and indicated there were 61% B220 $^{+}$ cells, 28% CD4 $^{+}$ cells, and 11% CD8 $^{+}$ cells. By 6 weeks, compartmentalization of HEVs, detected by PNA-d expression (MECA-79 staining), was also obvious in their concentration in the T-cell zone (Figure 1, D and E).

The postnatal expression of lymphoid chemokine mRNAs was evaluated by *in situ* hybridization with digoxigenin-labeled anti-sense probes (Figure 2). At 4 days, no chemokines were detected; at 7 days, CCL21 mRNA was only apparent in flat-walled lymphatic vessels (Figure 2A). By 10 days, it tended to be located at the edge of the NALT and by 21 days was present in the flat-walled vessels and also in those with the appearance of HEVs (Figure 2J). By 6 weeks, CCL21 mRNA was almost exclusively in HEV-like structures. The expression pattern of CCL21 in the NALT differed from that in the LN in its absence from stromal cells. CCL19 mRNA was rarely apparent at 7 days in the center of the NALT (Figure 2B), quite prominent at 10 days, and by 21 days had attained its adult geography and approximate intensity at the edge of the NALT, which correlates with the distribution of T cells (Figure

Figure 2. Developmental analysis of NALT chemokine mRNA expression detected by *in situ* hybridization. NALTs were isolated at different times in the postnatal period and exposed to digoxigenin-labeled probes for CCL21 (A, D, G, J, M), CCL19 (B, E, H, K, N), or CXCL13 (C, F, I, L, O). Scale bars, 100 μ m.

2K). CXCL13 mRNA was detected as early as 7 days but was dispersed through the NALT (Figure 2C); by 21 days, clear compartmentalization was apparent that coincided with the B-cell staining noted above in areas that were distinct from T cells or T-cell area-defining chemokines CCL21 and CCL19 (Figure 2L). CXCL13 mRNA was also evaluated by quantitative real-time PCR (Figure 3A). The kinetics were similar to those seen by *in situ* hybridization. A dramatic change occurred between 3 and 4 weeks. This could be a reflection of exposure to food antigens (after weaning), but interestingly, it also coincides with a dramatic increase in LT- α and LT- β mRNA (Figure 3B).

Expression of PNAd Continues in the NALT throughout Development and Correlates with GlyCAM-1 and HEC-6ST Expression

Expression of markers that define HEVs were evaluated in the postnatal period. These included a core glycoprotein (GlyCAM-1), a modifying enzyme (HEC-6ST), and PNAd, the sulfated determinant detected by MECA-79 antibody. Kinetics were evaluated by *in situ* hybridization and real-time PCR for mRNA and by immunohistochemistry and immunofluorescence. GlyCAM-1 mRNA was detected by real-time PCR by 14 days (39-fold increase)

Figure 3. Developmental analysis of NALT mRNA detected by real-time PCR. CXCL13 (■), LT- α (●), LT- β (□), GlyCAM-1 (▲), and HEC-6ST (◆) mRNA continues to increase in the postnatal period in WT mice. The changes are expressed as relative differences compared to day 0 mice. NALTs were isolated and mRNA prepared and evaluated with primers in quantitative real-time PCR, as indicated in Materials and Methods.

and then showed a rapid increase (500-fold) at 4 weeks (Figure 3C). Similarly, GlyCAM-1 mRNA was difficult to detect reproducibly by *in situ* hybridization before 3 weeks but was quite apparent by 4 weeks (data not shown). The kinetics of early expression of HEC-6ST

mRNA were somewhat similar to GlyCAM-1 with full expression by 28 days as revealed by real-time PCR; however, there was a decline in the later times (Figure 3D). Similar HEC-6ST mRNA kinetics were apparent by *in situ* hybridization (data not shown). Interestingly, however, the protein was detected by 21 days, and remained easily detectable at later time points (Figure 4). At 7 days, there were very few vessels that were PNAd-positive by immunofluorescence and they were quite small (Figure 4). At that time, expression, as detected by immunohistochemistry, was predominantly abluminal (data not shown). By 14 days, staining was more apparent, but there were still very few vessels. The pattern was quite spotty, although a few vessels appeared as if they might be staining in both an abluminal and luminal pattern. Vessels showed a gradual increase in diameter with age. By 3 weeks, there was clear luminal and abluminal expression, and by 6 weeks, the majority of the vessels were large, with both luminal and abluminal expression in a pattern similar to that seen in peripheral LN. The full luminal and abluminal pattern correlated with the expression of HEC-6ST, as noted previously.^{10,20,23}

The NALTs of LT- α ^{-/-} and LT- β ^{-/-} Mice Differ in Cellular Composition from Those of WT Mice

A comparison of the morphological appearance and cellular composition of NALTs from WT and mutant mice between 6 weeks and 3 months of age revealed profound defects in the LT-deficient mice. These differences were apparent in the NALTs of LT- β ^{-/-}, and even more pronounced in the LT- α ^{-/-} mice (Figure 5). As noted above, WT NALT was an obvious structure that was densely compacted with cells. The LT- β ^{-/-} NALT was smaller and less densely populated (Figure 5B) and the LT- α ^{-/-} NALT was generally difficult to distinguish from surrounding tissue and was hypocellular (Figure 5C). This hypocellular appearance was confirmed by the cell yields obtained from the various mice. The average NALT cell yield was $\sim 2 \times 10^6$ for WT, 2×10^5 for LT- β ^{-/-}, and 4×10^4 for LT- α ^{-/-} mice. Cytospin preparations revealed that the cells from WT mice were generally mononuclear in appearance. Those of LT- β ^{-/-} mice included both mononuclear cells and others with the appearance of macrophages and immature polymorphonuclear leukocytes; those of LT- α ^{-/-} mice were almost all activated macrophages and polymorphonuclear leukocytes (data not shown). The phenotype of cells in the NALT as evaluated by flow cytometry confirmed that members of the LT family influenced the reduction in B cells. By immunofluorescence, the compartmentalization in the LT- β ^{-/-} NALT was not as obvious as in the WT NALT, and absent in the LT- α ^{-/-} NALT, with almost no remaining B cells (Figure 5; D to F).

Lymphoid Organ Chemokines Are Reduced in LT-Deficient NALT

Expression of the lymphoid chemokines was evaluated by *in situ* hybridization of NALT tissue from at least five

Figure 4. PNAd expression changes from undetectable, to abluminal, to luminal along with increases in HEC-6ST in the postnatal period in WT NALT. Immunofluorescent staining with MECA-79 (PNAd, red) and rabbit anti-HEC-6ST (green) as indicated in Materials and Methods. Scale bar, 100 μ m; **insets**, digital enlargements.

LT- $\beta^{-/-}$ and five LT- $\alpha^{-/-}$ mice, ranging from 6 weeks to 2.5 months old, and compared to WT. In all cases, expression of the chemokines was drastically reduced in NALTs of LT- $\beta^{-/-}$ and essentially absent in LT- $\alpha^{-/-}$

mice. CXCL13 and CCL21 were not detected. In two of five LT- $\beta^{-/-}$ mice, CCL19 was present, but reduced compared to WT and was not in any distinct compartment because its expression was scattered throughout the

Figure 5. Cellularity and compartmentalization in the adult WT and LT-deficient NALTs. LT-deficient NALTs are hypocellular and exhibit loss of T- and B-cell compartmentalization. LT- $\alpha^{-/-}$ NALTs are more severely affected than LT- $\beta^{-/-}$ NALTs. **A–C:** H&E staining. **D–F:** Immunofluorescence: anti-B220, green; anti-CD4, red. All mice are 2.5 months of age. Scale bars, 100 μ m.

Figure 6. Chemokine expression in adult WT and LT-deficient NALTs: CXCL13 (A–C), CCL19 (D–F), and CCL21 (G–I). LT-deficient NALTs exhibit reduced lymphoid chemokine mRNA expression compared to WT, evaluated by *in situ* hybridization. All mice are 2.5 months of age. Scale bars, 100 μ m.

NALT. The representative figure at 6 weeks (Figure 6) was typical of the five mice in each group, with occasional expression in LT- $\beta^{-/-}$ and none in the LT- $\alpha^{-/-}$ NALTs. These results indicate that the defect in the LT-deficient mice is not simply a developmental delay but represents a qualitative difference from WT mice. These reductions in the lymphoid chemokines that are so essential for trafficking in the lymphoid organs,²⁷ explain the severe disorganization of the NALTs in the LT-deficient mice and their reduction in total cell number.

Expression of Luminal PNAd and HEC-6ST Is Dependent on LT

GlyCAM-1 mRNA was quite apparent by *in situ* hybridization in blood vessels in WT adult NALT (Figure 7A). However, it was not detected in the NALT of LT-deficient mice (Figure 7, B and C). A dramatic difference was apparent when MECA-79 staining for PNAd was compared in the HEVs of adult WT and LT-deficient mice. As noted above (Figure 1E and Figure 4), HEVs of adult WT NALTs stained with MECA-79 in a pattern very similar to that seen in the LN, with pericellular, both abluminal and luminal staining. The reticular pattern of staining that is also seen in LN was apparent (Figure 7D). The pattern of MECA-79 staining in LT- $\beta^{-/-}$ NALT was quite different. It was less intense, the majority was abluminal, and there was no reticular staining (Figure 7E). There was almost no MECA-79 staining in the NALT of LT- $\alpha^{-/-}$ mice (Figure 7F). Immunofluorescent staining showed no HEC-6ST protein in either LT-deficient mouse compared to WT (Figure 7; G to I). The severe reduction in HEC-6ST

protein in the LT-deficient mice was confirmed by *in situ* hybridization, in which no HEC-6ST mRNA was detected (data not shown). The data obtained by *in situ* hybridization, immunohistochemistry, and immunofluorescence were confirmed by real-time PCR, which showed that there was much less GlyCAM-1 and HEC-6ST mRNA in LT- $\beta^{-/-}$ NALTs. GlyCAM-1 mRNA was 20-fold reduced in LT- $\beta^{-/-}$ NALT compared to WT, and 100-fold reduced in LT- $\alpha^{-/-}$ NALT. HEC-6ST mRNA was reduced fivefold in LT- $\beta^{-/-}$ NALT compared to WT, and 50-fold in LT- $\alpha^{-/-}$ NALT (Figure 8). These data emphasize the importance of the LT family in regulation of HEV PNAd both at the level of a core protein (GlyCAM-1) and a crucial modifying enzyme (HEC-6ST), and indicate that both LT- α and LT- $\alpha\beta$ contribute individually to HEV development.

NALT Function Is Deficient in LT- $\alpha^{-/-}$ and LT- $\beta^{-/-}$ Mice

The NALT function with regard to cytokines was analyzed by ELISPOT on day 20 after immunization and boosting with OVA in cholera toxin, and by real-time PCR on day 30. ELISPOT analysis performed after cells were cultured with OVA revealed that the WT NALT contained high numbers of IFN- γ -producing cells, but no IL-4 or IL-5. In two separate experiments, IFN- γ spot-forming cells were apparent in WT NALT (average 126/10⁶ cells), and not detectable in LT- $\alpha^{-/-}$ or LT- $\beta^{-/-}$ NALT. Data from one of these experiments is presented in Table 1. The reduction in the number of IFN- γ -producing cells is even more dramatic when the very low absolute number of lympho-

Figure 7. LT-deficient NALTs show reduced GlyCAM-1, PNAd, and HEC-6ST. NALTs were removed from 2.5-month-old adult WT, $LT\beta^{-/-}$, or $LT\alpha^{-/-}$ mice, and evaluated for GlyCAM-1 mRNA by *in situ* hybridization with a digoxigenin-labeled probe (**A–C**), PNAd by immunohistochemistry (**D–F**), and PNAd (MECA-79, red) and HEC-6ST (green) by immunofluorescence (**G–I**). Scale bars, 100 μ m.

cytes in the LT-deficient NALT is taken into account. Three times more NALTs from LT-deficient mice, compared to WT mice, were needed to generate the appropriate number of cells for each experiment. Spot-forming cells for all three cytokines were detected in CLNs and mesenteric LNs of WT mice. Although there were no cytokine-producing cells in $LT\beta^{-/-}$ CLN, there were a few in their mesenteric LNs. High numbers of spot-forming cells for all three cytokines were detected in WT,

$LT\beta^{-/-}$, and $LT\alpha^{-/-}$ spleens. These data indicate that intranasal immunization was sufficient for priming in the spleen. However, it did not prime cells in either $LT\alpha^{-/-}$ or $LT\beta^{-/-}$ NALT. Furthermore, intranasal immunization of $LT\beta^{-/-}$ mice did not result in activation of cytokine-producing cells in CLN, although cytokine-producing cells were induced in mesenteric LN.

Real-time PCR analysis of cells confirmed the conclusion that LT-deficient mice had altered cytokine profiles (Figure 9A). Cells from WT NALT 30 days after intranasal

Figure 8. LT-deficient adult NALTs exhibit reduced GlyCAM-1 and HEC-6ST expression, evaluated by real-time PCR; #, $P < 0.05$ value WT compared to $LT\alpha^{-/-}$ and $LT\beta^{-/-}$; *, $P < 0.01$ value $LT\beta^{-/-}$ compared to $LT\alpha^{-/-}$. All mice are 2.5 months of age.

Table 1. NALTs of LT-Deficient Mice Do Not Contain Cytokine-Producing Cells on Day 20 after Intranasal Immunization (ELISA Analysis)

Organ	Cytokine	WT	$LT\beta^{-/-}$	$LT\alpha^{-/-}$
NALT	IFN- γ	104	0	0
	IL-4	0	0	0
	IL-5	0	0	0
CLN	IFN- γ	181	0	N/A
	IL-4	58	0	N/A
	IL-5	135	0	N/A
MLN	IFN- γ	98	5	N/A
	IL-4	10	2	N/A
	IL-5	50	11	N/A
Spleen	IFN- γ	285	108	185
	IL-4	18	19	22
	IL-5	145	85	190

Cells were cultured with OVA for 48 hours for IFN- γ analysis, and 72 hours for IL-4 and IL-5 analyses. Spot-forming cells reported per 10^6 cells.

Figure 9. NALTs of adult LT-deficient mice show reduced function after immunization. Lower levels of IFN-γ expression after intranasal immunization, evaluated by real-time PCR; an average of three experiments with at least two mice per group done in duplicate; #, $P < 0.05$ value compared to unimmunized mice; *, $P < 0.01$ value compared to unimmunized mice (A). LT-deficient mice exhibit reduced serum anti-OVA IgG (B) and negligible vaginal anti-OVA IgA (C) 30 days after immunization as evaluated by ELISA assay. All mice are 2 to 3 months of age. A representative experiment with five mice per group.

immunization (but not cultured) showed an increase in mRNA levels of IFN-γ and IL-4 whereas cells from LT-deficient NALT did not, except for a 10-fold increase of IL-4 mRNA in LT-α^{-/-} NALT even though no IL-4 protein was detected. Data averaged from three experiments with at least two mice per group performed in duplicate are presented in Figure 9A.

Anti-OVA antibody titers were evaluated in two separate experiments with material from five mice of each group analyzed individually. By day 20, OVA-specific serum IgG was detected in all three groups (data not shown). By day 30, serum from WT mice had an anti-OVA titer of 2×10^{-6} in one experiment and 1×10^{-6} in another (Figure 9B). Serum antibody titers from LT-α^{-/-} and LT-β^{-/-} mice were considerably lower at 2 to 8×10^{-4} . Thus, both LT-α^{-/-} and LT-β^{-/-} mice produced serum IgG but at 50- to 100-fold lower titers than WT. These results are most likely because of the splenic responses (also apparent when cytokines were analyzed in the ELISPOT assays). OVA-specific IgA was present in vaginal washing of WT mice (1:40 or 1:80); LT-β^{-/-} vaginal anti-OVA IgA was undetectable in one experiment and exhibited a titer of 1:10 in the second experiment; OVA-specific IgA was undetectable in LT-α^{-/-} vaginal washing (Figure 9C). These data clearly indicate that, although the LT-deficient mice possess NALTs, these organs are almost completely defective with regard to their ability to respond locally, and in particular to generate a response at the vaginal site after intranasal immunization.

Discussion

The data provided here indicate that the NALT is uniquely appropriate to elucidate the process of lymphoid organ development. At birth the organ is present in only a rudimentary form and it does not reach its full adult cellular complement and organization until the time of weaning. Particularly interesting are the data regarding lymphoid chemokine expression. Although detectable at an early stage, their location in T- and B-cell areas is not apparent until 3 weeks of age. Thus, lymphoid chemokines are present before T- and B-cell compartmentalization occurs and only later attain their adult localization. This is somewhat surprising as one might expect the chemokines to first be in their compartments and thus attract B or T cells to those areas. This indicates that the organization of lymphoid tissues is more complex than a simple linear process.

Because the NALT is located in the mucosal region, we expected strong expression of MAdCAM, and somewhat low levels of PNAd. In fact, this was not the case, and MAdCAM was only weakly detected and only in a stromal pattern (data not shown), whereas PNAd in the adult NALT was quite intense, suggesting a pattern more similar to that of peripheral LNs. The developmental pattern of PNAd expression in the NALT was similar to peripheral LNs in that it was first abluminal, and then attained its mature pericellular luminal and abluminal pattern. Consistent with this was timing of expression of GlyCAM-1 in

which mRNA increased in the immediate postnatal period (39-fold increase by day 14) and even more dramatically than other mRNAs at 4 weeks and continued its rise. This suggests both an environmental influence and a developmental effect on GlyCAM-1 expression. Additional cytokines such as TNF- α could be influencing GlyCAM-1 production at the later time. It will be interesting to identify the regulation of other PNAd core proteins such as CD34. The expression of a crucial modifying enzyme, HEC-6ST, is also developmentally regulated in that its mRNA continued to increase in the immediate postnatal period and coincided temporally with luminal PNAd expression. These data are consistent with studies on HEC-6ST-deficient mice that indicate that luminal PNAd expression on LN HEVs is dependent on HEC-6ST.²⁰ Developmental regulation of the other modifying enzymes (FucTIV, FucTVII) and other sulfotransferases²⁸ and their contribution to abluminal PNAd expression is a topic for further study.

The data presented here indicate that LT- α and the LT- $\alpha\beta$ complex play complementary, crucial roles in NALT development and function. Harmsen and colleagues¹⁵ had previously demonstrated that the NALTs of LT- $\alpha^{-/-}$ mice were severely lymphopenic and disorganized (although only data from TNF- $\alpha^{-/-}$ /LT- $\alpha^{-/-}$ mice were shown). HEVs of LT- $\alpha^{-/-}$ NALTs have been reported to have reduced PNAd staining^{7,15} although no analyses of LT- $\beta^{-/-}$ NALTs have been reported, nor have the roles of the individual cytokines in their contributions to chemokines or PNAd in that organ. We have previously reported in the RIPLT transgenic mouse system that ectopic expression of LT gives rise to accumulations of cells with characteristics of lymphoid organs.²⁹ RIPLT- α expression was associated with abluminal PNAd whereas the RIPLT- $\alpha\beta$ complex was necessary for luminal PNAd and HEC-6ST expression.¹⁰ Here we confirm and extend those observations in the adult NALT in which LT- α expression (in LT- $\beta^{-/-}$ mice) was sufficient for abluminal PNAd but not for HEC-6ST, whereas LT- $\alpha^{-/-}$ mice that have no LT- α or LT- $\alpha\beta$ complex had almost no PNAd (or HEC-6ST). These data indicate that the NALT in the various LT-deficient mice may be useful in identifying which sulfotransferases generate abluminal PNAd. The data provided here also reveal a previously unidentified role for LT in the regulation of GlyCAM-1, a PNAd core protein.

We report here for the first time an analysis of lymphoid chemokine expression in the NALT and the fact that LT is responsible for that regulation. The reduced expression of lymphoid chemokines in LT-deficient NALTs is consistent with a previous report regarding expression of those chemokines in spleens of LT-deficient mice;⁹ in agreement with that report, the effects are somewhat more pronounced in LT- $\alpha^{-/-}$ than LT- $\beta^{-/-}$ mice.

The data presented in this communication indicate that the NALTs of LT-deficient mice are not only abnormal in appearance, cellular composition, and chemokine and adhesion molecule expression, but also exhibit functional defects. The ELISPOT data are consistent with the previously reported reduction in antigen-specific CD8⁺ T cells in LT- $\alpha^{-/-}$ NALTs after immunization with influenza PR8.¹⁵ The absence of cytokines in LT-deficient NALTs is

consistent with a defect in function of this organ. This is most likely because of the fact that there are fewer cells in the LT-deficient NALTs, and also because those cells that are there do not appear to be primed. The absence of cytokines in the CLN in the LT- $\beta^{-/-}$ suggests that the nonfunctional NALT does not even allow priming of that node. The absence of vaginal antigen-specific IgA suggests that the NALT of LT- $\alpha^{-/-}$ mice and even LT- $\beta^{-/-}$ mice that possess CLNs, does not prime for a reaction in the vagina to generate immune responses in that location after intranasal immunization. Because LT-deficient mice exhibit multiple defects in immune responses, it is possible that the vaginal IgA defect is also because of a local deficiency in the vaginal mucosa. However, recent evidence suggests that this is unlikely because LT- $\beta^{-/-}$ mice that retain a sacral LN can be primed through the vaginal route to respond to HSV-2.¹² Interestingly, after intranasal immunization, both strains of LT-deficient mice can generate systemic (although reduced) antibody serum responses as shown here and also locally in the lung.³⁰

In conclusion, the NALT is a unique organ, with some mucosal properties (M cells, location) but with profound differences—namely barely detectable MAdCAM-1, but strong PNAd expression. When the NALT is disorganized, generation of a vaginal IgA response after immunization is compromised. Whether the functional impairment in IgA production in LT-deficient mice is solely because of local disorganization of the NALT, or whether defective chemokine expression in mucosal tissue contributes to the altered immune function, can be further investigated in this model, therefore providing additional insight into the influence of cytokines on immune responses in mucosal environments.

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References

- Goeringer GC, Vidic B: The embryogenesis and anatomy of Waldeyer's ring. *Otolaryngol Clin North Am* 1987, 20:207–217
- Wu HY, Russell MW: Induction of mucosal immunity by intranasal application of a streptococcal surface protein antigen with the cholera toxin B subunit. *Infect Immun* 1993, 61:314–322
- Balmelli C, Roden R, Potts A, Schiller J, De Grandi P, Nardelli-Haeffliger D: Nasal immunization of mice with human papillomavirus type 16 virus-like particles elicits neutralizing antibodies in mucosal secretions. *J Virol* 1998, 72:8220–8229
- Liu XS, Abdul-Jabbar I, Qi YM, Frazer IH, Zhou J: Mucosal immunization with papillomavirus virus-like particles elicits systemic and mucosal immunity in mice. *Virology* 1998, 252:39–45
- Wu HY, Nikolova EB, Beagley KW, Eldridge JH, Russell MW: Development of antibody-secreting cells and antigen-specific T cells in cervical lymph nodes after intranasal immunization. *Infect Immun* 1997, 65:227–235
- Hameleers DM, van der Ende M, Biewenga J, Sminia T: An immunohistochemical study on the postnatal development of rat nasal-associated lymphoid tissue (NALT). *Cell Tissue Res* 1989, 256:431–438
- Fukuyama S, Hiroi T, Yokota Y, Rennert PD, Yanagita M, Kinoshita N, Terawaki S, Shikina T, Yamamoto M, Kurono Y, Kiyono H:

- Initiation of NALT organogenesis is independent of the IL-7R, LTbetaR, and NIK signaling pathways but requires the Id2 gene and CD3(-)CD4(+)CD45(+) cells. *Immunity* 2002, 17:31-40
8. Sacca R, Cuff CA, Ruddle NH: Mediators of inflammation. *Curr Opin Immunol* 1997, 9:851-857
9. Ngo VN, Korner H, Gunn MD, Schmidt KN, Riminton DS, Cooper MD, Browning JL, Sedgwick JD, Cyster JG: Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J Exp Med* 1999, 189:403-412
10. Drayton DL, Ying X, Lee J, Lesslauer W, Ruddle NH: Ectopic LT alpha beta directs lymphoid organ neogenesis with concomitant expression of peripheral node addressin and a HEV-restricted sulfotransferase. *J Exp Med* 2003, 197:1153-1163
11. Koni PA, Sacca R, Lawton P, Browning JL, Ruddle NH, Flavell RA: Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta-deficient mice. *Immunity* 1997, 6:491-500
12. Soderberg KA, Linehan MM, Ruddle NH, Iwasaki A: MAdCAM-1 expressing genital lymph node in the LTbeta deficient mouse provides a site for immune generation following vaginal HSV-2 infection. *J Immunol* 2004, 173:1908-1913
13. Rennert PD, Browning JL, Hochman PS: Selective disruption of lymphotoxin ligands reveals a novel set of mucosal lymph nodes and unique effects on lymph node cellular organization. *Int Immunol* 1997, 9:1627-1639
14. Browning JL, Ngam-ek A, Lawton P, DeMarinis J, Tizard R, Chow EP, Hession C, O'Brine-Greco B, Foley SF, Ware CF: Lymphotoxin beta, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. *Cell* 1993, 72:847-856
15. Harmsen A, Kusser K, Hartson L, Tighe M, Sunshine MJ, Sedgwick JD, Choi Y, Littman DR, Randall TD: Cutting edge: organogenesis of nasal-associated lymphoid tissue (NALT) occurs independently of lymphotoxin-alpha (LT alpha) and retinoic acid receptor-related orphan receptor-gamma, but the organization of NALT is LT alpha dependent. *J Immunol* 2002, 168:986-990
16. Csencsits KL, Jutila MA, Pascual DW: Nasal-associated lymphoid tissue: phenotypic and functional evidence for the primary role of peripheral node addressin in naive lymphocyte adhesion to high endothelial venules in a mucosal site. *J Immunol* 1999, 163:1382-1389
17. Hemmerich S, Butcher EC, Rosen SD: Sulfation-dependent recognition of high endothelial venules (HEV)-ligands by L-selectin and MECA 79, and adhesion-blocking monoclonal antibody. *J Exp Med* 1994, 180:2219-2226
18. Bistrup A, Bhakta S, Lee JK, Belov YY, Gunn MD, Zuo FR, Huang CC, Kannagi R, Rosen SD, Hemmerich S: Sulfotransferases of two specificities function in the reconstitution of high endothelial cell ligands for L-selectin. *J Cell Biol* 1999, 145:899-910
19. Hiraoka N, Petryniak B, Nakayama J, Tsuboi S, Suzuki M, Yeh JC, Izawa D, Tanaka T, Miyasaka M, Lowe JB, Fukuda M: A novel, high endothelial venule-specific sulfotransferase expresses 6-sulfo sialyl Lewis(x), an L-selectin ligand displayed by CD34. *Immunity* 1999, 11:79-89
20. Hemmerich S, Bistrup A, Singer MS, van Zante A, Lee JK, Tsay D, Peters M, Carminati JL, Brennan TJ, Carver-Moore K, Leviten M, Fuentes ME, Ruddle NH, Rosen SD: Sulfation of L-selectin ligands by an HEV-restricted sulfotransferase regulates lymphocyte homing to lymph nodes. *Immunity* 2001, 15:237-247
21. De Togni P, Goellner J, Ruddle NH, Streeter PR, Fick A, Mariathasan S, Smith SC, Carlson R, Shornick LP, Strauss-Schoenberger J, Russell JH, Karr R, Chaplin DD: Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* 1994, 264:703-707
22. Heritage PL, Underdown BJ, Arsenault AL, Snider DP, McDermott MR: Comparison of murine nasal-associated lymphoid tissue and Peyer's patches. *Am J Respir Crit Care Med* 1997, 156:1256-1262
23. Bistrup A, Tsay D, Shenoy P, Singer MS, Bangia N, Luther SA, Cyster JG, Ruddle NH, Rosen SD: Detection of a sulfotransferase (HEC-GlcNAc6ST) in high endothelial venules of lymph nodes and in high endothelial venule-like vessels within ectopic lymphoid aggregates: relationship to the MECA-79 epitope. *Am J Pathol* 2004, 164:1635-1644
24. Gunn MD, Ngo VN, Ansel KM, Ekland EH, Cyster JG, Williams LT: A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. *Nature* 1998, 391:799-803
25. Ngo VN, Tang HL, Cyster JG: Epstein-Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. *J Exp Med* 1998, 188:181-191
26. Hjelmstrom P, Fjell J, Nakagawa T, Sacca R, Cuff CA, Ruddle NH: Lymphoid tissue homing chemokines are expressed in chronic inflammation. *Am J Pathol* 2000, 156:1133-1138
27. Baggiolini M: Chemokines and leukocyte traffic. *Nature* 1998, 392:565-568
28. Hemmerich S, Lee JK, Bhakta S, Bistrup A, Ruddle NR, Rosen SD: Chromosomal localization and genomic organization for the galactose/N-acetylgalactosamine/N-acetylglucosamine 6-O-sulfotransferase gene family. *Glycobiology* 2001, 11:75-87
29. Kratz A, Campos-Neto A, Hanson MS, Ruddle NH: Chronic inflammation caused by lymphotoxin is lymphoid neogenesis. *J Exp Med* 1996, 183:1461-1472
30. Constant SL, Brogdon JL, Piggott DA, Herrick CA, Visintin I, Ruddle NH, Bottomly K: Resident lung antigen-presenting cells have the capacity to promote Th2 T cell differentiation in situ. *J Clin Invest* 2002, 110:1441-1448