Colonic patch and colonic SILT development are independent and differentially regulated events

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Intestinal lymphoid tissues have to simultaneously ensure protection against pathogens and tolerance toward commensals. Despite such vital functions, their development in the colon is poorly understood. Here, we show that the two distinct lymphoid tissues of the colon—colonic patches and colonic solitary intestinal lymphoid tissues (SILTs)—can easily be distinguished based on anatomical location, developmental timeframe, and cellular organization. Furthermore, whereas colonic patch development depended on CXCL13-mediated lymphoid tissue inducer (LTi) cell clustering followed by LTα-mediated consolidation, early LTi clustering at SILT anlagen did not require CXCL13, CCR6, or CXCRI. Subsequent dendritic cell recruitment to and gp38⁺VCAM-1⁺ lymphoid stromal cell differentiation within SILTs required LTα; B-cell recruitment and follicular dendritic cell differentiation depended on MyD88-mediated signaling, but not the microflora. In conclusion, our data demonstrate that different mechanisms, mediated mainly by programmed stimuli, induce the formation of distinct colonic lymphoid tissues, therefore suggesting that these tissues may have different functions.

INTRODUCTION

Secondary lymphoid organs, such as lymph nodes and Peyer’s patches, develop in the sterile environment of the uterus during embryonic life, whereas solitary intestinal lymphoid tissues (SILT) develop early after birth under the continuous exposure to commensals and potential pathogens. In general, lymphoid tissue development depends on the interaction between hematopoietic-derived lymphoid tissue inducer (LTi) cells and stromal lymphoid tissue organizer cells and the engagement of the lymphotoxin (LT) signaling pathway. LTi cells express membrane-bound LTα₁β₂, which engages LTβR on stromal organizers, inducing them to synthesize chemokines, adhesion molecules, growth factors, and survival signals that further attract and retain LTi cells. Lack of LTβR triggering, as observed in LTβR-, LTα-, or LTβ-deficient mice, results in the absence of most lymphoid tissues. Exceptions to this general model are found in the formation of mucosal-associated lymphoid tissue, which can develop independently of nuclear retinoic acid–receptor-related orphan receptor γ (RORγ)-dependent LTi cells, helix–loop–helix–protein inhibitor of DNA binding 2 (Id2)-dependent LTi cells and/or LT signaling (reviewed in ). Importantly, in most lymphoid tissues, the initial clustering of LTi cells occurs independently of LT signaling. Indeed, we have recently shown that such clustering, at peripheral lymph node anlagen, is dependent on the retinoic acid–mediated release of the homeostatic chemokine CXCL13. Immune cell function and lymphoid tissue development in the intestine, where opposing needs for host defense and nutrient uptake collide, must be tightly regulated. The lymphoid tissues that are available for host protection in the small intestine include Peyer’s patches and SILTs. Peyer’s patches develop during embryogenesis. Hematopoietic cells start colonizing the developing gut around day E12.5. Among these cells, receptor tyrosine kinase RET-expressing CD45⁺IL7Rα⁻CD4⁻CD3⁻CD11c⁺ lymphoid tissue initiator cells, which have a non-redundant role in Peyer’s patch development, encounter RET ligands and start clustering at discrete locations in the
In this study, we aimed to characterize the development of lymphoid tissue within the murine colon. Analysis of adult colons confirmed the presence of two distinct lymphoid tissues in the steady state, which were easily distinguished based on their relative position within the colon and the presence of organized T cell areas. Colonic patches composed of large B-cell follicles (on average, two follicles per colonic patch) and separate T cell areas were present within the submucosa of the colon between the two external (longitudinal and circular) muscular layers and the muscularis mucosae, which were delineated with anti-smooth muscle actin (αSMA) staining (Figure 1a, b, arrowhead). Their number averaged 3.4±0.9 per colon (mean±standard deviation; n=14, range 2–5). In addition, numerous small B-cell clusters, containing only a few T cells, could be detected in the lamina propria, i.e., from the muscularis mucosae toward the lumen of the colon (Figure 1a, b, arrow). Importantly, these latter structures, which localized predominantly in the distal colon (Supplementary Figure S1a online), strongly resembled the SILTs of the small intestine. Indeed, similar to the reported stages of SILT maturation in the small intestine,22,24 colonic SILTs could be found as a mere collection of CD4+ LTi cells or containing additionally, different degrees of B-cell accumulation and organization (Supplementary Figure S1b, c online).

Highlighting the different origin of colonic patches and colonic SILTs, we found their development to occur during different timeframes. Development of colonic patches started in embryonic life (Supplementary Figure S1d online), but was completed only after birth. At day 0 (day of birth), colonic patch anlagen could be observed as clusters of CD3−CD4+ RORγ+ LTi cells in the submucosa of the colon (Figure 1c). With age, these early clusters were progressively infiltrated by B and T lymphocytes, which were initially randomly distributed but by day 14 were organized into separate B and T cell areas (Figure 1c). Of note, colonic patch development led to the disruption of the muscularis mucosae (Figure 1c), thereby allowing contact of the colonic patch tissue with the colonic epithelium. In marked contrast, SILT development was entirely postnatal. By days 0 and 7, no clusters of LTi cells could be found in the lamina propria of the colon. Only by day 14, the full spectrum of SILTs, i.e., immature clusters of LTi cells as well as mature clusters with their single well-defined B-cell follicle and stages in between, could be detected (Figure 1d).

The cellular organization within colonic patches and colonic SILTs is distinct

The cellular organization of colonic patches and colonic SILTs also diverged significantly (Figure 2). Definitive colonic patches had separate B and T cell areas, which contained CD35+ follicular dendritic cells and CD11c+ dendritic cells, respectively (Figure 2a I–III, V). CD3+CD4+RORγ+ LTi cells persisted within the "mature" colonic patch (Figure 2a II and data not shown). Importantly, colonic patches also contained CD31+ vessels (Figure 2a II); a fraction of which expressed the adhesion molecule MadCAM-1 and showed the typical appearance of high endothelial venules (HEVs) (Figure 2a IV and data not shown). In contrast, mature SILTs had a single central B-cell follicle, which contained CD35+ follicular dendritic cells,
and only sparsely distributed T cells that did not form T cell areas (Figure 2b I–III, V). Indeed, colonic SILTs contained very few CD3+ T cells (Figure 2b II). The majority of the CD4+ cells present within SILTs were CD3− RORγ+ IL7Rα+ (phenotype which classifies them as LTi cells) and colocalized with CD11c+ dendritic cells in a “ring-like” pattern enclosing the SILT (Figure 2b II, V). Importantly, CD31+ vessels within the SILT usually did not express MAdCAM-1 (Figure 2b IV) and lacked the appearance of HEVs (data not shown), suggesting that lymphocytes do not leave the circulation directly toward the SILT but rather toward the lamina propria of the colon, which contains flat MAdCAM-1+ vessels (27 and data not shown). Both colonic patches as well as SILTs contained gp38+VCAM-1+ stromal cells (Figure 2a VI, b VI), which were absent in non-lymphoid tissue-associated regions of the colon (data not shown).

Early LTi cell attraction to colonic patch and SILT anlagen is LT independent

Having established clear differences between colonic patches and SILTs, we subsequently addressed whether the molecular requirements for their development also differed. Definitive formation of secondary lymphoid tissues depends on the engagement of the LTαβ LTβR signaling pathway.1–3 To test whether this pathway was involved in the development of colonic lymphoid tissues, we analyzed the colons of LTβ−/− mice in the first 2 weeks of age. At day 0, clusters of CD3− CD4+ RORγ+ LTi cells could be observed in the submucosa of the colon (Figure 3a). Importantly, these clusters were neither associated with the distal end of the colon (Supplementary Figure S2a online).

Regarding SILTs, at day 14, only small clusters of CD3− CD4+ RORγ+ LTi cells were observed in the lamina propria of the colon of LTα−/− mice (Figure 3b and data not shown). Importantly, these clusters were neither associated with...
LTI cells and with that for the definitive formation of colonic patches and SILTs. However, in both cases, i.e., in both colonic patch and SILT organogenesis, it is not required for the initiation phase of lymphoid tissue development, namely, the early attraction and clustering of LTI cells.

**CXCL13 is necessary for the clustering of LTI cells at colonic patch anlagen, but dispensable for their clustering at SILT locations**

Given that early LTI cell clustering at lymph node anlagen also occurs independently of LT signaling\(^2\)–\(^4\) in a manner recently reported to be dependent on retinoic acid-induced CXCL13 expression,\(^\text{14}\) we reasoned that lymphoid tissue development within the colon could also be dependent on CXCL13. Confirming this hypothesis, we found no colonic patch anlagen, typified by clusters of LTI cells, in CXCL13\(^{+/−}\) mice at day 0. Indeed, although CD45\(^+\) hematopoietic cells were clearly observed in the colon (Supplementary Figure S2b online), indicating that colon infiltration by immune cells is not defective in CXCL13\(^{+/−}\) mice, immune cell aggregates in the submucosa could not be found at any time point. In marked contrast, however, immune cell aggregates could easily be detected in the lamina propria by days 7 and 14 (data not shown and Figure 3c). These aggregates were composed of CD3\(−\)CD4\(^+\)ROR\(γ^+\) LTI cells as well as CD11c\(^+\) dendritic cells and B220\(^+\) B cells and were associated with gp38\(^+\)VCAM-1\(^+\) stromal cells (Figure 3c). Importantly, the immune cells within these SILTs failed to organize into separate domains (Figure 3c and Supplementary Figure S1c online); and such unorganized SILTs developed much earlier and in higher numbers than the organized SILTs of wild-type mice (data not shown and Supplementary Figure S1c online). Collectively, these data demonstrate that early colonic patch vs. colonic SILT development occurs via different molecular pathways; although CXCL13 is absolutely required for colonic patch development, it is dispensable for LTI cell attraction and clustering and subsequent SILT development in the lamina propria of the colon.

**Chemokine receptors on colonic LTI cells and chemokine expression in the colon**

CXCL13-independent LTI cell attraction and clustering in the lamina propria, suggested that other chemokine(s) could function as the initial inducer of colonic SILT development. To identify this potential chemokine, we assessed chemokine receptor expression on colon-derived LTI cells. Single-cell suspensions were obtained from E18.5 wild-type colons and analyzed by flow cytometry. As shown in Figure 4a, colonic LTI cells, which we identified as CD45\(^+\)CD4\(^+\)ROR\(γ^+\) LTI cells, expressed the homeostatic chemokine receptors CXCR4, CXCR5, and CCR7. Importantly, they did not express the chemokine receptor CCR9, which is used by activated B and T cells to infiltrate mucosal tissues such as the gut,\(^28,29\) but they did express CCR6, which is used by B cells for the same purpose.\(^30\)

To gain further insight into which chemokine(s) might be involved in SILT genesis, we assessed chemokine expression...
by PCR. Chemokine messenger RNA (mRNA) levels were measured in whole colon samples obtained at days 0, 7, and 14 from wild-type, LTα−/− and CXCL13−/− mice. As seen in Figure 4b, we observed no differences in CXCL12, CCL19, and CCL21 mRNA expression levels between the different genotypes. In contrast, although we had already established it not to be involved in early immune cell clustering in colonic SILTs, we found CXCL13 mRNA expression to be reduced in LTα−/− mice as compared with wild-type mice (CXCL13 mRNA was not detected in CXCL13−/− mice). Of interest, we found CCL20 transcript expression to be reduced in LTα−/− mice as compared with wild-type and CXCL13−/− mice at a time point in which SILTs are fully developed, i.e., day 14 post-partum.

CCR6 is neither required for colonic patch nor colonic SILT development
As B-cell recruitment into SILTs in the small intestine was shown to be dependent on a functional CCR6-CCL20 axis and LTα−/− mice, which failed to develop colonic lymphoid tissues, had reduced colonic CCL20 expression (Figure 4b), we hypothesized that this axis would also have a role in lymphoid tissue development in the colon. To test this hypothesis, we analyzed the colon of CCR6−/− mice for the presence of lymphoid tissues. In contrast to the small intestine, lymphoid tissue formation in the colon of these animals progressed normally when analyzed at day 14 (Figure 5). CCR6-deficient colonic patches had B-cell domains containing CD35+ follicular dendritic cells (Figure 5a I and III); T cell domains containing CD11c+ dendritic cells (Figure 5a II and V); CD3−CD4+ LTi cells that persisted at the border of these separate domains (Figure 5a II); and MAAdCAM-1+ HEVs dispersed within a network of gp38+VCAM-1+ stromal cells (Figure 5a IV and VI).

CCR6-deficient SILTs had well-defined B-cell follicles containing CD35+ follicular dendritic cell networks (Figure 5b I and III). The few CD3+ T cells present within SILTs were sparsely distributed among their parenchyma of gp38+VCAM-1+ stromal cells (Figure 5b II and VI). CD11c+ dendritic cells were accumulated, in a ring-like pattern, at the periphery (Figure 5b V). Similarly, vessels expressing MAAdCAM-1+...
were normally found at the periphery of SILTs rather than within them (Figure 5b IV). Collectively, these data showed that, in contrast to the small intestine, the CCL20-CCR6 axis is neither required for colonic SILT nor colonic patch development.

CXCR3-deficient SILTs develop in different locations

LTi cell responsiveness to CXCR3 ligands is contextual, as they do not migrate toward CXCL10 between embryonic days E12.5 and E14.5,14 but readily do so after birth (Supplementary Figure S3a online). This particularity suggested that CXCR3 ligands could be involved in the post-natal development of lymphoid tissues. Supporting this hypothesis, colonic CXCL10 mRNA levels increased significantly with age in wild-type mice, but failed to do so in LTα−/− and CXCL13−/− mice (Supplementary Figure S3b online). Therefore, to determine whether CXCR3 had a role in the development of colonic lymphoid tissues, we analyzed CXCR3-deficient colons. Both colonic patches and colonic SILTs developed in these mice (Figure 6). Colonic patches had separate B and T cell areas, containing CD35+ follicular dendritic cells and CD11c+ dendritic cells, respectively; MadCAM-1+ HEVs and gp38+ VCAM-1+ stromal cells were also present (Figure 6a). CXCR3-deficient SILTs also seemed to develop normally, with mature SILTs having well-defined B-cell follicles that contained CD35+ follicular dendritic cells (Figure 6b). Furthermore, they contained sparsely distributed CD3+ T cells; CD11c+ dendritic cells that colocalized with CD3−CD4+ LTi cells; and gp38+ VCAM-1+ stromal cells (Figure 6b). Significantly, a large fraction of CXCR3-deficient SILTs also included MadCAM-1+ vessels within their parenchyma (Figure 6b IV and Supplementary Figure S3c online). This feature contrasted with the organization of SILTs in wild-type mice, in which MadCAM-1+ vessels were usually not present within the SILT itself but rather at its periphery (Figure 2b and Supplementary Figure S3c online). Importantly, the number and maturation status of SILTs did not differ between wild-type and CXCR3−/− mice (Supplementary Figure S1c online) and MadCAM-1+ vessels were found equally represented among the parenchyma of the different SILT maturation stages in CXCR3−/− mice (Supplementary Figure S3c online). These data suggested that, while LTi cell clustering in the lamina propria of the colon may be altered in the absence of CXCR3-guided migration, colonic SILT development upon LTi cell clustering proceeded
Figure 5  Colonic patch and colonic solitary intestinal lymphoid tissue (SILT) development is independent of CCR6. Histological characterization of the colonic patches (a) and colonic SILTs (b) present in the colon of 14-day-old CCR6−/− mice. Serial sections of the colon were stained for: (I) CD4 (green), αSMA (red), and B220 (blue); (II) CD4 (green), CD31 (red), and CD3 (blue); (III) CD4 (green), CD35 (red), and B220 (blue); (IV) CD4 (green), MadCAM-1 (red), and Lyve-1 (blue); (V) CD4 (green), IL7Rα (red), and CD11c (blue); and (VI) CD4 (green), VCAM-1 (red), and gp38 (blue). Bars = (a) 100 μm and (b) 50 μm. At least three colons were entirely sectioned and the detected lymphoid tissues further analyzed.

Figure 6  CXCR3-guided lymphoid tissue inducer migration influences solitary intestinal lymphoid tissue (SILT) location within the colon’s lamina propria. Histological characterization of the colonic patches (a) and colonic SILTs (b) present in the colon of 14-day-old CXCR3−/− mice. Serial sections of the colon were stained for: (I) CD4 (green), αSMA (red), and B220 (blue); (II) CD4 (green), CD31 (red), and CD3 (blue); (III) CD4 (green), CD35 (red), and B220 (blue); (IV) CD4 (green), MadCAM-1 (red), and Lyve-1 (blue); (V) CD4 (green), IL7Rα (red), and CD11c (blue); and (VI) CD4 (green), VCAM-1 (red), and gp38 (blue). Bars = (a) 100 μm and (b) 50 μm. At least three colons were entirely sectioned and the detected lymphoid tissues further analyzed.
undisturbed. Altogether, although CXCR3 seemed dispensable for colonic patch development, during colonic SILT development CXCR3 appeared to be involved in defining the exact location of SILT formation within the lamina propria.

SILT maturation is dependent on non-microbial-induced MyD88 signaling

As lymphoid tissue development in the colon was largely post-natal and thus, occurred simultaneously with the establishment of the gastrointestinal microbiota, we hypothesized that its development could be influenced by the recognition of the colonizing bacteria. Therefore, to test the role of bacterial colonization in colonic lymphoid tissue development, we analyzed the lymphoid tissues present within the colons of germ-free mice. We found that, in these mice, colonic patches developed normally (data not shown). In contrast, SILT development seemed to be moderately influenced by the presence of commensal bacteria, as a larger proportion of the SILTs was found to be immature in germ-free mice as compared with animals maintained in specific pathogen-free (SPF) conditions (Figure 7a). Importantly, the total number of colonic SILTs did not differ between germ-free and SPF mice (Figure 7a), suggesting that LTI cell clustering in the lamina propria of the colon progresses independently of the intestinal microbiota. In these particular studies, we used C3H mice, which we found to have many more colonic lymphoid tissues as compared with C57BL/B6 mice (14.75±1.5 vs. 3.6±1.1 colonic patches in C3H (n = 4) and B6 (n = 7) animals, respectively; and Figure 7a vs. b). Therefore, to rule out the possibility that our data were being affected by the genetic background of the mice, we decided to verify them by analyzing C57BL/B6 mice in which bacterial colonization of the intestine had been prevented by treatment with a cocktail of antibiotics since embryonic day E14 until sacrifice at 2 weeks post-partum. This protocol clearly reduced the amount of bacteria present in the colon (Supplementary Figure S4 online). However, to our surprise it influenced neither the number nor the maturation status of colonic SILTs (Figure 7b). As bacterial-derived products are recognized by pattern-recognition receptors, such as TLRs, which signal mainly via the adapter molecule MyD88, we decided to further confirm our data by analyzing the development of SILTs in the colon of MyD88−/− mice. In these mice, colonic patches developed normally (Supplementary Figure S5a online and data not shown). SILT development, however, seemed arrested at an immature stage (Figure 7b and Supplementary Figure S5b online). MyD88-deficient SILTs were smaller and had fewer B cells as compared to wild-type SILTs (Figure 7b and Supplementary Figure S5b online); and, likely as a consequence of defective B-cell recruitment, had reduced CD35+ follicular dendritic cell networks (Supplementary Figure S5b online). Altogether, our data suggest that, in contrast to the small intestine, bacterial recognition in the colon does not have a major role in SILT maturation. Furthermore, they imply that MyD88-dependent B-cell recruitment and SILT maturation are not a consequence of bacterial recognition via TLRs.

DISCUSSION

In this study, we analyzed the development of lymphoid tissues within the murine colon. We showed that the steady-state colon contains two distinct lymphoid tissues: colonic patches, which are embryonically programmed, and SILTs, which develop postnatally and depend on the adapter molecule MyD88, but not microbial colonization, for their full maturation.

Colonic patches were located in the submucosa, between the external longitudinal and circular muscular layers and the muscularis mucosae. The muscularis mucosae was disrupted at sites of colonic patches allowing for contact of the colonic patch tissue with the colonic epithelial layer, which should permit the sampling of the colon’s luminal contents by the antigen-presenting cells present within the colonic patch. In contrast, SILTs were positioned in the lamina propria of the colon, i.e., from the muscularis mucosae toward the lumen, directly contacting the intestinal epithelium.

Colonic patches were composed of B and T cells, segregated into clearly distinct compartments, and LTI cells that persisted at the border of these compartments. B-cell follicles contained CD35+ follicular dendritic cells. T cell areas had CD11c+ conventional dendritic cells and MAdCAM-1+ HEVs. Non-hematopoietic gp38+ VCAM-1+ stromal cells were present throughout the colonic patch parenchyma. In contrast to the uniform and highly structured appearance of colonic patches, colonic SILTs appeared in different developmental/maturation stages. In the same animal, they could range from...
The programmed development of colonic patches started during embryonic life, as LTi cells and/or their precursors associated with MadCAM-1+ stromal organizer cells in the colonic submucosa. Similarly, as to the formation of other secondary lymphoid organs in mice such programmed development ceased only after birth, when infiltration of the colonic patch anlagen by B and T cells occurred. Importantly, B and T cells were initially randomly distributed within the colonic patch but subsequently segregated into separate compartments. This contrasts with Peyer’s patch development in the small intestine in which, on recruitment, B and T lymphocytes promptly segregate into their respective domains3 and therefore suggests that Peyer’s patch and colonic patch development is differentially regulated.

Developmental regulation of colonic patches mirrored the molecular cues used during lymph node development (reviewed in 1–3). LTi cells clustered in the submucosa of the colon in a CXCL13-dependent, LTα-independent manner. Whether early expression of CXCL13 at the colonic patch anlage is induced by retinoic acid, as shown for lymph node development,14 warrants further investigation. In the colonic patch anlagen, LTi cells likely interacted with and supported the differentiation of gp38+VCAM-1+ stromal cells via the engagement of the LT signaling pathway. Importantly, sustained colonic expression of the chemokines CXCL13, CCL20, and CXCL10 was controlled by the LT pathway (our data and 32). However, and in contrast with CXCL13, these two latter chemokines were not essential for colonic patch development.

SILT development, in contrast to colonic patch development, was entirely postnatal. By day 14, the complete developmental/maturation spectrum of colonic SILTs was present and included both small and large clusters of immune cells. Despite common features, such as dependence on the LT pathway,23,33,34 the process of SILT formation differed substantially from that of colonic patches. CXCL13 was not essential for colonic SILT development as numerous SILTs were found in CXCL13-/- mice. However, CXCL13-deficient SILTs were aberrantly formed as B-cell follicles never developed, despite B-cell recruitment. This situation contrasts with the development of SILTs in the small intestine in which absence of CXCL13 prevents B-cell recruitment into the few aberrant SILTs that develop.29 In addition, colonic SILTs also formed earlier and more abundantly in CXCL13-/- mice than in wild-type mice, which could reflect an increased number of circulating LTi cells as a result of absence of peripheral lymphoid tissues.3,36

The other striking difference between SILT development in the small intestine and colon is the independence on the CCR6-CCL20 axis for colonic SILT formation. Even though colonic LTi cells expressed CCR6 and CCL20 expression was reduced in mice unable to properly cluster immune cells in the colonic lamina propria, colonic SILTs developed normally in CCR6-/- mice.

Another chemokine receptor functionally expressed on LTi cells is CXCR3 (our data and 37). Even though in the absence of CXCR3, SILT development and organization was mostly normal, MadCAM-1+ vessels were regularly found in CXCR3-deficient SILTs, therefore suggesting that SILT localization might be altered in the absence of CXCR3-guided LTi cell migration. Alternatively, as chemokine receptors were found to be expressed on endothelial cells and their deficiency found to cause vascular malformations,38,39 it may be that abnormal development of the colonic vasculature leads to the presence of MadCAM-1+ vessels within the parenchyma of colonic SILTs.

Colonic SILT development is likely directed by numerous chemokines with overlapping roles and this may explain the inability to completely abrogate these structures in mice deficient for a single chemokine (receptor). Redundancy in the chemokine system may even explain some of the discrepancies in SILT development between small intestine and colon described here. In this regard, it is important to recognize that SILT development concurred with the onset of microbial colonization of the intestine and exposure to bacterial components induces multiple chemokines. Therefore, similar to the small intestine in which SILTs are smaller in germ-free mice,23,24 it was likely that the recognition of the colonizing bacteria would also promote the development of lymphoid tissues in the colon. In contrast, however, we found the overall process of colonic SILT development to be independent of the colonic microflora. Notwithstanding, we found SILT maturation, i.e., B-cell recruitment, follicular organization, and follicular dendritic cell network development, to require MyD88 signaling. As bacteria were not required for SILT development, this suggests that either IL1R signaling or the release of endogenous TLR ligands may induce the maturation of SILTs. The release of endogenous TLR ligands would reconcile the published observations showing a role for both TLR2/4 and MyD88 in colonic SILT maturation.25

In conclusion, we showed that the murine colon harbors two developmentally distinct lymphoid tissues. Colonic patches started their development during embryogenesis, whereas colonic SILTs developed only after birth in a unique way, dissimilar to the formation of colonic patches, lymph nodes, and small intestinal SILTs. Furthermore, we showed a hitherto unrecognized role for CXCR3 in the localization of SILTs within the lamina propria of the colon and a role for MyD88 in SILT maturation, which was independent of the colonic microflora. Altogether, the data presented here highlight the multitude of different molecular pathways involved in the genesis of lymphoid tissues and provide a useful framework for future assessment of intestinal lymphoid tissue development during inflammatory conditions.
METHODS
Mice. C57BL/B6, LTα−/−, CXCL13−/−, CCR6−/−, CXCR3−/−, and MyD88−/− mice were kept under SPF conditions; C3H mice were kept under SPF or germ-free conditions. For breeding and prenatal analysis purposes, breeding pairs were housed together overnight and the day of vaginal plug detection recorded as embryonic day E0.5. For post-natal analysis, the day of pup delivery was considered day 0. All experiments were approved by the Vrije Universiteit Scientific and Animal Ethics Committees.

Antibiotic treatment. To prevent bacterial colonization of the intestine, wild-type mice were treated with a “cocktail” of antibiotics containing streptomycin (5 g l−1), colistin (1 g l−1), ampicillin (1 g l−1), and sucrose (2.5%, wt/vol; all from Sigma-Aldrich, St Louis, MO) in the drinking water starting at embryonic day E14 and continuing until analysis at day 14 post-partum. Antibiotic treatment was renewed every week.

Immunofluorescence histology. Colonies were dissected at the appropriate age, embedded in optimum cutting temperature compound (Sakura Finetek, Alpen aan den Rijn, The Netherlands), and frozen in liquid nitrogen. Frozen blocks were cut into 7-μm thick serial sections. Detection of organized gut-associated lymphoid tissue was performed on every 10th section (20th section for adult colons) stained for CD4 and MadCAM-1 in combination with either B220 or CD45− mice were kept under SPF conditions; C3H mice were kept under SPF or germ-free conditions. For breeding and prenatal analysis purposes, breeding pairs were housed together overnight and the day of vaginal plug detection recorded as embryonic day E0.5. For post-natal analysis, the day of pup delivery was considered day 0. All experiments were approved by the Vrije Universiteit Scientific and Animal Ethics Committees.
DISCLOSURE
The authors declared no conflict of interest.

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