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Peripheral lymphoid tissues, such as lymph nodes and Peyer’s patches (PPs), are organs required for mounting highly efficient immune responses to small quantities of Ag. The compartmentalization of the cellular components involved in the immune response into distinct zones supports the function of these tissues; however, little is known about how this compartmentalization is achieved. In this study, we analyzed neonatal PP development and present evidence that the CD3+ IL-7Rα+ PP inducer cells that initially play a pivotal role in the formation of the PP anlagen are involved in the formation of B and T cell zones in neonatal mice. PP inducer cells migrate between these zones by undergoing chemokine receptor switching.

Peyer’s Patch Inducer Cells Play a Leading Role in the Formation of B and T Cell Zone Architecture

Reiko Nakagawa,*1 Atsushi Togawa,*1 Takashi Nagasawa,† and Shin-ichi Nishikawa*

Peripheral lymphoid tissues, such as lymph nodes and Peyer’s patches (PPs), are organs required for mounting efficient immune responses (1, 2). Because the quantity of foreign Ags that the immune system encounters and must respond to is minute in comparison with the quantity of host proteins, it is essential that the immune response recruit Ag-reactive cells to the Ag-containing area and redistribute these Ag-primed cells to other regions (3, 4). PLTs are organs in which all of these processes are performed in a highly efficient manner. The tissue architecture of all PLTs is similar, although their structures are variable (5). It is well established that PLT architecture is a result of the intricate compartmentalization of cells into distinct zones, such as the B and T cell zones. Thus, one major question is how this compartmentalization is regulated before Ag-induced restructuring begins. We previously reported that the complex tissue architecture of PPs with distinct B and T cell zones results from a simple PP anlage during the neonatal period (6); however, the mechanisms regulating this process remain unknown. It was previously demonstrated that the segregation of B and T cell zones in the spleen can be reinduced by the injection of PLT inducer (LTi) cells, suggesting a role for LTi cells in compartmentalization (7–9). Therefore, this study sought to determine whether LTi cells were involved in the initial compartmentalization of the PLT under normal conditions and, if so, how these cells regulate the process of cellular compartmentalization.

In comparison with the neonatal process of establishing initial compartmentalization, the earliest processes that take place in PLT anlage formation are understood to a considerable extent for both LN and PPs (10–13). Although LNs and PLTs differ in their molecular requirements for initiating organogenesis, LTαβ and LTi cells that express LTαβ are key components regulating anlage formation, the former at the molecular level and the latter at the cellular level (13–17). LTi cells diverge from the lymphocyte differentiation pathway in the fetal liver (15, 18), and two transcriptional regulators, Id2 and RORγt, have been identified as essential for the lineage commitment of LTi cells (19–23). Although LTi cells share many common molecules, such as IL-7Rα and c-Kit, with lymphocyte progenitors, they do not undergo rearrangement of the Ig or TCR genes (24, 25). After the emergence of LTi cells in the fetal liver, the cells migrate to regions where the PLTs are formed; however, this process is poorly understood (12, 16, 21, 26, 27). Once LTi cells reach the appropriate PLT anlage, interactions between lymphoxygen (LTαβ), which is present on the cell surface, and the surrounding stromal cells induce the generation of lymphoid tissue organizer (LTo) cells (28, 29). LTi and LTo cells express an array of interacting molecules (10, 11, 13, 30), such as LTαβ and the LTβ receptor (14, 24, 27, 31–38), IL-7Rα and IL-7 (16, 17, 25, 28, 39, 40), integrin αββ7 and MadCAM1 (15, 23, 24), and CXCR5 and CXCL13 (41–45). The complementarity of LTi and LTo cells allows for their interaction, thereby expanding the realm of the PLT anlagen by recruiting more LTi cells. When LTi cells were first defined, their role was thought to be restricted to PPs and LNs, but subsequent work has shown that LTi cells are involved in the organogenesis of other PLTs in similar contexts (40, 46). Accordingly, the more general term LTi is used, rather than PPi or LNi.

The fate of LTi cells after PLT anlage formation remains another important topic of research (47). Lane and colleagues (7–9, 48–50) suggested that LTi cells have distinct roles after the completion of PLT organogenesis that affect cellular compartmentalization and the adaptive T cell response. One line of evidence also suggests that in vitro, LTi cells further differentiate into other cell lineages, including NK cells and dendritic cells, when provided with the appropriate signals (15, 18). Recently, Vonarbourg et al. (51) reported that the transition from LTi to NK cells occurs in vivo because of the downregulation of the molecular signatures specific to LTi cells. This phenotypic shift led to use of the term “immunological chameleon” to describe LTi cells (52). Because

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Abbreviations used in this article: E, embryonic (day); FDC, follicular dendritic cell; FZ, follicular zone; HEV, high endothelial venule; IFZ, interfollicular zone; LN, lymph node; LT, lymphoxygen; LTi, lymphoid tissue inducer; LTo, lymphoid tissue organizer; P, postpartum (day); PFZ, parafollicular zone; PLT, peripheral lymphoid tissue; PP, Peyer’s patch; PPi, PP inducer; PPo, PPi–PP organizer.

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LTi cells are thought to have multiple functions, it is possible that these cells are involved in the initial compartmentalization of LTs. Thus, we addressed this question in PPs and present evidence that CD3−IL-7Ra+ PP inducer (PPi) cells are implicated in the construction of B and T cell zones during the neonatal stage. We further show that the complex migration pattern of PPi cells throughout distinct zones is mediated by chemokine receptor switching.

Materials and Methods

Mice

C57BL/6, C.B17/lecr-SCID Jcl, and BALB/c A Jcl-Nude mouse strains were purchased from Japan CLEA. JH−/− mice were prepared from Quasi mice (53). BAC- transgenic RORγt-Cre mice were generated as described previously (54). CXCR4floxed mice (55), CXCR4floxed mice (56), CXCL12-GFP knockin mice (57), ROSA26A-EYFP mice (58), and Tie2-Cre mice (59) have been described previously. Female and male mice were mated overnight, and females with a vaginal plug were considered pregnant; thus, noon on the day when the vaginal plug was found was calculated as 0.5 d post coitum. All animal experiments were carried out in accordance with the RIKEN guidelines for animal and recombinant DNA experiments.

Abs

Pacific Orange-conjugated anti-CD45 (30-F11), Alexa Fluor 488-conjugated purified donkey polyclonal anti-IL-7Rα IgG(1H1), Alexa Fluor 594-conjugated purified donkey polyclonal anti-rat IgG(1H4+L), Alexa Fluor 647-conjugated purified chicken polyclonal anti-rat IgG(1L), Alexa Fluor 680-conjugated purified rabbit polyclonal anti-GFP, and Alexa Fluor 790–conjugated streptavidin were purchased from Invitrogen. eFluor 450–conjugated anti-CD3 (17A2), APC-conjugated, Alexa Fluor 700–conjugated, and biotin-conjugated anti-IL-7Rα (47R34), biotin-conjugated anti-CXCR4 (2B11), biotin-conjugated anti-CCR7 (4B12), biotin-conjugated IgG2a (eB2R2a), biotin-conjugated anti-CCR7 (eB15SD9), biotin-conjugated anti-CD19 (eB19D3), biotin-conjugated anti-human IgG Fc, and purified rat monoclonal anti-ROTYt (AFKJS-9) were purchased from eBioScience. APC Cy7-conjugated anti-CD4 (GD1.5), PE-conjugated anti- integrin α4β7 (DATK32), APC-conjugated anti-CD120 (PK136), biotin-conjugated anti-ICAM1 (3E2), biotin-conjugated anti-CXCR5 (2G8), biotin-conjugated anti-CD3 (145-2C11), biotin-conjugated or PE-conjugated anti-CD11c (HL3), APC-conjugated anti-CD19 (6D5), purified rat monoclonal anti-CD16/32 (2.4G2), purified rat monoclonal anti-VCAM1 (429[MVCAM.A]), purified rat monoclonal anti-MadCAM-1 (MECA-89), purified rat monoclonal anti-B220 (RA3-6B2), and PE-Cy7-conjugated streptavidin were purchased from BD Biosciences. Alexa Fluor 647–conjugated VCAM1 [429 (MVCAM.A)] was purchased from BioLegend. Purified goat polyclonal anti-mouse IL-7Rα, biotin-conjugated goat polyclonal anti-mouse CXCL13, and mouse monoclonal anti–TGF-β1/β2 (1D11) (10 mg/kg) was i.p. injected at days P1, P2, and P3 after birth. FACS analysis was performed at P4 (60, 61).

Preparation of single-cell suspensions for flow cytometry and cell sorting

Single-cell suspensions of the whole gut from embryonic mice and the PP from neonatal mice were prepared as previously described (6). Cells were stained with anti-CD45, anti-CD3, anti-CD4, anti–integrin α4β7, anti–IL-7Rα, anti-VCAM1, anti-ICAM1, and anti–chemokine receptor (CXCR4, CXCR5, CCR7, or control IgG2a) Abs with 7-aminocoumarin C (BD Biosciences). After staining for cell surface Ags, fixation and permeabilization were performed using the BD Cytofix/Cytoperm Kit (BD Biosciences Pharmingen), and the cells were then stained with anti-ROTYt. Stained cells were analyzed or sorted using a FACS Canto II or FACS Aria II (BD Biosciences), respectively. The culture conditions for the sorted inducer cells on OP9 stromal cells were described previously (16). A concentration of 10 mg/ml was used for all of the recombinant cytokines in this study (murine stem cell factor, murine IL-7, human TGF-β1, and human/mouse/ rat activin A).

Immunohistochemistry

Whole-mount immunostaining was performed as previously described, with slight modifications (6). Briefly, samples were fixed with 4% paraformaldehyde in PBS, dehydrated, and then blocked with endogenous peroxidase with 3% H2O2 in methanol. The small-intestine specimens were then blocked in 10% normal mouse serum (NMS) (1.5% Trition X-100 in PBS) for 1 h at 4°C and then incubated with PBST-BSA containing 1 mg/ml of the biotinylated anti-IL-7Rα, anti-VCAM1, anti-CD3, anti-CD19, anti-B220, or anti-CR1CR2 Abs overnight at 4°C. After washing five times in PBST-BSA for 1 h at 4°C, the primary Ab was detected using the R.T.U. Streptavidin-HRP and DAB Peroxidase Substrate Kit (Vector Laboratories) and then observed by microscopic microscopy (Leica).

For whole-mount immunofluorescence, the dehydrated small intestines were first blocked by incubating twice in PBST-BSA for 1 h at 4°C and then incubated with PBST-BSA containing 1 mg/ml of the anti–IL-7Rα goat polyclonal Ab, anti-VCAM1 rat mAb, biotinylated anti-CD3 mAb, anti-CD19 rat mAb, anti-ROTYt rat mAb, biotinylated anti-CXCL13 goat polyclonal Ab, or anti-MadCAM1 rat Ab. As we could not find any appropriate Abs for CXCL12 staining, we used CXCL12-GFP knockin mice (57) and the Alexa Fluor 488–conjugated purified anti-GFP rabbit polyclonal Ab. After washing five times in PBST-BSA for 1 h at 4°C, the primary Ab was detected with Alexa Fluor 488–conjugated purified donkey polyclonal anti-goat IgG(H+L), Alexa Fluor 594–conjugated purified donkey polyclonal anti-rat IgG(H+L), Alexa Fluor 647–conjugated purified chicken polyclonal anti-rat IgG(H+L), or Alexa Fluor 594–conjugated streptavidin. For the immunostaining of cryosections, samples were fixed with 4% paraformaldehyde in PBS for 1 min. After a brief wash, the sections were blocked by incubating twice in PBST-BSA for 1 h at room temperature and then incubated with PBST-BSA containing 1 mg/ml anti-B220 and biotinylated anti-CD3 Abs overnight at 4°C. After washing three times in PBST-BSA for 10 min at room temperature, the primary Ab was detected with Alexa Fluor 488–conjugated purified donkey polyclonal anti-rat IgG(H+L) and Alexa Fluor 594–conjugated streptavidin. Fluorescent signals were detected by confocal microscopy using the LSM710 microscope (Carl Zeiss).

Preparation of LTβR-Fc

The cording process of the extracellular domain of LTβR was conjugated to a human IgG Fc domain (14). The cDNA for the fusion protein was inserted into the pCAG-IGP plasmid under the control of the chicken β-actin promoter region. This plasmid was stably transfected into the 293F cell line, using the 293 transfection reagent (Invitrogen) under puromycin selection. The LTβR-Fc–producing 293F clones were cultured in CELLLINE (BD Bioscience) with FreeStyle 293 medium (Invitrogen). Secreted LTβR-Fc protein was purified and concentrated using a HiTrap Protein G HP column (GE Healthcare Life Sciences) and then sterilized with Millipore GV (Millipore). Purified LTβR-Fc was diluted in PBS, and ~ 20 mg/kg of the protein was i.p. administered to neonatal mice at day 4 post partum (P4).

Neutralization of TGF-β signaling

Mouse monoclonal anti–TGF-β1/β2 (1D11) (10 mg/kg) was i.p. injected at days P1, P2, and P3 after birth. FACs analysis was performed at P4 (60, 61).

Results

The neonatal process of cell compartmentalization establishment in PPs

The first PP primordium formed during embryonic development is composed of simple cell aggregates, and we have previously shown that this structurally simple PP anlage gains complexity and eventually develops into an organ with distinctive zones comprising different cell types (6). This process involves the intricate regulation of cell distribution but is independent of Ag stimulation. As shown in Fig. 1A, PP patterns at P7 consist of the following: 1) the follicular zone (FZ), where CXCL13 is expressed and B cells and follicular dendritic cells (FDCs) are concentrated; 2) the parafollicular zone (PFZ), where MadCAM1 high endothelial venule (HEV) and PP cells that are marked by RORγ expression are concentrated; and 3) the interfollicular zone (IFZ), where CXCL12 is expressed and CD3+ T cells are distributed. In contrast, all of these molecules and cells are evenly distributed in the PP anlagen in embryonic day E17.5 embryos. Thus, the basic tissue organization of PPs is achieved during the first 7 d of the neonatal period.

We next determined which cell types play a leading role in this neonatal process by analyzing the expression of molecular markers during the first 7 neonatal days (Fig. 1B). During this period, B cells accumulated in and were distributed throughout the FZ, CRI/CRI FDCs emerged in the FF, and T cells accumulated in and were distributed throughout the PFZ and IFZ. In addition, we detected events that were previously unreported. The most notable obser-
vation involved the behavior of IL-7Ra⁺ PPi cells. In the embryo, these cells are homogeneously distributed throughout the entire PP anlage, and these cells then segregate into multiple clusters corresponding to their prospective FZs and relocate to the outer zone of the FZ (6, 25, 28, 39, 43). Notably, IL-7Ra⁺ PPi cell migration to these two zones preceded the accumulation of lymphocytes into the same regions. Thus, we hypothesized that PPi cells are involved in the establishment of both B and T cell zones.

IL-7Ra⁺ cells in neonatal PPs correspond to PPi cells

To determine whether PPi cells play a leading role in the formation of the B and T cell zones, we first confirmed that the IL-7Ra⁺ cell population shown in Fig. 1 represented PPi cells, as IL-7Ra is widely expressed on immature lymphocyte precursors (62). To do so, we performed flow cytometry on cells from PPs to evaluate the expression of IL-7Ra and RORγ; the expression of RORγ has been used to distinguish PPi cells from T cells, which are also known to express IL-7Ra (21, 47, 62). The PP regions of P7 mice were isolated, dissociated, and analyzed by flow cytometry (Fig. 2), and the results indicate that most, if not all, of the CD3⁻ integrin αβ⁺ cells in the PP express RORγ, thus confirming the identity of these cells as PPi cells. In contrast, all of the CD3⁺ cells were RORγ negative. Thus, PPi cells could be distinguished from T cells by the expression of these markers. Although both CD3⁺ and CD3⁻ cells express IL-7Ra, the expression levels of IL-7Ra in postnatal life is confined to HEV. Accumulation of B and T cells in PPs occurs only after birth. At P7, B and T cell zones are completely segregated into FZ and IFZ, respectively. Disturbance of the homogeneity in the cellular distribution of PP anlage is first discerned as formation of clusters in which PPi accumulate. These clusters develop rapidly to FZ in which B and FDCs are concentrated. At P4, distribution of B and FDCs remains in FZ, whereas PPi disappears from FZ and is concentrated in PFZ. V CAM1 expression coincides with the distribution of PPi, suggesting that the PPi is the main inducer of V CAM1. T cells are distributed from PFZ to IFZ. Each picture is a representative result confirmed by at least two independent experiments. Original magnification ×25.
Chemokine receptor switching in PPi cells is essential for PPi cell relocation to the outer zone of the FZ

One intriguing finding was the relocation of PPi cells from the FZ to the outer zone (Fig. 1). Because PPi cells are CXCR5+ as reported in our previous studies (43), this relocation would be inconsistent with the observation that the chemokine CXCL13 is expressed in the FZ (Fig. 1A). To better understand the mechanism underlying the behavior of PPi cells, we analyzed chemokine receptor expression on PPi cells during neonatal development. As shown in Fig. 4, the expression of CXCR4, CXCR5, and CCR7 among the CD3+ IL-7Rα+ cell population was examined, and CXCR4 expression could be detected at P4 after CXCR5 downregulation. This CXCR5 downregulation occurred soon after birth, whereas CXCR4 expression was induced 1 d later (Supplemental Fig. 1B). Of interest, CXCR4 expression was detected in LTi cells in the LNs during the embryonic stage, whereas the downregulation of CXCR5 occurred during the neonatal stage, as is the case for PPi cells (Supplemental Fig. 1A). Thus, the expression of CXCR4 and CXCR5 is regulated independently in a tissue-specific manner. Nonetheless, as CXCL12 expression was observed in the IFZ (Fig. 1A), the relocation of PPi cells during the neonatal stage is consistent with the differential expression of chemokine receptors.

The molecular mechanism of chemokine receptor switching in PPi cells

Severe suppression of PP organogenesis during the neonatal stage (Fig. 3A) suggests that LTαβ signaling plays a role in chemokine receptor expression in PPi cells. To investigate this possibility, we analyzed chemokine receptor expression and distribution on PPi cells on days 1 and 2 after the injection of LTβR-Fc on P4, which is when chemokine receptor switching in PPi cells begins. LTβR-Fc injection reduced the increased expression of CXCR4 on P4 (Fig. 3D). However, CXCR5 expression remained negative, suggesting that the expression of CXCR4 and CXCR5 is regulated independently. In addition to the suppression of CXCR4 expression, the PPi cells located at the rim of the FZ at P4 do not migrate to the IFZ but rather return to the central part of the FZ (Fig. 3C). Thus, LTαβ signaling appears to be involved in the switching of chemokine receptor expression. Nevertheless, the block in LT signaling resulted in the disappearance of PPi cells from the PP the following day (Fig. 3C). However, as shown in Fig. 3B, this block in LT signaling did not have a substantial effect on CXCL12 expression, which suggests that the expression pattern of CXCL12 may be regulated independently of LTαβ.

PPi cells do not respond to LTαβ, as these cells express LTαβ, but not the LTαβ receptor. Hence, the inhibition of CXCR4 expression on PPi cells via the block in LT signaling was most likely indirect, presumably through the suppression of PPo cells or other cells that are reactive to LTαβ. In addition, as shown in Supplemental Fig. 2, all of the major cell types in the PP express LTαβ, so the effect of LTαβ on PPi cells remains unclear. However, because receptor switching occurred in scid/scid mice and JH−/− mice (Supplemental Fig. 4A), it is unlikely that lymphocytes are involved in this process.

Next, we investigated which factors induce CXCR4 expression on PPi cells. To do so, we tested the effects of various cytokines on PPi cells in vitro. TGF-β treatment resulted in the expression of CXCR4 in our cell culture system (Fig. 5A). Similarly, a previous study showed that TGF-β can induce CXCR4 expression in monocyte-derived dendritic cells (64). Of interest, CXCR5 was also expressed on the PPi cells expressing CXCR4, which suggests that these two chemokine receptors are differentially regulated. However, activin A, which is a member of the TGF-β family, did not affect its expression (Fig. 5A), and daily injections of an anti–TGF-β mAb (100 μg per neonate) (60) between P0 and P2 failed to inhibit receptor switching (Fig. 5B). These data indicate that the signals involved in chemokine receptor switching in the embryo warrant further investigation.

Chemokine receptor switching on PPi cells is essential for relocation

To confirm that chemokine receptor switching was involved in the relocation of PPi cells, we conditionally deleted the cxcr4 gene in RORγt+ cells by generating cxcr4floxt−/− × rorgt+/− mice (54, 56) (Supplemental Fig. 3A, 3B). The expression of the cxcr4 gene was almost completely disrupted in RORγt+ cells from these mice (Fig. 6). As expected, CXCR5 and CCR7 downregulation was observed.
We then analyzed the localization of IL-7Rα+ cells in the PP strain, and the results shown in Fig. 7 indicate that IL-7Rα+ PPI cells remained in the FZ and failed to relocate to the PFZ in the neonatal PP in the absence of CXCR4. Thus, chemokine receptor switching is required for the relocation of PPI cells from the FZ to the outer zones.

PPi cell relocation is required for the formation of the T cell zone

Lane and colleagues (7, 8) suggested that LTi cells play a role in the segregation of the T and B cell zones in the spleen. Thus, we used the mouse strain described above to evaluate the role of PPI cells in the formation of T cell zones while leaving other processes intact. By comparing the PP structures in normal mice with those in conditional knockout mice, we observed that T cell distribution in the mutant mice was markedly different from that in control mice (Fig. 7). In normal PPs, T cells are located in both the PFZ and IFZ, with the PFZ more densely populated. In mutant mice, however, T cells were found in the central regions of the PPs. An analysis of the cells dissociated from the PPs indicated that CD3+ cells in CXCR4 flox/+ and CXCR4 flox/− mice decreased significantly (23.9 ± 2.3% and 7.7 ± 0.18%, respectively; Fig. 6), indicating a reduction in overall T cell accumulation (Fig. 7). However, no difference in the ratio of CD3+/CD45+ cells in the LNs was noted between CXCR4 flox/+ and CXCR4 flox/− mice (51.4 ± 4.4% and 51.2 ± 7.2%, respectively), which indicated that the deletion of CXCR4 by RORγt-Cre did not affect the total number of peripheral CD3+ cells. In addition, PPI cells relocated normally in nu/nu mice lacking T cells (Supplemental Fig. 4B), suggesting that T cells may not play an important role in T cell zone formation. Thus, PPI cell relocation is likely required to direct T cell distribution to the IFZ.

FIGURE 3. Defective B cell accumulation in the FZ in PPs of mice that received one shot of LTαβ blocker at birth. (A) Mice were injected with LTβR-hFc chimeric protein or control human Ig at P1 and dissected at P8. Top panels show a gross view of PPs of control and treated mice. The PP from the injected mouse is flat and usually difficult to discern without staining. The PP from the treated mouse can be visualized by staining with Abs to molecular markers. The middle and bottom panels show, respectively, MadCAM1 and B220 expression. Ab binding is developed by using DAB. Both stainings show clearly the segregation of FZ in the treated mouse (Fig. 1). However, the B cell accumulation in this region of segregated FZ is severely inhibited by the treatment. Each image is representative of data confirmed by at least two independent experiments. Original magnification ×15. (B–D) The LT signal was blocked by injecting a recombinant decoy receptor, LTβR-Fc chimeric protein, at P3, P4, or P5. (B and C) The architecture of PPs was analyzed at P5, P6, and P7 by immunohistological study. Although PPI is eventually depleted from the PP by this treatment (data not shown), PPI remains in the PP at ≥2 d after treatment. Note that PPI relocation to PFZ is blocked by this treatment. (B) Upper, Green: CXCL12, red: CXCL13, blue: MadCAM1. Lower, Green: IL-7Rα, red: VCAM1. (C) Upper, Green: IL-7Ra, red: CD3, blue: CD19. Lower, Green: IL-7Ra, red: VCAM1. Scale bars, 100 μm. Each image is representative of data confirmed by at least two independent experiments. (D) PPI cells were isolated from the treated mice and analyzed for expression of chemokine receptors. Although CXCR4 expression of PPs is induced at P4, it is completely suppressed by this treatment. This result suggests that the maintenance of CXCR4 expression requires LT signal. Each panel is a representative result confirmed by at least three independent experiments with four to eight neonatal mice.
In contrast, the processes occurring in the FZ, such as B cell accumulation and FDC formation, were less affected in these mouse strains. In addition, no difference in CXCL13 localization was observed between normal and CXCR4 knockout mice (Supplemental Fig. 3C). Although the PFZ in mutant mice did not contain PPi cells, the distribution of CD11c+ cells (data not shown) and the formation of MadCAM+ vessels in this region occurred normally. Together, these results indicate that PPi cell relocation is specifically required for T cell distribution to T cell zones.

Discussion
The aim of this study was to elucidate the mechanism underlying initial cellular compartmentalization in PLTs, which includes the formation of segregated B and T cell zones. Although chemokines and their receptors have been implicated in the distribution of cells to specific zones, previous analyses have mostly investigated these processes in adult PLTs, in which the basic structure has already been established (3, 65, 66). As shown in this study as well as our previous studies, initial compartmentalization in the PPs is completed within the first neonatal week (6). Thus, studies of perinatal processes are instrumental to understanding the mechanisms underlying the formation of compartmentalized regions in PLTs. In the spleen, LTi cells play an essential role in the segregation of B and T cells and are involved in the functional activity of the T cell zone (7, 8). However, the regulation of normal PLT development and the role that LTi cells play during this process remain unresolved. To our knowledge, our study is the first to describe the neonatal process of PLT development.

FIGURE 4. Chemokine receptor switch of PPi during neonatal PP development. Expression of three chemokine receptors—CXCR4, CXCR5, and CCR7—of PPi was examined at E17.5 and P4. During this period, PPi undergoes a switch of chemokine receptors from CXCR4-CXCR5-CCR7+ to CXCR4+CXCR5-CXCR7+. Each panel is a representative result confirmed by at least three independent experiments performed with four to eight neonatal mice.

FIGURE 5. Induction of CXCR4 in PPi by TGF-β1. (A) PPi isolated from embryonic intestine was cultured on OP9 feeder layer in the presence of stem cell factor. The cells were stimulated by either TGF-β1 or activin A. TGF-β1, but not activin A, can induce CXCR4 in PPi. Each panel is a representative result confirmed by at least three independent experiments performed with 8 to 20 neonatal mice. (B) However, neonatal injection of anti–TGF-β1/β2 mAb (1D11) failed to inhibit CXCR4 induction in mice. Each panel is a representative result confirmed by at least two independent experiments performed with four neonatal mice.
By carefully analyzing the initial process of cell compartmentalization in PPs, we observed that IL-7Rα+ PPi cells play a leading role in the formation of both B and T cell zones. In the embryo, PPi cells are distributed homogeneously throughout the entire PP anlage (6, 25, 28, 39, 43). At the time of birth, this homogeneous PPi cell cluster breaks up into smaller clusters (Fig. 1B). B cells are attracted to these clusters, and the mature FZ containing FDCs is formed around P4. Immediately after clustering in the FZ, PPi cells translocate from the FZ to the outer zones, which is where T cells are attracted. The establishment of PPi cells in the T cell zones is consistent with previous studies showing that LTi cells are present in the T cell zones of the spleen (7). Our study shows that this final localization of LTi cells is the result of the intricate control of LTi cell localization. Furthermore, the observed migration pattern led us to hypothesize that PPi cells are involved in the formation of not only the PP anlagen but also the B and T cell zones.

The mechanism for how PPi cells are initially distributed homogeneously throughout the entire PP anlage and then suddenly break up to form segregated clusters remains unknown (6, 25, 28, 39, 43). However, it is known that these clusters develop into FZs by attracting B cells. When we blocked the interaction between PPi and PPo cells by administering a single injection of LTβR-Fc at P1, which is when cluster formation starts, B cell accumulation in the FZs was inhibited for many months (Fig. 3A). Thus, the interaction between PPi and PPo cells in these clusters promotes the formation of FZs.

**FIGURE 6.** Strategy for conditional deletion of the cxcr4 gene in RORγt+ cells. Specific deletion of the cxcr4 gene in PPi. CD3+ T cells in PPs as well as in LNs are essentially CXCR4 negative. Nonetheless, this result shows that CD3+ T cells in this strain of mouse may have normal activity to distribute to PLTs. In contrast, although basically all PPi cells at P7 express CXCR4, the CXCR4 expression is inhibited almost completely. Each panel is a representative result confirmed by at least three independent experiments performed with two to four neonatal mice.

**FIGURE 7.** Essential role of chemokine receptor switch of PPi in PP development. The cxcr4 gene was conditionally deleted in RORγt+ cells, and the process of PP organogenesis was compared in control (CXCR4\textsuperscript{flox/+}) and mutant (CXCR4\textsuperscript{flox/-}) mice. The relocation of IL-7Rα+ PPi was not observed in mutant mice at P7. Despite this defect, B cell accumulation, development of FDCs, and formation of MadCAM1+ HEV proceeded normally. In contrast, the distribution of CD3+ cells from PFZ to IFZ is inhibited to a great extent. Green: IL-7Rα, red: VCAM1. Scale bars, 100 μm. Each image is a representative result confirmed by at least two independent experiments.
formation of B cell zones by attracting B cells into the FZ. Of note, the transient inhibition of LTβ signaling at P1 resulted in a long-lasting effect, which strongly suggests that the PPi cell clusters in the FZs are required not only to attract B cells but also to trigger subsequent processes that establish tissue organization and enable the constant recruitment of B cells to the FZ in later life. Because PPi cells migrate from the FZ after B cell accumulation, PPi cells are not involved in the maintenance of the final tissue organization that is required for constant recruitment of B cells. Previous studies demonstrated that FDCs and B cells are the major factors that maintain the function of the FZ in mature PLTs (44, 67). Thus, it is likely that FZ development occurs in two steps. In the first step, the interactions between PPi and PPO cells induce the initial accumulation of B cells into PPi cell clusters. This accumulation then leads to the interaction between FDCs and B cells, which regulates sustained B cell recruitment. Although CXCL13 is the major chemokine involved in this process throughout life, the cells involved in CXCL13 induction and expression shift from PPi/PPO cells to B cells/FDCs.

Previous studies have demonstrated that LTI cells exist in the T cell zones and affect both the segregation of T and B cells and the adaptive T cell response (7, 49, 50). However, the process leading to LTI cell localization in the T cell zone remains unclear. In this study, we demonstrate that in PPs, LTI cell localization occurs through the elaborate control of chemokine receptor expression on PPi cells. In the initial stage of the neonatal process, CXCR5 on PPi cells and CXCL13 on PPO cells have a central role in directing PPi cell localization (43). In contrast, at P3–4, the time at which B cells enter the FZ and FDCs emerge, PPi cells leave the FZ to migrate to the outer zones, even though the pattern of chemokine expression remains consistent (CXCL13 in the FZ and CXCL12 in the PFZ to IFZ). Therefore, the chemokine expression pattern cannot account for PPi cell behaviors; rather, this behavior occurs through the switching of chemokine receptors on PPi cells from CXCR5 to CXCR4. Moreover, we provide evidence that PPi cell relocation is dependent on CXCR4, as the conditional knockout of CXCR4 in RORγt+ PPi cells resulted in the complete failure of PPi relocation. Strikingly, most PPi cells were retained in the FZ even after the accumulation of B cells and the development of FDCs (Fig. 7). Moreover, we found that T cell distribution throughout the IFZ was disturbed in this mouse strain, and this result is consistent with a previous study using LTββ knockout mice, which suggested that LTI cells are involved in T cell zone formation (31, 33, 35, 35–38, 68). In this study, we provide clear evidence that PPi cells migrate to the T cell zone to induce activity that attracts T cells. Taken together, these results indicate that PPi cells play a role in all of the key processes during PP organogenesis, namely, the formation of the anlage, B cell zone, and T cell zone.

Many questions concerning the initial process of compartmentalization remain unanswered. One important question is the following: How is chemokine receptor switching induced in PPi cells? The results presented in Fig. 3 demonstrate that this switch was inhibited by the blockade of LTββ signaling. However, this result does not necessarily indicate that the LTββ signal is sufficient for the switch. The LTββ signal is present during embryonic PP organogenesis (25), but the receptor switch does not occur prior to P1. Because the LTββ signal is the fundamental basis for PPi cell interactions with stromal cells (11, 13–16, 28), it is likely that additional signals are specifically induced in the FZ to activate the switch. As this switch is concomitant with the accumulation of B cells into the FZ, we also investigated the possibility that B cells were involved in the chemokine receptor switch. As shown in Supplemental Fig. 4A, however, chemokine receptor switching on PPi cells was shown to occur in scid/scid as well as JH−/− mice (61, 69). In addition, we examined PPi cell relocation in nu/nu mice that lack T cells and found that PPi cells relocated normally in this strain (Supplemental Fig. 4B). Thus, lymphocyte accumulation in the FZ is not required for receptor switching. Similarly, FDCs do not induce receptor switching because FDC development was suppressed in these strains. At present, the cell population responsible for receptor switching remains unknown, but our results suggest that these cells are induced in the FZ during the first 3 d of the neonatal period.

The addition of TGF-β to PPi cells in vitro induced the same chemokine receptor switching as that observed in vivo (Fig. 5A) (64). In contrast, the addition of activin A had no effect on chemokine receptor expression on PPi cells (Fig. 5A). However, daily injections of an anti-TGF-β mAb (100 μg per neonate) (60) from P0 to P2 failed to inhibit receptor switching (Fig. 5B). Thus, the cells and signals involved in this switch should be investigated in future studies.

Under culture conditions with IL-7, CXCR5 expression remains even though CXCR4 is induced by TGF-β stimulation, which indicates that CXCR4 and CXCR5 expression is differentially regulated. However, the specific cells and signals involved in this switch should be investigated in future studies.

Of note, chemokine receptor switching appears to be specific to the PPs. In LNs, CXCR4 is already expressed during the embryonic stage, and CXCR4 expression does not coincide with the down-regulation of CXCR5 (Supplemental Fig. 1A). Thus, the mechanisms underlying chemokine receptor expression may vary significantly between different PLTs. Moreover, the role of CXCR4 in the migration of LTI cells to the T cell zone in other PLTs remains unknown, although knocking out CXCR4 in RORγt+ cells had almost no effect on the organogenesis of the LNs or spleen (Supplemental Fig. 3D). Thus, further analysis is necessary to define the behaviors of LTI cells during the compartmentalization of cells in PLTs. Moreover, the histological architecture differs between PPs and LNs; PPi lack afferent lymphatics, whereas LNs are sandwiched between afferent and efferent lymphatics (70, 71). The current results represent another example of the diverse mechanisms underlying the organogenesis of lymphoid tissues. Taken together, these data indicate the existence of multiple pathways to attain the tissue architecture of PLT. Because the cellular basis for the formation of B and T cell zones in other PLTs remains unknown, it is difficult to extrapolate our results to the organogenesis of other LTs. Nonetheless, additional studies on LT development should take this functional role of LTI cells into account. In particular, it would be interesting to investigate the role of inducer cells in various stages of chronic inflammation (72–74) because the course of chronic inflammation varies according to the incidental conditions and may induce phenotype switching of LTI cells, as shown in this study.

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Disclosures

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