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*J Immunol* 2009; 182:2610-2619; doi: 10.4049/jimmunol.0801141

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Chemokine Receptor CXCR5 Supports Solitary Intestinal Lymphoid Tissue Formation, B Cell Homing, and Induction of Intestinal IgA Responses

Sarvari Velaga,* Heike Herbrand,* Michaela Friedrichsen,* Tian Jiong,† Martina Dorsch,‡ Matthias W. Hoffmann,† Reinhold Förster,* and Oliver Pabst†‡

Solitary intestinal lymphoid tissue (SILT) comprises a spectrum of phenotypically diverse lymphoid aggregates interspersed throughout the small intestinal mucosa. Manifestations of SILT range from tiny lymphoid aggregates almost void of mature lymphocytes to large structures dominated by B cells. Large SILT phenotypically resemble a single Peyer’s patch follicle, suggesting that SILT might contribute to intestinal humoral immune responses. In this study, we track the fate of individual SILT in vivo over time and analyze SILT formation and function in chemokine receptor CXCR5-deficient mice. We show that, in analogy to Peyer’s patches, formation of SILT is invariably determined during ontogeny and depends on CXCR5. Young CXCR5-deficient mice completely lack SILT, suggesting that CXCR5 is essential for SILT formation during regular postnatal development. However, microbiota and other external stimuli can induce the formation of aberrant SILT distinguished by impaired development of B cell follicles in CXCR5-deficient mice. Small intestinal transplantation and bone marrow transplantation reveal that defect follicle formation is due to impaired B cell homing. Moreover, oral immunization with cholera toxin or infection with noninvasive Salmonella fail to induce efficient humoral immune responses in CXCR5-deficient mice. Bone marrow transplantation of CXCR5-deficient recipients with wild-type bone marrow rescued B cell follicle formation in SILT but failed to restore full humoral immune responses. These results reveal an essential role of CXCR5 in Peyer’s patch and SILT development and function and indicate that SILT do not fully compensate for the lack of Peyer’s patches in T cell-dependent humoral immune responses. The Journal of Immunology, 2009, 182: 2610–2619.

Received for publication April 7, 2008. Accepted for publication December 18, 2008.

The Journal of Immunology

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0801141
cells. This signal triggers the organizer cells to secrete the chemokine CXCL13 that signals back to the CXC receptor CXCR5, thereby augmenting the expression of lymphotxin-α, lymphotxin-β receptor, CXCL13, or CXCR5. In addition to LTIC, CXCR5 is expressed by B cells and regulates B cell homing and follicle formation in lymphoid organs (15, 16). In particular, B cell homing into PP depends on CXCR5, since in PP a unique type of high endothelial venules localized in the B cell follicles displays luminal CXCL13 and facilitates the direct entry of B cells (17).

The development of SILT shares important features with PP development. Like PP, SILT are absent in the intestines of lymphotoxin-β receptor-deficient mice (4, 18). Moreover, SILT, as well as PP, fail to form in mice lacking the orphan nuclear receptor RORγt, which is required for the development and maintenance of LTIC (19).

The chemokine receptor CCR6 is expressed on B cells in ILF. Transition of CP to ILF, which is mainly characterized by the influx of B lymphocytes, is triggered through expression of CCR6 ligands by epithelial cells (10) and blocked in CCR6-deficient mice. In contrast, the overall number of CP and accumulation of dendritic cells are unaffected in these mutants (20). Lymphocyte influx into PP is also reduced in CCR6-deficient mice, resulting in smaller PP (21). Another chemokine receptor strongly influencing the phenotype of SILT is CCR7. Absence of CCR7 signaling results in heavily enlarged SILT that are, however, unchanged in frequency and cellular composition when compared with wild-type mice. This phenotype is conferred by bone marrow-derived cells and is independent of the presence of intestinal bacteria, suggesting that hypertrophy of SILT in CCR7-deficient mice does not depend on external stimulation (9).

In this study, we show that just like PP formation, regular initiation of SILT development depends on CXCR5. However, pathways independent of CXCR5 can be triggered in CXCR5-deficient mice through microbiota and other stimuli resulting in the formation of SILT lacking B cell follicles. Impaired follicle formation results from defect homing of CXCR5-deficient B cells. SILT in CXCR5-deficient mice fail to support robust IgA responses upon Salmonella infection or oral immunization with cholera toxin.

Bone marrow reconstitution of CCR5-deficient recipients with wild-type bone marrow cells rescues B cell deficiency in SILT but fails to restore the induction of IgA, indicating that SILT do not fully compensate for the lack of PP in CXCR5-deficient mice in T cell-dependent humoral immune responses.

**Materials and Methods**

**Mice and bone marrow chimera**

BALB/c, C57BL/6, C57BL/6-Ppcre (designated here as Ly5.1 mice), C57BL/6-Tg(ACTbEGFP)10Obf1 (designated here EGPFP mice), B6.Cg-Igh-1B /IPI1-Pplac322 (designated here as Igh7 mice), B6.Jtg-6wt/wt (designated here as μMT-H2B), and B6.Cg-B1r+1st103 (designated for at least 15 generations to C57BL/6 mice (designated here as CXCR5-deficient mice) were bred at the central animal facility of the Hannover Medical School under specific pathogen-free (SPF) conditions. C57BL/6 mice were additionally purchased from Charles River. CXCR5-deficient germfree mice were generated and bred at the central animal facility at the Hannover Medical School and analyzed at the age of 8, 10, or 20 wk. For generation of bone marrow chimeras, recipients were lethally irradiated with a single dose of 10 Gy and transplanted with 10⁶ congeneric bone marrow cells purified by discontinuous Lympholite-M gradient centrifugation. Chimeras were analyzed 8 wk after transplantation. Chimerism was above 90% for all chimeras used in the immunization experiments. All animal experiments have been performed in accordance with institutional guidelines and have been approved by the local committees.

**Oral immunizations and Salmonella infections**

For all infection experiments, an attenuated, noninvasive streptomycin-resistant derivative of the Salmonella enterica serovar typhimurium strain SL1344, which is referred to as SL1344 arak ssrB (22, 23), was used. Bacteria were grown in Luria-Bertani broth until an OD of 1.35 was reached, washed twice with PBS, and suspended to density of 10⁹ bacteria/100 μl of PBS. Mice continuously received streptomycin (5 mg/ml) via the drinking water starting 1 day before infection and were inoculated orally with 100 μl of bacterial suspension with a feeding needle. The number of inoculated bacteria was confirmed by serial plating of appropriate dilutions onto Luria-Bertani agar plates and overnight incubation. Intestinal colonization with Salmonella was confirmed by weekly plating of fecal IgA levels in the serum and ileum washes and intestinal washes were analyzed 8 days after infection. For oral immunization, mice received 20 μg of cholera toxin (Sigma-Aldrich) in 100 μl of PBS by gavage and were analyzed 10 days after immunization. Intestinal washes were collected by flushing 10 cm of the small intestine with 400 μl of PBS containing 0.1 mg/ml trypsin inhibitor, 50 nM EDTA, and 0.1% BSA.

**ELISA**

Ninety-six-well Nunc microprep plates were coated with 1 μg/ml cholera toxin or Salmonella LPS (Sigma-Aldrich) and incubated at 4°C overnight. Plates were washed once with PBS/0.05% Tween 20 and blocked with PBS/0.5% BSA/0.1% Tween 20 and incubated for 1 h at 37°C. Plates were thoroughly washed with PBS/0.05% Tween 20 and incubated with alkaline phosphatase-conjugated Abs appropriately diluted in PBS/0.5% BSA/0.1% Tween 20 for 1 h at 37°C. Plates were washed with PBS/0.05% Tween 20 and before adding 3, 5, 5'-tetramethylbenzidine dihydrochloride (Sigma-Aldrich). Absorbances were measured at 450 nm.

**Intestinal surgery**

Mouse vascularized small bowel transplantation was performed as previously described (24), with some modifications: C57BL/6 Ly5.1 mice were used as donors and congenic CXCR5-deficient Ly5.2 mice as recipients. Bacteria were grown in Luria-Bertani broth until an OD of 1.35 was reached, washed twice with PBS, and suspended to density of 10⁹ bacteria/100 μl of PBS. Mice continuously received streptomycin via drinking water starting 1 day before infection and were inoculated orally with 100 μl of bacterial suspension with a feeding needle. The number of inoculated bacteria was confirmed by serial plating of appropriate dilutions onto Luria-Bertani agar plates and overnight incubation. Intestinal colonization with Salmonella was confirmed by weekly plating of fecal IgA levels in the serum and ileum washes and intestinal washes were analyzed 8 days after infection. For oral immunization, mice received 20 μg of cholera toxin (Sigma-Aldrich) in 100 μl of PBS by gavage and were analyzed 10 days after immunization. Intestinal washes were collected by flushing 10 cm of the small intestine with 400 μl of PBS containing 0.1 mg/ml trypsin inhibitor, 50 nM EDTA, and 0.1% BSA.

**Antibodies**

The following Abs and conjugates were used in this study: goat anti-mouse IgA-peroxidase (Zymed Laboratories), goat anti-mouse IgM-peroxidase (Nordic Immunology), rat anti-mouse IgG-peroxidase (Jackson ImmunoResearch Laboratories), anti-CXCR5, anti-CCR5 (clone 12G5; Caltag Laboratories), anti-CD19-allophycocyanin (clone M1/70.15; Caltag Laboratories), anti-CD117-biotin (clone 104), anti-TER-119-biotin, anti-CD11c-biotin (clone N418; BioLegend), rat anti-mouse IgG1-peroxidase (Zymed Laboratories), and rat anti-mouse IgG2a-peroxidase (Zymed Laboratories). The following Abs and conjugates were used in this study: goat anti-mouse IgA-peroxidase (Zymed Laboratories), goat anti-mouse IgM-peroxidase (Nordic Immunology), rat anti-mouse IgG-peroxidase (Jackson ImmunoResearch Laboratories), anti-CD4 (clone RM4-5), anti CD19-allophy-26, and anti-CD3 (clone 17B7; Caltag Laboratories), and anti-CD4-PE (Southern Biotechnology Associates), anti-CD11b-PE (clone M1/70.15; Caltag Laboratories), anti-CD117-PE-cyanocarbonyl-biotin (clone ACK2; eBioscience), anti-IgL-1-biotin (clone A20; Chemicon International), anti-IgD, anti-GR-1, anti-CD4, anti-B220, anti-CD3, and anti-CXCR5 were provided by E. Kremmer (GSF National Research Center for Environment and Health, Munich, Germany). Anti-B220, anti-CD3, and anti-CD4 were coupled to Cy3 (Jackson ImmunoResearch Laboratories), FITC (Sigma-Aldrich), Cy5 (GE Healthcare), or Pacific Orange (Invitrogen).
Cell preparation and flow cytometry

Adult mice were sacrificed by CO2 inhalation and cervical dislocation. The small intestines were flushed with PBS and opened along the mesenteric border. SILT were cut out with a stab knife (5.0-mm blade; Fine Science Tools) using a stereo microscope. To obtain single-cell suspensions, SILT were placed in PBS containing 3% FCS and passed through a nylon mesh. Lineage-positive cells were excluded by incubation with a mixture of biotinylated Abs directed against CD3, B220, CD11c, CD11b, TER-119, and GR-1 that were detected by streptavidin conjugated to PerCP. Dead cells were excluded by 4',6-diamidino-2-phenylindole (DAPI) staining. Data were acquired using an LSR II flow cytometer (BD Biosciences) and analyzed using FACSDiva software (BD Biosciences) or FlowJo (Tree Star).

Immunofluorescence microscopy

Immunohistochemistry was performed as described previously (7). In brief, isolated small intestines were flushed with cold PBS, opened along the mesenteric site, and 2-cm-long pieces were flattened on filter paper. Tissue was frozen in OCT on dry ice. Eight-micrometer cryosections were prepared, air dried, and fixed for 10 min in ice-cold acetone. Sections were rehydrated with TBST (0.1 M Tris (pH 7.5), 0.15 M NaCl, and 0.1% Tween 20) and blocked with TBST containing 5% rat or mouse serum depending on the source of Ab. Sections were incubated with different sets of Abs diluted in TBST supplemented with 2.5% rat or mouse serum. Sections were washed with TBST containing 1% FCS and nuclei were stained with DAPI and covered with MOWIOL. Fluorescence images were made using an Axios1 microscope and analysisIS D software (Olympus) and composite images were taken using a Zeiss Axiovert 200M microscope and analyzed with Axiovision software.

Statistical analysis

Cholera toxin and Salmonella-specific Ig levels were normalized to the levels observed in wild-type mice. Statistical analysis was performed using GraphPad Prism software. To test the significance of data, we used an unpaired Student’s t test. Data are expressed as means ± SD. Statistical differences of the mean values are indicated as follows: *, p < 0.05 and **, p < 0.01.

Results

SILT constitute stable lymphoid aggregates in vivo

Analyzing the frequency of SILT present under various experimental conditions, we observed that the total number of SILT per small intestine is invariant (7, 9). This suggests that SILT, once established during postnatal development, might not newly form or disappear. However, definitive proof of this concept requires the tracking of individual SILT in the same animal in vivo over time. To this aim, we established a new method to visualize SILT. Adult wild-type C57BL/6 mice were lethally irradiated and reconstituted with bone marrow cells expressing the GFP in all cells (GFP-chimera). A, left image. SILT can easily be identified from the luminal side as fluorescent dome-shaped structures with reduced height compared with surrounding villi. More dispersed fluorescent cells can be observed within the villi. A, right image. From the serosal side PP and SILT of GFP-chimeras appear as brightly fluorescent aggregates. Some SILT are marked by white arrows and PP appear as large multinodular structures. B, Stability of SILT pattern over time. The upper micrograph shows the distribution of SILT in the small intestine of a GFP-chimera exposed under anesthesia by laparotomy (day 0). Three weeks after surgery mice were sacrificed and the distribution of SILT in the same gut segment was reanalyzed (day 21). Dotted white lines facilitate identification of SILT pattern; white arrows indicate SILT that was spotted only at the second but not the first inspection of the small intestine. C, Summarized results for six jejunal gut segments derived from six individual mice analyzed at intervals of 3 wk. Sections depicted in B refer to mouse 2 in the table.

Expression of the chemokine receptor CXCR5 in SILT cells

Development of PP during gestation as well as homing of naive B cells into mature PP depend on the chemokine receptor CXCR5 (15, 17). To delineate a potential role for CXCR5 in SILT development and homeostasis, we analyzed the expression of CXCR5 in SILT cells. SILT were microdissected from the intestines of BALB/c mice and analyzed for the expression of c-Kit, CD4, and CXCR5 on lineage− (lin−: CD3−B220−CD11b−CD11c−).
judged by quantitative real-time PCR (data not shown). ROR
we observed that both CD4

FIGURE 2. Expression of CXCR5 in SILT cells. A–D, Expression of CXCR5 was analyzed by flow cytometry. Single-cell suspensions were prepared from SILT microdissected from the small intestines of BALB/c mice and stained for CXCR5, c-Kit, CD4, and a lineage mixture containing Abs directed against CD3, B220, CD11c, CD11b, TER-119, and GR-1. A, CD4 expression defines two subpopulations of c-Kit+lin− SILT cells. B–D, Histograms illustrate weak expression of CXCR5 on lin− c-Kit+CD4+ cells (B), profound levels of CXCR5 on CD19+ cells (C), and no detectable CXCR5 expression on lin− c-Kit+CD4+ cells (D). Light gray lines indicate isotype control stainings. E, Location of c-Kit+CD4+CD3− cells in SILT. Horizontal sections from the small intestine of a C57BL/6 mouse were stained for c-Kit (blue), CD4 (green), and CD3 (red). c-Kit+CD4+CD3− cells (marked by white arrows) constitute a subpopulation of all c-Kit+ cells present in SILT.

also been reported to be expressed in lin− c-Kit+ lamina propria cells (25). In any case, our data reveal that at least two distinct populations of cells present in SILT, i.e., CD19+ B cells and lin− c-Kit+CD4+ cells, express CXCR5, suggesting that CXCR5 might fulfill several tasks in SILT formation and function.

CXCR5 is essential for regular SILT formation during postnatal development

Formation of PP during fetal development depends on CXCR5 (15). Penetration of this phenotype is subject to genetic background variations (26). However, we never observed any macroscopically discernible PP in the colony of CXCR5-deficient mice backcrossed to the C57Bl/6 background for at least 15 generations that was used throughout this study (data not shown). Impaired PP development can be rescued by adoptive transfer of wild-type LTIC isolated from fetal mesenteric lymph nodes into newborn CXCR5-deficient mice, indicating that CXCR5-sufficient LTIC can induce lymphoid organ development (27). We thus speculated that CXCR5 might serve an inductive function during SILT development. Yet, when analyzing cryosections of small intestines of adult 8- to 10-wk-old CXCR5-deficient mice, lymphoid aggregates were readily detectable, indicating that SILT can develop independent of CXCR5 (Figs. 3 and 4C). Moreover, the density of structures, i.e., the number of structures per area, closely matched the density of SILT observed in wild-type mice (data not shown). In contrast, at 4 wk of age, SILT were readily detectable in the small intestines of wild-type but not CXCR5-deficient mice (Fig. 3). Thus, CXCR5 appears to be indispensable for the regular developmental program generating SILT during early postnatal development. However, CXCR5-independent mechanisms can lead to the generation of lymphoid aggregations in the intestines of 8-wk-old CXCR5-deficient mice.

Initiation of SILT formation does not depend on microbial stimulation as revealed by the presence of normal numbers of SILT in germfree mice (9). However, microbial stimulation drives the
compared with 8-wk-old germfree CXCR5-deficient mice (Fig. 4A). An increase in the number of lymphoid aggregates in 20-wk-old germfree CXCR5-deficient mice was evident under SPF conditions. B cell follicles were designated class A, structures containing up to 20% of B cells were designated class B, and a discernible B cell core was designated class C. SILT displaying a prominent B cell follicle constituting >50% of the overall cell number were designated class D (Fig. 4A). For technical reasons, B cells in SILT were routinely identified by using anti-B220 stainings. However, B220-expressing cells in SILT could also be stained using anti-IgD Abs, indicating that the vast majority of all B220 staining cells in SILT are indeed naive B cells (data not shown and Ref. 7). Classifying the spectrum of SILT in C57BL/6 wild-type mice as described above yields a two-dimensional histogram (Fig. 4B), illustrating that in wild-type mice an increase in size coincides with a higher B cell content (Fig. 4B) as previously reported (7).

SILT in CXCR5-deficient intestines fail to accumulate B cells

SILT in adult 8-wk-old CXCR5-deficient mice encompassed a size spectrum of structures that was shifted in favor of large-sized SILT, i.e., SILT classified to size classes IV, V, and VI, when compared with wild-type mice (cf Fig. 4, A and C). The cellular composition of SILT in CXCR5-deficient mice displayed striking differences compared with wild-type controls. In wild-type mice, the majority of SILT contains a substantial number of B cells and are thus classified as classes C and D (Fig. 4B). In contrast, SILT in CXCR5-deficient mice were almost completely devoid of B cells as evidenced by the prevalence of class A and B structures (Fig. 4C). The size spectrum of SILT in 20-wk-old germfree CXCR5-deficient mice resembled that of CXCR5-deficient mice kept under SPF conditions (Fig. 4D). However, germfree CXCR5-deficient mice showed a further decreased content of B cells as evidenced by an increased proportion of class A structures compared with CXCR5 mutants kept under SPF conditions (cf Fig. 4, C and D). This suggests that microbial stimulation contributes to the accumulation of B cells in SILT of CXCR5-deficient mice. In CXCR5-deficient mice, the impact of intestinal microflora on the composition of SILT is not as obvious as in wild-type mice (9), as a pronounced shift toward B cell-depleted structures is already evident under SPF conditions.

Reduction of class C and D structures in CXCR5-deficient mice might be attributable to the incapability of CXCR5-deficient B cells to enter the structures. In a previous study, we could show that following small bowel transplantation B cells rapidly exchange between host and graft tissue and immunofluorescence microscopy revealed that the vast majority of such B cells localized in SILT (7). Thus, to evaluate the role of CXCR5 in the homing of B cells to SILT, we performed small bowel transplantations. Small intestinal fragments isolated from Ly5.1+ wild-type donors were grafted into congenic Ly5.2+ CXCR5-deficient recipients. The spectrum of SILT present in the wild-type graft 60 days after transplantation closely resembled those observed in the CXCR5-deficient host intestine or in untreated CXCR5-deficient mice (cf Figs. 5, A and B, to 4C). Immunofluorescent microscopy revealed that the few scattered B cells present in the SILT of the grafted intestine were of host origin (B220+Ly5.2+Ly5.1+; Fig. 5C), whereas the majority of c-Kit+ cells were of donor origin (cKit+Ly5.2−).
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Ly5.1+ (Fig. 5C). Thus, following intestinal transplantation, SILT B cells egress from the grafted tissue and cannot be replaced by host CXCR5-deficient B cells, even though a CXCR5-sufficient population of c-Kit+-expressing cells is retained in the graft. We therefore suggest that the shift in the SILT spectrum in the graft tissue directly reflects the impaired homing of host CXCR5-deficient B cells into SILT.

To confirm this hypothesis by an independent experimental approach, we transplanted irradiated wild-type Ly5.1+ recipients with equal mixtures of bone marrow derived from CXCR5-deficient Ly5.2+ mice and B cell-deficient Ly5.1+ mice. In these chimeras, all B cells will lack CXCR5, whereas any other cell types will contain a substantial number of CXCR5-sufficient wild-type cells. Consistent with our observations obtained by small bowel transplantations, SILT in such mixed bone marrow chimeras were almost completely devoid of B cells (Fig. 6, A and B). In yet another approach, we reconstituted CXCR5-deficient recipients with equal mixtures of wild-type Ly5.1+ and CXCR5-deficient Ly5.2+ bone marrow cells. In the resulting chimeras, CXCR5-sufficient and CXCR5-deficient hematopoietic cells can be distinguished using congenic markers, allowing us to directly compare the ability of wild-type and CXCR5-deficient cells to settle into SILT. In such chimeras, we observed the formation of B cell follicles and furthermore confirmed that the vast majority of B cells in SILT originated from CXCR5-sufficient Ly5.1 B cells (purple cells in Fig. 6C that express Ly5.1 (blue) and B220 (red)). This indicates that lack of CXCR5 expression on B cells is sufficient to cause the scarcity of B cells observed in SILT of CXCR5-deficient mice and suggests that CXCR5 expression on B cells is required for B cell homing into SILT.

CXCR5-deficient mice fail to mount efficient humoral responses to intestinal Ags

The general lack of PP and B cell follicles in SILT in CXCR5-deficient intestines prompted us to investigate humoral immune responses elicited by intestinal Ags in these mice. To this end, CXCR5-deficient mice and wild-type controls were orally immunized with a single dose of cholera toxin. After 10 days, the levels of cholera toxin-specific Igs in serum and intestinal washes were determined. In CXCR5-deficient mice, the amount of cholera toxin-specific IgA in the intestine was significantly decreased to ~40% of the wild-type level. Strikingly, Ag-specific Ig levels in the serum almost matched those of wild-type controls (Fig. 7A). To extend this observation, we next tested the immune response of CXCR5-deficient mice toward the attenuated noninvasive Salmonella enterica serovar typhimurium strain SL1344 aroA ssrB. CXCR5-deficient mice and wild-type controls were orally infected with 108 CFU of Salmonella SL1344 aroA ssrB and continuously treated with antibiotics via the drinking water to allow for a persistent colonization of the intestine. Infected mice shedded Salmonella with the feces until sacrifice, indicating persistent colonization (data not shown). Interestingly, in CXCR5-deficient mice, infection with Salmonella yielded significantly decreased levels of pathogen-specific IgA in both, intestinal washes and serum compared with wild-type mice (Fig. 7B). Total levels of serum IgM, IgG, and IgA as well as intestinal IgA were unchanged in CXCR5-deficient mice (data not shown and Ref. 28).

Impaired IgA responses in face of rescued follicle formation in SILT of CXCR5-deficient mice reconstituted with wild-type bone marrow

As described above, transplantation of irradiated CXCR5-deficient recipients with mixtures of wild-type and CXCR5-deficient bone...
Impaired humoral responses in CXCR5-deficient mice. CXCR5-deficient mice (CXCR5<sup>−/−</sup>) and wild-type mice (WT) were orally immunized with 20 μg of cholera toxin (A) or orally infected with 10<sup>8</sup> attenuated Salmonella (B). After 10 or 28 days, respectively, serum titers of Ag-specific IgM, IgG, and IgA as well as intestinal IgA were determined by ELISA. A. Following immunization with cholera toxin, CXCR5 deficiency led to a 2-fold reduction of Ag-specific IgA in the intestinal washes but did not change the levels of Ag-specific IgM in the serum (CXCR5<sup>−/−</sup>: n = 13; wild type: n = 12; results are representative of three individually performed experiments). B. In contrast, oral infection with Salmonella provoked a significantly reduced humoral response in CXCR5<sup>−/−</sup> mice as evidenced by reduced pathogen-specific Ig in serum and reduced IgA in intestinal washes (CXCR5<sup>−/−</sup>: n = 5; wild type: n = 6; results have been normalized to the Ig levels observed in wild-type mice; significance was revealed by unpaired Student’s t test: *p < 0.05 and **p < 0.01).

FIGURE 8. Transfer of wild-type bone marrow rescues follicle formation in SILT but not efficient production of cholera toxin-specific IgA in CXCR5<sup>−/−</sup> mice. The spectrum of SILT in bone marrow chimeras was determined as described for Fig. 4. Bone marrow cells from wild-type mice were injected i.v. into lethally irradiated CXCR5<sup>−/−</sup> mice and vice versa (wild type to CXCR5<sup>−/−</sup>: n = 12; CXCR5<sup>−/−</sup> to wild type: n = 8). A. SILT in wild-type mice transplanted CXCR5<sup>−/−</sup> bone marrow cells resembled that of untreated CXCR5 mutants. (176 SILT analyzed pooled from nine mice). B. Conversely, in CXCR5<sup>−/−</sup> mice reconstituted with wild-type bone marrow cells, the spectrum of SILT was shifted toward smaller structures containing B cell follicles, thereby resembling the situation in wild-type mice (403 SILT analyzed pooled from 12 mice; cf. with Fig. 4B). C. Eight weeks after transplantation, bone marrow chimeras were orally immunized with 20 μg of cholera toxin (CXCR5<sup>−/−</sup> recipients receiving wild-type bone marrow: n = 6; wild-type mice receiving CXCR5<sup>−/−</sup> bone marrow: n = 6; isogenic transplantations for CXCR5<sup>−/−</sup>: n = 5; isogenic transplantations for wild-type mice: n = 4; results have been pooled from two independent experiments and normalized to the Ig levels observed in isogenic wild-type transplantations). Ten days after immunization, intestinal washes were collected and the amount of cholera toxin-specific IgA was determined by ELISA. Intestinal washes of CXCR5<sup>−/−</sup> mice contained reduced amounts of IgA irrespective of reconstitution with wild-type or CXCR5-deficient bone marrow. The results depicted are representative of two individual experiments; *p < 0.05.

Consistent with this, wild-type recipients of CXCR5-deficient bone marrow that have poor B cell follicles in SILT still show a higher intestinal IgA titer in response to oral immunization with cholera toxin than CXCR5 mutants transplanted with wild-type bone marrow. In contrast, total IgM, IgG, and IgA levels in the serum showed no differences between CXCR5-deficient mice reconstituted with wild-type bone marrow cells and vice versa (data not shown). These results show that bone marrow transplantation is not sufficient to restore the induction of cholera toxin-specific IgA responses, indicating that defects other than the lack of B cells in SILT of CXCR5-deficient mice prevent the efficient induction of intestinal IgA.

In conclusion, results presented in this study reveal that like PP development, initiation of SILT formation in the endogenous tissue window crucially depends on CXCR5. However, in contrast to PP development, CXCR5-independent formation of aberrant SILT can be triggered by microbial and other stimuli. Still, B cell
homing and follicle formation in both PP and SILT utilize a CXCR5-dependent mechanism. Moreover, CXCR5-deficient mice show reduced humoral responses to T cell-dependent Ags, indicating that SILT do not fully compensate for the lack of PP in these mice.

Discussion

The anatomical location and the number of classical secondary lymphoid organs such as lymph nodes and PP are determined during gestation. Therefore, postnatal events may alter the phenotypic appearance of secondary lymphoid organs, but will not affect their location or number. In contrast, tertiary lymphoid tissue may form ectopically at any time in the adult organism once appropriate location or number. In contrast, tertiary lymphoid tissue may form in Ref. 14). In this study, we provide direct evidence that the positioning and density of SILT are fixed. Using a newly developed experimental approach, we tracked individual SILT in vivo. We observed that in adult mice >95% of all SILT could be retrieved a second time after a time period of 3 wk. Even though at present we cannot exclude that changes in the pattern of SILT would be detectable after longer time periods, these results argue for a stable position of SILT. A low level of uncertain structures might have been overlooked for technical reasons during the first inspection of the small intestine. In contrast to stable SILT patterns, the phenotypic manifestation of SILT is highly dynamic. In particular, microbial stimulation induces a shift in the spectrum of SILT toward follicle-containing structures (8–10). This situation resembles the situation in PP, which like SILT are hypoplastic in germfree mice and rapidly recruit mature lymphocytes upon microbial stimulation (29–31). Based on these observations, we suggest that SILT share decisive features, i.e., fixed positioning and number, with secondary lymphoid organs but not with tertiary lymphoid tissue.

A critical step in lymphoid organogenesis is the interaction of LTIC and mesenchymal organizer cells that allows for the growth of the organ anlage. The chemokine receptor CXCR5 constitutes an essential element in the interaction of LTIC and organizer cells. Consequently, PP as well as inguinal and sporadically cervical lymph nodes are absent from CXCR5-deficient mice (15, 26, 32).

However, CXCR5 does not represent a general prerequisite for lymphoid organogenesis since other lymph nodes form normally in these mice. In this manuscript, we report that 4-wk-old CXCR5-deficient mice entirely lack SILT in the small intestine, suggesting that like PP, regular development of SILT depends on CXCR5. However, during aging, SILT develop independent of CXCR5 and adult CXCR5-deficient and wild-type mice have a comparable density of SILT. The CXCR5-independent mechanism is supported by microbial stimulation and the development of SILT is delayed in germfree compared with SPF-kept CXCR5-deficient mice. Still, SILT eventually form in germfree CXCR5-deficient mice, indicating that stimuli other than microbial stimuli can trigger SILT formation. This compensatory mechanism does not involve the chemokine receptor CCR7. Despite the complete absence of all peripheral lymph nodes in CXCR5/CCR7 double-deficient mice (32), the number of SILT in these mutants matches that of the wild-type and single-deficient mouse strains (data not shown). SILT in these double-deficient mice like in CCR7-deficient mice (9) occasionally contains multiple nodules which renders their distinction from PP difficult. The chemokine receptor CCR6 was found to be expressed by the majority of B cells and lin- c-Kit+ cells present in SILT (20). Furthermore, it was shown that bacterial recognition by the innate receptor NOD1 that is expressed on epithelial cells is sufficient to induce ILF formation in a mechanism depending on CCR6 (10).

The hypothesis that lin- c-Kit+ cells in SILT might recapitulate lymphoid organogenesis postnatally (19, 33) is one of the most distinguishing features of PP and SILT may be their divergent time of development. Normal development of PP occurs during gestation, whereas SILT develops postnatally. The adoptive transfer of CXCR5-sufficient LTIC into newborn CXCR5-deficient mice leads to the formation of macroscopically visible follicular structures in the small intestine that share features of both PP and SILT (27). lin- c-Kit+ cells in SILT strikingly resemble LTIC in their phenotype and ontogeny. In this light, the results obtained by Finke et al. (27) can be perceived as support to the hypothesis that lin- c-Kit+ cells in SILT might induce intestinal immune responses to Salmonella
CXCR5 in the developmentally programmed induction of SILT

Additional complexity is added to the system considering the contribution of B1 cells to the intestinal IgA production. Peritoneal B1 cells express CXCR5 and require this receptor for homing into the peritoneal cavity (37, 49). In consequence, CXCR5-deficient mice as well as mice lacking the CXCR5 ligand CXCL13 harbor largely reduced B1 cell populations in the peritoneal cavity. However, B1 cells are unlikely to contribute to the induction of T cell-dependent anti-cholera toxin responses. We therefore suggest that impaired cholera toxin-specific IgA response in CXCR5-deficient mice might reflect the lack of PP. Interestingly, PP would play a nonredundant role in that other mechanisms, including follicle-sufficient SILT, cannot fully compensate for the lack of PP. A possible explanation for these on first-view contradictory observations might relate to the type of Ag investigated. PP as well as SILT are equipped with M cells, allowing for the efficient uptake of particulate material from the intestinal lumen. However, only PP but not SILT harbor dedicated T cell areas that are likely to be required for the induction of T cell-dependent anti-cholera toxin IgA responses, indicating that SILT might not be able to support T cell-dependent IgA responses (40).

In conclusion, our results indicate that SILT might be unable to fully compensate for the lack of PP during the induction of anti-cholera toxin IgA responses. Instead, PP and SILT might serve nonredundant functions in the intestine and both compartments might be particularly equipped to handle different types of Ag (40). Moreover, we demonstrate a nonredundant function for CXCR5 in the developmentally programmed induction of SILT during early postnatal development as well as SILT homeostasis.

Acknowledgments

We thank Dirk Bumann for providing Salmonella SL1344 aroA srxB and Sabrina Dähne and Tim Worbs for critically reading this manuscript and immunofluorescent microscopy.

Disclosures

The authors have no financial conflict of interest.

References


