Molecular Basis for Hematopoietic/Mesenchymal Interaction during Initiation of Peyer's Patch Organogenesis

By Kenya Honda,*‡ Hiroyasu Nakano,‖¶ Hisahiro Yoshida,* Satomi Nishikawa,* Paul Rennert,** Koichi Ikuta,§ Masakatsu Tamechika,¶ Kazuhito Yamaguchi,§§ Tetsuo Fukumoto,‡‡ Tsutomu Chiba,‡ and Shin-Ichi Nishikawa*

*From the *Department of Molecular Genetics, the ‡Department of Gastroenterology, and the §Department of Medical Chemistry, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan; the ‖Department of Immunology, Juntendo University, School of Medicine, Tokyo 113-8421, Japan; ¶Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, Tokyo 101-0062, Japan; the **Department of Immunology, Biogen, Incorporated, Cambridge, Massachusetts 02142; and the ‡‡Department of Anatomy and the §§Institute of Laboratory Animals, Yamaguchi University School of Medicine, Ube 755-8505, Japan*

Abstract

Mice deficient in lymphotoxin β receptor (LTβR) or interleukin 7 receptor α (IL-7Rα) lack Peyer's patches (PPs). Deficiency in CXC chemokine receptor 5 (CXCR5) also severely affects the development of PPs. A molecular network involving these three signaling pathways has been implicated in PP organogenesis, but it remains unclear how they are connected during this process. We have shown that PP organogenesis is initiated at sites containing IL-7Rα$^+$ lymphoid cells and vascular cell adhesion molecule (VCAM)-1/intercellular adhesion molecule (ICAM)-1 expressing nonlymphoid elements. Here we characterize these lymphoid and nonlymphoid components in terms of chemokine signals. The lymphoid population expresses CXCR5 and has a strong chemotactic response to B lymphocyte chemoattractant (BLC). Importantly, chemokines produced by VCAM-1$^+$ICAM-1$^+$ nonlymphoid cells mediate the recruitment of lymphoid cells. Furthermore, we show that these VCAM-1$^+$ICAM-1$^+$ cells are mesenchymal cells that are activated by lymphoid cells through the LTβR to express adhesion molecules and chemokines. Thus, promotion of PP development relies on mutual interaction between mesenchymal and lymphoid cells.

Key words: lymphoid tissue • lymphotoxin • interleukin 7 • chemokines • chemotaxis

Introduction

Secondary lymphoid tissues including the spleen (Spl),[1] LNs, and Peyer's patches (PPs) are thought to serve as focal regions that mediate and facilitate the cell–cell interactions required for Ab formation. To understand how the highly organized structures of these organs are formed, it is important to identify the cellular components and intercellular signals that comprise the initial phase of organogenesis. Molecular and genetic analyses have revealed a complex network of molecules involved in lymphoid organogenesis (1, 2). As for PP organogenesis, one group of important signaling molecules are TNF family molecules. Mice deficient in lymphotoxin (LT)α/β heterotrimer and its receptor LTβR fail to develop organized PPs (3, 4, 5). Moreover, the role of LTα homotrimer and its receptor TNFRp55 was also implicated (6). Another group is IL-7R, members of the cytokine receptor family. Mice lacking expression of IL-7Rα, γc, and their downstream target, Janus kinase (JAK)3, were defective in PP development (7, 8). Interestingly, these two groups of molecules are required within a narrow time window during the development of PPs (9, 10). B lymphocyte chemoattractant (BLC) and its receptor,

Address correspondence to Kenya Honda, the Department of Molecular Genetics, Graduate School of Medicine, Kyoto University, Syogoin-Kawaharacho 53, Sakyo-ku, Kyoto 606-8507, Japan. Phone: 81-75-751-4161; Fax: 81-75-751-4169; E-mail: khonda@virus.kyoto-u.ac.jp

[1]*Abbreviations used in this paper:* BLC, B lymphocyte chemoattractant; CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; E, embryonic day; ELC, EBV-induced molecule 1 ligand chemokine; FDC, follicular dendritic cell; ICAM, intercellular adhesion molecule; LT, lymphotoxin; MAdCAM, mucosal addressin cell adhesion molecule; PDGFR, platelet-derived growth factor receptor; PP, Peyer's patch; RT, reverse transcription; Spl, spleen; SLC, secondary lymphoid organ chemokine; VCAM, vascular cell adhesion molecule.

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CXC chemokine receptor 5 (CXCR5), have also been shown to be required for PP organogenesis, as the disruption of these genes resulted in abnormal PP development (11, 12).

It is likely that LT influences lymphoid organogenesis through the induction of adhesion molecules and chemokines. It was shown that the LTα homotrimer can act on endothelial cells to induce vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 (13). Although unable to stimulate endothelial cells, the LTα/β heterotrimer can induce adhesion molecules on nonendothelial stromal cell components (14, 15). Furthermore, a recent study has established a critical role for TNFRp55 and LTβR signaling in regulating the expression of homeostatic chemokines such as BLC, EBV-induced molecule (EBI)-1 ligand chemokine (ELC), and secondary lymphoid organ chemokine (SLC) in secondary lymphoid organs (16). Although these studies have greatly enhanced our knowledge on the signaling networks involved in PP organogenesis, the cellular interactions underlying the network remain largely obscure. Interestingly, B lymphocytes have been demonstrated as an indispensable source of LTα/β heterotrimers (11, 17, 18). In a study on a transgenic model expressing BLC, lymphoid neogenesis was observed in a B cell–dependent manner (19). However, as PP development remained intact in the absence of T and B lymphocytes (20), it must be assumed that cells other than mature lymphocytes are receiving or producing organogenic signals.

We have previously reported that developing PPs can first be detected at around gestational day 16 as clusters of VCAM-1$^+$ICAM-1$^+$ (V^+I^+) mesenchymal-like cells and CD3$^-$IL-7Rα$^+$ lymphoid cells (20). As the CD3$^-$IL-7Rα$^+$ population in the embryonic gut is capable of expressing LTα/β and both IL-7Rα and LTα/β are essential for PP organogenesis, it is likely that this population represents a PP inducer (10). We further hypothesized that LTα/β expressed in PP inducers activate surrounding nonlymphoid cells which function as a PP organizer to regulate subsequent processes such as recruitment of various hematopoietic cells to the PP anlage (21, 22). Additional support for this proposed mechanism was obtained from a study of helix-loop-helix inhibitor Id2$^{-/-}$ mice (23). In Id2$^{-/-}$ mice, both the differentiation of CD4$^+$CD3$^-$IL-7Rα$^+$ cells and the formation of V^+I^+ cell clusters are inhibited. This notion fits well with the hypothesis that CD4$^+$CD3$^-$ cells play a role in LN organogenesis (24).

In this study, to further promote our understanding of molecular and cellular events involved in the initial phase of PP development, we isolated and characterized CD4$^+$CD3$^-$IL-7Rα$^+$ lymphoid and V^+I^+ nonlymphoid populations using cell-surface markers and flow cytometry. We show that migration and activation of CD4$^+$CD3$^-$IL-7Rα$^+$ cells are regulated by V^+I^+ cells. We find that these V^+I^+ cells are mesenchymal and in turn are activated by CD4$^+$CD3$^-$IL-7Rα$^+$ cells through LTβR to express VCAM-1, ICAM-1, BLC, and ELC. Our results indicate that mutual activation of lymphoid and mesenchymal cells occurs in PP anlagen, and that these interactions are the underlying mechanisms of PP organogenesis.

Materials and Methods

Mice. C57BL/6 and LTα$^{-/-}$ mice were purchased from Japan SLC, Inc. and The Jackson Laboratory, respectively. Establishment of Id2$^{-/-}$ and IL-7Rα$^{-/-}$ mice was reported previously (23, 25). Matings were conducted overnight, and noon of the day vaginal plugs were observed was designated as embryonic day (E)0.5. All animal procedures described in this study were performed in accordance with the guidelines for animal experiments of Kyoto University Graduate School of Medicine.

Construction of Fusion Proteins. An expression construct encoding a fusion of the extracellular domain of murine LTβR (amino acids 1–222) and Fc portion of human IgG_1 (LTβR-Ig) was made by PCR amplification of the former with CGGAATTCTGGTCAGCCGAGCCGAAAGG and CGGGATCCATTGCTCCTGGCTCTGGGGG as 5′ and 3′ primers, respectively. The PCR product was subcloned into the EcoRI and BamHI sites of a pBSK vector containing the Fc portion of human IgG1 (provided by B. Seed, Harvard Medical School, Boston, MA) and the resulting LTβR-Ig fragment was transferred into a mammalian expression vector pMKITneo (provided by K. Maruyama, Tokyo Medical Dental University, Tokyo, Japan). COS-7 cells were transfected with pMKITneo/LTβR-Ig by lipofection and fusion proteins were purified using protein-G Sepharose columns (Amersham Pharmacia Biotech). A fusion protein consisting of the extracellular domain of mTNFRp55 (amino acids 1–212; TNFRp55-Ig) was prepared similarly with TCAGAATTCCGGACATGGGTCTCCCCACC and TCAGGATCCGCAGTACCTGAGTCCTGGGG as primers. Human-IgG Fc fragment (ChromPure™; Jackson ImmunoResearch Laboratories) was used as a control.

Abs. Anti–VCAM-1 mAb (RAV2; a gift from T. Kina, Kyoto University) was labeled with Alexa™ 488 or Alexa™ 594 (Molecular Probes, Inc.). PE- and FITC-conjugated anti–ICAM-1 mAb (3E2), FITC- or biotin-conjugated anti-CD4 mAb (RM4-5), and PE-conjugated anti-CD3e mAb (2C11) were purchased from BD PharMingen. Biotin conjugated Abs for mucosal addressin cell adhesion molecule (MAdCAM)-1 (MECA-89), CD45 (30-F11), CD40 (3/23), B220 (RA3-6B2), CD35 (8C12), CD23 (B3B4), CD11c (HL3), CD11b (M1/70), and PECAM-1 (MEC13.3) were purchased from BD PharMingen. mAbs for VE-cadherin (VECD-1), platelet-derived growth factor receptor (PDGFR)-α (APA-5), PDGFR-β (APB-5), Flk-1 (AVAS12), and IL-7Rα (A7R34; reference 26) were prepared and labeled in our laboratory. Anti-LTβR mAb (AFH6; reference 27), anti–BP-3 mAb (a gift of M.D. Cooper, Howard Hughes Medical Institute, University of Alabama, Birmingham, Alabama), and anti-FDC–M1 mAb (a gift of G. Kelsoe, Duke University Medical Center, Durham, NC) were biotinylated in our laboratory.

For blocking IL-7Rα in vivo, 3 mg of A7R34 was injected intraperitoneally into pregnant mice. Polyclonal rat IgG (Sigma-Aldrich) was injected as a control.

Isolation of CD4$^+$CD3$^-$IL-7Rα$^+$ Cells and V^+I^+ Cells from Embryonic Intestine. For preparation of CD4$^+$CD3$^-$IL-7Rα$^+$ cells, embryonic small intestines freed from mesenteries were treated with 2 U/ml of dispase (GIBCO BRL) at 37°C for 15 min, shaken vigorously manually, and washed with ice-cold HBSS/1% BSA.

For preparation of V^+I^+ cells, minced embryonic small intestines were digested at 37°C for 1 h with 100 U/ml each of colla-

genase types I and IV (GIBCO BRL) in HBSS containing 2.5 mM $CaCl_2$, 10 mM Hepes, pH 7.5, and DNase I (50 μg/ml; Sigma-Aldrich) and then incubated in dissociation buffer (GIBCO BRL) in a shaking water bath for 15 min. Cell suspensions were passed through glass wool column to eliminate the epithelial cells (28).

Cell suspensions were incubated in mouse sera to block nonspecific Ab binding and stained with labeled mAbs. For isolation of gated cells by a FACS Vantage™ (Becton Dickinson), enrich mode followed by normal-R mode sorting was performed while being gated to exclude small debris, propidium iodide (Sigma-Aldrich)-positive dying cells, and other sources of background interference. Isolated cells were obtained at a purity of >98%. In some experiments, $CD4^+$ cell isolation from embryonic intestines was performed with Dynabeads Mouse CD4 (L3T4) and DETACHaBEAD Mouse CD4 (Dynal) according to the manufacturer's protocol. Until E17.5, all of the $CD4^+$ cells in the intestine were $CD3^-$IL-7Rα^+. Purity of the isolated cells was >90%. The yield of V^+I^+ cells and $CD4^+$ cells from each mouse embryonic small intestine was around 5×10^2 and 5×10^3, respectively.

Flow Cytometry Analysis. Three-color analyses of V^+I^+ cells were performed with Alexa™ 488–conjugated anti–VCAM-1, PE-conjugated anti–ICAM-1, and biotinylated mAbs followed by allophycocyanin-conjugated streptavidin (Molecular Probes, Inc.). Biotin-conjugated isotype-matched mAbs (BD PharMingen) were used as controls. After staining, fluorescence intensity was measured on samples of 10^6 cells by FACS Vantage™ (Becton Dickinson).

In the analysis with LTβR-Ig, $CD4^+$ cells were cultured in various concentrations of murine rIL-7 (a gift from T. Sudo, Toray Basic Research Laboratories, Kanagawa, Japan) in RPMI 1640/5% FCS for 6 h. Cells were suspended in HBSS/2% mouse serum/rat anti–mouse CD32/16 mAb (2.4G2; BD PharMingen) to block Fc receptor binding, stained with LTβR-Ig, and followed by detection with PE-labeled donkey anti–human IgG (Jackson ImmunoResearch Laboratories).

Immunohistochemistry. Whole-mount immunohistostaining was performed as described previously (20). In brief, specimens were fixed 1 h in 2% paraformaldehyde (pH 7.4) on ice, washed with PBS, serially dehydrated with methanol, blocked for intrinsic peroxidase activities in 0.3% of H_2O_2, and rehydrated. After incubation with PBSMT (1% skim milk, 0.3% Triton X-100 in PBS) to block nonspecific bindings, specimens were incubated in primary Abs overnight, and washed in PBST (0.3% Triton X-100 in PBS). After incubation with horseradish peroxidase (HRP)-conjugated secondary reagents, color reactions were carried out with diaminobenzidine and nickel chloride.

For immunofluorescence microscopy, fetal intestines were embedded in OCT compound, snap frozen in liquid nitrogen, and stored at −80°C. Cryostat sections (10-μm thick) were dried and fixed with acetone. Slides were preincubated for 30 min in PBS/0.05% Tween 20/2% BSA/2% heat-inactivated goat serum before incubation with conjugated Abs in a humidified chamber for 1 h at room temperature. After three washes in PBS, confocal microscopy was performed using a Leica TCS-NT laser scanning microscope and Leica image software.

RNA Expression Studies. For Northern analysis, 5 μg of poly(A)$^+$ RNA of fetal mouse intestine was separated by electrophoresis in a 1.0% agarose-formaldehyde gel and transferred onto filters. Radioactive DNA probes for BLC and ELC were prepared by random-primed labeling of a 527-bp cDNA fragment of mouse BLC (nucleotides 9–535) and a 693-bp cDNA fragment of mouse ELC (nucleotides 16–708). The blot was hybridized with a probe, and visualized by the BAS 5000 system (Fuji Film and Photo, Inc.). Full-length glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as a control for quantitating amount loaded.

Whole-mount in situ hybridization was conducted as described previously (29). Fetal small intestines were isolated and fixed in cold 4% paraformaldehyde/PBS overnight. After washing, the intestines were treated with 100 μg/ml proteinase K (Sigma-Aldrich), and hybridized with digoxigenin-labeled RNA probes. After hybridization, samples were treated with RNase A (Sigma-Aldrich), incubated with alkaline phosphatase–conjugated antidigoxigenin Ab (Boehringer) at 4°C overnight, and developed with a reaction mixture for alkaline phosphatase containing polyvinyl alcohol.

Reverse Transcription PCR Analyses. Total RNA was isolated from sorted cells using ISOGEN (Nippon gene). First-strand cDNA was produced from 300 ng total RNA by reverse transcription (RT) using oligo-dT primers (SuperScript Preamplification System; GIBCO BRL) following the manufacturer's protocols. PCR amplifications were performed with specific primers for CD4, IL-7Rα, CD3ε, CXCR5, CC chemokine receptor (CCR)7, ELC, and BLC. GAPDH signals were used to calibrate differences in the amount of starting cDNA between samples. Specific primers were as follows: CD4/S, ACTGCATCAGGAAGTGAACCTTG; CD4/AS, TCTCACTGAGGAGCCTCTTGAT; CD3ε/S, AGATTGTCTGCCAGCCACCTTG; CD3ε/AS, GGACCTGTGCCAATGATTACAG; IL-7Rα/S, GTCCATTATCCAAACTGAGTCG; IL-7Rα/AS, CATCGGAGGACTCCATTCACTG; CCR7/S, AGAATACCACGGTGGACTACA; CCR7/AS, GCAGCTGCTATTGGTGATGTT; CXCR5/S, ATGAACTACCCACTAACCCTG; CXCR5/AS, AGGTGAACCAGGCTCTAGTTT; ELC/S, CTGCCTCAGATTATCTGCCAT; ELC/AS, GCCAGAGTGATTCACATCTCT; BLC/S, TGAGGCTCAGCACAGCAACG; BLC/AS, CTTGAGCATTCCCTCTCAGCT; GAPDH/S, ATGGTGAAGGTCGGTGTGAACGGATTTGGC; and GAPDH/AS, GCATCGAAGGTGGAAGAGTGGGAGTTGCTG.

In Vitro Chemotaxis. Isolation of B cells from Spl was performed following the essentially same procedure as described previously (30), except that anti-CD43 mAb (BD PharMingen) coated anti–rat IgG Dynabeads (Dynal) were used. Flow cytometry analysis confirmed that the purity of isolated B cells was >94% $CD19^+$ cells. Chemotaxis assays were performed in polycarbonate membrane, 6.5-mm diameter, 5-μm pore size Transwell cell culture chambers (Corning Costar Corp.). 100 μl aliquots of cells (2×10^4/ml) suspended in RPMI/2% FCS were added to the upper chambers and chemokines were added to the lower wells. The cells were allowed to migrate for 2 h at 37°C in a 5% CO_2 incubator, after which the number of cells that migrated to the lower chamber were counted by flow cytometry. Murine ELC and SLC were obtained from R&D Systems and murine BLC from Dako.

Electron Microscopy. FACS® sorted cells were fixed immediately with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature. Cells were then washed and postfixed in 1% osmium tetroxide for 1 h at 4°C. After further washing, cells were dehydrated in a graded series of alcohol and embedded in Epon812. Ultrathin sections (90–150 nm) were cut on a microtome (LKB 8000 Ultratome) and routinely stained with uranylacetate and lead citrate for examination with a 200 CX transmission electron microscope (JEOL) operating at 80 kV.

Results

Chemokine Reactivity of PP Inducers. We previously reported that embryonic intestinal $CD3^-IL\text{-}7R\alpha^+$ cells produce $LT\alpha/\beta$ and play an essential role in PP organogenesis (10) and adopted the term "PP inducer" to designate this population. Approximately half of these PP inducer cells also express CD4, which has been implicated as a marker for the population involved in the induction of mesenteric LN (24). Although it is still unclear whether only $CD4^+$ cells or both $CD4^+$ and $CD4^-$ cells are PP inducers, this study focuses on the $CD4^+$ population to allow comparison with the study by Mebius et al. (24).

At E16.5, $CD4^+$ cells begin to form segregated clusters in embryonic intestine, in contrast to the scattered pattern over the gut that is observed up to E15.5. By E17.5, increasing numbers of $CD4^+$ cells are recruited to PP anlagen (Fig. 1 A). We attempted to investigate the molecular mechanisms underlying this clustering process. If the migration of $CD4^+$ cells is regulated by specific chemokines, the corresponding chemokine receptors should be expressed in the PP inducers. Among the characterized receptors, CXCR5 and CCR7 have been suggested to participate in the accumulation of PP inducers. In particular, CXCR5 was a likely candidate, because mice genetically deficient in this gene possess few PPs (12). RT-PCR analyses revealed that both chemokine receptors are expressed in $CD4^+CD3^-IL\text{-}7R\alpha^+$ cells sorted from the intestine (Fig. 1 B). Moreover, in vitro chemotactic analyses showed that their ligands, BLC, ELC, and SLC, all induced migration of $CD4^+CD3^-IL\text{-}7R\alpha^+$ cells (Fig. 1 C). Furthermore, the magnitude of response of $CD4^+CD3^-IL\text{-}7R\alpha^+$ cells to BLC was comparable to that observed for B lymphocytes (Fig. 1 D). It was likely that the overall higher frequency in $CD4^+CD3^-IL\text{-}7R\alpha^+$ cells than in B cells reflected a high basal motility in $CD4^+CD3^-IL\text{-}7R\alpha^+$ cells. Similar results were obtained with $CD4^+CD3^-IL\text{-}7R\alpha^+$ cells from E15.5 intestines. These data show that PP inducers are highly competent to respond to chemokines.

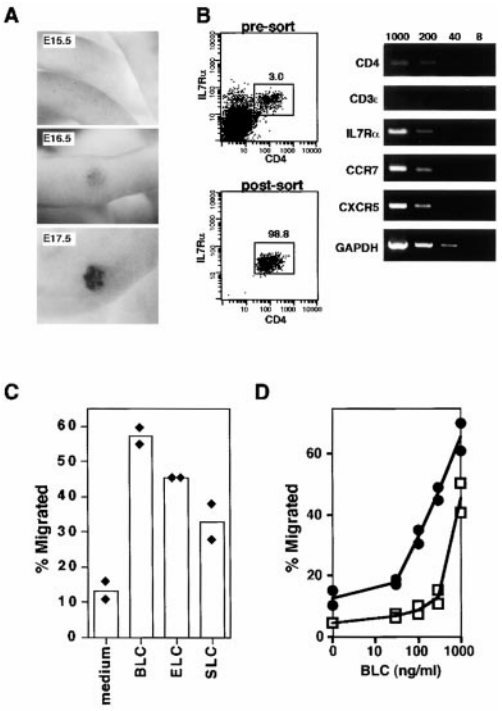

Figure 1. Chemotactic activity of BLC, ELC, and SLC on $CD4^+CD3^-IL\text{-}7R\alpha^+$ cells. (A) Whole-mount immunostaining analysis of E15.5, E16.5, and E17.5 gut with anti-CD4 mAb. (B) RT-PCR analysis on CXCR5 and CCR7 in FACS®-sorted $CD4^+CD3^-IL\text{-}7R\alpha^+$ cells from intestine at E17.5. The numbers of cells subjected to RT-PCR are 1,000, 200, 40, and 8 cells from left to right in each panel. Specific primers for CD4, IL-7Rα, and CD3-ϵ were used as positive and negative controls. (C) $CD4^+$ cells were prepared with Dynabeads and placed in the chemotaxis chamber in duplicates. ELC and SLC maximally attracted $CD4^+$ cells at 100–300 ng/ml. Results are expressed as the percentage of input cells migrating to the lower chamber containing BLC (1 μg/ml), ELC (300 ng/ml), or SLC (300 ng/ml). Data from individual wells are shown as filled diamonds and means as bars. The results are representative of three independent experiments. (D) Chemotactic response to BLC by $CD4^+$ cells from intestine at E17.5 (filled circles) or B cells from Spl (open squares). Lines represent the averages of duplicated Transwells. The results are representative of at least three independent experiments.

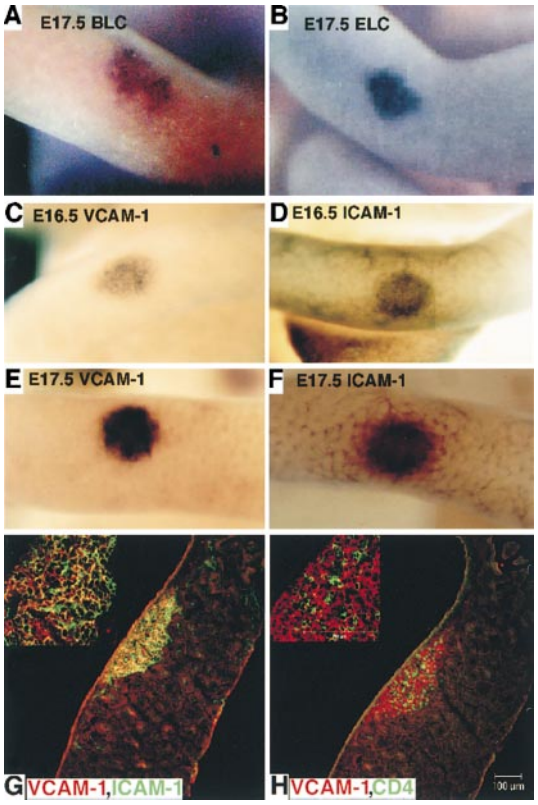

Figure 2. Expression of BLC, ELC, VCAM-1, and ICAM-1 in PP anlagen. Whole-mount in situ hybridization analysis of BLC (A) and ELC (B) expression in E17.5 intestines. Whole-mount immunostaining analysis of E16.5 (C and D) and E17.5 (E and F) gut with mAbs against VCAM-1 (C and E) and ICAM-1 (D and F). Cryostat sections of intestines at E17.5 were double labeled with Abs against VCAM-1 (Alexa™ 594; red) in combination with (G) anti–ICAM-1 (FITC; green) or (H) anti-CD4 (FITC; green) (original magnification: ×200) and subjected to confocal microscopic analysis. The insets are included to show the morphology of the V^+I^+ cells and $CD4^+$ cells (original magnification: ×630).

To investigate the involvement of these chemokines in the clustering of PP inducers, their expression in the embryonic intestine was analyzed by whole-mount in situ hybridization. ELC and BLC were both expressed in developing PPs at E17.5 (Fig. 2, A and B), but not at E15.5 (data not shown), supporting their involvement in PP inducer accumulation.

Isolation of V^+I^+ Cells. We next tried to identify the cell population that expresses chemokines. At E16.5, when $CD4^+$ clusters first appear, expression of ICAM-1 and VCAM-1 are detectable among the nonlymphoid elements of the PP anlagen (Fig. 2, C and D). Marked increase in expression levels of VCAM-1 and ICAM-1 by E17.5 coincide perfectly with an increase in the number of recruited $CD4^+$ cells (Fig. 1 A, and Fig. 2, E and F). Confocal microscopic analysis revealed the presence of cells coexpressing VCAM-1 and ICAM-1 in the PP anlagen, although single-positive cells could also be detected (Fig. 2 G). Although none of the VCAM-1^+ cells coexpress CD4, they are located within close vicinity of the $CD4^+$ cells (Fig. 2 H). This led us to predict that these V^+I^+ cells are the chemokine-expressing cells observed by whole-mount in situ hybridization.

To examine this possibility, we attempted to develop a method to isolate the V^+I^+ cells in the PP anlagen in order to measure their chemokine expression. Enzymatic treatment of the intact gut as described in Materials and Methods was found to result in efficient dissociation of the cells without affecting the ability to detect VCAM-1/ICAM-1. As shown in Fig. 3, A and B, a V^+I^+ population appears at E16.5 to E17.5, consistent with the results of immunohistochemical staining of whole-mount intestines. The V^+I^+ population comprises only ~0.5% of dissociated cells from the entire embryonic intestines. As this fraction could not be detected in cells from embryonic intestines in IL-$7R\alpha^{-/-}$ (Fig. 3, C and D), $LT\alpha^{-/-}$, or $Id2^{-/-}$ (data not shown) mice lacking PPs (3, 8, 23), this V^+I^+ population should represent cells existing in developing PPs.

Expression of ELC and BLC by V^+I^+ Population. Using highly purified V^+I^+ populations (5×10^3 cells), we analyzed expression of BLC and ELC by RT-PCR. Other populations from E17.5 embryonic intestines were also investigated. The purity of corresponding populations in each sorted preparation was >98% (data not shown). As can be seen in Fig. 3 E, V^+I^+ cells expressed both BLC and ELC mRNA. Of note is that ELC was detected only in the V^+I^+ population, suggesting that V^+I^+ cells may form specific foci to attract cells that are competent to respond to ELC. Although BLC expression was observed in VCAM-1 single-positive cells in addition to V^+I^+ cells, our histological analysis demonstrated that BLC-producing cells were concentrated in PP anlage (Fig. 2 A). Taken together, V^+I^+ cells in the PP anlagen likely form focal regions rich in the chemokines capable of recruiting PP inducer cells.

Molecular Requirements for Induction of V^+I^+ Cells. We next investigated whether formation of V^+I^+ cells in the gut requires LT and IL-7Rα signals. Pregnant mice were given a single injection of either blocking mAb to IL-7Rα

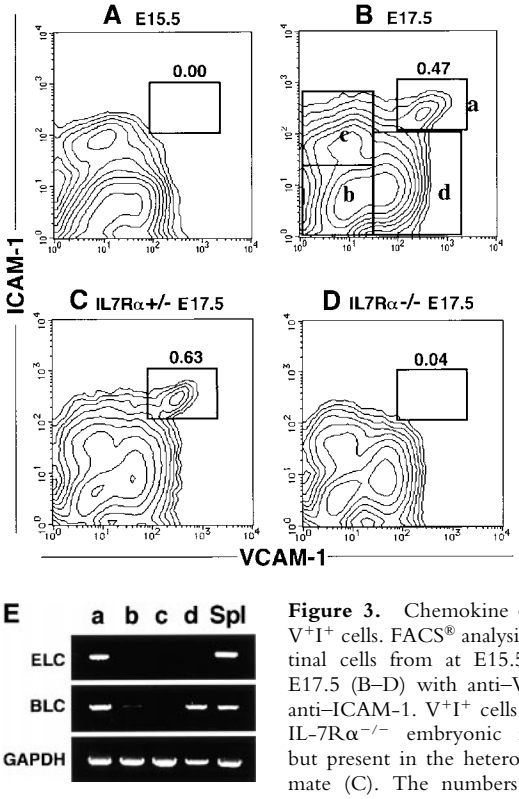

Figure 3. Chemokine expression by V^+I^+ cells. FACS® analysis of the intestinal cells from at E15.5 (A) and at E17.5 (B–D) with anti–VCAM-1 and anti–ICAM-1. V^+I^+ cells are absent in IL-$7R\alpha^{-/-}$ embryonic intestine (D) but present in the heterozygous littermate (C). The numbers indicate the percentage of the V^+I^+ population. (E) RT-PCR analysis of transcripts encoding ELC and BLC in FACS®-sorted V^+I^+ population (a) and other populations (b to d) from E17.5, as indicated in B. Reverse-transcribed mRNA from Spl tissue was analyzed as positive control. PCR with GAPDH primers is shown as control for the cDNA content of individual samples. Similar results were obtained from three independent experiments.

(A7R34; reference 26), LTβR-Ig, or TNFRp55-Ig at day 15.5 of gestation and cells from the intestines of embryos at E17.5 were analyzed for VCAM-1 and ICAM-1 expression by flow cytometry. Control mice were treated with equal amounts of human-IgG Fc fragment or polyclonal rat IgG. As expected, after in utero treatment with A7R34 or LTβR-Ig, depletion of the V^+I^+ population was observed, whereas the corresponding population from control mice remained unaffected (Fig. 4 A). Concurrent with the depletion of this population, PP development was completely blocked. Therefore, both IL-7Rα and LTβR signaling are indispensable for the formation of V^+I^+ cells. mAb staining and RT-PCR analysis showed that these cells indeed express LTβR (Fig. 4 B, and data not shown). On the other hand, TNFRp55-Ig failed to block PP development (data not shown), though this treatment consistently suppressed the proportion of V^+I^+ cells. The same results were obtained by treatment with higher (twofold) amounts of TNFRp55-Ig (data not shown). Such a partial effect of TNFRp55-Ig on PP organogenesis was consistent with previous results on TNFRp55-deficient mice (6).

We have previously reported that PP inducers are an essential source of LTα/β for PP development, and suggested the role of IL-7Rα signal for its induction. Indeed, exposure of pregnant mice to A7R34 at E15.5 suppressed

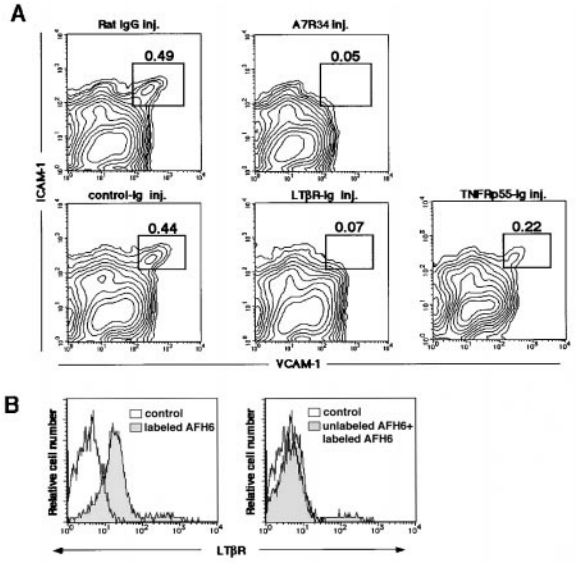

Figure 4. Signaling through IL-7Rα and LTβR are required for induction of V^+I^+ cells. (A) Either A7R34 (3 mg), LTβR-Ig (250 μg), or TNFp55-Ig (250 μg) was injected (inj.) to pregnant mice at E15.5, and cells were prepared from embryonic intestines at E17.5 and analyzed by FACS®. Control mice were treated with equal amounts of human-IgG Fc fragment or polyclonal rat IgG. The numbers indicate the percentage of the cell population positive for both VCAM-1 and ICAM-1. (B) Expression of LTβR in gated V^+I^+ cells was measured by flow cytometry. Cells were incubated with or without labeled anti-LTβR mAb (AFH6). To confirm signal specificity, blocking with unlabeled AFH6 was performed (right).

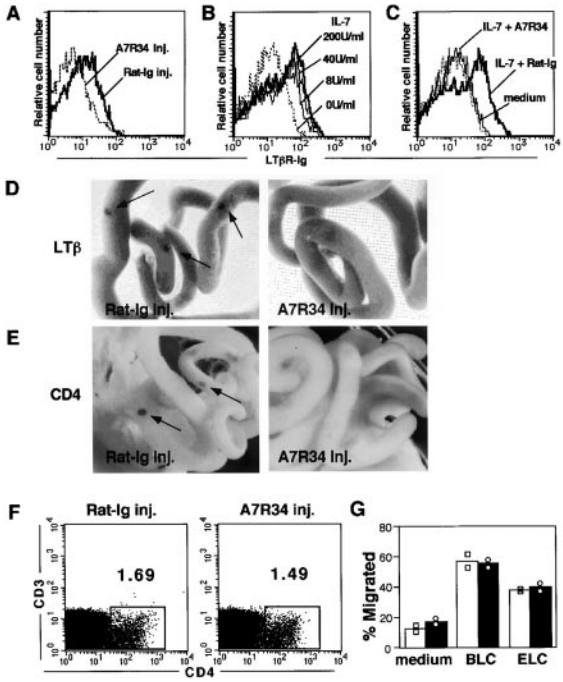

Figure 5. IL-7 induces LTα/β upregulation in PP inducers. (A–C) Histograms show LTβR-Ig staining in $CD4^+$ cells. (A) $CD4^+$ cells from E17.5 intestine after in utero treatment with A7R34 or control rat IgG at E15.5 were analyzed. inj., injected. (B) $CD4^+$ cells from E15.5 intestine were analyzed after in vitro cultures in the absence or presence of rIL-7 at indicated amounts for 6 h. (C) Cells were preincubated with 3 μg/ml A7R34 or equal amounts of polyclonal rat IgG and then incubated with rIL-7 (40 U/ml) for 6 h. (D–G) E17.5 intestines after in utero treatment with either A7R34 or control rat IgG at E15.5 were analyzed. (D) Whole-mount in situ hybridization analysis of LTβ expression. Arrows indicate positive spots. (E) Whole-mount immunostaining with anti-CD4 mAb. Arrows indicate accumulation of $CD4^+$ cells. (F) The number of $CD4^+CD3^-$ cells in the intestine was measured by FACS®. (G) Chemotactic response of $CD4^+$ cells from intestines after treatment with either control rat IgG (white bars) or A7R34 (black bars) to BLC or ELC.

LT expression in $CD4^+$ cells of the PP anlage (Fig. 5, A and D). To test whether IL-7 directly induces LTα/β expression, $CD4^+$ cells sorted from embryonic intestines were incubated in vitro with rIL-7. This stimulation with rIL-7 resulted in induction of LTα/β in a dose-dependent manner (Fig. 5 B). The induction was inhibited by preincubation of the cells with A7R34 (Fig. 5 C). It thus appears likely that V^+I^+ cells are induced by LTα/β on the surface of PP inducers that are expressed in response to IL-7Rα signals.

Cellular Interactions Required for Chemokine Expression. In concert with the failure of V^+I^+ cell formation in PP anlagen, a notable defect in accumulation of $CD4^+$ cells was observed in A7R34-treated intestines (Fig. 5 E). Under these conditions, the distribution of $CD4^+$ cells in the gut at E17.5 resembled that observed at E15.5, although the number of $CD4^+CD3^-$ cells in the intestine measurable by FACS® was comparable to that in the control intestine (Fig. 5 F). Treatment with A7R34 was found not to affect directly the migratory response of $CD4^+$ cells to BLC and ELC (Fig. 5 G). These results suggested that the signaling pathway from IL-7R to LTα/β is required for the expression of BLC and ELC in V^+I^+ cells. A recent study on splenic follicle formation also showed that LTα/β signals upstream of BLC and ELC (16). We measured the RNA levels of BLC and ELC in A7R34- or control rat IgG–treated embryonic intestines by Northern blot analysis and whole-mount in situ hybridization. Pregnant mice were given intravenous injections of A7R34 at E15.5 and intestines were collected at E17.5. Compared with mice that received control rat IgG, ELC message was not detected in the intestines of A7R34-treated embryos (Fig. 6 A). Although BLC expression was detectable in the A7R34-treated embryos, its expression level was also reduced markedly by this treatment. Likewise, ELC and BLC in the PP anlage were not observed by in situ hybridization in intestines of A7R34-treated embryos (Fig. 6 B). These results strongly suggest that LTα/β production by PP inducers in response to the ligand for IL-7R is requisite for chemokine expression by V^+I^+ cells.

Moreover, at E17.5 when chemokine producing regions have already formed, treatment with A7R34 still inhibited the expression of ELC and BLC, as measured by in situ hybridization (data not shown). This result indicates that IL-7R/LTα/β signaling is also required for the maintenance of chemokine expression in the PP anlage.

Evidence for Mesenchymal Origin of V^+I^+ Cells. Recent studies have shown that stromal cells, dendritic cells, and endothelial cells are sources of chemokines (30, 31). To de-

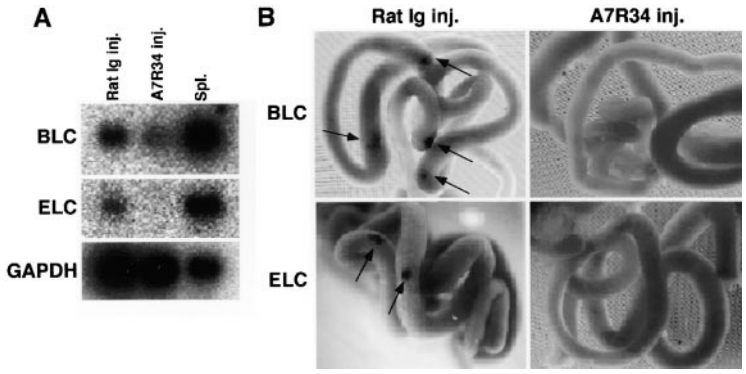

Figure 6. Signal pathway from IL-7R to LTα/β regulates expression of BLC and ELC. (A) Northern blot analysis of BLC and ELC mRNA from E17.5 intestines after treatment with either A7R34 or polyclonal rat IgG at E15.5. GAPDH was used as control to quantitate amount of RNA loaded. Total RNA isolated from Spl tissue was loaded as positive control. inj., injected. (B) Whole-mount in situ hybridization analysis of BLC and ELC expression in E17.5 intestines after treatment with A7R34 or control rat IgG at E15.5. Several spots (arrows) positive for expression of BLC and ELC are visualized in the control intestine.

termine the lineage derivation of V^+I^+ cells, we checked for expression of various cell surface markers. Flow cytometry analyses showed that V^+I^+ cells were negative for CD45 (a marker for hematopoietic cells), CD11c (a marker for dendritic cell), and VE-cadherin (a marker for vascular endothelial cells) expression, whereas they expressed both PDGFRα and PDGFRβ (Fig. 7 A, and Table I). Although PDGFRα and PDGFRβ are singly expressed in a variety of cell lineages (32, 33, 34), coincident expression of both rarely occurs in nonmesenchymal lineages, suggesting a mesenchymal origin for V^+I^+ cells.

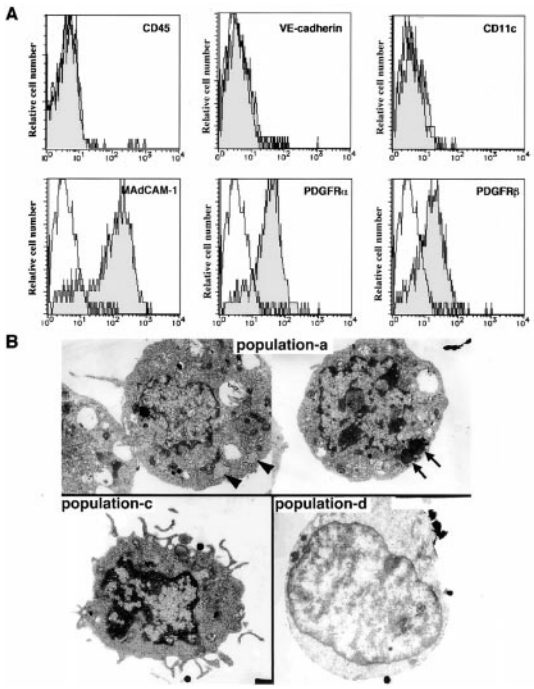

Figure 7. V^+I^+ cells are of a mesenchymal lineage. (A) Three-color FACS® analysis of various cell surface molecules (CD45, CD11c, VE-cadherin, PDGFRα, PDGFRβ, and MAdCAM-1; filled histograms) or isotype-matched mAbs (open histograms) on gated V^+I^+ cells. (B) Representative transmission electron microscopic appearance of cells in the populations indicated in Fig. 3 B obtained from the gut at E17.5. FACS®-sorted cells were fixed and embedded for sectioning. Arrowheads and arrows indicate well-developed endoplasmic reticulum and lipid droplets, respectively.

To determine if indeed this is the case, we prepared V^+I^+ cells as single cell suspensions and analyzed them by electron microscopy along with other fractions (population c and d in Fig. 3 B) obtained from the gut at E17.5 (Fig. 7 B). Pale euchromatic nuclei and relatively rich cytoplasm were common features of the population a (V^+I^+ cells) and d cells. Compared with fewer cytoplasmic component in population d cells, however, V^+I^+ cells had well-developed endoplasmic reticulum (arrowheads), many vacuoles, and occasional lipid droplets (arrows). These morphologic features suggested high secretory activity and resembled cells from lymphoid organ environments such as thymic mesenchymal cells (35) or bone marrow reticular cells (36). In contrast, population c cells showed many cytoplasmic projections that are characteristic to dendritic cells. About 60%

Table I. *Phenotypic Characterization of V^+I^+ Cell*

Surface antigen	Expression
CD45	−
CD11c	−
CD11b	−
B220	−
CD3	−
CD4	−
CD40	+
IL-7Rα	−
VE-cadherin	−
Flk-1	−
PECAM-1	−
VCAM-1	High
ICAM-1	High
MAdCAM-1	High
PDGFRα	+
PDGFRβ	+
BP-3	−
FDC-M1	−
CD35	−
CD23	−

of population c cells actually express CD11c (data not shown).

Taken together, we concluded that V^+I^+ cells were likely from the mesenchymal lineage. Mutual interaction between the hematopoietic PP inducer and the mesenchymal V^+I^+ cells appears to form the major framework of cellular interaction involved in PP anlagen formation.

Discussion

Histological events that characterize the initial phase of PP organogenesis are the appearance of segregated regions expressing VCAM-1 and ICAM-1 and concomitant accumulation of various hematopoietic cells. Among the hematopoietic lineages that appear in the PP anlage, IL-7Rα^+CD3$^-$ cells are likely to be the most important for PP formation, as only this population expresses the LTα/β requisite for PP organogenesis (10). However, little is known about how these cells congregate in the PP anlage, although a variety of chemokines have been implicated in the accumulation of hematopoietic cells. Recently, Mebius et al. demonstrated that CD4$^+$CD3$^-$ cells in the embryonic mesentery express CXCR5 (24). Here we elucidate the molecular mechanisms regulating cell–cell interaction required for PP organogenesis, with particular emphasis on the involvement of chemokines.

Consistent with previous reports, CD4$^+$CD3$^-$IL-7Rα^+ cells sorted from the embryonic gut were found to express the BLC receptor, CXCR5. Moreover, we found that CCR7, the receptor for ELC and SLC, is also expressed by this population. Thus, PP inducers express the receptors for homeostatic chemokines and we confirmed that both receptors are functional by in vitro migration assays. Surprisingly, in response to BLC, PP inducers demonstrated a comparable level of chemotactic activity to that of B cells, which have been considered the specific target of BLC (30). In situ hybridization analyses revealed that ELC and BLC expression become detectable concurrently in PP anlagen. Taken together, it is highly likely that these two chemokines play a role in the clustering of PP inducers.

Previous studies showed that null mutations of BLC or CXCR5 result in reduction of the number of PPs in addition to defects in organized peripheral lymphoid tissue formation (11, 12). This strongly supports involvement of BLC/CXCR5 in PP induction, but the partial phenotype suggests that CCR7 might compensate this defect to some extent. Despite the specific localization of ELC expression in PP anlagen, CCR7 null mice develop normal numbers of PPs (37), indicating that BLC is likely the major chemokine regulating migration of the PP inducer.

If BLC and ELC are the molecules responsible for accumulation of PP inducers, a possible scenario is that chemokine-producing regions are established before the involvement of PP inducers. Thus, the IL-7Rα–dependent process of PP induction would occur after accumulation of PP inducers in the preestablished PP anlage. However, we showed here that production of chemokines as well as VCAM-1/ICAM-I expression are dependent on IL-7Rα–induced production of LTα/β by PP inducers. This indicates that chemokine expression, which we believe requisite for the migration of PP inducers, are in turn induced by PP inducers themselves. Moreover, although chemokine producing regions have already formed at E17.5, maintenance of this region appears dependent on LTα/β stimulation. Hence, it is likely that PP inducers are the cells which act on surrounding tissues to induce chemokines, and IL-7Rα and LTα/β are molecules involved in upstream regulation of chemokine production in the PP anlagen.

We investigated whether or not V^+I^+ cells are the producers of ELC and BLC. For this purpose, we established conditions to allow dissociation of V^+I^+ cells from embryonic intestines and performed flow cytometry analysis which enabled us to address the following points. First, V^+I^+ cells express LTβR and thus could be the target of PP inducers. Furthermore, they produce ELC and BLC. Although VCAM-1 single-positive cells also express low levels of BLC, V^+I^+ cells appear to be the sole population expressing ELC. Third, multicolor analysis of this population revealed expression of both PDGFRα and PDGFRβ, but no hematopoietic or endothelial markers, a feature that is rarely found except among mesenchymal lineages. Morphological analysis of purified V^+I^+ cells indicated that they shared the characteristics of stromal cell components supporting lymphoid organs such as thymic (35) and bone marrow reticular cells (36). Taken together, these observations support that V^+I^+ cells belong to the mesenchymal lineage and are activated by PP inducers to express VCAM-1, ICAM-1, ELC, and BLC; conversely all of these molecules have the potential to attract PP inducers and other hematopoietic cell lineages to the PP anlage. Thus, in the sense that V^+I^+ cells are an active component of PP organogenesis rather than a passive target of the PP inducer, our terminology "PP organizer" appears appropriate (21, 22).

Interestingly, V^+I^+ cells express MAdCAM-1 and CD40, two molecules that are rarely observed in the mesenchymal lineage. We speculate that expression of these molecules may reflect representation by V^+I^+ of an activated state of mesenchymal cells. Another active mesenchymal like cell is the follicular dendritic cell (FDC) for which extensive, but to date yet inconclusive, attempts have been undertaken to identify its derivation. All of the above molecules have been reported to be expressed in FDCs, thus suggesting a relationship between V^+I^+ cells and FDCs. However, other FDC-specific markers such as FDC-M1 or CD35 could not be detected in V^+I^+ cells. In any case, determination of the lineage derivation of FDCs remains an important issue.

A striking feature of PP inducers and organizers is the extent to which they are mutually complementary. PP inducers express LTα/β to activate PP organizer, while PP organizers express chemokines to activate PP inducers. This interdependent relationship in which both components are at the same time activator and responder raises a question that remains still unanswerable; namely, what is

the initial event for PP induction? The answer to this question might also explain how the position of PP anlagen is determined. In an earlier review, we presented a model that the first event for PP induction is the activation of PP inducers by the ligand for IL-7Rα, which should be expressed by cells distinct from either PP inducer or organizers (22). If this is the case, we need to identify which cells express the IL-7Rα ligand and how expression of this ligand is induced in the specific region from which PP anlagen develop. However, our extensive attempts to pinpoint expression of IL-7 and thymic stroma–derived lymphopoietin (TSLP) in the embryonic intestine have so far failed. In any case, the above model requires involvement of two cellular components to trigger the first event, with subsequent inducer/organizer interaction.

An attractive explanation for this process involving only two cellular compartments is the "reaction-diffusion model" (38), proposing that PP position is determined not entirely by program but by interaction between two diffusible factors, one of which is essential for initiating the developmental process. Such a mechanism has been implicated in various situations such as stripe formation in fish (39), and more recently, determination of hair follicle anlagen (40). This model, though simpler, is difficult to prove.

Whatever the mechanisms underlying the triggering process, we think that interaction between PP inducer and PP organizer starts in a few cells, but the segregated PP anlagen become detectable rapidly within 24 h. This process is reminiscent to the process of delayed-type hypersensitivity (DTH) reaction in the skin, in which antigen-specific T cells enter in the antigen injected site and recruit many other cells within 24 h. Indeed, previous studies demonstrated that even a single antigen-reactive T cell can induce this response (41). Likewise, once a small interaction unit with PP inducer and PP organizer are stimulated, it rapidly spreads to a large region by recruiting more and more inducers in the region. Such a similarity suggests that the inflammatory process may be a prototype from which the mechanisms underlying PP organogenesis are derived.

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