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Adaptation of Solitary Intestinal Lymphoid Tissue in Response to Microbiota and Chemokine Receptor CCR7 Signaling

Oliver Pabst, Heike Herbrand, Michaela Friedrichsen, Sarvari Velaga, Martina Dorsch, Günter Berhardt, Tim Worbs, Andrew J. Macpherson, and Reinhold Förster

Besides Peyer’s patches, solitary intestinal lymphoid tissue (SILT) provides a structural platform to efficiently initiate immune responses in the murine small intestine. SILT consists of dynamic lymphoid aggregates that are heterogeneous in size and composition, ranging from small clusters of mostly lineage-negative cells known as cryptopatches to larger isolated lymphoid follicles rich in B cells. In this study, we report that in chemokine receptor CCR7-deficient mice SILT is enlarged, although unchanged in frequency and cellular composition compared with wild-type mice. This phenotype is conferred by bone marrow-derived cells and is independent of the presence of intestinal bacteria. Remarkably, particularly small-sized SILT predominates in germfree wild-type mice. Colonization of wild-type mice with commensal bacteria provokes an adjustment of the spectrum of SILT to that observed under specific pathogen-free conditions by the conversion of pre-existing lymphoid structures into larger-sized SILT. In conclusion, our findings establish that intestinal microbes influence the manifestation of gut-associated lymphoid tissues and identify CCR7 signaling as an endogeneous factor that controls this process. The Journal of Immunology, 2006, 177: 6824–6832.

In the intestine, the divergent needs of host defense and nutrient uptake collide: whereas the efficient exclusion of microorganisms and viruses requires a tight epithelial barrier, the absorption of nutrients from the intestinal lumen favors a thin and permeable boundary. A broad arsenal of immune mechanisms and compartments has developed to maintain this vital equilibrium, including both inductive and effector functions. The most prominent inductive sites in the intestine are Peyer’s patches (PP) that are characterized by several aggregated B cell follicles with an overlying follicle-associated epithelium containing M cells. M cells actively sample Ags from the intestinal lumen that are transported into PP (1). Within the subepithelial dome of PP, Ags are taken up by dendritic cells (DC). Upon stimulation, Ag-loaded DC migrate to the interfollicular T cell zone and into follicles of PP (2). This allows T cell priming, and, as a consequence, the differentiation of naive B cells into mature IgA-secreting effector cells. In a chemokine receptor-dependent process, these activated B cells subsequently populate the intestinal lamina propria (3, 4).

Recent work extended this dogmatic model and proposed class switching to occur in the lamina propria itself (5), as well as in alternative inductive sites in the murine intestine, such as isolated lymphoid follicles (ILF) (6). The architecture of ILF resembles that of a single follicle observed in PP (6), suggesting that both types of structures might serve similar purposes. Indeed, there is evidence that ILF, like PP, are capable of generating gut-homing plasma cells (7, 8) and support class switch recombination (9).

Besides these anatomical and functional similarities, PP and ILF both depend on related, but distinct mechanisms and molecules for their generation: the formation of PP is initiated during gestation and propagated by the interaction of lymphoid tissue-inducer cells (LTIC) with mesenchymal organizer cells that receive signals via the chemokine receptor CXCR5 and the lymphotixin-β receptor, respectively (for review, see Refs. 10 and 11). Consequently, the temporally restricted disruption of lymphotixin signaling during gestation interferes with PP development, resulting in PP-deficient mice. Similarly, PP fail to develop in CXCR5-deficient mice, and adoptive transfer of CXCR5-competent LTIC into newborn CXCR5-deficient recipients has been suggested to rescue the formation of PP (12). Although the generation of ILF shares many features with that of PP, e.g., the requirement of lymphotixin-β receptor signaling, ILF emerge only postnatally. Additionally, ILF, but not PP development can be induced by bone marrow transplantation of adult ILF-deficient lymphotixin α mutants with wild-type bone marrow (13). This suggests that compared with the development of PP, ILF formation is more flexible and temporarily not restricted to prenatal development of the organism.

Besides PP and ILF, yet another type of lymphoid structures has been described in the small intestine, termed cryptopatch (CP). CP are small clusters of mostly lineage-negative cells expressing the stem cell factor receptor cKit (CD117) and the IL-7R α-chain (CD127) (14). CP are localized at the base of intestinal crypts and have been suggested to breed intraepithelial lymphocytes (15). However, more recent work has cast doubt on this function, as evidenced by the lack of RAG gene activity in CP (16) and the presence of intraepithelial lymphocytes in the absence of CP (17) as well as by fate-mapping studies using transgenic knock-in mice expressing a reporter gene under the control of the retinoic acid orphan receptor γt (18).

We have reported recently that in a comprehensive survey of lymphoid aggregations in the murine intestinal wall, only a minority of structures obeys the criteria commonly used to define ILF or CP. In contrast, we observed that aggregated lymphoid...
structures in the murine intestine are dynamic, and that their phenotype depends on external stimuli as well as mouse strain-specific genetic influences (17). In consequence, we proposed that CP and ILF do not constitute separate entities of lymphoid structures, but represent polarized aspects of a continuous spectrum of structures that we refer to as solitary intestinal lymphoid tissue (SILT). A related idea was discussed by Eberl (19), suggesting that lineage-negative cells present in CP might represent the adult counterparts of LTIC driving the maturation of CP into ILF.

In this study, we show that SILT formation is disturbed in the absence of CCR7 signaling, resulting in heavily enlarged structures resembling PP. This phenotype can be induced in wild-type or reverted in CCR7-deficient mice by corresponding bone marrow transplantsations. We demonstrate that intestinal bacteria support a shift in the spectrum of SILT toward larger structures containing more B cells compared to those observed in germfree animals. In contrast, germfree CCR7-deficient mice possess a similar array of SILT as observed under nongermfree conditions, suggesting that hypertrophy of SILT in CCR7-deficient mice does not depend on external stimulation. Regardless of the manipulation applied, the number of SILT in the intestine remains invariant, whereas their morphology is highly dynamic. This result supports the concept of SILT as anlagen-dependent secondary lymphoid organ equipped with cellular and molecular mechanisms that allow a rapid response to environmental challenges.

Materials and Methods

Mice

Animals were bred at the central animal facility of Hannover Medical School under specific pathogen-free conditions or purchased from Charles River Laboratories. LTR-, CCR7-deficient, and plp/plp mice on a C57BL/6 genetic background have been described elsewhere (20–22). CCR7-deficient mice on a BALB/c genetic background were bred at Hannover Medical School by backcrossing of CCR7-deficient mice on a mixed 129SV: BALB/c background to BALB/c mice for seven generations. All cell-deficient mice on a BALB/c background (mb-l/Cre) have been described previously (23) and were bred at the Gesellschaft für Biotechnologische Forschung. Antibiotic treatment was started during gestation by supplementing the drinking water with 1.3 mg/ml sulfamethoxazole and 0.26 mg/ml trimethoprim. Antibiotics were exchanged every second day until sacrifice.

Germfree mice

Germfree C57BL/6 wild-type mice were bred at the Universitätsklinik Zürich, and colonized for different time periods by housing with specific pathogen-free mice in the same cage. All colonized and germfree mice were analyzed at the age of 5 mo irrespective of the time of colonization. CCR7-deficient germfree mice on a C57BL/6 background were generated and bred at the central animal facility of Hannover Medical School. Germfree status of experimental mice was verified regularly by three independent methods. First, fecal pellets and caecal contents of sentinel mice were analyzed at the age of 5 mo irrespective of the time of colonization. CCR7-deficient germfree mice on a C57BL/6 background were generated and bred at the central animal facility of Hannover Medical School. Germfree status of experimental mice was verified regularly by three independent methods. First, fecal pellets and caecal contents of sentinel mice were analyzed at the age of 5 mo irrespective of the time of colonization. CCR7-deficient germfree mice on a C57BL/6 background were generated and bred at the central animal facility of Hannover Medical School. Germfree status of experimental mice was verified regularly by three independent methods. First, fecal pellets and caecal contents of sentinel mice were analyzed at the age of 5 mo irrespective of the time of colonization. CCR7-deficient germfree mice on a C57BL/6 background were generated and bred at the central animal facility of Hannover Medical School. Germfree status of experimental mice was verified regularly by three independent methods. First, fecal pellets and caecal contents of sentinel mice were analyzed at the age of 5 mo irrespective of the time of colonization.

Bone marrow chimera

Bone marrow reconstructions were performed with mice on a C57BL/6 genetic background. Recipients were irradiated lethally with a single dose of 10 Gy and reconstituted by i.v. injection of 1 × 10^7 congeneric sex-matched bone marrow cells purified by discontinuous Lympholyte M gradient centrifugation. Reconstitution was checked using congeneric markers after 6 wk, and animals were analyzed 7–9 wk after reconstitution.

Adaptive transfers

Donor cells were isolated from spleens of wild-type and CCR7-deficient mice on a BALB/c genetic background by mincing organs through a nylon mesh. Lymphocytes were enriched by purification using a discontinuous Lympholyte M gradient. A total of 3 × 10^7 cells was injected into the tail vein of B cell-deficient syngenic recipients. Adaptive transfers were repeated after 1 wk, and animals were analyzed 1 wk after the second transfer. Competitive adaptive transfer experiments of differentially labeled splenocytes were performed, as described previously (25).

Antibodies

The following Abs and conjugates were used in this study: anti-TCRβ FITC, anti-TCRγδ PE (Caltag Laboratories), anti-CD4 PerCP, anti-CD8α allophycocyanin-Cy7, anti-CD11c-bio, anti-CD21 FITC, anti-CD23 allophycocyanin, anti-CD35 PE, anti-CD62L allophycocyanin, anti-CD262L PE (BD Biosciences), anti-CD19 PE (Southern Biotechnology Associates), anti-CD117 and anti-CD117 allophycocyanin (eBioscience), anti-CD7α (clone A7R34; Naturoc), anti-IL-7Ra (clone A7R34; Naturoc), and anti-Ly5.1 FITC and anti-Ly5.2 PE (Cymbus Biotechnology). Anti-CD8α (clone RM CD8), anti-CD3 (clone 17A2), anti-IgD (clone HB250), and anti-B220 (clone B4216) Abs were provided by E. Kremmer (GSP National Research Center for Environment and Health, Munich, Germany) and conjugated to FITC, biotin, Cy3, and Cy5, as recommended by the manufacturer (Amersham). Biotinylated Abs were detected by streptavidin- Alexa 488 (Molecular Probes) or streptavidin-PerCP (BD Biosciences). Unconjugated anti-IL-7Ra and anti-cKit Abs were recognized by mouse anti-rat Cy3 conjugates (Jackson Immunoresearch Laboratories).

Immunoistochemistry

Immunohistochemistry was performed, as described previously (17). For analysis of distribution and cellular composition of lymphoid aggregations, composite images were automatically assembled using a motorized Axiovert 200M microscope (Zeiss) with an autofocus module and KS500 software (Zeiss) (17).

Flow cytometry

Preparation of single-cell suspensions of mesenteric lymph node and PP as well as isolation of intraepithelial and lamina propria lymphocytes were performed, as described (17). To isolate ILF, washed intestines were opened longitudinally and individual ILF were dissected from the lumen side with a scalpel under a stereomicroscope. ILF were minced through a nylon mesh and directly used for flow cytometry. Cells were stained using the Abs described above. Lineage-positive cells were excluded using a mixture of biotinylated Abs directed against CD3, CD19, CD11b, CD11c, and TER-119 that was recognized by streptavidin coupled to PerCP. Dead cells were excluded from further analysis by gating on DAPI-negative cells. Flow cytometry data were acquired on an LSRII (BD Biosciences) and analyzed using FACS Diva Software (BD Biosciences) or WinList (Verity Software House).

Automated image analysis and statistical analysis

Image processing and analysis were performed using the open source software ImageJ (National Institutes of Health). Lymphoid aggregations were manually defined as regions of interest for the subsequent automated image analysis by a custom ImageJ plugin (for download: http://www2.mh-hannover.de:4457/5420.html): single-color channels were generated from RGB images and subjected to a user-defined thresholding before pixel areas were measured. The correlation of pixel areas to cell numbers was verified by manually counting the number of B220- and cKit-positive cells in 100 arbitrarily chosen lymphoid aggregations. Typically, results obtained by automated image analysis and manual cell counting differed <5%.
Statistical analysis was performed on the original data before classification into five classes using GraphPad Prism 4.0 software using nonparametric two-tailed Mann-Whitney U test. Statistical differences are indicated for the mean values and variances separately as follows: *, p < 0.05; **, p < 0.005; ***, p < 0.001.

Results

Classification of small-sized lymphoid aggregations in the small intestine

Lymphoid aggregations in the small intestine that we refer to as SILT display a heterogeneous phenotype in respect to size and cellular composition (13, 17). We have reported recently a systematic experimental approach to analyze these structures by the use of automated immunofluorescence analysis of large coherent areas of the intestinal wall (17). Because the major cellular constituents of SILT are B cells and lineage-negative stem cell factor-receptor (cKit)-positive cells, these two markers were used throughout this study to classify lymphoid aggregations. To this end, horizontal sections through the crypt zone were stained for nuclei (DAPI), B cells (anti-B220), and cKit+ cells (Fig. 1A). Composite images were assembled by automated immunofluorescence microscopy, and the frequency of aggregated structures in the crypt zone was determined. According to their size, lymphoid structures were assigned into one of the five following classes: structures that occupy an area of up to 5,000, 10,000, 15,000, 20,000, or >20,000 μm² at the level of the crypt zone were designated as classes I to V, respectively (Fig. 1, A and B). The contribution of cKit+ and B220+ cells to the overall cellularity was determined by automated image analysis (Fig. 1B; see also Materials and Methods). Importantly, the size of structures correlated with the content of both cKit+ and B220+ cells, with small structures (class I) displaying a high percentage of cKit+ cells and a low proportion of B220+ cells. Vice versa, large structures (class V) are distinguished by a lower content of cKit+ cells, but harbor a higher percentage of B220+ cells (Fig. 1C). Therefore, a classification of SILT based on size of the structure, content of cKit+ cells, or content of B220+ cells yields comparable results when a sufficient number of SILT is included. In this study, we used all three parameters to classify SILT in various manipulated mice and obtained equivalent results. Because we observed that the size represents the most unbiased characteristic, subsequent Figs. 2, 5, 6, and 7 will display results obtained by a size-based classification.

CCR7-deficient mice display atypically hyperplastic SILT

We and others have reported previously that any type of lymphoid aggregation is absent in the intestine of lymphotoxin α mutants (17, 26). To further characterize the requirements of other signaling molecules expressed on LTIC for SILT organogenesis, we analyzed the phenotype of SILT in CCR7-deficient and plt/plt mice that lack the CCR7 ligands CCL19 and CCL21-Ser. When analyzing horizontal sections through the crypt zone, we observed no that lack the CCR7 ligands CCL19 and CCL21-Ser. When analyzing horizontal sections through the crypt zone, we observed no significant difference in the frequency of SILT in CCR7-deficient mice compared with wild-type controls (52 ± 7 SILT/cm² in C57BL/6 wild-type mice; 54 ± 9 SILT/cm² in CCR7-deficient mice on a C57BL/6 background; 54 ± 6 SILT/cm² in plt/plt mice on a C57BL/6 background, n = 5 each; see also Fig. 2A). Vertical sections revealed that the overall architecture of SILT in CCR7-deficient and plt/plt mice resembles typical wild-type ILF with a prominent B cell follicle, a subepithelial dome occupied by DC, and T cells scattered throughout the structure (Fig. 2B and data not shown). We next used the classification system described above to analyze the phenotype of SILT in CCR7-deficient and plt/plt mice in comparison with wild-type animals (Fig. 2C). Interestingly, we observed that CCR7-deficient mice almost exclusively display SILT that fall into categories IV and V (Fig. 2C).

Because the phenotype of SILT depends on the genetic background (6), we investigated the influence of genetic background alterations on SILT morphology observed in CCR7-deficient mice. Similarly to our results obtained on the C57BL/6 background, the spectrum of SILT observed in CCR7-deficient mice on a BALB/c or mixed C57BL/6:129SV:BL6/C background was consistently shifted to larger-sized structures (data not shown). Moreover, such mice occasionally displayed exceptionally huge SILT, with an average diameter of >200 μm and more than one follicle, which do generally not occur in wild-type mice, and consequently cannot be distinguished from PP by simple morphological criteria (data not shown). Notably, such multifollicular SILT were not present in CCR7-deficient mice on a C57BL/6 background, suggesting that hypertrophy of SILT is even more pronounced in CCR7-deficient mice on a BALB/c and mixed genetic background. Irrespective of the genetic background analyzed, structures resembling CP (classes I and II) are almost entirely absent in CCR7-deficient mice (Fig. 2C and data not shown). This suggests that the pattern of differently sized SILT observed in wild-type animals is dramatically shifted in CCR7 mutants, now including exceptionally huge structures resembling PP.

![Figure 1](http://www.jimmunol.org/Downloadedfrom/fig1.jpg)

**FIGURE 1.** Classification of small-sized lymphoid aggregations in the small intestine. A, Horizontal sections through the crypt zone were stained for cKit (green), B220 (red), and nuclei (blue). B, Representative examples for lymphoid aggregates of an area of up to 5,000, 10,000, 15,000, 20,000, or >20,000 μm² that are classified as classes I to V, respectively. Individual structures were analyzed for the frequency of cKit- and B220-positive staining cells by automated image analysis. Therefore, single channels representing cKit- and B220-positive stained areas were extracted from RGB images (upper row) and converted into black and white images (middle and lower panels). C, Structures of classes I to V show a decreasing contribution of cKit-positive stained areas and an increasing contribution of B220-positive staining areas. Boxes depict the 75 and 25 percentiles. The mean value is indicated by horizontal bars within the boxes. Classes I to V are depicted by increasing greyscale intensity.
To track the phenotype of SILT observed in adult CCR7-deficient mice back to early stages of development, we analyzed the presence and phenotype of SILT 7 and 14 days after birth in wild-type and CCR7-deficient mice on a BALB/c background. In both wild-type and mutant mice, we observed SILT 7 days after birth. However, at both time points, SILT structures have more frequently been spotted in CCR7-deficient mice and usually contained a core of B220-expressing cells that was only sporadically present in SILT of wild-type controls (Fig. 3), suggesting that hypertrophy and accumulation of B cells in SILT occur early after birth.

In CCR7-deficient mice, the cellular composition of PP resembles that of SILT

We next used flow cytometry to investigate the cellular composition of SILT in wild-type and CCR7-deficient mice on a C57BL/6 genetic background in more detail. Individual SILT were dissected from the surrounding tissue under a stereomicroscope, and single-cell suspensions were prepared. Importantly, this approach only allows the isolation of SILT recognizable under a stereomicroscope from the luminal surface of the small intestine. Consequently, such cell suspensions are exclusively derived from large-sized SILT (classes IV and V) that are characterized by a prominent B cell follicle and a corresponding bulgelike morphology. We found that B cells residing in wild-type class IV and V SILT as well as CCR7-deficient SILT are of the conventional B2 cell phenotype (CD19+/CD21+/CD23high/CD5-; Fig. 3 and data not shown). Likewise, CD4+ and CD8+ T cells in both wild-type and CCR7-deficient SILT are largely L-selectin (CD62L) negative, suggesting that large SILT contain only few naive T cells (Fig. 4 and data not shown). However, whereas in wild-type mice PP and SILT display a clearly different cellular composition, this difference is less pronounced in the corresponding CCR7-deficient structures. In particular, wild-type PP contain a significantly higher percentage of naive (CD62L+) CD4+ T cells compared with wild-type SILT, whereas in CCR7-deficient mice both compartments are scarce in naive T cells (Fig. 4). In conclusion, the cellular

**FIGURE 2.** CCR7-deficient and plt/plt mice harbor hypertrophied SILT. A, Horizontal sections through the crypt zone of 8- to 10-wk-old wild-type, plt/plt, and CCR7-deficient mice, all on a C57BL/6 background, were stained for nuclei (blue) and B cells (anti-B220, red); SILT are marked by arrows. B, Vertical sections through SILT in wild-type and CCR7-deficient mice reveal a comparable architecture in both strains with a prominent B cell core (anti-B220, red), interspersed T cells (anti-CD3, blue), and an overlying dome region containing DC (anti-CD11c, green). C, The size distribution of SILT observed in specific pathogen-free wild-type, plt/plt, and CCR7-deficient mice was assessed, as described for Fig. 1. Classes I to V are depicted by increasing greyscale intensity, and classes not detected are indicated by Ø. Numbers in parentheses indicate the number of mice and SILT analyzed. Statistical differences between the mean size of SILT present in each group are indicated as follows: *, p < 0.05; **, p < 0.005.

To track the phenotype of SILT observed in adult CCR7-deficient mice back to early stages of development, we analyzed the presence and phenotype of SILT 7 and 14 days after birth in wild-type and CCR7-deficient mice on a BALB/c background. In both wild-type and mutant mice, we observed SILT 7 days after birth. Despite this, SILT structures have more frequently been spotted in CCR7-deficient mice and usually contained a core of B220-expressing cells that was only sporadically present in SILT of wild-type controls (Fig. 3), suggesting that hypertrophy and accumulation of B cells in SILT occur early after birth.

**FIGURE 3.** SILT are present early after birth in both wild-type and CCR7-deficient mice. Horizontal sections through the intestinal wall of wild-type and CCR7-deficient mice on a BALB/c background were analyzed 7 and 14 days after birth for the presence and phenotype of SILT. Sections were stained using anti-cKit (blue) and anti-B220 (red) Abs. SILT structures were more frequently observed in CCR7-deficient mice compared with age-matched wild-type controls. They usually contained a dense follicular core of B220-expressing cells that was only sporadically observed in SILT of 7- or 14-day-old wild-type mice.
composition of SILT observed in CCR7-deficient mice strikingly resembles that of PP observed in these mutants, and likewise is very reminiscent of large SILT (classes IV and V) observed in wild-type mice.

Bone marrow reconstitution induces/reverts the phenotype of SILT

Previous reports showed that ILF and CP can be induced in lymphotoxin α mutants lacking such structures by transplantation with wild-type bone marrow (13, 26). We therefore generated bone marrow chimeras using wild-type and CCR7-deficient mice as recipients and/or donors and analyzed the phenotype of SILT 7–9 wk after reconstitution. We observed that bone marrow transplantation as such did not significantly affect the spectrum of SILT in wild-type mice that received wild-type bone marrow (Fig. 5). In contrast, wild-type recipients that received bone marrow from CCR7-deficient donors almost completely recapitulated the phenotype observed in genuine CCR7 mutants. Conversely, reconstitution of CCR7 mutants with wild-type bone marrow converted the SILT phenotype largely to the wild-type pattern. In contrast, bone marrow transplantation with wild-type cells failed to revert the spectrum of SILT in plt/plt recipients (Fig. 5). In addition, we performed similar experiments using lymphotoxin α mutant mice on a C57BL/6 genetic background as recipients for both wild-type and CCR7-deficient bone marrow. As expected, our results confirmed previous reports demonstrating the generation of lymphoid aggregates in those chimeric animals reconstituted with wild-type bone marrow. Moreover, we observed that the phenotype of SILT induced by transplantation of CCR7-deficient bone marrow into either lymphotoxin α mutants or wild-type recipients is almost identical (Fig. 5). These results suggest that lack of CCR7 expression on hemopoietic cells causes hypertrophy of SILT, whereas CCR7 ligands need to be produced by nonhemopoietic cells to maintain a wild-type-like array of SILT.

Perturbed exit from the periphery does not significantly contribute to SILT hypertrophy in CCR7-deficient mice

It has been reported recently that CCR7 is required for the exit of lymphocytes from the periphery (27, 28). Because this phenomenon might contribute to SILT hypertrophy in CCR7 mutants, we analyzed the accumulation of wild-type and CCR7-deficient B cells in an adoptive transfer model using B cell-deficient recipients. In B cell-deficient mice, we observed normal frequencies of SILT that were reduced in size compared with wild-type mice. Adoptive transfer of splenocytes from either wild-type or CCR7-deficient donor animals (twice $3 \times 10^7$ splenocytes/recipient) resulted in the accumulation of >20% of the adoptively transferred B cells in the recipients’ spleens after 2 wk (25.5 ± 6.0 vs 22.0 ± 5.3% B cells in the recipients’ spleens using wild-type and CCR7-deficient donors, respectively; $n = 4$ mice analyzed). However, in neither case did we observe a significant accumulation of transferred B cells in SILT (3.2 ± 2.7 vs 3.3 ± 2.6% B220-positive cells in the recipients’ SILT using wild-type and CCR7-deficient donors).
FIGURE 6. Commensal bacteria progressively drive the maturation of SILT. Germfree C57BL/6 mice were colonized with commensal bacteria for the period of time indicated and analyzed for the frequency and phenotype of SILT. Bars represent the contribution of class I- to class V-type SILT to all lymphoid structures in the small intestine. Classes I to V are marked by roman numbers and depicted by increasing greyscale intensity; classes not detected are indicated by Ø. Numbers in parentheses indicate the number of mice and SILT analyzed. Statistical differences were tested for each time point compared with germfree mice and are indicated in the bottom lines. Differences between mean values and variances are indicated separately (mean/variance): *, p < 0.05; ***, p < 0.001.

donors, respectively; n = 4 mice analyzed). Similarly, we observed that 3 days after a competitive transfer of differently labeled splenocytes from wild-type and CCR7-deficient donors into syngenic wild-type recipients, no preferential accumulation of cells of either donor genotype occurred in SILT (data not shown). These data suggest that SILT hypertrophy in CCR7-deficient mice is not caused by perturbed egress of lymphocytes.

Commensal bacteria stimulate the maturation of SILT in the small intestine

CP have been reported to be present in germfree mice (14), whereas controversial observations were published regarding the presence of ILF in such animals. Hamada et al. (6) initially observed ILF in germfree mice, whereas other reports suggested that ILF only develop upon stimulation by intestinal bacteria (13) and changes in the commensal flora have been correlated with ILF hypertrophy (29, 30). We observed that germfree C57BL/6 mice predominantly contain SILT with an average diameter of ~90 μm corresponding approximately to an area of 6300 μm^2 in horizontal sections through the crypt zone (classes I and II) and lack large-sized SILT (classes IV and V; Fig. 6). However, germfree mice possess a few class III structures containing up to 60% of B cells that may be organized in a compact follicular structure and thus fit the original description of ILF. To analyze the dynamics of SILT transition during colonization of germfree mice, we generated a set of age-matched germfree and colonized C57BL/6 mice. Germfree mice were colonized by housing them together with specific pathogen-free sentinel mice in the same cage. All animals were analyzed at an age of 5 mo. After 4, 8, 16, 22, 41, and 71 days of colonization, the formerly germfree mice were sacrificed, and the frequency and phenotype of SILT were analyzed. We observed 54 ± 8 SILT/cm^2 in the proximal small intestine in germfree animals compared with 52 ± 7 SILT/cm^2 in age-matched mice that had been colonized for 41 days. Likewise, no significant differences in the density of lymphoid structures per sectioned area were observed at any other time point analyzed or in the distal small intestine (data not shown). These findings suggest that colonization of germfree mice does not result in the de novo formation of aggregated lymphoid structures in the intestine. Instead, colonization of germfree mice induced profound changes in the phenotype of already existing SILT, i.e., these mice harbored larger structures with a higher content of B220-positive cells in comparison with germfree animals (Fig. 6 and data not shown). The average size of SILT increased with time after colonization, reaching a plateau after 41 days. From this time point on also, the SILT spectrum remained invariant and was indistinguishable from the one observed in specific pathogen-free sentinel animals (data not shown).

This indicates the following: 1) an assimilation of the intestinal flora of test and sentinel animals, and 2) that this particular microbiota mixture created the given SILT spectrum that is by and large identical with that observed earlier (17) (compare Figs. 2 C and 6). The microbiota-induced increase in average SILT size was paralleled by increasing frequencies of B cells in SILT, suggesting that the influx of B cells drives the size increment of these structures.

Considering the major influence of commensal bacteria in the intestine on the phenotype of SILT, we asked whether the spectrum observed in specific pathogen-free CCR7-deficient mice is influenced by the presence of intestinal bacteria to a similar extent. For this, we generated long-term antibiotic-treated and germfree CCR7-deficient mice. Importantly, under both conditions, the phenotype of SILT retained the CCR7 mutant-specific profile and did not shift to the spectrum observed in germfree wild-type animals (Fig. 7; compare with Figs. 2 C and 6), demonstrating that intestinal commensal bacteria do not significantly contribute to the phenotype of SILT in these mutants. Moreover, we did not observe any differences in the numbers of aerobic or anaerobic bacteria that could be cultured from faeces of specific pathogen-free CCR7-deficient and wild-type mice (data not shown).

These results demonstrate that the number of SILT does not depend on either functional CCR7 signaling or the presence of microbiota, suggesting that SILT cannot be generated de novo.

Consequently, SILT, like PP, should be regarded as genuine secondary lymphoid organs that adapt to the presence of microbiota, resulting in a shift in the spectrum of SILT.

Discussion

The spatial integration of Ag-sampling mechanisms provided by M cells, Ag-presenting DC, as well as extensive B and T cell regions in PP provides a very efficient platform to handle intestinal
negative cells termed CP, qualifying such tissue as a bona fide secondary lymphoid organ rather than tertiary lymphoid tissue (17). In support of our hypothesis, we demonstrate in the present study that in CCR7-deficient mice as well as in germfree mice challenged with commensal bacteria, only the phenotype, but not the overall number of SILT is subject to changes. Our findings, however, do not rule out the possibility that neogenesis of additional SILT-like lymphoid aggregations may develop in the intestine under pathological conditions, for example, in consequence of drug- or pathogen-induced inflammation. Notably, any studies that led to the suggestion that lymphoid aggregations in the small intestine and colon could form de novo either used sections along the crypt villus axis or were based on whole mount inspections of the intestine using a stereomicroscope. In contrast, we used horizontal sections through the crypt zone in combination with automated image acquisition and analysis. This method avoids any risk that small structures escape detection, and thus allows determining the total number of aggregated structures in the intestine without any bias due to the size of the clusters analyzed. This comprehensive view facilitates a clear-cut recording of differences in the size and frequency of lymphoid clusters in the small intestine.

The cellular composition of SILT in CCR7-deficient mice resembles that of PP in these mutants, and on occasion exceptionally huge SILT can even be detected by macroscopic inspection of entire intestines. SILT in CCR7-deficient mice belong almost exclusively to SILT of classes IV and V and cannot be distinguished from the correspondingly classified SILT in wild-type mice, suggesting that CCR7 deficiency does not elicit structural abnormalities, but rather disrupts an apparently negative feedback loop normally preventing massive influx of naive B cells.

Recently, it has been reported that lack of CCR7 impairs the establishment of central tolerance in these mice, thereby leading to inflammatory infiltrates and tertiary lymphoid tissue formation in various organs (37). However, for several reasons, these findings are unlikely to provide an explanation for the hypertrophy of SILT in the intestine: 1) The number of lymphoid clusters in the intestine of CCR7-deficient mice is unchanged compared with wild-type controls, which rules out considerable neogenesis; 2) No CP-like tissue can be detected in CCR7-deficient mice, indicating the conversion of all of these structures into class IV and V SILT; and 3) SILT hypertrophy occurs invariably early after birth, obeying an endogenous genetic disposition, and does not progress during aging. In addition, by adoptive transfer experiments, we found that impaired emigration from peripheral tissues is unlikely to account for hypertrophy of SILT in CCR7-deficient animals.

Bone marrow reconstitution with CCR7-deficient or wild-type cells is sufficient to interconvert the spectrum of SILT observed in wild-type and CCR7-deficient mice, respectively, indicating that CCR7 function on hemopoietic cells is of pivotal importance for SILT manifestation. Similarly, the pattern of SILT observed in plt/plt mutants lacking the CCL19 and CCL21-Ser gene phenotype resembles the spectrum present in CCR7 mutants. However, in contrast to CCR7-deficient mice, SILT hypertrophy in plt/plt mice cannot be reverted by transplantation of wild-type bone marrow. This suggests that both the CCR7-ligand production by a nonhemopoietic cell in the SILT and the subsequent immigration of a CCR7+ cell of hemopoietic origin are required for limiting the expansion of SILT.

We and others have reported earlier that CCR7 signaling is required for lymphoid organogenesis, as evidenced by the lack of all peripheral lymph nodes in CXCR5/CCR7-double-deficient, but not either single-deficient mouse mutant (21, 38). Consistently, in addition to naive lymphocytes and DC, CCR7 is also expressed on LTIC isolated from fetal mesenteric lymph nodes (21). However,
in contrast to the lack of peripheral lymph node in CXCR5/CCR7-double-deficient mice, the number of macroscopically discernable nodular structures in the intestine, i.e., PP, is increased compared with CXCR5 single-deficient mice (0–1 PP in CXCR5-deficient mice; n = 24 vs 4–5 PP in CXCR5/CCR7-double-deficient mice, n = 15; p < 0.001). Considering data presented in this work, such lymphoid nodules in CXCR5/CCR7-double-deficient intestines might not necessarily represent proper PP but, alternatively, giant SILT forming in the absence of CCR7-dependent size regulation.

Interestingly, lineage-negative cells in SILT phenotypically resemble LTIC, a cell population active in lymph node and PP development during embryogenesis, and it has been hypothesized that these cells might postnatally recapitulate a developmental pathway that to date has been attributed to prenatal processes (19). In line with this hypothesis, we observed that cells isolated from the intestinal lamina propria, thereby including SILT-resident cells, contain CD4+ c-kit+ lineage cells (hereafter referred to as lineage cells) of which almost 50% express CCR7 (data not shown). In addition, SILT-derived lineage cells bind lymphotoxin-β receptor fusion proteins indicative of lymphotoxin expression by these cells (O. Pabst, unpublished observations). Therefore, lineage+ cells in SILT are equipped with several molecules that are critical for lymphoid organogenesis. Postnatal progression of lymphoid organogenesis in turn is distinguished by the massive recruitment of T and B cells into primordial organ anlagen, a process that is highly reminiscent of SILT transition, qualifying lineage+ cells in SILT as prime candidates to coordinate SILT adaption in response to microbial stimulation. Involvement of CCR7 in this process might either allow the settlement of these regulatory cells into SILT or alternatively constitute an intrinsic requirement for their proper function, i.e., lineage+ cells might depend on CCR7 signaling inside SILT triggering their regulating function. In either case, the presence of CCR7-competent cells in SILT is mandatory for the regulation of B and T cell influx provoked by external stimuli, and in the absence of CCR7+ cells SILT will be overloaded with lymphocytes, building giant aggregates resembling PP. A central function of lineage-restricted lymphocytes. CCR7 constitutes a co-receptor, with IL-7R, Thy1.1+ T cells西甲of IL-7R+ T cells and TNF receptor I function.

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Disclosures
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