

# Lymphoid Tissue Inducer Cells in Intestinal Immunity

I. I. Ivanov · G. E. Diehl · D. R. Littman (✉)

Howard Hughes Medical Institute, Skirball Institute of Biomolecular Medicine,  
 New York University School of Medicine, New York, NY 10016, USA  
*littman@saturn.med.nyu.edu*

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**Abstract** During fetal development, lymphoid tissue inducer cells (LTis) seed the developing lymph node and Peyer's patch anlagen and initiate the formation of both types of lymphoid organs. In the adult, a similar population of cells, termed lymphoid tissue inducer-like cells (LTi-like cells), supports the formation of organized gut-associated lymphoid tissue (GALT) in the intestine, including both isolated lymphoid follicles (ILFs) and cryptopatches (CPs). Both LTi and LTi-like cells require expression of the transcription factor ROR $\gamma$ t for their differentiation and function, and mice lacking ROR $\gamma$ t lack lymph nodes, Peyer's patches, and other organized GALT. In ILFs and cryptopatches, LTi-like cells are in close contact with different populations of intestinal dendritic cells (DCs), including a subpopulation recently shown to extend dendrites and sample luminal microflora. This interaction may allow for communication between the intestinal lumen and the immune cells in the lamina propria, which is necessary for maintaining homeostasis between the commensal microflora and the intestinal immune system. The potential functional implications of the organization of LTi-like cells, DCs, and lymphocytes in the lamina propria are discussed in the context of maintenance of homeostasis and of infectious diseases, particularly HIV infection.

## 1

### Introduction

In the intestine, a balance must be maintained between potentially harmful bacteria and an extensive network of cells that constitute the gut-associated lymphoid tissues (GALT). The mechanisms that govern this homeostasis remain poorly understood. When the homeostasis is compromised, the outcome can range from inflammatory bowel disease to food allergies to inflammation-associated malignancies. In this review, we discuss recent studies that shed light on the organization of immune cells in the intestine and provide clues as to how these interact with luminal microorganisms. We describe the role for an orphan nuclear receptor, ROR $\gamma$ t, in the development of lymphoid tissue inducer (LTi) cells during fetal life and of intestinal cryptopatch (CP) cells postnatally, resulting in the genesis of secondary lymphoid organs and tertiary lymphoid follicles, respectively. We also review recent studies showing that dendritic cells (DCs) communicate with the intestinal lumen and transport bacteria from the lumen to the lamina propria (LP). Finally, we propose a model that describes how sensing of microbial content by specialized DCs that extend processes across epithelia can regulate the immune response to the microflora while maintaining the integrity of the intestinal epithelium.

A single layer of intestinal epithelial cells protects the internal organs from more than 700 species of resident gut bacteria totaling approximately  $10^{14}$  cells [1–4]. The mucosal immune system contains and regulates this permanent “infection,” but it also supports it, because of the advantages provided by the microorganisms. The immune system must sense changes in the composition of the microflora. These changes may alert the host to the presence of pathogenic bacteria and activate prompt defense mechanisms. It remains unclear, however, how host defenses can discriminate between commensal and pathogenic bacteria, because they share antigens and express identical Toll-like receptor (TLR) ligands. The intestinal immune system must also have the capacity to recognize and become tolerized against food antigens. Thus the mucosal immune system must handle a number of “nonself” antigens differently—some antigens will induce regulatory or tolerogenic responses, others will be sequestered, and yet others will induce different types of protective immune responses.

## 2

### Development of Organized GALT Structures

The GALT consists of secondary lymphoid organs [Peyer’s patches (PPs) and the mesenteric lymph nodes (MLNs)], tertiary organized lymphoid tissues

[CPs and isolated lymphoid follicles (ILFs)], dispersed cells [intraepithelial T lymphocytes (IELs) and LP T and B lymphocytes], and an organized network of specialized DCs. In addition, Paneth cells, specialized epithelial cells at the base of the intestinal crypts, secrete antimicrobial peptides that exert their action in the lumen of the intestine. Together, these diverse cells and organized lymphoid structures are thought to regulate the microflora while also defending the host from breaches in epithelial integrity and preventing inflammatory responses against harmless antigens. How these different functions are accomplished remains poorly understood, but it is clear that there must be extensive communication between the immune system components within the intestinal mucosa. In addition, the epithelial cell compartment is also regulated by interactions with components of the mucosal immune system. Recent studies with genetically modified mice have advanced our understanding of the organization and relationship between GALT components. However, the mechanisms and mediators involved in the cross talk between these components have yet to be elucidated.

In general, the PPs, ILFs, and CPs have been ascribed different functions based on their developmental and structural differences. However, several studies, as outlined below, have observed redundant or overlapping functions between PPs and ILFs. CP function remains undetermined, although our recent studies suggest that these cells are similar to the LT<sub>i</sub> cells that, in the fetus, are required for development of all lymph nodes and PPs. The CP cells, like fetal LT<sub>i</sub> cells, are characterized by the expression of the orphan nuclear hormone receptor ROR $\gamma$ t, which is necessary for the development of all three types of the organized GALT within the LP, as well as for development of lymph nodes [5, 6].

## 2.1

### Functions of ROR $\gamma$ t in Lymphoid Organogenesis and T Cell Development

ROR $\gamma$  and the closely related ROR $\gamma$ t isoform are retinoic acid receptor-related transcription factors for which no ligand has yet been identified. These proteins are encoded by overlapping transcripts with alternative start sites and differ in sequence at their amino termini. During T cell development, ROR $\gamma$ t is expressed in CD4<sup>+</sup>CD8<sup>+</sup> (double positive, DP) thymocytes, the precursors for CD4<sup>+</sup> helper and CD8<sup>+</sup> cytotoxic T cells. In our early studies, we found that forced ROR $\gamma$  expression in T cells resulted in inhibition of NFAT-mediated induction of IL-2 transcription. This was due to competition for NFAT binding sites on DNA by ROR $\gamma$  [7]. In a separate study, He et al. isolated ROR $\gamma$ t in a screen for cDNA clones that inhibited Fas-mediated activation-induced cell death in a T cell line [8]. This most likely reflects the ability of ROR $\gamma$ t to block

NFAT-dependent induction of FasL. In mice lacking expression of ROR $\gamma$  and ROR $\gamma$ t, survival of DP thymocytes was dramatically reduced, and fewer T cells differentiated to maturity. This was not related to a defect in FasL expression, but, instead, was due to the requirement for ROR $\gamma$ t to direct expression of the antiapoptotic factor Bcl-xL in DP thymocytes. Forced expression of Bcl-xL in the mutant mice rescued development of thymocytes and restored normal T cell numbers in the periphery [9]. We have proposed that the function of ROR $\gamma$ t in the thymus is to prolong the life span of DP thymocytes, giving them the opportunity to be selected into the mature T cell pool by interaction of their receptors with MHC molecules and thus avoiding “death by neglect.” Because the T cell receptor  $\alpha$  locus can undergo sequential V to J segment rearrangements, resulting in expression of new TCRs, a longer life span increases the opportunity for selection by host MHC-peptide complexes. Indeed, in the absence of ROR $\gamma$ t, only proximal V $\alpha$  to J $\alpha$  rearrangements were observed, resulting in a limited TCR repertoire [10].

A second major function of ROR $\gamma$ t, revealed in the analysis of the mutant mice, is to direct the development of lymph nodes and PPs. None of these secondary lymphoid organs developed in the absence of this nuclear receptor, although development of splenic follicles was normal [9]. A similar phenotype was observed in mice lacking the inhibitory HLH transcription factor Id2 [11]. In the absence of either ROR $\gamma$ t or Id2, there was a loss of the fetal CD4<sup>+</sup> CD3<sup>-</sup> IL-7R $\alpha$ <sup>+</sup> cells proposed by Mebius and Nishikawa to be involved in development of lymphoid tissues [12–15]. These cells, now named lymphoid tissue inducer (LTi) cells, are best defined by the expression of ROR $\gamma$ t [5]. LTi cells can be readily visualized in and isolated from mice in which a GFP reporter has been inserted at the start site of the ROR $\gamma$ t gene [5]. They have been observed in embryos as early as day 12.5 of gestation, in aggregates in regions where lymph nodes and PPs will develop. Many, but not all, LTi cells express high levels of CD4. In addition to IL-7R $\alpha$ , they also express the lymphotoxin  $\alpha_1\beta_2$  heterotrimer (LT), the related TNF family member TRANCE, and the chemokine receptors CXCR5 and CCR7.

Lymph node and PP development in the fetus has been proposed to be initiated by localized production in mesenchymal “organizing” centers of the chemokines CXCL13/BLC, the CXCR5 ligand, and CCL19/ELC or CCL21/SLC, the CCR7 ligands. The LTi cells then migrate toward the organizer, where they induce a cascade of events by LT-mediated triggering of the LT $\beta$  receptor and the downstream NF- $\kappa$ B pathway through NIK, a kinase deficient in lymphoid organ-defective *aly/aly* mice. This results in upregulation of the integrin ligands ICAM-1 and VCAM-1 on the stroma as well as expression of chemokines that recruit B and T cells and more LTi cells in a positive feedback loop. Finally, the cells in the newly formed clusters reorganize to form an organized lymph

**Table 1** Development of LNs and organized GALT as well as induction of sIgA production in different mouse models

Model	PP	MLN	PLN	ILFs	CPs	LP B cells	sIgA
LT $\alpha^{-/-}$	—	—	—	—	—	—	—
LT $\beta$ R $^{-/-}$	—	—	—	—	—	—	—
<i>aly/aly</i>	—	—	—	—	+	—	—
LT $\beta^{-/-}$	—	+	+/-	—	—	—	—
TNF $^{-/-}$	Less	+	+	+	+	+	+
TNFR-I $^{-/-}$	Less/small	+	+	—	+	++	Low
TNFR-II $^{-/-}$	+	+	+	+	+	+	+
TRANCE $^{-/-}$	+	—	—	NR	NR	NR	NR
ROR $\gamma^{-/-}$	—	—	—	—	—	NR	NR
IL-7R $\alpha^{-/-}$	—	+	+	Atrophied	+ (less)	NR	NR
$\gamma_c^{-/-}$	—	—	—	—	—	Very few	NR
Id2 $^{-/-}$	—	—	—	—	—	NR	NR
IL-7 $^{-/-}$	—	—	—	NR	NR	NR	NR
$\mu$ MT	+ (small)	+	+	—	+	+	+ (half)
RAG-KO	+ (small)	+	+	—	+ (large)	—	—
AID $^{-/-}$	+	+	+	++	+	+ (IgM+)	—
				Hyperplasic			
Germ-free	+ (small)	+	+	—	+	Few	Very low
TCR $\beta$ X $\delta^{-/-}$	+	+	+	+	+	+	+

NR, not reported

node or PP structure with B cell follicles, T cell areas, and specialized vasculature [16]. This series of events is thought to apply in general to development of lymphoid organs, but there are some differences as to which gene products are required for each type of organ, as listed in Table 1. Although the early inductive events are now reasonably well characterized, there remain many details to be worked out. For example, the LT signal, although essential for lymph node development, is not sufficient to induce expression of ICAM-1 and VCAM-1 and the development of the lymph nodes; therefore, the LT $\alpha$  cells are clearly providing other essential signals [5]. It is also not yet known how the mesenchymal cell response to LT $\alpha$  cells results in subsequent organization of lymphoid tissues.

A third function that we can now ascribe to RORY $\gamma$  is its requirement in the development of tertiary lymphoid tissues in the lamina propria in small intestine and colon. We observed RORY $\gamma$ -expressing cells in both CPs and

ILFs, and these structures were absent in the ROR $\gamma$ t-deficient mice. Because ROR $\gamma$ t<sup>+</sup> cells in the lamina propria have features similar to those of LTi cells, we believe that they perform inductive functions similar to those that occur during fetal development of secondary lymphoid organs. We will describe studies on the functions and potential relationship between CPs and ILFs and speculate as to the potential role of ROR $\gamma$ t<sup>+</sup> LTi-like cells in the genesis of ectopic lymphoid follicles in autoimmune diseases.

Fetal LTi cells and adult LTi-like cells thus play a central role in the development of secondary lymphoid organs and organized GALT and require the expression of ROR $\gamma$ t. ROR $\gamma$ t-deficient animals differ significantly from other models that lack organized GALT and/or peripheral lymph nodes (PLNs) (see Table 1), such as *LT $\alpha$ <sup>-/-</sup>*, *LT $\beta$ <sup>-/-</sup>*, *LT $\beta$ R<sup>-/-</sup>*, and *aly/aly* or *NIK<sup>-/-</sup>* animals. In the latter models, the defects are at later stages of lymphoid organ development, whereas the ROR $\gamma$ t (and Id2) deficiencies result in early abrogation of inducer cell development.

## 2.2

### Structure and Function of Organized GALT Structures

To simplify the discussion of immune responses, mucosal immune sites are customarily divided into inducer (or inductive) and effector sites. The inducer sites are generally identified as secondary lymphoid organs, which in the intestine are the PPs and MLNs. The effector sites are within the LP, where there are populations of B and T lymphocytes as well as multiple populations of DCs and other myeloid cells, and the epithelium, where the IELs reside. It is thought that antigens come in contact with the immune system in the inductive sites, where they are delivered either actively by DCs or passively through M cells. Upon antigen presentation in the inductive site, lymphocytes differentiate into effector cells and migrate to effector sites. This separation of inducer and effector sites rests on the assumption that induction of immune responses occurs only in organized lymphoid follicles of secondary lymphoid organs (PPs and MLNs in the intestine), whereas effectors (plasma cells and activated T cells) accumulate and act in the LP.

Recent findings suggest that this conceptual separation of inductive and effector sites is not so clear-cut in vivo. For example, PPs and intact MLN architecture have been reported to be dispensable for antigen-specific B cell responses and IgA production [17, 18]. However, other types of lymphoid aggregates in the intestine, such as ILFs, may also serve as inductive sites, and these have different developmental requirements than PPs. The appearance of functional ILFs may be dependent on signals received by other GALT structures, such as CPs, or DCs in the LP. In addition, the LP, which was considered an

exclusive effector site, may have an additional role in B cell activation and class switching [19]. The primary argument for the exclusive inductive capacity of PPs and MLNs was that these were thought to be the only sites where antigen gained access to the mucosal immune system, which is required for the inductive phase of an immune response. PPs contain follicular-associated epithelium (FAE) with specialized intestinal epithelial cells, the M cells, which sieve antigen from the lumen and deliver it to subpopulations of DCs. These DCs can then present the antigen in the context of a germinal center (GC) reaction in the PP or migrate to the MLN and induce immune responses there. However, recent studies have described at least four alternative means of antigen uptake directly into the LP, thus circumventing the PP. M cells were also found in ILFs [20] as well as dispersed in the villus intestinal epithelium [21]. Thus antigen can potentially gain access to the mucosa through both ILFs and villus M cells. Other studies have demonstrated that LP DCs can extend dendrites through the epithelial tight junctions and sample luminal antigens directly [22, 23]. Although it is thought that migration of DCs to the MLN is required to induce an immune response [24], it remains possible that antigen presentation or induction of immune functions can occur directly within the LP. Finally, epithelial cells can directly transfer IgG-bound antigen by using the intestinal neonatal Fc receptor (FcRn) as a shuttle [25], thus delivering the antigen into the LP, where it will most likely be taken up by phagocytic immune cells.

Despite the lack of a clear functional delineation between inducer and effector sites, the existence of distinct immune structures within the intestines is well established. We discuss recent advances in our understanding of their structure and function during immune responses below.

### 2.2.1

#### **Peyer's Patches**

The Peyer's patch (PP) is the largest organized lymphoid tissue of the small intestine. The central structures are B cell follicles, which are usually multiple and large. The number of B cell follicles defines the size of the PP. In mice PPs contain fewer than 10 follicles, usually 3–4, whereas human PPs may be several centimeters long and contain many tens of follicles [26]. T cells are also present in the PP. The T cell areas are around high endothelial venules (HEVs), between the B cell follicles. The luminal side of the PP is lined with a specialized epithelium, called follicle-associated epithelium (FAE). FAE lacks crypts or villi and, in contrast to the columnar villous epithelium, is cuboid, has few goblet cells, and does not contain secretory cells such as Paneth cells. The major feature of FAE is the presence of specialized epithelial cells called M cells. M cells are derived from adjacent crypts and have specialized microfolds

instead of microvilli on their luminal surface, which allow for transcytosis of luminal antigens [27]. On the PP side M cells possess large pockets that are tightly associated with PP immune cells, represented by clusters of DCs, CD4<sup>+</sup> T cells, and B cells [28, 29]. M cells participate in the formation of a regional environment bellow the FAE that differs in composition of immune cells from the rest of the PP and the villous epithelium. This region is also known as the subepithelial dome (SED). M cells are the major portals of antigen entry into the mucosa and thus direct and deliver the antigen to immune cells in the SED [30–32]. Pathogenic microorganisms, such as virulent *Salmonella* species, *Yersinia* species, *Shigella flexneri*, Poliovirus, and reoviruses may take advantage of the M cells to gain access to the intestinal mucosa.

As in other immune inductive sites, PP DCs are probably responsible for initiating and directing the subsequent immune response. In the PP, several populations of DCs have been described [33]. The first is found directly below the SED. These cells are CD11c<sup>+</sup>, DEC205<sup>+</sup>, and M342<sup>+</sup> and are also found in the B cell follicle outside of GCs. These cells are mainly immature myeloid DCs (CD11b<sup>+</sup> CD8 $\alpha$ <sup>+</sup>) [34] and appear to take up antigens transported by M cells. The second population is found in the intrafollicular region and is seen in close proximity with T cells. These cells are mainly lymphoid DCs (CD11b<sup>+</sup> CD8 $\alpha$ <sup>+</sup>) [34] and also express DEC205 and M342, which correlate with DC maturation. “Double negative” DCs (CD11b<sup>+</sup> CD8 $\alpha$ <sup>+</sup>) are found in both locations.

DCs in the PP induce intestine-specific immune responses. Surface phenotypic analysis of CD11c<sup>+</sup> DC populations revealed that PP DCs express higher levels of MHC class II molecules, but similar levels of costimulatory and adhesion molecules, compared with splenic DCs [35]. DCs isolated from the spleen induce a Th1-biased response characterized by high levels of IFN $\gamma$  production. In contrast, DCs isolated from PPs, especially those of the myeloid lineage, induce a Th2 response that includes elevated IL-4 and IL-10 and reduced IFN $\gamma$  production by T cells [34, 35]. In addition, stimulation with CD40L or RANKL leads to IL-10 production by DCs from PPs, but IL-12 production by DCs from lymph nodes [34–36]. Thus, there exist significant functional differences between DCs from different tissues.

One of the most prominent roles of the PP is the formation of GCs after antigenic stimulation, with subsequent production of IgA. Class switching to IgA occurs in the GCs, and the resulting plasmablasts migrate out of the PP to the LP to form plasma cells and produce sIgA [37, 38]. B cells play a major role in the organization of the PP in general and the FAE in particular, because B cell-deficient mice have very small PPs, FAE and M cell numbers [39].

PPs also play a major role in the activation of T cells and their homing to the LP. Because PPs are on the major route of lymphocyte recirculation, small



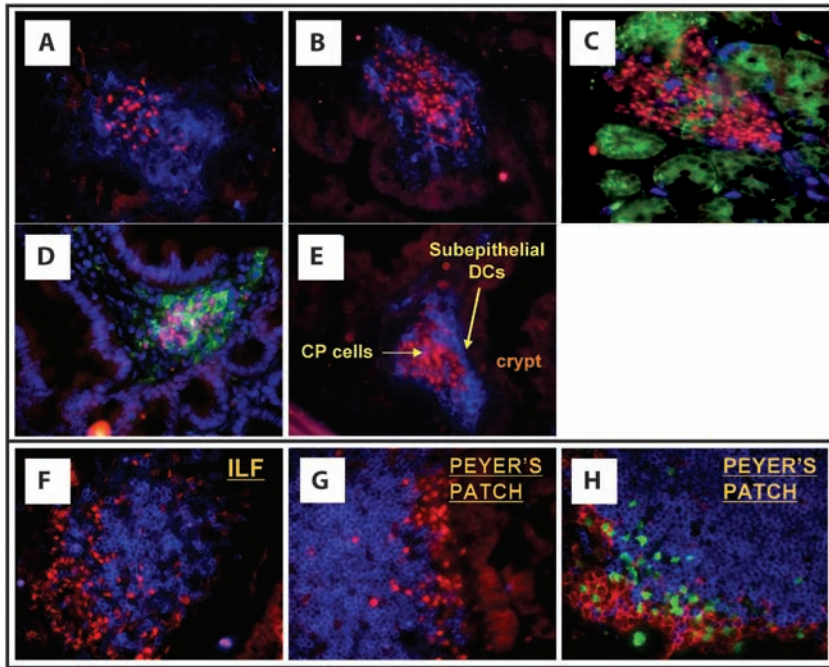
naïve T cells enter the T cell areas of the PP using L-selectin and CCR7 [40]. After antigen stimulation, secretion of retinoic acid by intestinal DCs causes the upregulation on T cells of the gut homing receptors  $\alpha 4\beta 7$  and CCR9 and downregulation of E selectin ligands [41], allowing the T cells to preferentially migrate to the LP.

### 2.2.2

#### Isolated Lymphoid Follicles

ILFs are relatively large lymphoid aggregates found throughout the LP, in both small intestine and colon, with the highest density in the antimesenteric wall of the small intestine [20, 42]. They are most abundant in the distal ileum [42], which may be related to their developmental requirement for intestinal flora. ILFs are composed of a single B cell follicle and thus resemble a small PP. Their similarity to PPs is further underlined by the presence of a GC and M cells. A recent study implied that ILFs can serve as inductive sites for mucosal immune responses, especially following signals from pathogenic bacteria [43]. ILFs are composed of a large central cluster of B cells surrounded by a ring of  $\text{ROR}\gamma^+\text{c-kit}^+\text{IL-7R}\alpha^+$  cells (see below and Fig. 1F). In addition, a large number of DCs are present in the ILF [20] (Fig. 2). Besides size and general morphology, ILFs appear postnatally [44], whereas PPs develop during late fetal life. Although both ILFs and PPs require  $\text{LT}\beta\text{R}$  signaling for development, ILFs, but not PPs, require  $\text{LT}\beta\text{R}$  signaling for maintenance through adulthood: Treatment of adult mice with  $\text{LT}\beta\text{R}$ -Ig eliminates ILFs, but not PPs or MLNs [44]. In addition, ILF development requires stimulation by commensal intestinal microflora [42]. Thus ILFs were absent in germ-free mice, which have small PPs [20], but ILF development was induced by recolonization with normal flora [42]. Furthermore, ILF hyperplasia correlated with the increased commensal bacterial load in activation induced cytidine-deaminase (AID)-deficient mice that cannot class switch and therefore lack IgA. The ILF hyperplasia was abolished when bacterial load was decreased by antibiotic treatment [45]. Together, these studies suggest that ILFs form continuously throughout adult life, in response to the commensal microflora, and that  $\text{ROR}\gamma^+$  cells are essential for their induction.

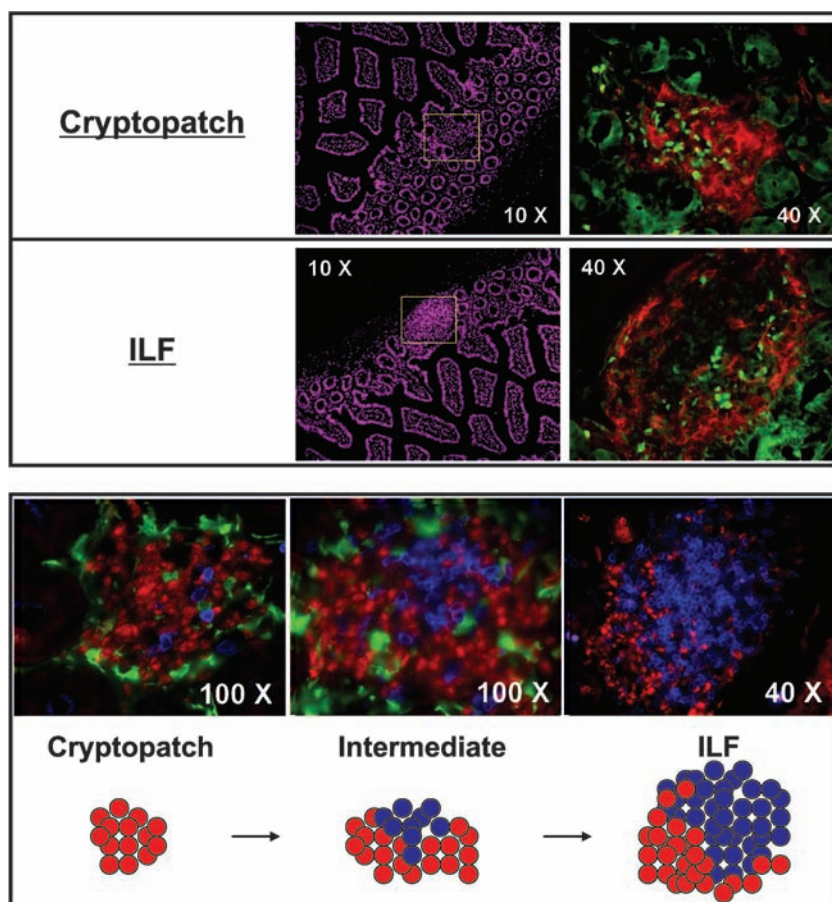
ILFs may be functionally redundant or complementary to PPs. They may contribute to the production of antigen-specific sIgA [17] or may serve as inductive sites of pathogen-specific immune responses *in vivo* [43]. One possibility is that ILFs form in the absence of PPs, when IgA levels are low, or in response to the microflora to supplement levels of sIgA, thus serving as a second line of defense and aiding the PP. Another possible role for ILFs is that they are responsible for the induction of sIgA against bacterial stimuli, whereas



**Fig. 1A–H** ROR $\gamma$ <sup>+</sup> cells act as inducers of all organized GALT in small intestinal LP. Different sized cryptopatches (CPs) (A–C) are present at the bottom of villi near the crypt areas (D, E). Cryptopatches contain large clusters of ROR $\gamma$ <sup>+</sup> cells (red) surrounded by CD11c<sup>+</sup> DCs (blue in A, B, and E and green in D). Very few B and T cells are present in CPs (C). ROR $\gamma$ <sup>+</sup> cells are also present in ILFs (F) and around B cell follicles in the PP (G, H). All sections are at  $\times 40$  magnification. Colors: A, B, E Red (ROR $\gamma$ ), blue (CD11c); C red (ROR $\gamma$ ), blue (CD3), green (B220); D red (ROR $\gamma$ ), blue (DAPI), green (CD11c); F, G red (ROR $\gamma$ ), blue (B220); H green (ROR $\gamma$ ), blue (B220), red (CD11c)

PPs and MLNs are more important in the production of antigen-specific sIgA after oral immunization [17, 18, 43].

Most studies point to a function of ILFs as sensors of intestinal microflora, receiving signals from the lumen and probably transmitting these signals to the mucosal immune system. Thus the close association of ILFs with DCs and ROR $\gamma$ <sup>+</sup>c-kit<sup>+</sup> cells would allow ILFs to receive signals from the DCs that sample the lumen of the intestine. The presence of hundreds of organized lymphoid structures throughout the LP, as opposed to the limited number of PPs, would allow for faster and more efficient sampling and activation of local immune responses. Keeping the immune response localized would also



**Fig. 2** Cryptopatches and ILFs. *Top and middle panels:* Cryptopatches are small lymphoid aggregates consisting mainly of  $\text{ROR}\gamma^t$  lymphoid tissue inducer-like cells and dendritic cells (DCs). ILFs are large aggregates that are readily detectable with DAPI staining and consist of a large B cell follicle surrounded by  $\text{ROR}\gamma^t$  inducer-like cells and DCs. Sections were obtained from the small intestine of an adult  $\text{ROR}\gamma^t$ -GFP-KI mouse.  $\text{ROR}\gamma^t$  cells (green) and DCs (red) were stained with antibodies against GFP (Alexa 488) and CD11c (PE). *Left panels:* DAPI staining only. *Bottom panel:* Different stages of ILF development. In this model  $\text{ROR}\gamma^t$  cells in the cryptopatch receive signals from DCs and induce stromal cell production of chemokines, such as CXCL13, to recruit B cells and form ILFs. Structures intermediate between CPs and ILFs, containing small clusters of B cells, are often seen in the LP. Sections were obtained from the small intestine of a heterozygous  $\text{CX}_3\text{CR1}$ -GFP-KI mouse. Staining is with anti- $\text{ROR}\gamma^t$  Cy3 (red) and B220-APC for B cells (blue). In the first two panels  $\text{CX}_3\text{CR1}^+$  DCs (green) were stained with an antibody against GFP (Alexa 488)

reduce the potential damage caused by inflammation, therefore maintaining the integrity of the intestinal barrier. Additionally, ILF-based immune responses may precede those in PPs and MLNs, which would require more time to develop because of the requirement for cell migration to and from these sites.

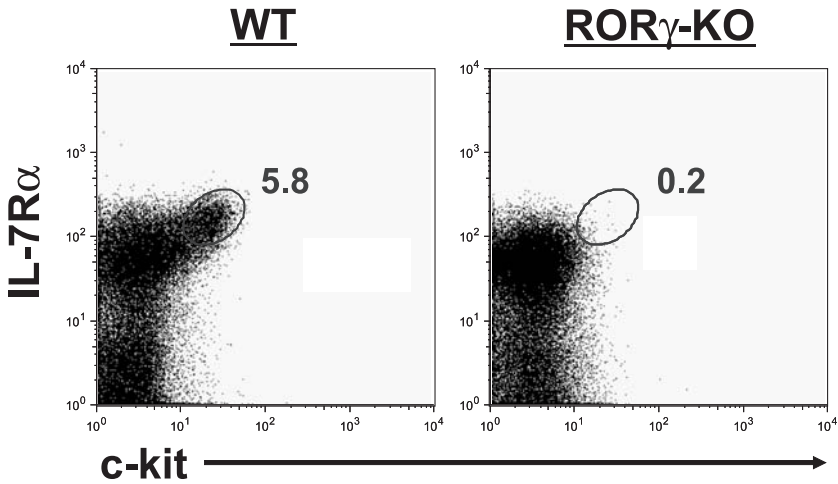
### 2.2.3

#### Cryptopatches

Cryptopatches (CPs) were first described as clusters of  $c\text{-kit}^+ \text{Lin}^-$  lymphoid-like cells and DCs in the LP of the small intestine [46]. Their name is derived from their location in proximity to the bottom of crypt villous areas. CPs are distinguished from ILFs by the lack of both B and T lymphocytes [20]. It has been estimated that about 1,500 CPs accumulate over time in the adult mouse intestine [46, 47].

The exact function of CPs is currently unknown. Because the major lymphoid-like cells in CPs express the lymphoid precursor markers  $c\text{-kit}$  and  $\text{IL-7R}\alpha$ , CPs were postulated to be involved in the extrathymic pathway of IEL differentiation. Initial reconstitution experiments, as well as transfer of  $\text{Lin}^- c\text{-kit}^+ \text{IL-7R}\alpha^+$  CP cells from nude mice into *scid* mice, supported this hypothesis, as IELs were generated in the immunodeficient host [48]. However, we have recently shown that, in immune-competent mice, CP cells do not give rise to IELs. This conclusion was made possible by the finding that the orphan nuclear receptor  $\text{ROR}\gamma\text{t}$  is selectively expressed in the lymphoid-like CP cells in the adult small intestine and is required for the development of CPs.  $\text{ROR}\gamma\text{t}^{-/-}$  mice completely lack CPs ([6], Fig. 3), but there is no reduction in the number of  $\text{TCR}\gamma\delta$  IELs. The reduced  $\text{TCR}\alpha\beta$  IEL numbers were due to compromised thymic output in the mutant mice, and these cells were restored upon forced expression of  $\text{Bcl-xL}$ , despite continued absence of CP cells. To determine which cells are precursors of the IEL, we performed fate-mapping experiments. When  $\text{ROR}\gamma\text{t-Cre}$  transgenic mice were crossed with  $\text{ROSA26-GFP}$  reporter mice that express GFP only after Cre-mediated excision of a transcriptional stop signal, all DP-derived T cells, including  $\text{TCR}\alpha\beta$  IELs, and all CP cells expressed GFP. In contrast, when the  $\text{ROSA26-GFP}$  mice were crossed to  $\text{CD4-Cre}$  mice (in which Cre expression is present in DP cells and  $\text{CD4}$  lineage T cells but not CP cells), GFP could only be seen in the  $\text{TCR}\alpha\beta$  IELs, but not in CP cells. These experiments supported the finding that most, if not all, of the  $\text{TCR}\alpha\beta$  IEL are derived from  $\text{CD4}^+ \text{CD8}^+$  DP thymocytes rather than from CP cells [6].

In addition, the fate-mapping studies indicated that the  $\text{ROR}\gamma\text{t}^+$  fetal LTi cells and adult CP cells have no apparent lineage progeny. This finding suggests that  $\text{ROR}\gamma\text{t}^+$  LTi cells and CP cells are terminally differentiated cells



**Fig. 3** ROR $\gamma$ -KO mice lack CP cells. No c-kit<sup>hi</sup>IL-7R $\alpha$ <sup>hi</sup> CP cells are detected in ROR $\gamma$ -KO and ROR $\gamma$ t-KO (not shown) mice. Representative staining of total LP lymphocytes is shown. The plots were gated on CD45<sup>+</sup> lymphocytes. The numbers indicate percentage of CD45<sup>+</sup> lymphocytes

whose function is to induce lymphoid structures in the appropriate microenvironment. Once secondary lymphoid organs are induced during fetal development, LTi cells appear to no longer be necessary. In contrast, the ROR $\gamma$ t<sup>+</sup> CP cells are thought to be continuously replenished in adult animals. Reconstitution experiments have shown that CPs and ILFs in LT $\alpha$  and common  $\gamma$  chain ( $\gamma_c$ )-deficient mice can be reconstituted by the transfer of wild-type bone marrow (BM), indicating that adult BM can be a source of precursors for LTi-like cells [49, 50].

## 2.3

### Cryptopatches as Organizing Centers for Localized Mucosal Responses

In adult mice heterozygous for the ROR $\gamma$ t<sup>GFP</sup> allele, expression of the GFP reporter was limited to DP thymocytes and to cells in the lamina propria of the small and large intestine. In the LP, all Lin<sup>-</sup>c-kit<sup>+</sup>IL-7R $\alpha$ <sup>+</sup> CP cells [48] were GFP<sup>+</sup>. We also utilized a monoclonal antibody against ROR $\gamma$ /ROR $\gamma$ t to assess its expression in wild-type mice. Combining these two approaches revealed that CPs consist almost exclusively of ROR $\gamma$ t<sup>+</sup> cells tightly clustered and surrounded by DCs (Fig. 1A–E). ILFs contain a peripheral layer of ROR $\gamma$ t<sup>+</sup> cells in addition to the major B cell follicle (Fig. 1F) and may thus represent a later stage in GALT development than the CP. Interestingly, ROR $\gamma$ t<sup>+</sup> cells were also

found surrounding B cell follicles in the PP (Fig. 1G,H), providing a potential mechanistic and functional link between all three types of organized GALT. These structural features suggest that ILFs form from CPs. In support of this hypothesis we identified structures similar to the ones described by Pabst et. al. [51], which appear to be an intermediate between ILFs and CPs. These structures contain mostly ROR $\gamma$ <sup>+</sup> cells and DCs but also have small clusters of B cells. The existence of these intermediate structures suggests a developmental relationship between ILFs and CPs and they may represent activated CPs that are in the process of recruiting B cells to develop into an ILF (unpublished data, Fig. 2, and [51]). The intermediate structures described here may also correspond to the small B220<sup>+</sup> clusters reported by Lorenz et al. and labeled “immature ILFs” [42], to distinguish them from the much larger mature ILFs.

The temporal development of the two types of structures also hints that ILFs may be derived from CPs. In the mouse, CPs first develop around 1–2 weeks of age and ILFs are not observed until the colonization of the intestine by microflora around weaning time (3–4 weeks) [20, 46].

In addition, as can be seen from Table 1, mice deficient in organized GALT lack either both ILFs and CPs or only ILFs. For example *aly/aly*, IL-7R $\alpha$ , and RAG-KO mice have CPs but lack ILFs, but there is no mouse model that specifically lacks CPs while still preserving ILFs. The ILF-like aggregates in RAG-KO mice [20] are most likely enlarged CPs and not ILFs because they do not contain any B cells. It is possible that the CPs are hyperactivated in the RAG-KO LP and recruit, through a positive feedback loop, the only available lymphoid-like cells in this environment. As a result, these ROR $\gamma$ <sup>+</sup> cells form large CPs instead of ILFs.

CPs may thus activate the intestinal stroma to recruit B cells and form ILFs and in this way participate in an integrated mucosal immune network. In the case of fetal LT $\alpha$ i cells, developmentally timed signals are likely to activate the inducer function of these cells (such as lymphotoxin  $\alpha_1\beta_2$  and other still undefined mediators) and subsequent development of lymph nodes and PPs. If ROR $\gamma$ <sup>+</sup> CP cells have a similar inducer function, it is likely that this is regulated by environmental signals rather than by developmental timing. Environmental signals emanating in the lumen may be transmitted to the ROR $\gamma$ <sup>+</sup> cells through the surrounding layer of DCs. This may induce the recruitment of B cells and generation of ILFs. In support of the hypothesis that ROR $\gamma$ <sup>+</sup> cells help recruit naïve B cells to LP, ROR $\gamma$ -KO mice lack CPs and have very few B cells in the LP (I.I. Ivanov and D.R. Littman, unpublished data). Correspondingly B cells accumulate in the spleen and peritoneal cavity (our unpublished data and [52]). Additionally, naïve B cells require intact LT $\beta$ R-signaling on stromal cells for their recruitment to the LP [53, 54]. Together, these data suggest that LT $\beta$ R-signaling on cryptopatch stroma, most likely



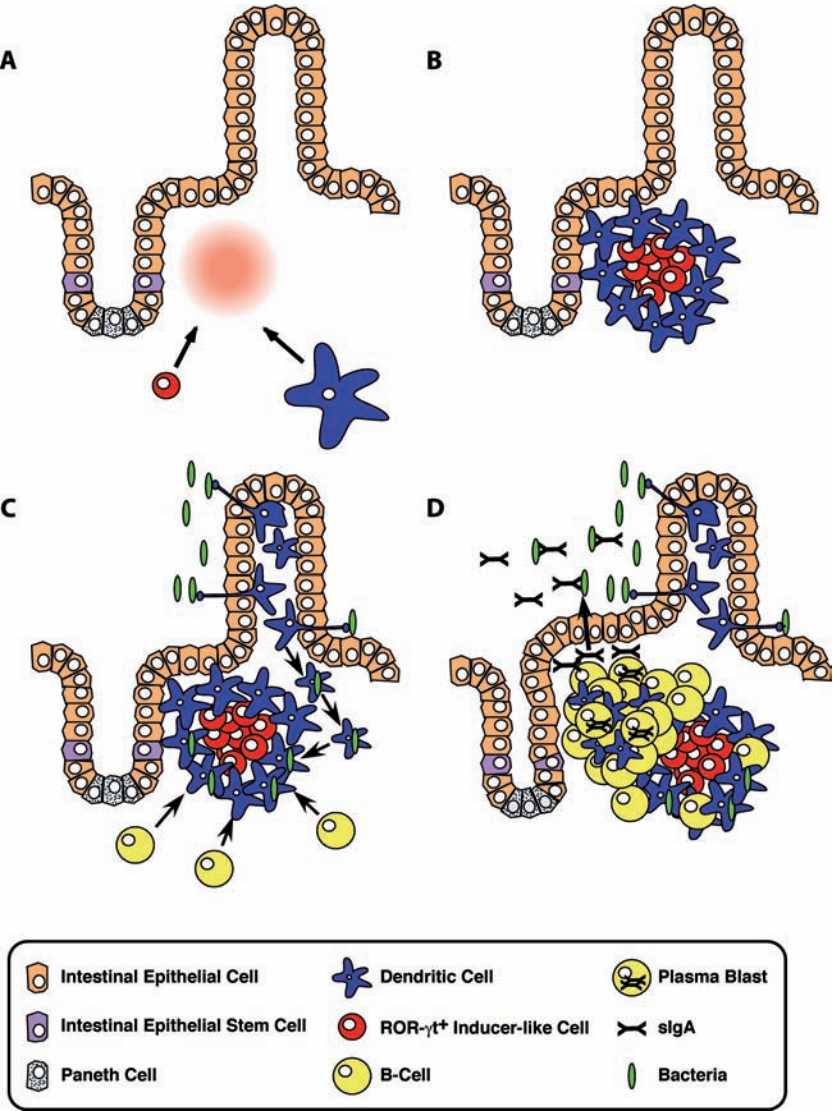
induced by ROR $\gamma$ <sup>+</sup> CP cells, is necessary for the recruitment of naïve B cells to the LP. Formation of ILFs would then allow for production of sIgA.

DCs probably have an essential role in CP function. Large clusters of DCs are present at the periphery of the CP, surrounding the ROR $\gamma$ <sup>+</sup> cells as shown in Fig. 1. These DCs are located directly in the subepithelial space of the crypts and may be the first to receive signals from penetrating bacteria or bacteria present in the crypts, which would be a signal for a failure in immune protection. Alternatively, DCs may transmit signals from the lumen by direct sampling of its content. The latter was suggested by the presence at the periphery of the CP of a population of DCs that express the fractalkine receptor CX<sub>3</sub>CR1, as can be seen in Fig. 2. These DCs were recently demonstrated to form a dense network under the basal lamina and to project dendrites into the lumen of the intestine, especially in the terminal ileum [22]. The CX<sub>3</sub>CR1-expressing DCs can transport bacteria into the LP with these transepithelial dendrites [22]. DCs that transport bacteria into the lumen may then transmit the information to the CP as well as to the MLN. In our model, this transport may induce the differentiation of CP to ILFs. Interestingly, mature ILFs, are mostly present in the terminal ileum [42], which is colonized by microflora and is the only region of the small intestine that contains these transepithelial dendrites. Figure 4 represents schematically a model of the possible function of CPs and ILFs.

The strategic location of CPs at the base of crypts may indicate that these structures have functions in addition to inducing differentiation of B cell follicles. For example, in response to luminal signals, possibly mediated by the epithelium-associated network of DCs, CPs may induce innate immune responses in the nearby crypt epithelium. Following signals from the lumen, ROR $\gamma$ <sup>+</sup> CP cells or associated DCs may thus modify the function of Paneth cells by inducing secretion of bioactive molecules (e.g., defensins), or they may participate in the regeneration of epithelium from epithelial stem cells in the crypt. The latter is an especially attractive possibility in light of the finding of Medzhitov and colleagues that TLR-mediated signaling supplied by the intestinal microflora is required for regeneration of the intestinal epithelium after chemical or radiation-induced damage [55].

### 3 Dendritic Cells, a Double-Edged Sword

In the model that we propose (Fig. 4), DCs continuously survey the intestinal lumen for commensal and pathogenic microorganisms and communicate with other cells in the lamina propria and the epithelium to activate innate







**Fig. 4A–D** Model of cryptopatch (CP) function in sIgA production. **A** The initial stages and order of cell recruitment in CP and ILF formation are unclear. ROR $\gamma$ <sup>+</sup> LTi-like cells and DCs are recruited to the base of villi. The signals for this recruitment may initiate from the DCs, the ROR $\gamma$ <sup>+</sup> LTi-like cells, or the stroma. However, ROR $\gamma$ <sup>+</sup> cells were shown to be the major inducer population in fetal lymph node and PP anlagen and may also play a similar role in the adult. **B** A CP, consisting of ROR $\gamma$ -expressing LTi-like cells as well as dendritic cells, is formed. **C** CPs may receive signals from the lumen through the sampling action of subepithelial DCs. DCs may deliver bacterial antigens to the CP in addition to migrating to mesenteric lymph nodes. Activation of CP cells may lead to induction of B cell recruitment signals from the stroma. **D** The recruited B cells form clusters, resulting in ILF formation. B cells are then activated and class-switch to IgA. IgA-producing cells may differentiate into plasmablasts/plasma cells in the lamina propria to secrete sIgA into the lumen.

host defense mechanisms. In this model, activation of the DCs by microbial products induces recruitment of B cells as well as some LP T cells into the CP, resulting in formation of ILFs. This could be due to direct action of CP-associated DCs on B and T cells, to activation of CP stromal cells by the DC, or to DC-mediated activation of the ROR $\gamma$ <sup>+</sup> CP cells, which, in turn, would activate the stromal cells, resulting in lymphocyte recruitment. An important outcome would be the production of sIgA, which limits the concentration of bacteria in the intestinal crypts. The introduction of antigen would cause the maturation of a CP into an ILF and allow for a rapid and localized immune response. Additionally, DCs will migrate to the MLN to induce both mucosal and systemic immune responses.

The direct detection of antigens by DCs allows for rapid recognition of pathogens. The DCs form a complex network throughout the intestine and can rapidly transmit danger signals and recruit immune effector cells. However, in some pathogenic settings, such as HIV infection, this network and direct recruitment may lead to an enhancement of infection. DCs can bind HIV through the cell surface C-type lectin DC-SIGN, which results in virus uptake and enhanced infection of cocultivated T cells [56, 57]. This interaction may also allow intact virus to exploit the trafficking properties of DC to disseminate from the mucosa to secondary lymphoid organs [56, 57]. In addition, the interaction of intestinal DC with effector memory T cells could explain the sustained loss of these cells that has been reported in HIV and SIV infections.

Activated memory T cells express the chemokine receptor CCR5. This is a major coreceptor for both HIV and SIV, making memory T cells a primary target for both viruses. Mattapallil et al. used quantitative PCR to show that at the peak of SIV infection 30%–60% of CD4<sup>+</sup> memory T cells throughout the body are infected with the virus. Most of these infected cells disappear within

4 days [58]. As a consequence, over one-half of all memory CD4<sup>+</sup> T cells in SIV-infected macaques are destroyed directly by the virus during the acute phase of infection.

Because of the constant activation by oral antigens and the surveillance necessary in the GI tract, a much higher percentage of T cells in the gut are memory T cells and an easy target for HIV infection. In 1995, the massive depletion of CD4<sup>+</sup> T cells in the gut was first reported [59]. It wasn't until 1998 that studies in macaques demonstrated how quickly this depletion occurred. Veazey et al. reported that 14–21 days after infection with SIV there was a dramatic decrease in CD4<sup>+</sup> T cells in the gut [60]. Strikingly, at this point in infection there was no decrease in blood or lymph node CD4<sup>+</sup> T cells. In addition, the decrease of T cells seems to be a direct effect of the virus, as peak virus production in the GI tract in SIV infection coincides with peak number of infected CD4<sup>+</sup> T cells [61]. There is a corresponding depletion of CD4<sup>+</sup> cells in other mucosal areas, as similar depletion has been observed in the vaginal and lung mucosa [62, 63]. It was further demonstrated that most infected cells were not recently activated T cells but were resting memory cells [61].

The findings with SIV were recently correlated with two studies of HIV-infected patients. In HIV<sup>+</sup> patients, there is a significant and preferential depletion of mucosal CD4<sup>+</sup> T cells compared with peripheral blood CD4<sup>+</sup>. This depletion occurs mainly in the LP instead of in the PP in all stages of disease [64, 65]. As in macaques infected with SIV, this depletion occurs preferentially within CCR5<sup>+</sup> CD4<sup>+</sup> T cells [65].

Cross-sectional analysis of a cohort of primary HIV-1 infection subjects showed that although chronic suppression of HIV-1 permits near-complete immune recovery of the peripheral blood CD4<sup>+</sup> T cell population, a significantly greater CD4<sup>+</sup> T cell loss persists in the GI mucosa, despite up to 5 years of fully suppressive therapy [64].

It is formally possible that DC-SIGN-mediated uptake of HIV represents a mechanism for long-term retention of infectious HIV particles within DCs. Intestinal DCs therefore represent a potentially sizable reservoir for HIV and may be important in the transport and dissemination of the virus. Local dissemination in the lamina propria could involve not only T helper cells but also the lymphoid-like CP cells. In mouse, about one-third to one-half of these RORγ<sup>+</sup> cells express CD4, and these cells also express a variety of chemokine receptors that can function as coreceptors for HIV entry. The equivalent cell population in humans has not yet been described, but it is highly likely to be present, because ILFs have been identified in human lamina propria. If these cells also express CD4, they are highly likely to be targets of HIV within the intestine, and this may contribute to pathogenesis of viral infection. Understanding the contribution of intestinal DCs and the RORγ<sup>+</sup>

CP cells to initiation of immune responses in the LP may therefore lead to important insights for designing strategies to eliminate HIV from the body.

## 4

### **LTi Cells and Ectopic Lymphoid Follicles in Autoimmunity**

Several autoimmune diseases, including Crohn's disease, rheumatoid arthritis, and type I diabetes, are marked by formation of ectopic lymphoid organ-like structures within the affected tissues. The contribution of such ectopic follicles to disease pathogenesis remains unexplored. The role of LTi cells or related inducer cells (such as those found in CPs) in these lesions has yet to be explored. Forced expression of CXCL13/BLC in pancreatic islet cells resulted in the local formation of lymphoid follicles, but this was independent of LTi cells, because follicles were also observed in  $ROR\gamma^{-/-}$  mice ([66] and unpublished collaborative result). However, CXCL13 expression by stromal cells is induced by  $LT\beta R$  signaling. As LTi-like cells are the producers of  $LT\alpha\beta$ , this system may bypass the requirement for the cells. In autoimmune diseases, inflammatory stimuli may result in activation of LTi or CP-like cells, particularly in the intestine, and this could induce excessive follicle formation. Consistent with this notion, the number of ILFs is increased in dextran sulfate-induced colitis in mice [67] and also in Crohn's disease [68] and ulcerative colitis [69] in humans. Such tertiary lymphoid tissue could have a central role in autoimmune disease. Because nuclear receptors are readily amenable to pharmacological manipulation, inhibition of  $ROR\gamma$  function in vivo may be achievable and may provide a means to control inflammatory bowel disease.

## 5

### **Conclusions**

The intestinal immune system is a dynamic environment. By necessity, many types of detection and inductive sites must exist throughout the intestine. The intestinal immune system is constantly bombarded with different types of antigens, which it must rapidly identify and respond to accordingly. If the antigen is a food antigen, the immune system must become tolerant; if the antigen is a non-pathogenic commensal bacteria, the immune system must work to contain it in the lumen of the intestine; if the antigen is a pathogenic bacteria or virus, the immune system must initiate a protective immune response. The DC-CP-ILF axis may play a major role in integrating signals

from the lumen or the crypt, thus allowing for the rapid detection of and response to a variety of antigens while limiting the response to a local region. This would allow for control of pathogenic microbes while stronger responses in either the PP or MLN are being generated. Consequently, this will result in faster containment as well as limit the potential damage to the delicate balance of the intestine.

The mechanisms that are involved in sampling microorganisms and mounting appropriate responses can potentially also be turned against the host, resulting in autoimmune inflammatory bowel disease or in persistent infections. A better understanding of the role of the intestinal DC-CP-ILF axis in immune responses will be necessary to determine its importance in human disease.

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