Immunity, Vol. 17, 31–40, July, 2002, Copyright ©2002 by Cell Press

Initiation of NALT Organogenesis Is Independent of the IL-7R, LTβR, and NIK Signaling Pathways but Requires the Id2 Gene and $CD3^{-}CD4^{+}CD45^{+}$ Cells

Satoshi Fukuyama,[1,5] Takachika Hiroi,[1] Yoshifumi Yokota,[2] Paul D. Rennert,[3] Manabu Yanagita,[1] Naotoshi Kinoshita,[1] Seigo Terawaki,[1] Takashi Shikina,[1] Masafumi Yamamoto,[1,4] Yuichi Kurono,[5] and Hiroshi Kiyono[1,6,7,8]

[1]Department of Mucosal Immunology
Research Institute for Microbial Diseases
Osaka University
Suita, Osaka, 565-0871
[2]First Department of Biochemistry
Fukui Medical University
Matsuoka, Fukui, 910-1193
Japan
[3]Biogen Incorporated
Cambridge, Massachusetts 02142
[4]Department of Oral Medicine
School of Dentistry at Matsudo
Nihon University
Matsudo, Chiba, 271 8587
[5]Department of Otolaryngology
Faculty of Medicine
Kagoshima University
Kagoshima, 890-8520
Japan
[6]Immunobiology Vaccine Center
The University of Alabama at Birmingham
Birmingham, Alabama 35294
[7]Division of Mucosal Immunology
Department of Microbiology and Immunology
The Institute of Medical Science
The University of Tokyo
Tokyo, 108-8639
Japan

Summary

Initiation of nasopharyngeal-associated lymphoid tissue (NALT) development is independent of the programmed cytokine cascade necessary for the formation of Peyer's patches (PP) and peripheral lymph nodes (PLN), a cytokine cascade which consists of IL-7R, LTα1β2/LTβR, and NIK. However, the subsequent organization of NALT seems to be controlled by these cytokine signaling cascades since the maturation of NALT structure is generally incomplete in those cytokine cascade-deficient mice. NALT as well as PP and PLN are completely absent in $Id2^{-/-}$ mice. NALT organogenesis is initiated following the adoptive transfer of $CD3^{-}CD4^{+}CD45^{+}$ cells into $Id2^{-/-}$ mice, constituting direct evidence that $CD3^{-}CD4^{+}CD45^{+}$ inducer cells can provide an IL-7R-, LTα1β2/LTβR-, and NIK-independent tissue organogenesis pathway for secondary lymphoid tissue development.

[8]Correspondence: kiyono@biken.osaka-u.ac.jp

Introduction

In rodents, nasopharyngeal-associated lymphoid tissue (NALT) is an organized lymphoid structure found on both sides of the nasopharyngeal duct dorsal to the cartilaginous soft palate and is considered analogous to the Waldeyer's ring in humans (Kuper et al., 1990, 1992). NALT consists of follicle-associated epithelium (FAE), high endothelial venules (HEV) and T cell- and B cell-enriched areas. Numerous M cells, specialized for antigen sampling, are present in NALT and Peyer's patches (PP) FAE (Jeong et al., 2000). The structural similarity of NALT and PP suggests that NALT is an important inductive site for mucosal immunity in the upper respiratory tract, much like PP in the intestinal immune system. NALT has been shown to be an important site for the generation of high-affinity memory B cells (Shimoda et al., 2001). Nasal immunization with antigen (Ag) and cholera toxin (CT) as a mucosal adjuvant induces Ag-specific Th2-type responses for the generation of Ag-specific IgA-producing cells in the nasal passages and distal mucosal sites, including the genitourinary and intestinal tracts (Imaoka et al., 1998; Kurono et al., 1999; Yanagita et al., 1999). In spite of accumulated evidence demonstrating the unique structure and the importance of NALT in the mucosal immune system, little is known concerning the cellular and molecular mechanisms that control NALT development.

Lymphotoxin (LT) is a critical signaling molecule for secondary lymphoid tissue organogenesis. Lymph nodes (LN) and PP are absent in LTα-deficient ($LT\alpha^{-/-}$) mice (De Togni et al., 1994). LTα forms LTα1β2 heterotrimers that can transduce an activation signal through the LTβ receptor (LTβR) to organize secondary lymphoid tissues (Crowe et al., 1994; Rennert et al., 1996, 1998). Our finding that the LTα1β2 heterotrimer could be antagonized by an LTβR-immunoglobulin (LTβR-Ig) fusion protein demonstrated that the timing of secondary lymphoid tissue development was regulated during embryogenesis. When LTβR-Ig was administered to pregnant mice at embryonic day 18 (E18), the offspring lacked PP but had unaltered LN formation. If the fusion protein was delivered from E12 and thereafter, PP as well as popliteal, axillary, and inguinal LN were absent, with only mesenteric LN and cervical LN retained (Rennert et al., 1997; Yamamoto et al., 2000). In IL-7 receptor α chain-deficient ($IL\text{-}7R\alpha^{-/-}$) mice, only the formation of PP was impaired (Adachi et al., 1998). Blocking of IL-7Rα function by a single injection of the antagonistic mAb on E15.5 resulted in PP-deficient offspring with unaltered LN (Yoshida et al., 1999). These findings indicate that well-programmed cytokine-mediated PP and LN formation is initiated during embryogenesis.

Recently a model describing the development of PP was proposed (Honda et al., 2001). It was shown that lymphoid lineage $IL\text{-}7R^{+}CD3^{-}$ cells considered to be PP inducers express CXC chemokine receptor 5 (CXCR5) and are capable of producing membrane-associated LTα1β2 heterotrimer (Honda et al., 2001), while in contrast, mesenchymal lineage vascular cell adhesion mole-

cule (VCAM)-1$^+$ and intercellular adhesion molecule (ICAM)-1$^+$ PP organizers express LTβR (Honda et al., 2001). Following stimulation signals provided via IL-7R, PP inducers express LTα1β2 to activate PP organizers via LTβR, while PP organizers produce chemokines such as B lymphocyte chemoattractant (BLC) and EBV-induced molecule 1 ligand chemokine (ELC) to stimulate PP inducers via CXCR5 and the CC chemokine receptor (CCR) 7 (Honda et al., 2001). The reciprocal interaction between inducer and organizer cells via chemokine and cytokine receptors is essential for the formation of PP, and the loss of any part of either of the signaling programs is sufficient to disrupt secondary lymphoid tissue development, as evidenced by the loss of PP in LTβR$^{-/-}$ and IL-7Rα$^{-/-}$ mice (Futterer et al., 1998; Adachi et al., 1998). *Aly/aly* mice which have defective NIK function also fit this paradigm, as recent analyses have established that NIK is essential to the mediation of TNFR family signal transduction, including that of LTβR (Nakano et al., 1996; Shinkura et al., 1999; Yin et al., 2001). Thus, *aly/aly* mice lack PP and LN (Miyawaki et al., 1994) since the NIK mutation inhibited reciprocal interaction between PP inducers and organizers via LTα1β2 and LTβR. Further evidence in support of this model comes from studies showing that mice which lack the CD3$^-$CD4$^+$CD45$^+$ inducer cells due to genetic deletion of the Id2 or RORγ genes also completely lack PP and LN (Sun et al., 2000; Yokota et al., 1999).

In this study, we have examined and compared the contribution of tissue organogenesis-associated cytokine signals provided via IL-7R, LTβR, and NIK for the formation of NALT and PP. Our novel findings constitute direct evidence that CD3$^-$CD4$^+$CD45$^+$ cells can provide an IL-7R-, LTα1β2/LTβR-, and NIK-independent tissue organogenesis pathway for the initiation of a secondary lymphoid tissue development. Thus, the formation of NALT differs markedly from that of other lymphoid tissues such as PP and LN.

Results

Postnatal Development of NALT

In normal mice, NALT is a bell-shaped accumulation of lymphoid cells (Figures 1C and 2A). NALT-associated HEV express PNAd, an addressin phenotype distinct from the mucosal addressin cell adhesion molecules (MAdCAM)-1$^+$ that characterizes the HEV of PP (Csencsits et al., 1999). To determine when NALT developed, we immunohistologically analyzed PNAd in wild-type mice of various ages. NALT formation, indicated by the expression of PNAd and an accumulation of mononuclear cells, was not observed during embryogenesis or in newborn nasal tissue (Figure 1A, and data not shown), whereas PP with associated HEV were already developed (Figure 1E). Instead, PNAd$^+$ HEV with associated lymphocytes were detected bilateral manner in nasal tissue beginning at 1 week postpartum (Figures 1B and 1D) and was strongly expressed in 8-week-old mice (Figure 1C), as compared with isotype control mAb (Figure 1H). As expected, the size of PP tissue continued to increase with the increasing age of the mice (Figures 1E–1G). These findings suggested that the initiation of tissue organogenesis is chronologically different in NALT and PP.

Development of NALT in Secondary Lymphoid Tissue-Deficient Mice

To investigate the involvement of lymphoid tissue organogenesis-related signals provided by IL-7/IL-7R, LTα1β2/LTβR, and NIK in the formation of NALT, we next examined mice lacking PP and/or LN: IL-7Rα$^{-/-}$, LTα$^{-/-}$, LTβ$^{-/-}$, *aly/aly* mice, and mice treated in utero with LTβR-Ig fusion protein. NALT was detected in all PP null or PP/LN null mouse strains (Figures 2B–2E, 2G). However, the size of NALT was smaller in IL-7Rα$^{-/-}$, LTα$^{-/-}$, LTβ$^{-/-}$, and *aly/aly* mice compared with NALT of wild-type mice (Figure 2). Furthermore, we compared the NALT formation in mice treated in utero with that of those treated at birth with LTβR-Ig. PP-deficient mice that had been treated in utero with LTβR-Ig were observed to form NALT (Figure 2E). Since our study demonstrated that the initiation of NALT formation occurred 1 week after birth (Figure 1), it was formally possible that in utero-administered fusion protein might drop below a minimum effective dose prior to the onset of NALT development postnatally. Therefore, we next administered LTβR-Ig fusion protein into newborn mice and observed that they too developed NALT (Figure 2F). These results suggest that the induction of NALT formation requires a cytokine signaling cascade independent from those of PP since tissue organogenesis signals provided by IL-7R, LTα1β2/LTβR, and NIK pathways are not essential for the initial stage of NALT development.

Detection of PNAd$^+$ HEV in NALT of PP-Deficient Mice

PNAd$^+$ HEV was detected in the NALT of IL-7Rα$^{-/-}$, LTα$^{-/-}$, LTβ$^{-/-}$, and *aly/aly* mice as well as in that of mice treated with LTβR-Ig in utero and at birth (Figure 3). PNAd expression on NALT in these mice further confirmed that NALT formation is independent of the cytokine pathways that trigger PP and LN formation. However, the levels of PNAd expression in the NALT of these PP- and/or LN-deficient mice seemed to be lower than in wild-type mice (Figures 3A and 3C). Although the expression of PNAd was influenced by nasal immunization with CT in LTα$^{-/-}$ mice, the level was still low when compared with wild-type mice (Figures 3A and 3D). These results suggest that at least a part of the PNAd expression in NALT is regulated under IL-7R and LTα1β2/LTβ R-NIK signals.

Role of L-Selectin/PNAd for the Formation of NALT

Inasmuch as the molecular interaction of L-selectin and PNAd has been shown to be a key step for the migration of lymphoid cells into NALT (Csensits at al., 1999), we thought it important to examine whether NALT formation is influenced by the blockade of this adhesion molecule interaction. To directly address the role of L-selectin in the development of NALT, we analyzed NALT formation in L-selectin$^{-/-}$ mice. Gross histology revealed that though NALT was present in L-selectin$^{-/-}$ mice (Figures 3J and 3K), it was smaller than that of normal control mice. Further, the treatment of C57BL6/c mice with anti-PNAd mAb (MECA79) resulted in the reduction of the size of NALT (Figures 3L and 3M). These results indicate the possible contribution of other adhesion molecules that might compensate for the homing of NALT lymphocytes in L-selectin$^{-/-}$ mice and anti-PNAd mAb-treated

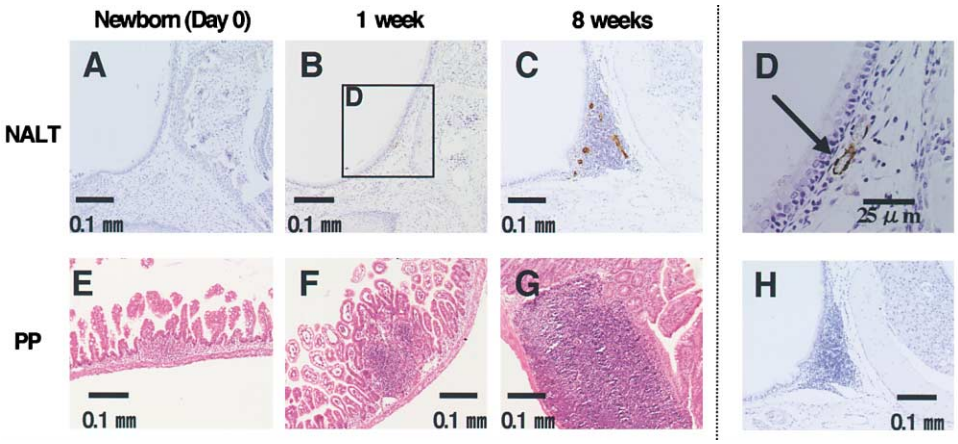

Figure 1. Chronological Analysis and Comparison of the Development of NALT and PP

(A–D) Fixed tissue sections were stained with MECA 79 (rat anti-mouse PNAd mAb) to detect NALT HEV, followed by counterstaining with hematoxylin. (A) Nasal tissue from newborn mice (day 0) with no PNAd-expressing HEV. (B) The NALT anlagen from 1-week-old mice with small accumulation of lymphoid cells around single PNAd-expressing HEV in the nasal tissue. (C) 8-week-old murine NALT with numerous PNAd-expressing HEV. (D) Enlargement of the NALT anlagen of the 1-week-old mice from Figure 2B. Arrows point to PNAd-expressing HEV. (E–G) Fixed tissue sections were HE stained for the detection of PP. (E) PP in the intestine from newborn mice (day 0). (F) PP in the intestine of 1-week-old mice. (G) PP in the intestine of 8-week-old mice.
(H) Isotype matched irrelevant Ab (rat IgM, R4-22; PharMingen) reacted with nasal tissue of 8-week-old mice.

mice. However, these findings do not diminish the importance of PNAd/L-selectin interaction for the entry of lymphocytes into NALT.

Characterization of Lymphoid Cells Isolated from NALT

Since tissue organogenesis of NALT was maintained in PP null mice, we next characterized lymphocytes isolated from the NALT of LT$\alpha^{-/-}$, *aly/aly*, and strain-matched wild-type mice. Flow cytometry analysis of NALT mononuclear cells in the wild-type and *aly/+* mice revealed populations of B220^{+} B cells (70%–80%) and CD3^{+} T cells (14%–20%; Table 1), findings in agreement with those of our previous study (Hiroi et al., 1998). In contrast, a higher frequency (51%) of CD3^{+} T cells ($p < 0.05$) was noted in NALT lymphocytes isolated from LT$\alpha^{-/-}$ than from wild-type mice (19%; Table1). When NALT lymphocytes isolated from *aly/+* and *aly/aly* mice were compared, the frequency of B and T cells in *aly/+* and *aly/aly* mice was similar to that of wild-type and LT$\alpha^{-/-}$ NALT, respectively. The frequency of CD11c^{+} dendritic cells was higher in NALT isolated from *aly/aly* mice ($p < 0.05$) than in control *aly/+* NALT (Table 1). Despite the alteration in the frequency of B and T cells

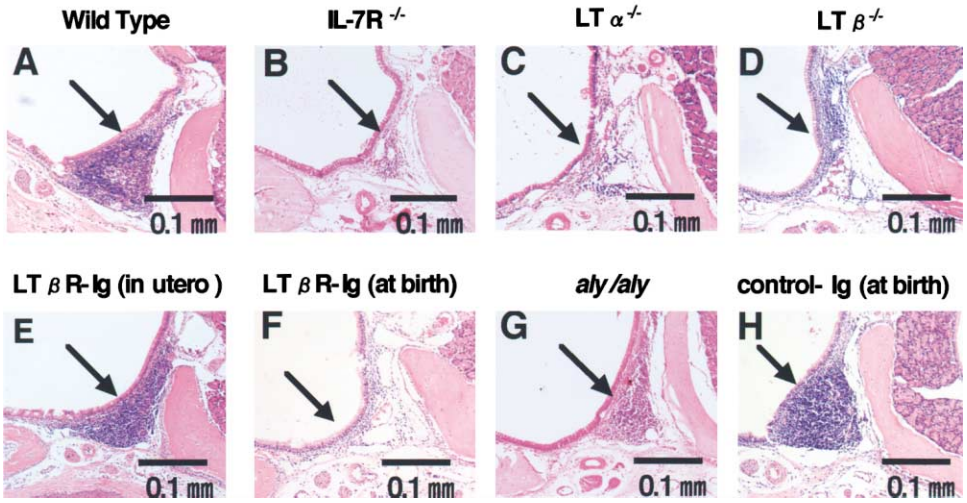

Figure 2. Development of NALT in Secondary Lymphoid Tissue-Deficient Mice

Paraffin sections with HE staining were prepared from 8-week-old mice.
(A) NALT of wild-type (WT) mice. (B) NALT of IL-7R$\alpha^{-/-}$ mice. (C) NALT of LT$\alpha^{-/-}$mice. (D) NALT of LT$\beta^{-/-}$mice. (E) NALT of mice treated with LTβR-Ig fusion protein in utero. (F) NALT of mice treated with LTβR-Ig fusion protein at birth. (G) NALT of *aly/aly* mice. (H) NALT of mice treated with control-Ig at birth. Arrows point to NALT.

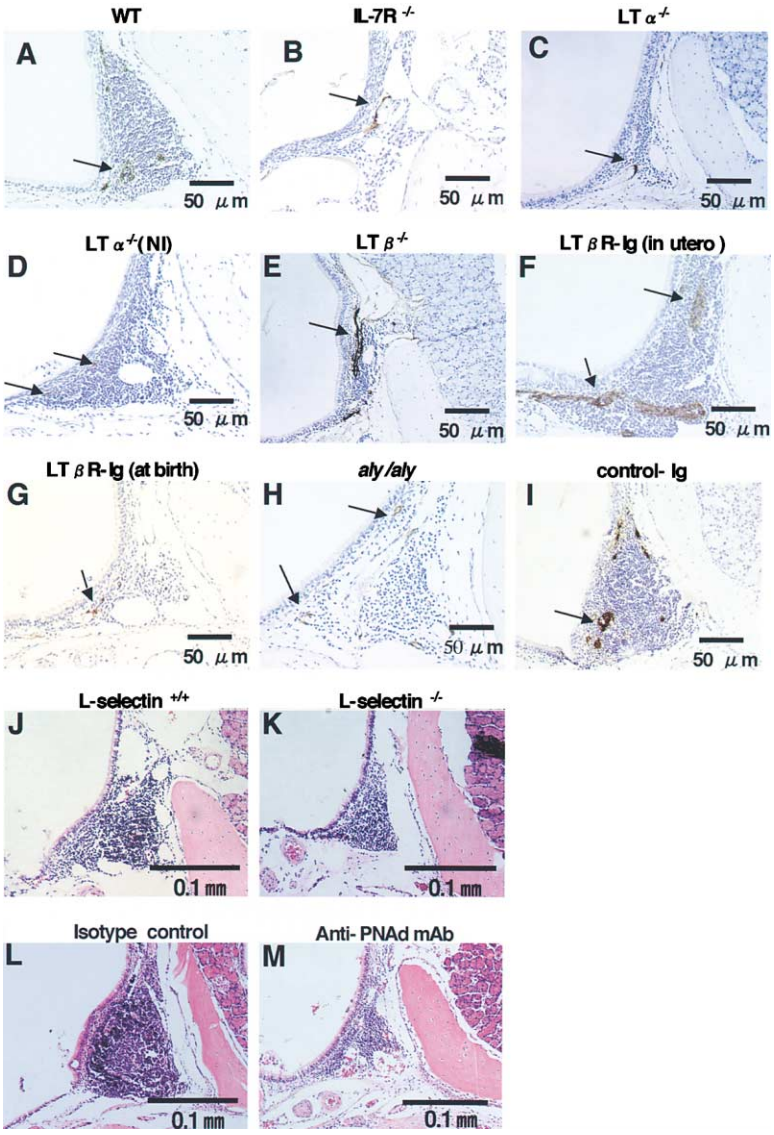

Figure 3. Role of PNAd and L-Selectin for the Development of NALT

PNAd$^+$ HEV was detected in NALT of mice lacking PP and/or LN (A–I). NALT was developed in L-selectin$^{-/-}$ mice and wild-type mice treated with anti-PNAd mAb (J–M).

(A–I) All fixed tissue sections isolated from 8-week-old mice were stained with MECA 79 (rat anti-mouse PNAd mAb) for the detection of HEV, followed by counterstaining with hematoxylin. Arrows point to PNAd-expressing HEV. (A) NALT of wild-type (WT) mice. (B) NALT of IL-7R$\alpha^{-/-}$ mice. (C) NALT of LT$\alpha^{-/-}$ mice. (D) NALT of LT$\alpha^{-/-}$ mice after nasal immunization (NI) with CT, as described in Experimental Procedures. (E) NALT of LT$\beta^{-/-}$mice. (F) NALT of mice treated with LTβR-Ig fusion protein in utero. (G) NALT of mice treated with LTβR-Ig fusion protein at birth. (H) NALT of *aly/aly* mice. (I) NALT of mice treated with control-Ig at birth.

(J–M) Paraffin sections with HE staining were prepared from background mice (J) and L-selectin$^{-/-}$ mice (K). A similar preparation was made for isotype control Ab- (L) and mAb anti-PNAd (M)-treated WT mice. For the treatment, WT mice were systemically treated with mAb anti-PNAd (MECA-79) or isotype control (rat IgM) once per week for 5 consecutive weeks.

in NALT lymphocytes from wild-type and PP-deficient mice, the ratio of CD4/CD8 in CD3$^+$ T cells did not change among these different mice groups (Table 1). Although there was some alteration in the ratio of B and T cells isolated from the NALT of wild-type and PP null mice, our findings demonstrate that lymphoid cells, including B cells, T cells and DC, accumulate in the NALT of PP-deficient mice (LT$\alpha^{-/-}$ and *aly/aly*).

Development of CD3$^-$CD4$^+$CD45$^+$ Cells in Infant NALT

CD3$^-$CD4$^+$CD45$^+$ cells are considered critical for secondary lymphoid tissue development (Honda et al., 2001; Sun et al., 2000; Yokota et al., 1999). Thus, we next searched for such cells in NALT isolated from infant mice. FACS analysis revealed the presence of CD3$^-$CD4$^+$CD45$^+$ cells in NALT of 2-week-old wild-type (0.32%; 20.8 $\times$ 10^3/mouse) and LT$\alpha^{-/-}$mice (0.22%; 4.40 $\times$ 10^3/mouse) (Figure 4). A similar population of CD3$^-$CD4$^+$CD45$^+$ cells (1.90%; 1.43 $\times$ 10^4/mouse) was found in the intestines of E18 embryos (Figure 4). It was interesting that a small population of CD3$^-$CD4$^+$CD45$^+$ cells was still present in infant NALT because these cells were hardly detectable in peripheral blood after birth (Mebius et al., 1997). This finding was further confirmed by immunohistological analysis. In 10-day-old wild-type and LT$\alpha^{-/-}$ mice, small numbers of CD3$^-$CD4$^+$ cells were noted in the bilateral NALT anlagen (Figures 4A–4C). Thus, CD3$^-$CD4$^+$ inducer cells were found at the initiation site of NALT organogenesis.

The Id2 Gene Is an Essential Molecule for the Organogenesis of NALT

The accumulation of CD3$^-$CD4$^+$CD45$^+$ cells in NALT anlagen suggested that these cells might be involved in NALT organogenesis. Therefore, we investigated nasal tissues in Id2$^{-/-}$ mice that lack this cell population (Yo-

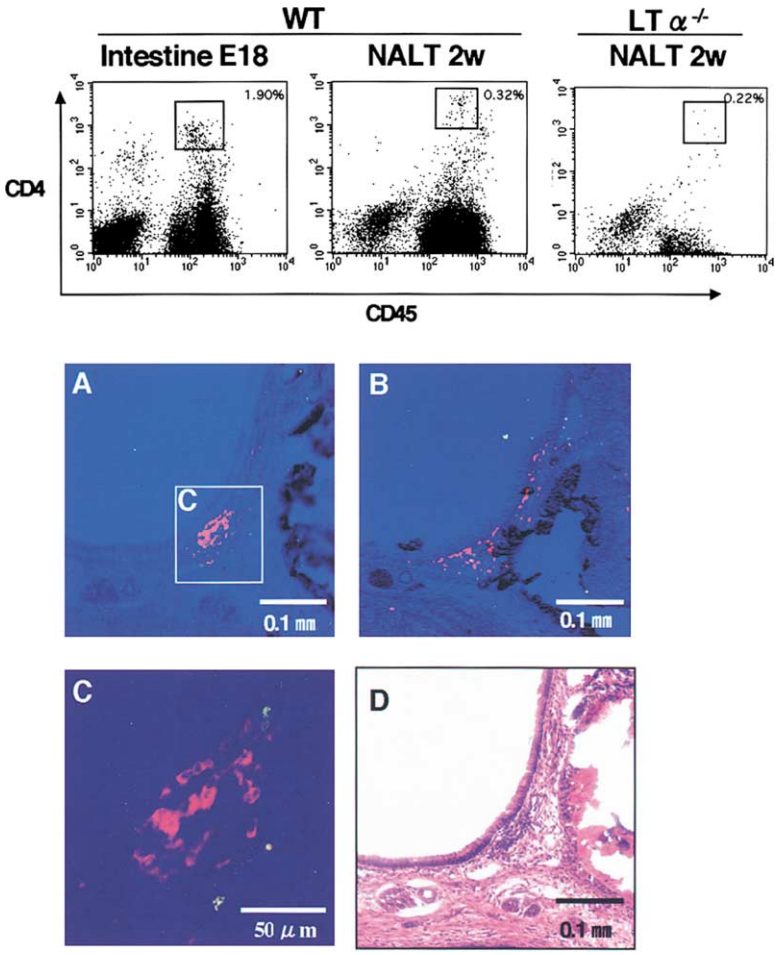

Figure 4. Presence of Inducer $CD3^-CD4^+CD45^+$ Cells in Infant NALT

Lymphoid cells were isolated from the intestines of E18 embryos and from NALT of 2-week-old wild-type (WT) and $LT\alpha^{-/-}$ mice. Cells were then first stained with FITC-anti-CD45, PE-anti-CD4, and biotin-anti-CD3 mAbs, and then with streptavidin-APC. The percentage of $CD3^-CD4^+CD45^+$ cells was determined by flow cytometry after gating for the CD3 negative fraction.

(A) The accumulation of $CD3^-CD4^+$ cells was detected in bilateral NALT anlage in 10-day-old WT mice and (B) $LT\alpha^{-/-}$ mice. (C) Enlargement of the NALT anlage of 10-day-old mice from (A). Fixed tissue was stained with FITC-anti-CD3 and PE-CD4 mAbs, and then examined by confocal microscopy. (D) A serial section of (A) was also stained by HE. A control experiment using confocal analysis was performed using spleen of adult WT mice stained with the same combination of the fluorescence-conjugated mAbs (data not shown).

kota et al., 1999) and found that $Id2^{-/-}$ mice were deficient in NALT (Figures 5A and 5B). Furthermore, PNAd expression was not observed in the $Id2^{-/-}$ mice, whereas this addressin was found in NALT of wild-type littermates (Figures 5D and 5E). In the next experiment, we nasally immunized $Id2^{-/-}$ mice with CT. Although the size of NALT was enlarged after the nasal immunization of $LT\alpha^{-/-}$ mice with CT (Figures 3C and 3D), there were no sign of NALT development or PNAd expression on vessels in the nasal tissue of nasally immunized $Id2^{-/-}$ mice (Figures 5C and 5F). These results further confirmed our finding that the development of NALT is defective in $Id2^{-/-}$ mice. The analysis of $Id2^{-/-}$ mice indicates that a population of inducer cells, i.e., $CD3^-CD4^+CD45^+$ cells, is involved in the development of NALT and that the organogenesis of NALT, like that of PP and LN, depends on the helix-loop-helix inhibitor Id2 (Yokota et al., 1999).

Table 1. Analysis of Lymphocytes in NALT Isolated from PP Null and Wild-Type Mice

	Total Numbers of Cell Isolated ($\times 10^4$)	Surface Marker-Positive Cells (%) (Number of Positive Cells $\times 10^3$)			Ratio of T Cell Subset
		CD3	B220	CD11c	CD4/CD8
Wild-type	26.5 ± 1.0	19.1 ± 1.5 (50.7 ± 4.6)	69.9 ± 4.6 (185.0 ± 10.9)	1.9 ± 0.5 (5.4 ± 1.3)	1.7 ± 0.2
$LT\alpha^{-/-}$	1.8 ± 0.3	51.2 ± 3.5* (7.2 ± 1.7*)	31.3 ± 4.7* (5.5 ± 0.9*)	2.9 ± 1.0 1.4 ± 0.1*)	1.9 ± 0.2
aly/+	4.2 ± 1.4	13.5 ± 1.0 (5.8 ± 1.4)	77.1 ± 5.2 (31.5 ± 4.1)	1.2 ± 0.2 (0.5 ± 0.2)	1.9 ± 0.2
aly/*aly*	3.3 ± 0.2	57.1 ± 4.0* (18.9 ± 1.9*)	31.1 ± 12.1* (10.2 ± 4.0*)	7.6 ± 0.8* (0.25 ± 0.2)	2.1 ± 0.1

Lymphocytes were isolated from NALT of PP deficient- and wild-type control-mice. Cells were then stained with FITC-, PE-, or APC-conjugated mAbs specific for CD3, CD4, CD8, CD11c, and B220 as described in Experimental Procedures. The percentage and the number of positive cells were determined by flow cytometry and represented mean ± SEM from three different experiments.
*$p < 0.05$.

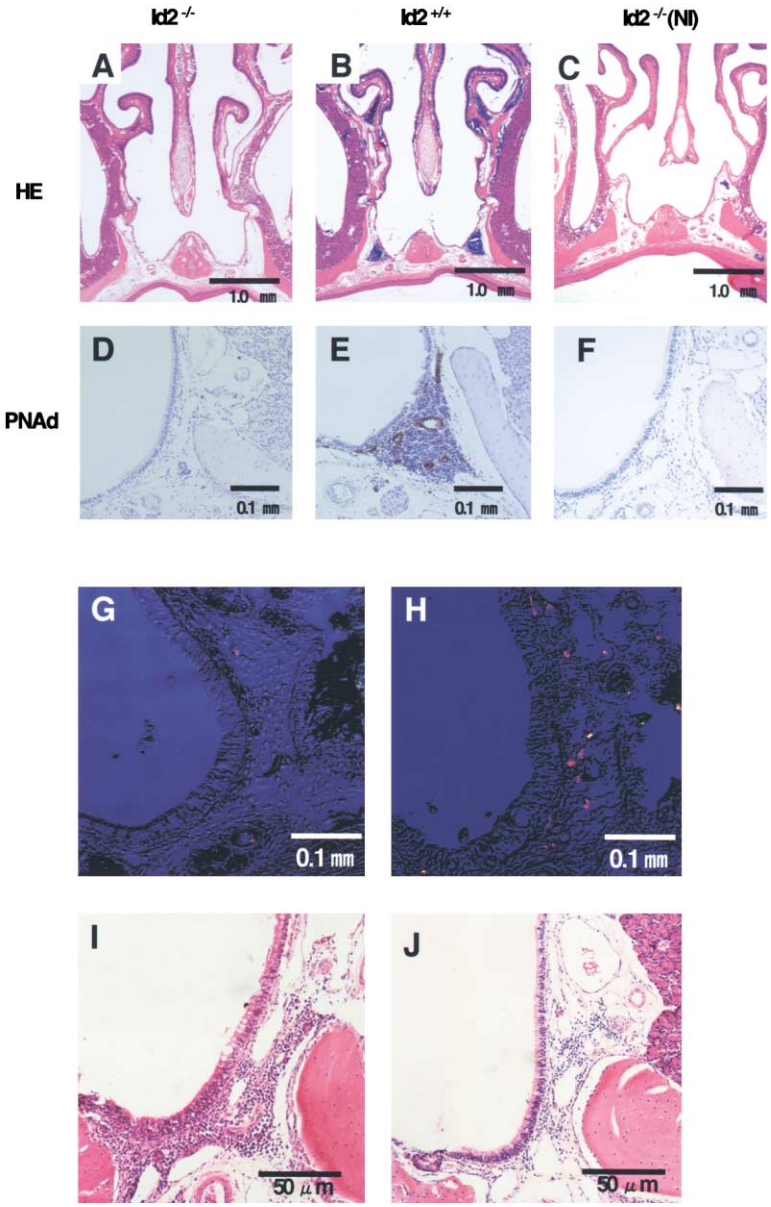

Figure 5. Essential Role of Id2 and $CD3^{-}CD4^{+}CD45^{+}$ Cells for the NALT Organogenesis

Id2 deficiency resulted in the lack of NALT formation (A–F). NALT organogenesis was induced in $Id2^{-/-}$ mice by the adoptive transfer of fetal liver or $CD3^{-}CD4^{+}CD45^{+}$ cells (G–J).
(A–C) Fixed tissue sections were stained with HE.
(D–F) Tissue sections were stained with MECA79, followed by counterstaining with hematoxylin. (A) NALT was absent in the nasal tissue of $Id2^{-/-}$mice. (B) NALT was intact in $Id2^{+/+}$ littermates. (C) NALT was not developed in $Id2^{-/-}$mice nasally immunized (NI) with CT. (D) PNAd expression of HEV in the nasal tissue was not detected in $Id2^{-/-}$mice. (E) HEV of NALT in $Id2^{+/+}$ littermates normally expressed PNAd. (F) PNAd expression was not developed in $Id2^{-/-}$mice after nasal immunization of CT.
(G and H) Fixed tissue was stained with FITC-anti-CD3 and PE-CD4 mAbs and then examined by confocal microscopy. (G) $CD3^{-}CD4^{+}$ cells were absent in the nasal tissue of $Id2^{-/-}$ infant mice. (H) $CD3^{-}CD4^{+}$ cells were found to have accumulated in the nasal tissue of $Id2^{-/-}$ infant mice 7 days after the adoptive transfer with fetal liver cells.
(I and J) The same tissues stained by HE. In these experiments, each group containing three $Id2^{-/-}$ mice was adoptively transferred with fetal liver cells or $CD3^{-}CD4^{+}CD45^{+}$ cells. Development of NALT-like structure was noted in all of these adoptively transferred mice. (I) A NALT-like structure was observed to have developed in $Id2^{-/-}$ mice 7 weeks after the adoptive transfer with fetal liver cells. (J) A NALT-like structure was noted in $Id2^{-/-}$ mice transferred with $CD3^{-}CD4^{+}CD45^{+}$ cells.

Organogenesis of NALT in $Id2^{-/-}$ Mice after Adoptive Transfer with Fetal Liver or $CD3^{-}CD4^{+}CD45^{+}$ Cells

In order to clarify the role of $CD3^{-}CD4^{+}CD45^{+}$ cells for the induction of NALT development, we first performed an adoptive transfer experiment of fetal liver cells from $Id2^{+/+}$ mice into $Id2^{-/-}$ mice (Figures 5G–5J). Seven days after the transfer of fetal liver cells into $Id2^{-/-}$ newborn mice, $CD3^{-}CD4^{+}$ cells had migrated into the site of NALT formation (Figures 5G and 5H) and, seven weeks after transfer, a NALT-like structure was detected (Figure 5I). Furthermore, the adoptive transfer of $CD3^{-}CD4^{+}CD45^{+}$ cells isolated from fetal intestine of wild-type mice into $Id2^{-/-}$ newborn mice led to the initiation of NALT-like structure in $Id2^{-/-}$ mice (Figure 5J). These findings are a direct demonstration that $CD3^{-}CD4^{+}CD45^{+}$ cells from fetal liver function to initiate lymphoid tissue organogenesis (e.g., NALT). However, the lymphoid structure induced by the adoptive transfer of $CD3^{-}CD4^{+}CD45^{+}$ cells was smaller than that induced by the adoptive transfer with fetal liver cells (Figures 5I and 5J), suggesting that additional cell types contribute to the full development of NALT tissue.

Discussion

This study provides persuasive and direct evidence that the mechanism of cytokine- and cytokine receptor-mediated organogenesis in NALT is distinct from that in PP and LN. First, NALT was shown to develop in various circumstances which are known to cause PP and LN deficiency, as for example, in $LT\alpha^{-/-}$, $LT\beta^{-/-}$, *aly/aly* mice, and mice treated in utero with LTβR-Ig. Since NALT tissue organogenesis begins after birth, we also treated mice with LTβR-Ig postnatally to ensure that the antagonist was present during the initial stage of NALT formation. NALT was still detected in mice

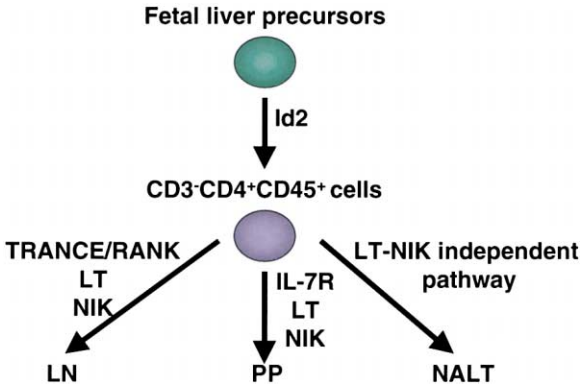

Figure 6. A Summary of the Proposed Organogenesis of NALT, PP, and LN

In the case of LN and PP, $CD3^-CD4^+CD45^+$($IL\text{-}7R\alpha^+$) cells are considered to be the inducer of secondary lymphoid tissue. Id2 is indispensable for the induction and the differentiation of the inducer cells from the fetal liver precursors. Following activation via IL-7R, these $CD3^-CD4^+CD45^+$ cells express $LT\alpha1\beta2$ heterotrimer, which then binds to LTβR for the subsequent signal transduction via NIK for the expression of necessary adhesion molecules and/or chemokines. The accumulation of lymphoid cells is induced by these homing-related molecules (e.g., PP). The development of $CD3^-CD4^+CD45^+$ cells in NALT also requires Id2, but the initiation of NALT organogenesis is independent of IL-7R, $LT\alpha1\beta2$/LTβR, and NIK signaling. It is not yet known which cytokines and corresponding receptors fill the roles of the IL-7R, $LT\alpha1\beta2$/LTβR, and NIK signaling pathways for the formation of NALT.

treated at birth with LTβR-Ig. LTβR is a critical signaling molecule for stromal cell activation at the site of PP and LN formation via signals transduced to NF-κB through NIK (Fagarasan et al., 2000; Honda et al., 2001; Yin et al., 2001). The ability of NALT to form in the absence of LTβR signaling and NIK function suggests that a unique signal exists to trigger NALT organogenesis (Figure 6). It was also interesting to note that the gross architecture of NALT varied among LT-deficient mice, mice treated in utero with LTβR-Ig, and mice treated after birth with LTβR-Ig, suggesting that the LTβR cytokine signaling cascade contributes to the maturation of cellularity and to the gross development of NALT.

IL-7Rα signaling also is essential for the initiation of PP organogenesis, since IL-7R engagement is thought to be required for $CD3^-CD4^+CD45^+$ cells to express $LT\alpha1\beta2$ (Adachi et al. 1998; Yoshida et al., 1999). NALT development in $IL\text{-}7R\alpha^{-/-}$ mice is further evidence that the signaling axis that induces NALT organogenesis is distinct from that which triggers PP formation. Although IL-7R signaling was not essential for the initiation of NALT organogenesis, the maturation of NALT structure was incomplete in $IL\text{-}7R^{-/-}$ mice when compared with wild-type mice (Figures 2 and 3). Other known signaling axes for secondary lymphoid organ formation include the TRANCE/RANK pathway for LN development (Kim et al., 2000). The potential role of this pathway for NALT genesis is not yet known.

$CD3^-CD4^+CD45^+$ cells expressing $LT\alpha1\beta2$ heterotrimers have been implicated as an inducer cell population for secondary lymphoid tissue formation including PP (Sun et al., 2000; Yokota et al., 1999). The observation that NALT development is distinct from that of PP led us to ask if the $CD3^-CD4^+CD45^+$ cells played a role in NALT organogenesis. The helix-loop-helix inhibitor Id2 is a key regulatory factor for the differentiation of $CD3^-CD4^+CD45^+$ cells from stem cells, and the absence of these cells in $Id2^{-/-}$ mice correlates with the loss of PP and LN (Yokota et al., 1999). We have now demonstrated that NALT is deficient in $Id2^{-/-}$ mice (Figure 5). $CD3^-CD4^+CD45^+$ cells have been shown to be derived from fetal liver cells in vivo (Mebius et al., 2001; Yoshida et al., 2001). Therefore, the adoptive transfer of fetal liver cells or $CD3^-CD4^+CD45^+$ cells from $Id2^{+/+}$ wild-type mice into $Id2^{-/-}$ newborn mice resulted in the migration of $CD3^-CD4^+CD45^+$ cells to the site of NALT formation and in the formation of NALT-like structure (Figure 5). This constitutes a direct, functional demonstration that $CD3^-CD4^+CD45^+$ cells deliver an organogenic signal to the site of secondary lymphoid organ formation such as NALT. The formation of other secondary lymphoid tissues absent from $Id2^{-/-}$ mice was not achieved using this protocol, as the transfer of cells occurred after birth, that is, after the developmental window during which LN and PP formation is initiated (data not shown). Thus, Id2-regulated $CD3^-CD4^+CD45^+$ cells are the common-ancestor (or inducer) cells for the organogenesis of NALT as well as PP, although the $CD3^-CD4^+CD45^+$ cells do not use the IL-7R-mediated $LT\alpha1\beta2$/LTβR cytokine signaling cascade for the onset of secondary lymphoid tissue formation in the upper respiratory tract. Our current effort is aimed at identifying the critical signaling cytokine and corresponding receptor used by $CD3^-CD4^+CD45^+$ cells for the initiation of NALT development.

It was interesting to note that PNAd expression was not observed in these NALT-like structures of $Id2^{-/-}$ mice adoptively transferred with fetal liver cells or $CD3^-CD4^+CD45^+$ cells (data not shown). These results suggest that additional factors including cytokine, chemokine, and/or adhesion molecule might be necessary for the maturation of NALT in $Id2^{-/-}$ mice adoptively transferred with fetal liver cells or $CD3^-CD4^+CD45^+$ cells. The finding that NALT is formed in L-$selectin^{-/-}$ mice and wild-type mice treated with anti-PNAd mAb also supports the hypothesis that multiple signals contribute to the NALT formation and subsequent development (Figure 3). Addressins were similarly absent from LN induced to form in $LT\alpha^{-/-}$ mice (Rennert et al., 1998), likewise suggesting that LTα and/or LTαβ regulate(s) PNAd (and MAdCAM-1) expression in developing NALT. Furthermore, the NALT-like structure of $Id2^{-/-}$ mice adoptively transferred with $CD3^-CD4^+CD45^+$ cells was smaller than the NALT formed after fetal liver cell transfer. This result suggests that an additional population of inducer/organizer type cells might be necessary for the formation of mature NALT in addition to $CD3^-CD4^+CD45^+$ cells.

There was a chronological difference between lymphoid development in the nasal tract and the intestine (Figure 1). NALT was not present during embryogenesis, whereas it is known that LN development begins as early as E11 and PP development on E16.5 in the embryonic mesentery and intestine, respectively (Rennert et al., 1997; Yoshida et al., 1999). Further, we could not detect $PNAd^+$ HEV, an important adhesion molecule for the recruitment of lymphocytes to NALT (Csencsits et al.,

1999), in the nasal tract of embryonic mice. NALT and PNAd-positive HEV were not present until 7 days after birth. MAdCAM-1 is expressed on the HEV of gut-associated lymphoid tissues, such as PP in mice (Berlin et al., 1993), and is identified as early as E16.5 at the site of PP organogenesis (Hashi et al., 2001). HEV of LN anlagen similarly expresses MAdCAM-1 starting early in development, with strong expression in newborn mice (Mebius et al., 1996). The expression of MAdCAM-1 is reportedly very low or absent from the HEV of adult NALT (Csencsits et al., 1999), and we found that the HEV of infant NALT (e.g., 7 days old) did not express MAdCAM-1 (data not shown). Thus, NALT formation is chronologically and immunohistochemically distinct from that of PP and LN. Adhesion molecule or addressin expression is thought to be a critical early step in the organogenesis of PP (VCAM-1, MAdCAM-1) and PLN (MAdCAM-1) (Adachi et al., 1997; Hashi et al., 2001; Kim et al., 2000; Mebius et al., 1996). These results suggest that PNAd expression may play a similar role in the development of NALT.

In keeping with a recent report, we found that NALT continued to express PNAd in the adult mouse (Csencsits et al., 1999). We found PNAd expressed on the HEV of NALT in adult LT$\alpha^{-/-}$, LT$\beta^{-/-}$, IL-7R$\alpha^{-/-}$, and *aly/aly* mice, although the intensity of PNAd expression was somewhat lower in these mice than in wild-type mice. The TNF receptor family regulates the expression of several adhesion (or homing) molecules and chemokines for the homing of lymphocytes to secondary lymphoid tissues and inflammatory lesions (Cuff et al., 1999), and these signals are likely transduced in part through NIK (Fagarasan et al., 2000; Nakano et al., 1996; Yin et al., 2001). Our results suggest that the expression of PNAd in NALT is in part regulated by LTα1β2/LTβR- and NIK-associated signaling events. Since the PNAd expression defect is particularly pronounced in LT$\alpha^{-/-}$ mice, an additional role for LTα3/TNF-R signaling should be considered.

Based on the results generated in the present study, two possibilities can be proposed to explain the differences between the mechanisms responsible for the organogenesis of NALT and PP. First, NALT formation could be preprogrammed to initiate IL-7R-, LTα1β2/LTβR-, and NIK-independent tissue organogenesis signaling events after nativity. Alternatively, NALT development may depend on triggering signals provided by inhaled environmental antigens after birth. Taken together, it is likely that NALT organogenesis is preprogrammed to be inaugurated following nativity via an undefined cytokine and corresponding receptor signaling cascade, and inhaled antigens may initiate or accelerate the signals in this cascade. In support of this proposal, PP formation is complete within a few days after birth (and exposure to luminal antigens), although the basic architecture of the tissue is formed during the gestational period (e.g., E15.5) (Hashi et al., 2001).

In summary, our results demonstrate that the formation of NALT is initiated independently of IL-7R, LTα1β2/LTβR, and NIK, but nonetheless relies on the presence of common inducer (ancestor) cells which are CD3$^-$CD4$^+$CD45$^+$ cells. In the case of LN and PP formation, these cells are triggered to express LTα1β2 heterotrimer, which activates LTβR$^+$ stromal organizer cells causing them to express VCAM-1, ICAM-1, or MAdCAM-1 (Honda et al., 2001). Although the organogenesis of NALT requires Id2 and its associated inducer CD3$^-$CD4$^+$CD45$^+$ cells, as does that of PP and LN, the initiation of NALT organogenesis is independent of IL-7R, LTα1β2/LTβR, and NIK signaling cascades (Figure 6). Unique, but not yet identified, molecules may be involved in the lymphoid tissue organogenesis of NALT. Thus, multiple cytokine receptor signaling pathways seem to be important for the organization of cellularity and for the gross development of NALT. Elucidation of the details of this unique signaling axis for NALT organogenesis, including the cytokines, chemokines, and adhesion molecules involved, may further our understanding of the alternative events that trigger secondary lymphoid tissue organogenesis.

Experimental Procedures

Mice

C57BL/6J Jcl mice and *aly/aly* mice (ALY/Nsc Jcl-*aly/aly*) were obtained from Japan Clea Co. (Tokyo, Japan). LT$\alpha^{-/-}$ (C57BL/6) mice and L-selectin$^{-/-}$ (B6/129S) mice were obtained from Jackson Laboratories (Bar Harbor, ME). IL-7R$\alpha^{-/-}$ (129/Ola$\times$C57BL/6) mice were kindly provided by Immunex Corp. (Seattle, WA) (Peschon et al., 1994). Id2$^{-/-}$ (129/Sv) mice were generated as described (Yokota et al., 1999). These strains were maintained in conventional housing at the Experimental Animal Facility of the Research Institute for Microbial Diseases, Osaka University (Osaka, Japan). The nasal tissue of LT$\beta^{-/-}$ mice was kindly provided by Dr. R. Flavell (Koni et al., 1997), Biogen Inc (Cambridge, MA). Pregnant wild-type mice were injected intravenously with 200 μg of LTβR-Ig fusion protein on gestational days 14 and 17. In some experiments, the peritoneal cavity of newborn mice was injected with LTβR-Ig fusion protein or control IgG (50 μg/mouse) according to the method described previously (Yamamoto et al., 2000). LTβR-Ig fusion protein and control IgG were also prepared as described elsewhere (Rennert et al., 1997, 1998).

Wild-type mice were treated with anti-PNAd mAb (50 μg/mouse, MECA-79, BD PharMingen, SanDiego, CA) or isotype control (rat IgM, PharMingen) once per week for 5 consecutive weeks.

Histology and Immunohistochemistry

The heads and the intestines of the mice were fixed in 4% paraformaldehyde. Adult nasal tissues isolated from 6- to 8-week-old mice were decalcified in EDTA solution for 10 days, and all tissues were embedded in paraffin. Frontal sections of the nasal tissues (5 μm) and intestinal sections with PP (5 μm) were stained with hematoxylin/eosin (HE). For immunohistochemical staining, deparaffinated sections (5 μm) were incubated with anti-PNAd mAb (MECA-79; PharMingen) according to the method described previously (Csencsits et al., 1999). The sections were then incubated with biotinylated secondary Ab (ZYMED, San Francisco, CA) and exposed to avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). After color development with diaminobenzidine, the sections were counterstained with hematoxylin.

For confocal microscopic analysis, the nasal tissues from 7-day-old mice were fixed in 4% paraformaldehyde and rapidly frozen in OCT emdedding medium (Sakura Finetechnical Co., Ltd., Tokyo, Japan). Cryostat sections (5 μm) were prepared and stained with FITC-anti-CD3ϵ (145-2C11; PharMingen) and PE-anti-CD4 (RM4-5; PharMingen) mAbs for 1 hr at room temperature. Histological analysis was performed using confocal microscopy (Bio-Rad Laboratories Inc., Hercules, CA).

Nasal Immunization

LT$\alpha^{-/-}$ and Id2$^{-/-}$ mice were immunized nasally with 10 μl of PBS containing CT (5 μg/mouse; Sigma Chemical, St. Louis, MO) once per week for 3 consecutive weeks. Nasal tissues were collected 7 days after final immunization.

Isolation and FACS Analysis of Mononuclear Cells

Mononuclear cells from NALT were isolated as described (Asanuma et al., 1995; Hiroi et al., 1998). In brief, the mononuclear cells were isolated by gentle teasing through stainless steel screens, and FACS analysis was performed (Hiroi et al., 1994, 1995). The mononuclear cell preparation was pretreated with anti-CD16/CD32 FcR mAb (2.4G2) and then stained with the appropriate fluorescence-conjugated anti-CD3ε (145-2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-B220 (RA3-6B2), anti-CD45 (30-F11), and anti-CD11c mAb (HL3) (all from PharMingen). Flow cytometry analysis and cell isolations were then performed using FACSCalibur and FACS Vantage (Becton Dickinson, San Jose, CA), respectively.

Adoptive Transfer Experiment of Fetal Liver Cells or $CD3^-CD4^+CD45^+$ Cells

Fetal liver cells were collected from E16 $Id2^{+/+}$ embryos, and $CD3^-CD4^+CD45^+$ cells were isolated from the fetal intestine (E18) of $Id2^{+/+}$ mice by using FACS Vantage (Becton Dickinson), as previously described, with some modifications (Yoshida et al., 2001). Isolated cells were cultured in medium containing RPMI 1640, 10% FBS, 50 μg/ml penicillin G, 50 U/ml streptomycin, 100 ng/ml IL-7 (Genzyme, Cambridge, MA), and 100 ng/ml stem cell factor (R&D Systems, Minneapolis, MN) in order to activate these cells (Yoshida et al., 1999). After 1 day of culture, these fetal liver cells (1.0×10^5) or $CD3^-CD4^+CD45^+$ cells (1.0×10^3) were injected into the peritoneal cavity of $Id2^{-/-}$ newborn mice. The nasal tissues obtained from 7-day-old $Id2^{-/-}$ mice adoptively transferred with fetal liver cells were analyzed by confocal microscopy (Bio-Rad Laboratories Inc., Hercules, CA). Histological analysis was also performed for the nasal tissues of 7- to 8-week-old $Id2^{-/-}$ mice adoptively transferred with fetal liver cells or $CD3^-CD4^+CD45^+$ cells.

Acknowledgments

This work was supported by a Grant-in-Aid for COE Research from the Ministry of Education, Science, Sports, and Culture of Japan; the Ministry of Health, Labor, and Welfare of Japan; and the Japanese Human Science Foundation. We thank the members of the Mucosal Immunology Group of Osaka University for their critical comments. We also thank Noriko Kitagaki for her technical help.

References

Adachi, S., Yoshida, H., Kataoka, H., and Nishikawa, S.-I. (1997). Three distinctive steps in Peyer's patch formation of murine embryo. Int. Immunol. *9*, 507–514.

Adachi, S., Yoshida, H., Honda, K., Maki, K., Saijo, K., Ikuta, K., Saito, T., and Nishikawa, S.-I. (1998). Essential role of IL-7 receptor alpha in the formation of Peyer's patch anlage. Int. Immunol. *10*, 1–6.

Asanuma, H., Inaba, Y., Aizawa, C., Kurata, T., and Tamura, S. (1995). Characterization of mouse nasal lymphocytes isolated by enzymatic extraction with collagenase. J. Immunol. Methods *187*, 41–51.

Berlin, C., Berg, E.L., Briskin, M.J., Andrew, D.P., Kilshaw, P.J., Holzmann, B., Weissman, I.L., Hamann, A., and Butcher, E.C. (1993). Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. Cell *16*, 185–190.

Crowe, P.D., VanArsdale, T.L., Walter, B.N., Ware, C.F., Hession, C., Ehrenfels, B., Browning, J.L., Din, W.S., Goodwin, R.G., and Smith, C.A. (1994). A lymphotoxin-beta-specific receptor. Science *264*, 707–710.

Csencsits, K.L., Jutila, M.A., and Pascual, D.W. (1999). 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. *163*, 1382–1389.

Cuff, C.A., Sacca, R., and Ruddle, N.H. (1999). Differential induction of adhesion molecule and chemokine expression by LTα3 and LTαβ in inflammation elucidates potential mechanisms of mesenteric and peripheral lymph node development. J. Immunol. *162*, 5965–5972.

De Togni, P., Goellner, J., Ruddle, N.H., Streeter, P.R., Fick, A., Mariathasan, S., Smith, S.S., Carlson, R., Shornick, L.P., Strauss-Schoenberger, J., et al. (1994). Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. Science *264*, 703–707.

Fagarasan, S., Shinkura, R., Nogaki, T., Ikuta, K., Tashiro, K., and Honjo, T. (2000). Alymphoplasia (aly)-type nuclear factor kappa B-inducing kinase (NIK) causes defects in secondary lymphoid tissue chemokine receptor signaling and homing of peritoneal cells to the gut-associated lymphatic tissue system. J. Exp. Med. *191*, 1477–1486.

Futterer, A., Mink, K., Luz, A., Kosco-Vilbois, M.H., and Pfeffer, K. (1998). The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. Immunity *9*, 59–70.

Hashi, H., Yoshida, H., Honda, K., Fraser, S., Kubo, H., Awane, M., Takabayashi, A., Nakano, H., Yamaoka, Y., and Nishikawa, S.-I. (2001). Compartmentalization of Peyer's patch anlagen before lymphocyte entry. J. Immunol. *166*, 3702–3709.

Hiroi, T., Fujihashi, K., McGhee, J.R., and Kiyono, H. (1994). Characterization of cytokine-producing cells in mucosal effector sites: $CD3^+$Tcells of Th1 and Th2 type in salivary gland-associated tissue. Eur. J. Immunol. *24*, 2653–2658.

Hiroi, T., Fujihashi, K., McGhee, J.R., and Kiyono, H. (1995). Polarized Th2 cytokine expression by both mucosal γδ and αβ T cells. Eur. J. Immunol. *25*, 2743–2751.

Hiroi, T., Iwatani, K., Iijima, H., Kodama, S., Yanagita, M., and Kiyono, H. (1998). Nasal immune system: distinctive Th0 and Th1/Th2 type environments in murine nasal-associated lymphoid tissues and nasal passage, respectively. Eur. J. Immunol. *28*, 3346–3353.

Honda, K., Nakano, H., Yoshida, H., Nishikawa, S., Rennert, P., Ikuta, K., Tamechika, M., Yamaguchi, K., Fukumoto, T., Chiba, T., et al. (2001). Molecular basis for hemotopoietic/mesenchymal interaction during initiation of Peyer's patch organogenesis. J. Exp. Med. *193*, 621–630.

Imaoka, K., Miller, C.J., Kubota, M., McChesney, M.B., Lohman, B., Yamamoto, M., Fujihashi, K., Someya, K., Honda, M., McGhee, J.R., et al. (1998). Nasal immunization of nonhuman primates with simian immunodeficiency virus p55gag and cholera toxin adjuvant induces Th1/Th2 help for virus specific immune responses in reproductive tissues. J. Immunol. *161*, 5952–5958.

Jeong, K.I., Suzuki, H., Nakayama, H., and Doi, K. (2000). Ultrastructural study on the follicle-associated epithelium of nasal-associated lymphoid tissue in specific pathogen-free (SPF) and conventional environment-adapted (SPF-CV) rat. J. Anat. *196*, 443–451.

Kim, D., Mebius, R.E., MacMicking, J.D., Jung, S., Cupedo, T., Castellanos, Y., Rho, J., Wong, B.R., Josien, R., Kim, N., et al. (2000). Regulation of peripheral lymph node genesis by the tumor necrosis factor family member TRANCE. J. Exp. Med. *192*, 1467–1478.

Koni, P.A., Sacca, R., Lawton, P., Browning, J.L., Ruddle, N.H., and Flavell, R.A. (1997). Distinct roles in lymphoid organogenesis for lymphotoxins α and β revealed in lymphotoxin β-deficient mice. Immunity *6*, 491–500.

Kuper, C.F., Hemeleers, D.M., Bruijntjes, J.P., van der Ven, I., Biewenga, J., and Sminia, T. (1990). Lymphoid and non-lymphoid cells in nasal-associated lymphoid tissue (NALT) in the rat. An immuno- and enzyme-histochemical study. Cell Tissue Res. *259*, 371–377.

Kuper, C.F., Koornstra, P.J., Hameleers, D.M., Biewenga, J., Spit, B.J., Duijvestijin, A.M., van Breda Vriesman, P.J., and Sminia, T. (1992). The role of nasopharyngeal lymphoid tissue. Immunol. Today *13*, 219–224.

Kurono, Y., Yamamoto, M., Fujihashi, K., Kodama, S., Suzuki, M., Mogi, G., McGhee, J.R., and Kiyono, H. (1999). Nasal immunizaton induces *Haemophilus influenzae*-specific Th1 and Th2 responses with mucosal IgA and systemic IgG antibodies for protective immunity. J. Infect. Dis. *180*, 122–132.

Mebius, R.E., Streeter, P.R., Michie, S., Butcher, E.C., and Weissman, I.L. (1996). A developmental switch in lymphocyte homing receptor and endothelial vascular addressin expression regulates lymphocyte homing and permits $CD4^+CD3^-$ cells to colonize lymph nodes. Proc. Natl. Acad. Sci. USA *93*, 11019–11024.

Mebius, R.E., Rennert, P., and Weissman, I.L. (1997). Developing lymph nodes collect $CD4^+CD3^-$ $LT\beta^+$ cells that can differentiate to

APC, NK cells, and follicular cells but not T or B cells. Immunity *7*, 493–504.

Mebius, R.E., Miyamoto, T., Christensen, J., Domen, J., Cupedo, T., Weissman, I.L., and Akashi, K. (2001). The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45(+)CD4(+)CD3(−) cells, as well as macrophages. J. Immunol. *166*, 6593–6601.

Miyawaki, S., Nakamura, Y., Suzuka, H., Koba, M., Yasumizu, R., Ikehara, S., and Shibata, Y. (1994). A new mutation, *aly*, that induces a generalized lack of lymph nodes accompanied by immunodeficiency in mice. Eur. J. Immunol. *24*, 429–434.

Nakano, H., Oshima, H., Chung, W., Williams-Abbott, L., Ware, C.F., Yagita, H., and Okumura, K. (1996). TRAF5, an activator of NF-κB and putative signal transducer for the lymphotoxin-beta receptor. J. Biol. Chem. *271*, 14661–14664.

Peschon, J.J., Morrissey, P.J., Grabstein, K.H., Ramsdell, F.J., Maraskovsky, E., Gliniak, B.C., Park, L.S., Ziegler, S.F., Williams, D.E., and Ware, C.B. (1994). Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. J. Exp. Med. *180*, 1955–1960.

Rennert, P.D., Browning, J.L., Mebius, R., Mackay, F., and Hochman, P.S. (1996). Surface lymphotoxin alpha/beta complex is required for the development of peripheral lymphoid organs. J. Exp. Med. *184*, 1999–2006.

Rennert, P.D., Browning, J.L., and Hochman, P.S. (1997). Selective disruption of lymphotoxin ligands reveals a novel set of mucosal lymph nodes and unique effects on lymph node cellular organization. Int. Immunol. *9*, 1627–1639.

Rennert, P.D., James, D., Mackay, F., Browning, J.L., and Hochman, P.S. (1998). Lymph node genesis is induced by signaling through the lymphotoxin β receptor. Immunity *9*, 71–79.

Shimoda, M., Nakamura, T., Takahashi, Y., Asanuma, H., Tamura, S., Kurata, T., Mizuochi, T., Azuma, N., Kanno, C., and Takemori, T. (2001). Isotype-specific selection of high affinity memory B cells in nasal-associated lymphoid tissue. J. Exp. Med. *194*, 1597–1607.

Shinkura, R., Kitada, K., Matsuda, F., Tashiro, K., Ikuta, K., Suzuki, M., Kogishi, K., Serikawa, T., and Honjo, T. (1999). Alymphoplasia is caused by a point mutation in the mouse gene encoding NF-κB-inducing kinase. Nat. Genet. *22*, 74–77.

Sun, Z., Unutmaz, D., Zou, Y., Sunshine, M.J., Pierani, A., Brenner-Morton, S., Mebius, R.E., and Littman, D.R. (2000). Requirement for RORγ in thymocyte survival and lymphoid organ development. Science *288*, 2369–2373.

Yamamoto, M., Rennert, P., McGhee, J.R., Kweon, M.-N., Yamamoto, S., Dohi, T., Otake, T., Bluethmann, H., Fujihashi, K., and Kiyono, H. (2000). Alternate mucosal immune system: organized Peyer's patches are not required for IgA responses in the gastrointestinal tract. J. Immunol. *164*, 5184–5191.

Yanagita, M., Hiroi, T., Kitagaki, N., Hamada, S., Ito, H., Shimauchi, H., Murakami, S., Okada, H., and Kiyono, H. (1999). Nasopharyngeal-associated lymphoreticular tissue (NALT) immunity: fimbriae-specific Th1 and Th2 cell-regulated IgA responses for the inhibition of bacterial attachment to epithelial cells and subsequent inflammatory cytokine production. J. Immunol. *162*, 3559–3565.

Yin, L., Wu, L., Wesche, H., Arthur, C.D., White, J.M., Goeddel, D.V., and Schreiber, R.D. (2001). Defective lymphotoxin-beta receptor-induced NF-kappa B transcriptional activity in NIK-deficient mice. Science *291*, 2162–2165.

Yokota, Y., Mansouri, A., Mori, S., Sugawara, S., Adachi, S., Nishikawa, S.-I., and Gruss, P. (1999). Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. Nature *397*, 702–706.

Yoshida, H., Honda, K., Shinkura, R., Adachi, S., Nishikawa, S., Maki, K., Ikuta, K., and Nishikawa, S.-I. (1999). IL-7 receptor α^+ $CD3^-$ cells in the embryonic intestine induces the organizing center of Peyer's patches. Int. Immunol. *11*, 643–655.

Yoshida, H., Kawamoto, H., Santee, S.M., Hashi, H., Honda, K., Nishikawa, S., Ware, C.F., Katsura, Y., and Nishikawa, S.-I. (2001). Expression of α4β7 integrin defines a distinct pathway of lymphoid progenitors committed to T cells, fetal intestinal lymphotoxin producer, NK, and dendritic cells. J. Immunol. *167*, 2511–2521.

Note Added in Proof

Since this manuscript was submitted, Randall and colleagues published similar findings on the independence of LTα for the organogenesis of NALT (Harmsen et al., 2002 [J. Immunol.]).