in the T84 cell line and in dendritic cells, and this localization differed from that of mCTs and nCT. Although dmCTs accumulate in the ON/E for a short period, this accumulation did not last and the dmCTs were not transferred into the OBs. Furthermore, nasal application of OVA, together with dmCT E112K/DEV and dmCT E112K/KDGL as adjuvants, resulted in only minimal induction of IgE Ab responses. Collectively, these results indicate that these newly created dmCTs are potent nontoxic mucosal adjuvants suitable for the induction of both mucosal and systemic immune responses in humans.

Although the advantages of nasal immunization have made it the route of choice for the administration of enterotoxins, one area of continuing concern has been the possibility that nasal vaccines could enter the CNS because of the anatomical proximity of the ON/E and OBs to the brain. This potential for neurotoxicity could, of course, rule out the use of enterotoxin-based mucosal adjuvants in humans. Indeed, it was reported that nLT and detoxified LT mutants also targeted the CNS of mice following nasal application (51). In addition, our previous study showed the potential toxicity of CT for the CNS (12). Once CT-B or another nasal enterotoxin vaccine has been associated with neurons via GM1 binding, it cannot be efficiently cleared, and it accumulates in neuronal tissues associated with the olfactory tract tissues (12).

Although our past studies provided evidence that the nCT that accumulates in the olfactory tissue after nasal administration did not lead to obvious pathologic changes in brain tissue (52), our more recent study showed that nasal application of nCT induced nerve growth factor-β, an indicator of nerve cell damage in the olfactory tissues of rhesus macaques (23). We still do not understand the full biological and pathogenic consequences of enterotoxin deposition in the CNS mediated by ganglioside GM1 binding of olfactory tissues; however, it has been shown that a human nasal influenza vaccine with nLT as mucosal adjuvant resulted in the side effect of Bell's palsy (14, 15). It is likely that the nLT was the active component in this vaccine leading to facial paralysis.

In this regard, we have created a second generation of mCTs that avoid transport to the CNS tissues. Our current study clearly shows that dmCTs were not transferred into the OBs, although they were bound and began to accumulate in the ON/E within 24 h. More importantly, the dmCTs that accumulated in the ON/E cleared after 24 h and were not seen in the OBs 7 days after the last immunization even though they were given three times in three consecutive administrations at weekly intervals. Furthermore, because the second generation of dmCTs was designed to avoid cAMP activation by failing to traffic to the ADP-ribosylation activity center, dmCTs did not induce any inflammatory responses despite their early accumulation in the ON/E. Indeed, nasal washes of mice given nasal dmCTs as mucosal adjuvants resulted in essentially no TNF-α production (data not shown). These findings strongly support the safety of newly developed second generation E112K/KDEV and E112K/KDGL dmCTs.

In addition to our current approach, which inhibits intracellular trafficking of the enterotoxin A subunit, others have developed a nCT-based safe and effective adjuvant which targets the Ig receptor on both naive and memory B cells to avoid GM1 ganglioside binding (53). Thus, the enzymatically active A1 subunit of nCT was combined with a dimer of an Ig binding element (DD) from *Staphylococcus aureus* protein A (CT-A1-DD) and coadministered with Ag. This CT-Al-DD adjuvant induced significant Ab and T cell responses without CNS toxicity (53). Furthermore, the same study showed that mCT-A1 E112K-DD failed to elicit adjuvanticity (53). In contrast, others as well as our studies have clearly shown that mCT E112K and dmCTs (E112K/KDEV and E112K/KDGL) retained their nasal adjuvant activities (10, 11, 22, 23). It is possible that the different outcomes between these studies is due to the GM1 ganglioside binding ability of the adjuvants that we have used (10, 13, 17). Thus, mCT and dmCTs target all nucleated cells including professional APCs such as dendritic cells and macrophages, whereas mCT-A1 E112K-DD is limited to binding to B cells. The precise cellular and molecular mechanisms of these new nasal adjuvants needs to be elucidated in future studies. Both dmCTs and CT-A1-DD adjuvants are potential candidates for the development of safe mucosal vaccines.

Past studies have shown that nCT induces marked increases in Ag-specific IgE Ab responses after nasal immunization (10). To develop a mucosal adjuvant that is both safe and effective, the potential for IgE Ab production must be reduced without sacrificing the ability to generate Ag-specific mucosal S-IgA and plasma IgG Ab responses. Our previous studies singled out mCT E112K as the safest and most effective CT-derived adjuvant because it supported Ag-specific S-IgA Ab responses with lower levels of total and anti-CT-B IgE Ab responses than those observed with other enterotoxin-derived adjuvants after nasal immunization (13). Furthermore, when used as a nasal adjuvant, the mCT-A E112K/LT-B chimera, a mCT E112K derivative, induced significantly lower levels of total and Ag-specific IgE Ab responses in the plasma of mice (32). In the current study, we have assessed whether newly created dmCTs induce Ag-specific IgE Ab responses. Our results show that nasal administration of dmCT E112K/KDEV or E112K/KDGL does not enhance IgE Ab responses. Thus, levels of OVA-specific IgE Ab responses were comparable to those elicited by mCT E112K. These results indicate that these dmCT E112K/KDEV and dmCT E112K/KDGL molecules likely avoid allergic reactions to the coadministered Ag and, thus, are promising candidates for being potent nontoxic mucosal adjuvants for human use.

In an attempt to circumvent the toxicity of enterotoxin adjuvants, we developed double mutants of nCT that both lacked the ADP-ribosyltransferase activity and expressed altered ER retention signals (dmCT E112K/KDEV and dmCT E112K/KDGL). To examine the efficacy of these dmCTs for the induction of Ag-specific mucosal S-IgA and systemic IgG Ab responses, we initially used a 5-μg dose of dmCT as nasal adjuvant. We chose this dosage because our previous studies showed that the induction of Ag-specific immune responses in both mucosal and systemic tissues of mice and nonhuman primates required a 10-fold higher dose of mCT E112K/S61F or mCT-A E112K/LT-B than of nCT (2, 10, 11, 23). Because our initial experiments showed dmCTs to be effective adjuvants at the 5-μg dose, we next tested whether 0.5 μg of the newly developed dmCTs could also elicit nasal adjuvanticity. Our results showed both dmCTs to be potent mucosal adjuvants, even when administered at a 10-fold lower rate. In contrast, at a dosage of 0.5 μg, mCT E112K failed to induce the high titers of Ag-specific immune responses seen with nCT.

Although we cannot currently offer a precise explanation for this outcome, it seems clear that the additional point mutation in mCT E112K improves nasal adjuvanticity. Because the structure of the CT molecule is thought to be key to its biological activity (13), there has been concern that the introduction of a point mutation into CT might change or destabilize its own structure and cause diminished mucosal adjuvanticity (13). However, our finding that second generation dmCTs retained nasal adjuvanticity even at lower doses suggests that the molecular structures of dmCTs may be unchanged or stable even after the introduction of double mutations. To explore that possibility, we are currently testing the crystal structures of these dmCTs.

The newly developed dmCTs showed their adjuvant activities through Th2-type cytokine production, especially via an IL-4-dependent mechanism. Thus, nasal immunization with OVA plus dmCT E112K/KDEV or dmCT E112K/KDGL failed to induce OVA-specific Ab responses in IL-4-deficient mice (data not shown). Interestingly, analyses of Th1- and Th2-type cytokine production by $CD4^+$ Th cells revealed that dmCT E112K/KDGL exhibited similar characteristics, especially in the responses of Th1-type cytokine synthesis. Thus, nasal administration of either nCT or dmCT E112K/KDGL resulted in higher levels of IFN-γ and IL-2 production than did that of mCT E112K or dmCT E112K/KDEV, although the levels were relatively lower than the IFN-γ and IL-2 production induced by anti-CD3 and -CD28 mAb treatment. In further support of the similar adjuvant properties shared by nCT and dmCT E112K/KDGL, their OVA-specific plasma IgG2a Ab responses, which are known to be associated with Th1-type cytokines, were significantly higher than those seen in mice given nasal mCT E112K or dmCT E112K/KDEV. We propose that these differences in cytokine profile and OVA-specific IgG subclass Ab responses are due to a difference in the intracellular trafficking of dmCT E112K/KDEV and dmCT E112K/KDGL. These findings indicate that dmCT E112K/KDGL may retain the prototype adjuvanticity of nCT, including the induction of cell-mediated immunity. Because nCT as a nasal adjuvant successfully induced CTL activity by $CD8^+$ T cells, we are currently testing cytokine production and CTL activity by $CD8^+$ T cells from mice given nasal dmCT E112K/KDGL as a mucosal adjuvant.

In summary, a newly developed second generation of both dmCT E112K/KDEV and dmCT E112K/KDGL retained adjuvant activity and elicited mucosal and systemic immunity to nasally coadministered Ags without exhibiting ADP-ribosyltransferase activity or participating in normal intracellular trafficking. Interestingly, like mCT E112K, dmCT E112K/KDEV showed dominant Th2-type cytokine responses. In contrast, like nCT, dmCT E112K/KDGL elicited not only Th2-type cytokine responses but also significantly higher Th1-type cytokine responses than did dmCT E112K/KDEV and mCT E112K. Although different in their immunobiological characteristics, both of our newly developed second-generation dmCTs show promise as effective and safe nasal adjuvants in mice. Future studies will determine whether these adjuvants are safe and effective in both nonhuman primates and humans.

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Disclosures

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CUTTING EDGE

Cutting Edge: Uniqueness of Lymphoid Chemokine Requirement for the Initiation and Maturation of Nasopharynx-Associated Lymphoid Tissue Organogenesis[1]

Satoshi Fukuyama, Takahiro Nagatake,* Dong-Young Kim,* Kaoru Takamura,* Eun Jeong Park,* Tsuneyasu Kaisho,† Norimitsu Tanaka,‡ Yuichi Kurono,‡ and Hiroshi Kiyono[2]**

CD3$^-$CD4$^+$CD45$^+$ inducer cells are required for the initiation of mucosa-associated organogenesis of both nasopharynx-associated lymphoid tissues (NALT) and Peyer's patches (PP) in the aerodigestive tract. CXCL13$^{-/-}$ mice and mice carrying the paucity of lymph node T cell (plt) mutation and lacking expression of CCL19 and CCL21 accumulate CD3$^-$CD4$^+$CD45$^+$ cells at the site of NALT but not of PP genesis. Although NALT was observed to develop in adult CXCL13$^{-/-}$ and plt/plt mice, the formation of germinal centers in CXCL13$^{-/-}$ mice was affected, and their population of B cells was much lower than in the NALT of CXCL13$^{+/-}$ mice. Similarly, fewer T cells were observed in the NALT of plt/plt mice than in control mice. These findings indicate that the initiation of NALT organogenesis is independent of CXCL13, CCL19, and CCL21. However, the expression of these lymphoid chemokines is essential for the maturation of NALT microarchitecture. The Journal of Immunology, 2006, 177: 4276–4280.

Nasopharynx-associated lymphoid tissue (NALT)[3] plays a pivotal role in the initiation of Ag-specific immune responses at both systemic and mucosal sites (1). Thus, NALT acts as an important inductive site for the generation of Ag-specific IgA-committed B cells (1). In addition, although NALT possesses a predominance of naive Th0 CD4$^+$ cells, the Ag-specific Th1 and/or Th2 immune responses can be induced in NALT through intranasal administration of Ags and mucosal adjuvants (e.g., cholera toxin) (1). Thus, NALT is thought to be a key secondary lymphoid structure for the upper respiratory tract.

The lymphotoxin (LT)βR signaling pathway is essential for the organogenesis of secondary lymphoid tissues, including peripheral lymph nodes (LN) and Peyer's patches (PP) (2). However, previous reports by our and other groups (3, 4) have demonstrated that NALT organogenesis, unlike that of other secondary lymphoid tissues, can occur independently of the LTβR signaling pathway. Inducer cells with phenotypes of CD3$^-$, CD4$^+$, and CD45$^+$ are required for the initiation of the organogenesis of NALT, PP, and LN (1–3). Lymphoid chemokines, including CXCL13, CCL19, and CCL21, have been shown to be important for the recruitment of CD3$^-$CD4$^+$CD45$^+$ cells to the PP anlagen (5, 6). Not surprisingly then, CXCR5$^{-/-}$ mice and CXCL13$^{-/-}$ mice lack PP and several types of LN such as inguinal and iliac LN (7). Although PP and LN are developed in mice carrying the paucity of LN T cell (*plt*) mutation, which are known not to produce CCR7 ligands, CCL19 and CCL21, a study using double mutants of CXCL13$^{-/-}$ and *plt/plt* mice revealed that CCL19, CCL21, and CXCL13 were cooperatively involved in the development of secondary lymphoid organs (7). However, little is known about the involvement of these lymphoid chemokines for the recruitment of CD3$^-$CD4$^+$CD45$^+$ cells to sites of NALT development.

To better understand the varying roles of lymphoid chemokines in the development of NALT and the maintenance of its architecture, we investigated the unique characteristics of NALT development using CXCL13$^{-/-}$ mice and *plt/plt* mice. Our results provide the first evidence that the initiation of NALT organogenesis is independent of lymphoid chemokines,

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[3] Abbreviations used in this paper: NALT, nasopharynx-associated lymphoid tissue; PP, Peyer's patch; LT, lymphotoxin; LN, lymph node; E17, 17-day-old embryo; LDM, laser microdissection; FDC, follicular dendritic cell; PNA, peanut agglutinin; NALTi, NALT inducer cell; PPi, PP inducer cell; MLN, mesenteric LN.

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including CXCL13, CCL19, and CCL21. Lymphoid chemokines have been shown to play an important role in the maturation of the microarchitecture of secondary lymphoid organs (8, 9), and our current findings show that these same chemokines are essential for NALT microarchitecture formation as well.

Materials and Methods

Mice

BALB/c and C57BL/6 mice were purchased from Japan SLC. The procedure for generating CXCL13$^{-/-}$ mice on a C57BL/6 background was reported previously (10). *Plt/plt* mice with a BALB/c background were provided from Drs. H. Nakano and T. Kakiuchi (Department of Immunology, Toho University School of Medicine, Tokyo, Japan) (11). CXCL13$^{-/-}$*plt/plt* mice were generated by intercrossing CXCL13$^{-/-}$ mice with *plt/plt* mice. PCR primers D4Mit237 (sense, 5′-TTCAAACTCATGAGTCTATGGGG-3′; antisense, 5′-ATATACACGTAGACTCGCACGC-3′) were used to determine the genome type of *plt/plt* and *plt/+* or +/+ (11).

Cell analysis and isolation by flow cytometry

Cells were isolated from the nasal tissues and intestines and then stained with the appropriate fluorescence-conjugated anti-CD3ε (145-2C11; BD Pharmingen), anti-CD45 (30-F11; BD Pharmingen), anti-CD4 (L3T4; BD Pharmingen), anti-B220 (RA3-6B2; BD Pharmingen), anti-CD11c (HL3; BD Pharmingen), anti-CXCR5 (2G8; BD Pharmingen), and/or anti-CCR7 (4B12; eBioscience) (3). Cells were then analyzed using a FACSCalibur flow cytometer (BD Biosciences), and data analysis was performed with CellQuest software (BD Biosciences). For the purification of CD3$^-$CD4$^+$CD45$^+$ cells from infant nasal tissues (10-day-old) and embryonic intestines (17-day-old embryos (E17)), we used an AutoMACS (Miltenyi Biotec) combined with a FACSAria cell sorter (BD Biosciences), as described previously (3).

Isolation of NALT anlagen for RT-PCR

Following the manufacturer's recommendations, we obtained RNA from NALT anlagen by using the laser microdissection (LMD) system (Leica Microsystems). Unfixed nasal tissues isolated from newborn, 7-day-old, 14-day-old, and 6-wk-old mice were frozen in liquid nitrogen. Samples were sectioned into 8-μm thicknesses and immediately fixed in 75% ethanol/diethylpyrocarbonate-treated water for 30 s. Sections were counterstained with toluidine blue (Wako Pure Chemical) for 30 s. After the dehydration with ethanol and xylene, the sections were dissected with a LMD system (Leica Microsystems). The site of NALT formation was captured from each tissue and lysed in TRIzol (Invitrogen Life Technologies) for quantitative RT-PCR using the LightCycler system (Roche Diagnostics) (12).

Primers and hybrid probes for real-time RT-PCR

The primers and hybrid probes used for PCR were as follows: the oligonucleotide primers specific for CXCR5 (sense, 5′-TTCTCCACCCAATGTACC-3′; antisense, 5′-AACCTCTGTCGTCATTCTC-3′), CXCR5 detection FITC-labeled probe (5′-ATTCTACGCACCAATGGGGAAGGAAGC CAACT-3′), and LightCycler Red 640-labeled hybrid probe (5′-GCCTGGG GAAAGCAAGATAGCAAAGTGGTCCTA-3′); the oligonucleotide primers specific for CCR7 (sense, 5′-ATGCTGGCTATGAGTTTC-3′; antisense, 5′-GCTGCTATTGGTGATGTT-3′), CCR7 detection FITC-labeled probe (5′-ATGATCACCTTGATGGCCTTGTTCCGCTCAAAG-3′), and LightCycler Red 640-labeled hybrid probe (5′-TGCGTGCCTGGAGCAAGG TACGGATGATAATGA-3′); the oligonucleotide primers specific for CXCL13 (sense, 5′-GAACAGGCATTTAGTGACAAC-3′; antisense, 5′-TTTTGGAAGCCTGCGTTTT-3′), CXCL13 detection FITC-labeled probe (5′-AATGTGAACTTGTAGCTCGTACTAACAAGAGG-3′), and LightCycler Red 640-labeled hybrid probe (5′-TTGCGAGATGGACT TCAGTTATTTTGCACC-3′); the oligonucleotide primers specific for CCL19 (sense, 5′-GCCAAGAACAAAGGCAACA-3′, antisense, 5′-CA CACTCACATCGACTCTCTA-3′), CCL19 detection FITC-labeled probe (5′-TGGCCCAGGAAACCAAGGACCA-3′), and LightCycler Red 640-labeled hybrid probe (5′-AAGAGAGGACCAGGCCTCCT-3′); the oligonucleotide primers specific for CCL21a (sense, 5′-ACAGACACAGCCCTCAA-3′; antisense, 5′-CATGAGGTGGCTGCTTT-3′), CCL21a detection FITC-labeled probe (5′-CCAGGAGATCCCCCACGAACTTC-3′), and LightCycler Red 640-labeled hybrid probe (5′-AGCTGGGTGGT TCACGGT-3′); and the oligonucleotide primers specific for GAPDH (sense, 5′-TGAACGGGAAGCTCACTGG-3′; antisense, 5′-TCCACCACCCT GTTGCTGTA-3′), GAPDH detection FITC-labeled probe (5′-CTGAG GACCAGGTTGTCTCCTGCGA-3′), and LightCycler Red 640-labeled hybrid probe (5′-TTCAACAGCAACTCCCACTCTTCCACC-3′). They were designed and produced by Nihon Gene Research Laboratories.

Immunohistochemistry

For confocal microscopic analysis, the nasal tissues and intestines were fixed in 4% paraformaldehyde for the preparation of cryostat sections (5 μm) (3). These tissues were then stained with appropriate fluorescence-conjugated mAb as described above. To assess the formation of the germinal center and follicular dendritic cell (FDC) network in NALT, the previously described nasal immunization protocol was used (3). NALT sections were incubated with biotinylated peanut agglutinin (PNA) (Vector Laboratories) and then stained with FITC-streptavidin (BD Pharmingen). To detect the FDC network, the serial sections were stained with anti-FDC-M1 (BD Pharmingen) and then visualized with FITC-conjugated anti-rat IgG (BD Pharmingen). Histological analysis was performed using a confocal microscope (Leica Microsystems).

Results and Discussion

Lymphoid chemokine family-independent accumulation of CD3$^-$CD4$^+$CD45$^+$ cells at the NALT anlagen

The lymphoid chemokines CXCL13, CCL19, and CCL21 were shown to be involved in the migration of CD3$^-$CD4$^+$CD45$^+$ inducer cells into the PP anlagen (5, 6). Our previous study showed that CD3$^-$CD4$^+$CD45$^+$ inducer cells accumulated at the site of NALT development in mice aged between 7 and 10 days (3). When these lymphoid chemokine-deficient mice were examined, we detected a cluster of the inducer cells at the site of NALT formation in the infant nasal cavity of 10-day-old CXCL13$^{-/-}$ mice, *plt/plt* mice, and CXCL13$^{-/-}$*plt/plt* mice in addition to 10-day-old C57BL/6 and BALB/c mice (Fig. 1*A*). The size of the CD3$^-$CD4$^+$ inducer cell cluster in the NALT anlagen of CXCL13$^{-/-}$ infant mice and *plt/plt* infant mice was similar to that observed in control C57BL/6 and BALB/c mice, respectively. When single-cell preparations from nasal tissues of 10-day-old CXCL13$^{-/-}$ mice, 10-day-old *plt/plt* mice, and 10-day-old CXCL13$^{-/-}$*plt/plt* infant mice were examined, CD3$^-$CD4$^+$CD45$^+$ cells were also found (Fig. 2*A*). The number of CD3$^-$CD4$^+$CD45$^+$ cells isolated from nasal tissues of CXCL13$^{-/-}$ mice, *plt/plt* mice, and CXCL13$^{-/-}$*plt/plt* mice did not differ significantly from that of controls (Fig. 2*B*). As one might expect based on the previous study (6), several cellular clusters of CD3$^-$CD4$^+$ inducer cells were observed in the intestine of 17-day-old embryos (E17) of C57BL/6 and BALB/c mice (Fig. 1*B*). In contrast, we could not detect any signs of an accumulation of CD3$^-$CD4$^+$ inducer cells in intestines isolated from E17 CXCL13$^{-/-}$ mice and CXCL13$^{-/-}$*plt/plt* mice (Fig. 1*B*). These results confirm those of a previous study (6) and indicate that the degree to which the initiation of tissue genesis depends on lymphoid chemokines can be used to distinguish NALT inducer cells (NALTi) (independent) from PP inducer cells (PPi) (dependent). Thus, CXCL13 is indispensable for the accumulation of CD3$^-$CD4$^+$CD45$^+$ PPi but not of NALTi in the respective tissue anlagen.

Unexpectedly, the accumulation of inducer cells was also observed in the mesentery of E17 CXCL13$^{-/-}$ mice and CXCL13$^{-/-}$*plt/plt* mice, indicating that CXCL13 is not essential for the formation of mesenteric LN (MLN), the other member of GALT (Fig. 1*C*). Furthermore, the development of MLN was conserved in CXCR5$^{-/-}$ mice and CXCR5$^{-/-}$CCR7$^{-/-}$ mice (7). Even among GALT, then, the migration of CD3$^-$CD4$^+$ cells into the specific tissue anlagen (e.g., PP and MLN) shows a variable dependence on CXCL13. Surprisingly, FACS analysis revealed that CD3$^-$CD4$^+$CD45$^+$ cells were observed in mononuclear cells isolated from E17 intestines of CXCL13$^{-/-}$ mice, *plt/plt* mice, and CXCL13$^{-/-}$*plt/plt* mice (Fig. 2*A*). The number and the

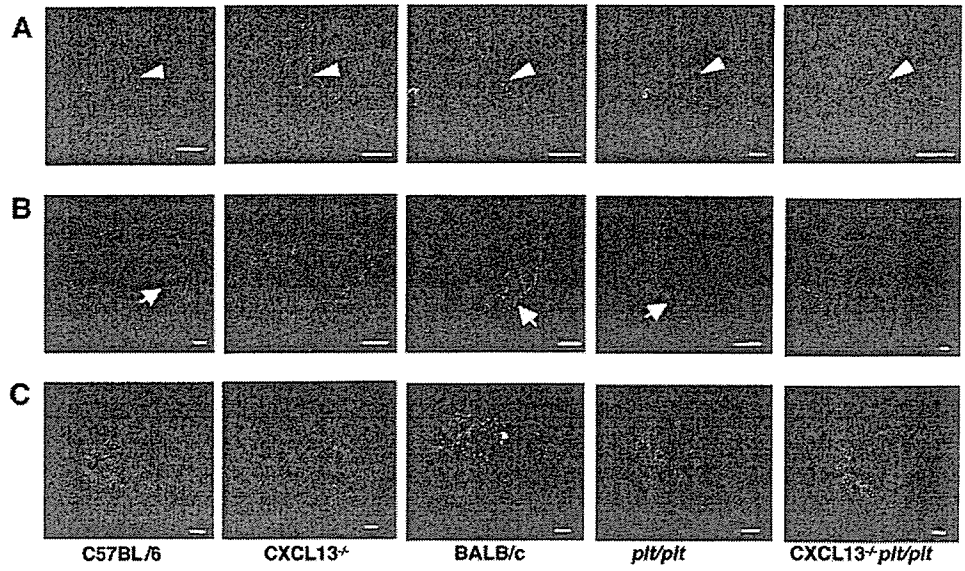

FIGURE 1. Histological analysis of infant nasal tissues and fetal intestines in lymphoid chemokine-null mice. *A*, Nasal tissues were isolated from 10-day-old C57BL/6, CXCL13$^{-/-}$, BALB/c, *plt/plt*, and CXCL13$^{-/-}$*plt/plt* mice. *B*, Fetal intestine was isolated from E17 of C57BL/6, CXCL13$^{-/-}$, BALB/c, *plt/plt*, and CXCL13$^{-/-}$*plt/plt* mice. *C*, Mesenteries were isolated from E17 C57BL/6, CXCL13$^{-/-}$, BALB/c, *plt/plt*, and CXCL13$^{-/-}$*plt/plt* mice. Frozen sections were incubated with anti-mouse CD3ε-FITC (green) and anti-mouse CD4-PE (red). Arrowheads in *A* and arrows in *B* indicate the accumulation of CD3$^-$CD4$^+$ cells at the anlagen of NALT and PP, respectively. Scale of bars, 100 μm.

frequency of CD3$^-$CD4$^+$CD45$^+$ cells in CXCL13$^{-/-}$ and *plt/plt* embryonic intestines did not differ significantly from those observed in control mice (Fig. 2). These results indicate that the migration of CD3$^-$CD4$^+$CD45$^+$ cells into embryonic intestine does not depend on lymphoid chemokines; however, CXCL13 is essential for directing the inducer cells at the site of PP anlagen.

These data demonstrate that CD3$^-$CD4$^+$CD45$^+$ NALTi can migrate to the site of NALT formation without lymphoid chemokines such as CXCL13, CCL19, and CCL21, which are known to be associated with the other lymphoid tissue genesis programs. Furthermore, the size of the CD3$^-$CD4$^+$CD45$^+$ cell cluster and the number of CD3$^-$CD4$^+$CD45$^+$ cells in infant nasal tissues did not change in lymphoid chemokine-deficient mice. Thus, the PP genesis-associated lymphoid chemokines may not have any involvement in the formation of the NALT anlagen operated by the NALTi. If that is the case, then our efforts should be focused on identifying the molecules that are at work in the migration of NALTi to the NALT anlagen.

The expression of chemokine receptors by NALTi

Inasmuch as the chemokine receptor family of CXCR5 and CCR7 has been shown to play a key role in the migration of PPi to the tissue genesis site (5), it was logical to next examine the use of the chemokines by NALTi. We first performed quantitative RT-PCR to examine the levels of CXCR5 and CCR7 expression by NALTi and PPi. For both chemokine receptors, levels expressed by NALTi were significantly lower than those expressed by PPi (Fig. 3*A*). Thus, CXCR5 and CCR7 expression by NALTi fell to levels that were barely detectable. The finding was further confirmed by FACS analysis, where PPi expressed CXCR5 and CCR7, especially the CD4^{high} fraction (Fig. 3*B*). However, NALTi expressed neither CXCR5 nor CCR7 (Fig. 3*B*).

The interaction of CXCR5/CXCL13 essentially promotes chemotactic activity for the clustering of PPi in the embryonic intestine (5). Furthermore, together with IL-7Rα signaling, CXCR5 is involved in the induction of LTα1β2 expression on PPi (7). In addition, CXCR5 signaling mediates the activation of β_1 integrin expressed on PPi for the interaction of VCAM-1$^+$ICAM-1$^+$ stromal cells at the PP anlagen (6). Although the multipotent function of CXCR5 expressed by PPi is required to initiate the development of PP, NALTi did not express CXCR5. Therefore, NALTi is thought to mediate the initiation of NALT organogenesis without a CXCR5/CXCL13-mediated signal. Furthermore, CCR7 expressed on CD3$^-$CD4$^+$CD45$^+$ cells are cooperatively involved in the organogenesis of PP and other LN (5, 7). Given that neither CCR7 nor CXCR5 is expressed on NALTi (Fig. 3, *A* and *B*), it is likely that the initial step of NALT organogenesis is completely independent of the lymphoid chemokine signaling mediated by the corresponding receptors of CXCR5 and CCR7.

Uniqueness in the production of lymphoid chemokines by NALT

To further support our findings using immunohistological analysis of the lymphoid tissue genesis in lymphoid chemokine-deficient mice, CXCL13-specific mRNA was rarely produced at the site of NALT formation of newborn and 7-day-old BALB/c and C57BL/6 mice (Fig. 3*C*). Likewise, the production of CCL19-specific mRNA at NALT anlagen was nil or extremely low in newborn mice. In contrast, we detected constitutive mRNA expression of CCL21 at the site of NALT development in mice newly born up to mice aged 6 wk (Fig. 3*C*). High levels of mRNA expression for CXCL13, CCL19, and CCL21 were also detected in the NALT of 6-wk-old mice (Fig. 3*C*). As NALT developed, the expression of lymphoid chemokines, including CXCL13 and CCL19, gradually increased.

CXCL13 and CCL19 have been shown to be produced by VCAM-1$^+$ICAM-1$^+$ stromal cells in the anlagen of PP (5). LTβR signaling through the alternative NF-κB pathway by the interaction of stromal cells and PPi is thought to induce CXCL13, CCL19, and CCL21 expression (2). In the case of NALT, neither CXCL13 nor CCL19 was expressed at birth, but the expression of both gradually increased as NALT matured. Thus, it is interesting to postulate that the initial triggering of CXCL13 and CCL19 production is induced by the cluster of NALTi accumulated at the NALT anlagen in the neonatal stage, with lymphoid cells gradually taking over the expression of these two chemokines.

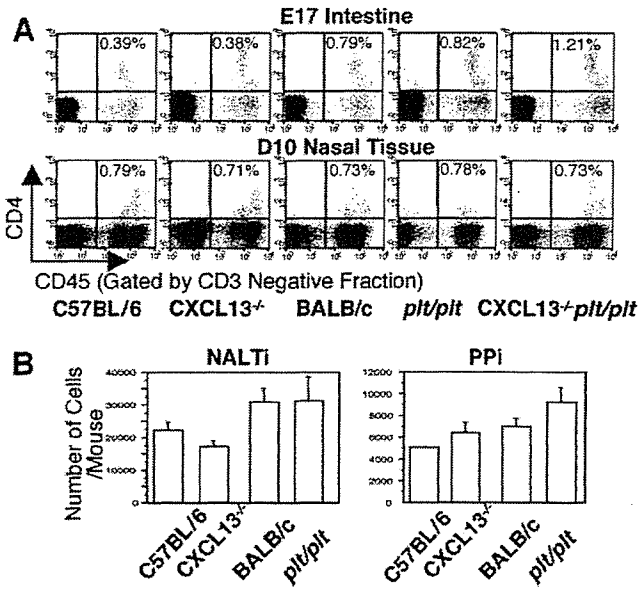

FIGURE 2. Analysis of inducer cells of lymphoid chemokine-null mice. Mononuclear cells were isolated from nasal tissues of 10-day-old mice and intestines of E17 mice. *A*, FACS analysis was performed to detect $CD3^-CD4^+CD45^+$ cells in C57BL/6, $CXCL13^{-/-}$, BALB/c, *plt/plt*, and $CXCL13^{-/-}$*plt/plt* mice. Results are representative of three independent experiments. *B*, The number of $CD3^-CD4^+CD45^+$ cells in day 10 (D10) nasal tissues (NALTi) and E17 intestines (PPi) in C57BL/6, $CXCL13^{-/-}$, BALB/c, and *plt/plt* mice. No significant differences were noted between lymphoid chemokine-null ($CXCL13^{-/-}$ and *plt/plt* mice) and control (C57BL/6 and BALB/c) mice. Significance was evaluated by an unpaired *t* test.

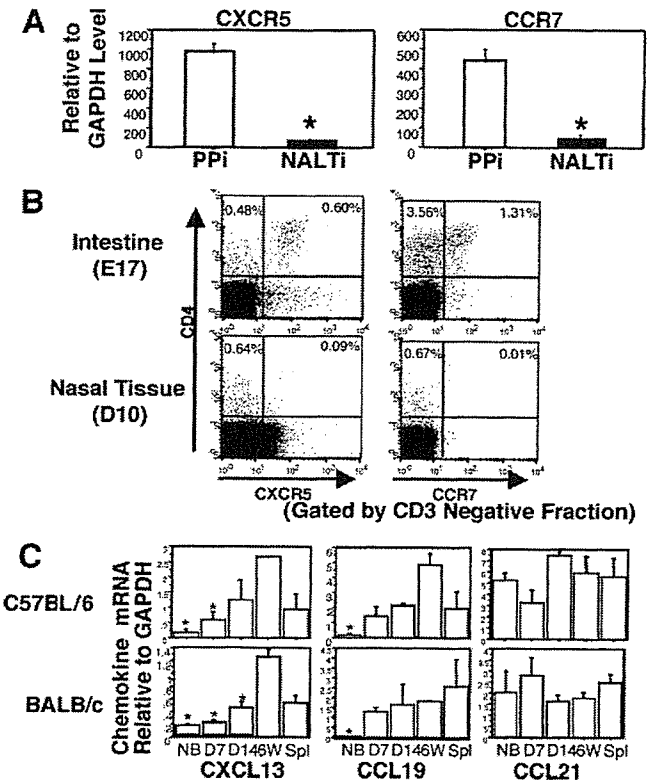

FIGURE 3. Analysis of the expressions of lymphoid chemokines and their corresponding receptors by NALTi and PPi and NALT anlagen. *A*, $CD3^-CD4^+CD45^+$ cells were sorted from mononuclear cells of day 10 (D10) nasal tissues (NALTi) and of E17 intestines (PPi) by FACSAria. Quantitative analysis of CXCR5 and CCL7 mRNA expression was performed using LightCycler. The expression of each chemokine receptor was normalized to the expression of GAPDH. RNA was extracted from three individual experiments per group. Significance was evaluated by an unpaired *t* test. *, $p < 0.05$. *B*, CXCR5 and CCR7 expression of $CD3^-CD4^+$ cells of D10 nasal tissues and E17 intestines were analyzed using FACSCalibur. *C*, Chronological analysis of lymphoid chemokine expression in NALT was performed using NALT anlagen of newborn (NB), 7-day-old (D7), and 14-day-old (D14) mice, NALT, and spleen of 6-wk-old (6W) C57BL/6 and BALB/c mice, which were isolated by a LMD system. Quantitative analysis of mRNA expression of lymphoid chemokines (CXCL13, CCL19, and CCL21) was performed using LightCycler. *, $p < 0.05$ compared with 6 wk via an unpaired *t* test ($n = 3$ on each time point).

CCL21 is produced by stromal cells in the T cell area, endothelial cells of high endothelial venules, and lymphatic vessels (7). Therefore, since the level of CCL21 expression is high at birth and remains high during the maturation stage related to the other two chemokines, stromal cells and endothelial cells in NALT, including anlagen and adult stages, seem to be a key source for the production of CCL21.

Microarchitecture of NALT in the lymphoid chemokine-null mice

Thus far, our data have demonstrated that the lymphoid chemokines CXCL13, CCL19, and CCL21 are not involved in the induction of NALT organogenesis. The role of lymphoid chemokines for the maintenance of mature NALT in adult mice should be investigated next. The total number of mononuclear cells in NALT of young adult $CXCL13^{-/-}$ mice and *plt/plt* mice was always lower than in normal mice (Fig. 4*A*). Of the various lymphoid cell subsets, the population of $B220^+$ B cell saw the greatest decrease, followed by $CD3^+$ cells and $CD11c^+$ cells in the NALT of $CXCL13^{-/-}$ mice (Fig. 4*A*). Furthermore, we sought to determine whether the migration of B1 and B2 cells into NALT might be altered in $CXCL13^{-/-}$ mice since the most obvious alteration was associated with the B cell subset. The level of CXCR5 expression by B1 cells in NALT was similar to that by B2 cells (our unpublished data). Although it has been established that a major subset of NALT B cells belong to B2 cells (13), both B1 and B2 cells were reduced in the NALT of $CXCL13^{-/-}$ mice (our unpublished data). These data indicate that CXCL13 is required for the migration of both B1 and B2 cells into NALT. Using confocal microscopic analysis, we further showed that the microarchitecture of the B cell area was destroyed in $CXCL13^{-/-}$ mice, leaving the NALT extensively occupied by T cells (Fig. 4*B*). The formation of a germinal center and FDC network was thus disrupted in the NALT of $CXCL13^{-/-}$ mice (Fig. 4*B*). In contrast, the T cell area was not observed in the NALT of *plt/plt* mice (Fig. 4*B*). The formation of the germinal center and the FDC network was intact in the NALT of *plt/plt* mice (Fig. 4*B*). These findings suggest that CXCL13 is involved in the recruitment of lymphocytes into NALT instead of the initiation of NALT tissue genesis. Furthermore, it was previously suggested that CXCL13 contributed to the subsequent microarchitecture formation of the B cell zone in NALT (9). However, it should be noted that B cell themselves are also capable of regulating the microarchitecture formation via the use of LT family-mediated signals (8). $LT\alpha1\beta2$-expressing B cells themselves promote the formation of follicles in secondary lymphoid organs (8). Therefore, B cell migration into NALT may also affect the disorganized follicles in the NALT of $CXCL13^{-/-}$ mice. In contrast, CCL19 and CCL21 preferentially promoted T cell migration into NALT

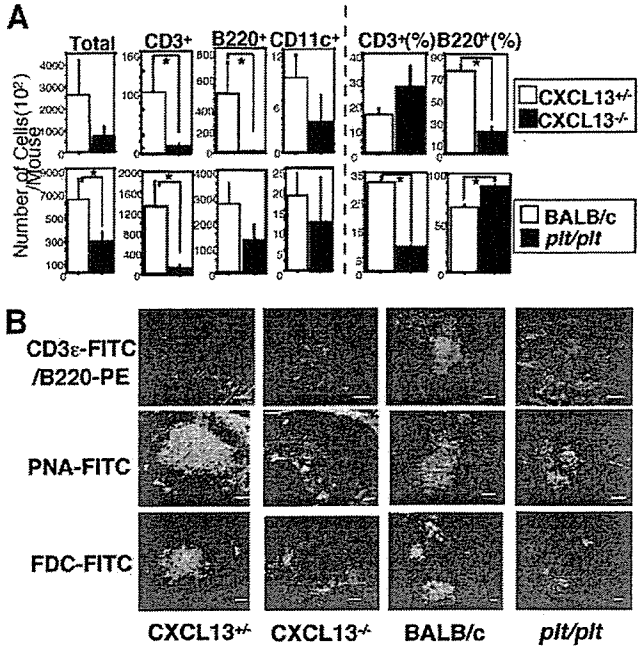

FIGURE 4. Microarchitecture of NALT in adult CXCL13$^{-/-}$ and *plt/plt* mice. *A*, Mononuclear cells isolated from the NALT of CXCL13$^{+/-}$, CXCL13$^{-/-}$, BALB/c, and *plt/plt* mice were analyzed using FACSCalibur. Data were obtained from three individual experiments. Significance was evaluated by an unpaired *t* test. *, $p < 0.05$. *B*, NALT was obtained from CXCL13$^{+/-}$, CXCL13$^{-/-}$, BALB/c, and *plt/plt* mice nasally immunized with cholera toxin. Frozen sections of NALT were incubated with anti-mouse CD3ε-FITC (green) and anti-mouse B220-PE (red) (*top panels*). The formation of a germinal center was analyzed by PNA-FITC (green) (*middle panels*). The network of FDC was stained by anti-FDC-FITC (green) (*bottom panels*). Scale of bars, 80 μm.

but were not involved in the genesis of tissue or the formation of microarchitecture like the germinal center and FDC network.

The cytokine signaling via LTβR induces the expression of CXCL13 by stromal cells in the B cell area for the recruitment of B cells into the follicular regions and the formation of germinal centers in spleen (7). This evidence provides a logical explanation as to why the microarchitecture of NALT is disorganized in mice lacking LTβR signaling (e.g., LTα$^{-/-}$ mice, LTβ$^{-/-}$ mice, and IκB kinase^{AA} mice) (3, 4, 14). Our data further support the findings by Ying et al. (15), which showed that the reduced production of CXCL13, CCL19, and CCL21 in LTα and LTβ deficiency resulted in the disorganization of NALT. Thus, not only do our results confirm the findings that CXCL13 is involved in the maintenance of the microarchitecture of NALT (9) (Fig. 4), they further show that it is not involved in the initiation of the tissue genesis (Figs. 1–3). The analysis of *plt/plt* mice showed that CCL19 and CCL21 promote T cell migration to NALT. CXCL13 also plays an essential role in the formation of the germinal center and FDC network, whereas CCL19 and CCL21 are not involved.

Our findings demonstrate that the lymphoid chemokine family interactions of CXCR5/CXCL13 and CCR7/CCL19 and CCL21 are not essential for the initiation of NALT genesis associated with the NALTi migration into the NALT anlagen. However, as our current study demonstrates, these lymphoid chemokines do play key roles in the creation and maintenance of NALT structure in adult mice. The latter finding is in total agreement with the recent study by Rangel-Monero et al. (9), which showed CXCL13, CCL19, and CCL21 were required for the organization of NALT. Our further examinations suggested that the lymphoid chemokine interactions of CXCR5/CXCL13 and CCR7/CCL19 and CCL21 provide distinct signals for the initiation of tissue genesis, as well as recruitment of lymphoid cells and subsequent microarchitecture formation of different mucosa-associated lymphoid tissues located in the aero-digestive tract.

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Disclosures

The authors have no financial conflict of interest.

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