Chemokine regulation of naïve T cell traffic in health and disease

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Abstract

A central feature of the immune response is the precise spatio-temporal convergence of T cells and antigen presenting cells (APC) in particular microenvironments within secondary lymphoid organs (SLO). CCR7 and its ligands CCL19 and CCL21 have been identified as the gatekeepers for both naïve T lymphocytes and dendritic cells (DC) to these defined anatomical compartments. A new perception on the regulation of lymphocyte traffic in lymph nodes (LN) has come from observations that sphingosine-1-phosphate (S1P) receptor agonists affect T cell entry and exit from these organs. Recent developments in intravital microscopy (IVM) techniques reveal unexpected autonomous random motion of lymphocytes within secondary lymphoid tissues, and provoke questions about the mechanisms that guide their compartmental navigation.

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1. Introduction

The world of microbial pathogens and tumors contains a nearly infinite number of antigenic determinants. The adaptive immune system has evolved to cover this enormous spectrum of antigens by equipping lymphocytes with unique antigen receptors, which are capable of recognizing millions of antigens. As an organism’s capacity to generate and maintain a large lymphocyte population is inevitably limited, the number of lymphocyte with any given antigenic specificity must be restricted in order to guarantee maximal antigen-receptor diversity. For example, in the case of CD8+ T cells in the mouse it has been estimated that the precursor frequency of naïve T cells is in the range of 1 in $2 \times 10^5$, or 100–200 cells per mouse [1]. This poses an immense challenge on the immune system, namely to assure that no invading pathogen escapes detection while maintaining manageable precursor numbers. Two basic principles facilitate these requirements: first, antigen from the periphery is acquired by antigen presenting cells (APC), and then displayed in secondary lymphoid organs (SLO), including peripheral (PLN) and mesenteric lymph nodes (MLN), spleen, Peyer’s patches (PP), appendix, and tonsils. The routes by which antigen gains access to SLO are variable. One major mechanism involves uptake by dendritic cells (DC) in peripheral sites, and subsequent migration of DC via lymphatics or blood to PLN and spleen, respectively. However, free antigen may also enter SLO by this route or, in the case of PP, by transcytosis from the intestinal lumen via M cells. Second, naïve T cells restlessly recirculate through SLO in order to scan APC for the presence of cognate peptide antigen presented on MHC molecules (reviewed in [2,3]). Antigen encounter in the context of appropriate MHC molecules results in cell division and a >1000-fold expansion of naïve T cells, as well as to their differentiation into effector cells, which eliminate the pathogen from the organism (reviewed in [4]). This expansion is followed by a retraction phase during which the majority of effector cells undergo apoptosis, leaving behind a stable population of antigen-specific memory cells (reviewed in [4,5]).

The encounter of antigen-loaded APC and naïve T cells is arguably the central event of the adaptive immune response. Therefore, characterization of the molecular mechanisms that guide APC and T cells to the appropriate microenvironment is critical for understanding how the immune system works. During the past few years, experimental evidence has accumulated demonstrating that chemokines and their
receptors are the key elements that direct lymphocytes and APC to distinct anatomical areas in secondary lymphoid organs. Subsequently, the direct contact of APC and T cells leads to naïve T cell priming (reviewed in [6,7] as well as Gunn in this issue of Seminars in Immunology). CCR7 and its ligands CCL21 (also known as 6Ckine; eotaxin-2; secondary lymphoid-tissue chemokine or SLC; thymus-derived chemotactic agent-4 or TCA-4) and CCL19 (Epstein Barr virus-induced ligand chemokine-1 or ELIC; macrophage inflammatory chemokine-3α or MIP-3α) have emerged as the molecular gatekeepers of naïve T cell entry into lymph nodes (LN) from the blood stream. These two chemokines also mediate the migration of mature APC from the periphery to lymph nodes via afferent lymph vessels. However, it remains incompletely understood how T cells and APC navigate within T cell zones of SLO in order to establish physical contact. While chemokines are certainly involved in establishing and maintaining the anatomic organization of SLO, recent data using two-photon (2P) microscopy of intact lymph nodes have revealed new and unprecedented insights into the rules that govern the movement of T cells within T cell zones of PLN [8–10]. These findings raise questions on the role of chemokine gradients in T cell migration within these specialized microenvironments. Furthermore, the recent identification of pharmacologic agents that act on sphingosine-1-phosphate (SIP) receptors has opened a new field on how T cell migration to, and exit from PLN is regulated and may be pharmacologically manipulated (reviewed in [11]). This review will focus on the role of the CCR7–CCL21/CCL19 axis in the orchestration of the primary immune response, explore its pathophysiologic role, and discuss recent insights into the rules of extravascular T cell locomotion in PLN.

2. Molecular mechanisms of T cell entry into PLN

2.1. Sites of T cells entry into PLN

In principle, there are two possible ports of lymphocyte passage into PLN, namely from the blood stream or via afferent lymph vessels. Blood-borne lymphocytes transmigrate through specialized postcapillary venules which are called high endothelial venules (HEV) due to their cuboidal shape (reviewed in [12]). HEV are found in all SLO, with the exception of the spleen, where lymphocytes emigrate via blood sinusoids in the marginal zone. Morphological studies have found an abundance of lymphocytes within the vascular wall of HEV, predominantly in the paracortical areas of LN [13]. We have recently extended these findings by employing fluorescence intravital microscopy (IVM) of mouse inguinal LN HEV [14]. Based on this method, we have described five branching orders of the venular tree within this organ—postcapillary and small collecting venules in the paracortex (orders III–V) and large collecting venules in the medulla (orders I–II), which drain into the epigastric vein [15,16].

Lymphocytes mainly interact with orders III–V HEV in the paracortex [16].

The second route by which lymphocytes may enter PLN is via initial migration into peripheral tissue, such as the skin, and subsequent egress from these organs via afferent lymphatics into draining LN. Studies performed in sheep have shown that afferent lymphatics of cutaneous LN almost exclusively contain antigen-experienced T cells, whereas thoracic duct lymph contains mainly naïve and only a small fraction of memory/effector T cells [17]. Based on these experiments it was proposed that naïve T cells would enter PLN mainly via HEV, whereas effector/memory T cells preferentially travel through non-lymphoid tissues. However, experiments in other species, such as the rat, revealed that many antigen-experienced T cells are well capable of direct interaction and transmigration through HEV [18]. As will be discussed below, these discrepancies can be explained by the more recent discovery of different subsets of memory T cells, which display differential migratory properties [19].

2.2. The molecular mechanisms of T cell–HEV interaction

There is ample experimental evidence that leukocyte migration from the blood stream into tissues occurs not in a random, but rather a highly controlled manner, mediated by consecutive molecular interactions, termed the multistep adhesion cascade [2,3,20]. In the case of naïve T lymphocytes in PLN HEV, the first step, tethering and rolling, is mediated by T cell-expressed L-selectin, which binds to peripheral node addressin (PNAd) displayed on HEV (Fig. 1). PNAd comprises a group of fucosylated, sulfated and sialylated LewisX–decorated molecules, including CD34, GlyCAM-1, sgp200, and podocalyxin [21–24]. Firm adherence of rolling naïve T cells in HEV is primarily mediated by the β2 integrin lymphocyte function-associated antigen (LFA)-1 (CD11a/CD18 or αLβ2 integrin), which binds to intercellular adhesion molecules (ICAM)-1 and -2 on HEV [16]. However, integrins are usually found in an inactive or low-affinity confirmation on the surface of leukocytes, and require an activation signal to undergo conformational change, which renders them highly active. Integrin triggering on rolling lymphocytes in HEV is pertussis toxin (PTX)-sensitive, implicating G protein-linked signaling, most likely by chemokine receptors, in lymphocyte recirculation [16,25].

Several lines of evidence have established CCL21 and CCL19 as the main triggers of LFA-1-mediated T cell arrest in HEV. Both CCL21 and CCL19 bind to CCR7, which is highly expressed on naïve T cells, and both are potent chemoattractants for naïve T cells in vitro. They also promote rapid integrin activation on rolling naïve T cells in flow chamber assays [26–32]. These effects are stronger on naïve than on memory T cells or B cells. Furthermore, desensitization of naïve T cells with CCL21, but...
Fig. 1. Schematic model of the multi-step adhesion cascade facilitating the entry of naïve T cells into PLN from the blood. The first step of T cell–HEV interaction, rolling, is mediated by binding of T cell-expressed L-selectin to PNAd displayed on HEV. The N-terminal C-type lectin domain of L-selectin specifically recognizes the 6-sulfated, sialylated, and fucosylated tetrasaccharide Lewis X (sLeX), a core-1-linked glycan that decorates the endothelial sialomucins CD34, podocalyxin, GlyCAM-1, and sgp200, and is recognized in humans and mice by mAb MECA-79. Evidence from HEC-GlcNAc-6-O-sulfotransferase (the enzyme responsible for sulfation of LeX)-deficient mice suggests that alternative L-selectin ligands may exist on HEV, whose exact biochemical nature remains to be determined [24]. These ligands are not recognized by MECA-79. In the second step, CCL21, and perhaps CCL19, which are presumably presented by GAGs on the HEV surface, bind to CCR7 on rolling T cells. CXCR4–CXCL12 interaction has also been proposed to play an accessory role in the T cell trafficking to PLN [47]. Chemokine binding to their respective receptors elicits intracellular G protein-linked signals, which induce a rapid conformational change in the β2-integrin LFA-1 on T cells (adapted from [115–117]). LFA-1 in its active form then binds to endothelial ICAM-1 and/or ICAM-2, which mediates firm arrest of rolling T cells.

In the mouse, there are two functional CCL21 proteins that differ by one amino acid in position 65, and have been termed, accordingly, CCL21-ser and CCL21-leu ([34–35] reviewed by Gunn in this issue). When transgenically expressed in mice, both forms of CCL21 show equivalent biological activity in terms of T cell attraction [36]. While CCL21-ser mRNA and protein are constitutively expressed at high levels in PLN HEV as well as in stromal cells in the T cell zones, CCL21-leu expression is predominantly found in lymph vessels in non-lymphoid tissues [28,36,37]. In the case of CCL19, there are multiple transcribed pseudogenes in the murine genome, but only the ELC-atg (scya-19) gene encodes functional CCL19 protein [37]. CCL19 mRNA is also expressed by T zone stromal cells, and to a lesser degree by dendritic cells, but not by HEV [37,38].

Comprehension of the pivotal role of CCR7 and its ligands in T cell recirculation has come from gene-targeted and spontaneous mutant mouse strains, respectively [39,40]. In the absence of CCR7, naïve T cells are almost completely inhibited from migration into PLN and PP, whereas B cell homing occurs at a frequency of 20–50% of that of wild-type cells [39]. PLN in CCR7−/− mice lack the characteristic distribution of B cell follicles and T cell rich areas as observed in wild-type mice; rather, they display an irregular distribution of B and T cells in the paracortex, with the occurrence of prominent B cell follicles with enlarged germinal centers in only some PLN. T cells in the spleen are spread throughout the marginal sinuses and the red pulp, instead of localizing to the periarteriolar lymphocyte sheath (PALS). This phenotype is similar to a spontaneous mutant mouse strain, called paucity in lymph node T cells (plt [40,41]). In plt/plt mice, the CCL21-ser and ELC-atg genes are deleted ([34–37,42] as well as Gunn in this issue), resulting in smaller LN and PP with reduced numbers of naïve T cells, but apparently normal B cell numbers, and a compensatory increase in naïve T cells in the spleen [41,42]. PLN in plt/plt mice exhibit an absence of recognizable T cell zones as well as an increased number of T cells in and around B cell follicles. When naïve T cells from wild-type mice were adoptively transferred into plt/plt mice homing to PLN and PP was drastically reduced, while B cell homing was at normal levels. IVM studies revealed that naïve T cells roll but not CXCL12, blocks their firm adhesion in PLN HEV [33].
IVM, Tcm interact with PLN HEV in an effect similar to that observed in CCL21-deficient mice in this issue). As shown by Campbell and co-workers in this volume (38), CCR7-deficient mice do not arrest in plt/plt PLN HEV (33). However, injection of CCL21 into the dermis of plt/plt mice led to its accumulation in draining PLN, where it was present on the luminal surface of HEV. Thus, LFA-1-mediated naïve T cell sticking in these vessels was restored (33). A similar effect was observed when CCL19 was used instead of CCL21 (38). CCR7-deficient mice do not mount contact hypersensitivity (CHS) or delayed type hypersensitivity (DTH) reactions, and show impaired antibody responses (39). The plt/plt mice reveal an increased sensitivity to mouse hepatitis virus infections (42). Surprisingly, however, they show delayed, yet enhanced T cell immune responses, including CHS reactions (43). A possible explanation for this apparently diverse biologic behavior between CCR7−/− and plt/plt mice could be the observed shift of the T cell response from PLN to the spleen in plt/plt mice, as the enhanced responses were substantially suppressed in splenectomized plt/plt mice. Moreover, whereas DC in CCR7+/− mice are unable to enter cutaneous lymphatic vessels, in plt/plt mice they can reach PLN, because lymph vessel expression of CCL21-leu remains intact in plt/plt PLN. Thus, DC can localize to the superficial cortex and thereby interact with T cells (43).

2.3. Memory T cell homing to PLN

Whereas previously naïve and memory T cells were mainly distinguished based on expression of surface markers that differentiate between these subsets, but not between different memory T cell subsets, recent evidence suggests that there is also considerable heterogeneity within the antigen-experienced T cell population. This has been demonstrated in functional and phenotypic terms, including the expression of adhesion molecules and chemokine receptors on these cells. A seminal paper by Sallusto et al. recently proposed the existence of at least two separate populations of memory T cells in humans, which are defined by the expression of LN homing molecules (19), so-called central memory T cells (Tcm) that express l-selectin and CCR7, and effector memory T cells (Tem) that are negative for these antigens. Subsequent studies in mice have confirmed the existence of such populations and have demonstrated that Tcm preferentially localize to SLO, whereas Tem are found predominantly in non-lymphoid organs, such as lungs, liver, or the intestinal epithelium (44–46) and Campbell and co-workers in this volume). As shown by IVM, Tcm interact with PLN HEV in an l-selectin and a CCR7-dependent manner (46).

Taken together, CCR7 and its ligands CCL21 and CCL19 are important signals mediating the firm adhesion of naïve and central memory T cells in PLN HEV. As both chemokines are capable of inducing integrin activation of rolling T cells in vitro and in vivo, their relative importance in this process has yet to be determined. Gene targeted mice with a selective deficiency in CCL19 and/or one or both CCL21 genes will give insights into this question.

2.4. A role for CXCR4/CXCL12 in lymphocyte homing to PLN?

Besides CCR7 ligands, CXCL12 and its receptor CXCR4 have recently been implicated to play a (minor) role in T cell migration to PLN (47). The residual homing observed for wild-type T cells in plt/plt recipient mice was abolished when CXCR4-deficient donor T cells were used. However, CXCR4−/− T cells showed normal homing behavior in wild-type mice. Furthermore, CXCL12 protein, but not mRNA expression could be detected in PLN HEV. Thus, this CCR7-independent homing pathway may participate in trafficking of T cells with low or absent CCR7 expression, such as for certain memory T cell populations.

3. Chemokine transport from the periphery to PLN and presentation to leukocytes

As mentioned above, we and others have observed that intracutaneously administered chemokines are rapidly transported to HEV in draining PLN (reviewed by Rot et al. in this issue). Fluorescently-tagged or radiolabeled chemokines, such as CCL2 or CCL21, are detectable in HEV in situ within minutes after injection (33,48). The rapidity of this process suggests that the lymphatic system is the primary access route of these molecules to HEV. In an elegant study, Gretz et al. characterized the morphological basis for the transport of lymph-borne, soluble factors into and within PLN (49). Low molecular weight molecules, including chemokines, enter the subcapsular sinus, and are then channeled along a meshwork of collagen fibers in the fibroblastic reticular cell (FRC) network towards HEV. The FRC network forms conduits, which radiate out from HEV towards the subcapsular sinus. Each reticular fiber is ensheathed by FRC and is thus physically separated from the interstitial space of the cortical parenchyma. This barrier prevents low molecular weight molecules from freely diffusing into the B and T cells areas, but rather shunts them directly to perivascular spaces in immediate vicinity of HEV. Once chemokines reach this submucosal compartment, they are translocated via intracellular transport vesicles to the luminal surface of HEV (33,38,48). Here, they are presented to leukocytes in the blood stream establishing alternative multi-step adhesion cascades.

The concept of endothelial chemokine transcytosis was first introduced by Middleton et al. whose ultrastructural studies showed that intracutaneously injected chemokines, such as CXCL8 (IL-8) and CCL5 (RANTES), are bound at the abluminal surface of venular endothelial cells (50) and Rot et al. in this issue). The bound chemokines are then internalized into caveolae (plasmalemmal vesicles) and transported across the endothelial cell to the luminal surface (50). Similarly, Baekkevold et al. described that CCL19 mRNA in human tonsils was expressed by perivascular cells, but not HEV; nevertheless, CCL19 protein was detectable.
by electron microscopy in distinct intracellular vesicles and at leukocyte-HEV interfaces [38]. Intracutaneously injected CCL19, like CCL21, accumulated in HEV both inside the cells and on their luminal surface, and was able to restore T cell sticking in PLN HEV of plp/pl mice [33,38]. These data suggest that under physiologic conditions CCL19 secreted by stromal cells may be transcytosed by HEV and presented on their surface.

The molecular mechanisms of chemokine transcytosis in endothelial cells are still incompletely understood. Candidate molecules for chemokine transport and presentation in blood vascular and lymphatic endothelial cell surfaces include the chemokine receptors D6 and D and the Duffy antigen/receptor for chemokines (DARC) as well as glycosaminoglycans (GAGs; reviewed in [51] as well as by Rot et al. in this issue). D6 and DARC are both promiscuous in binding a large variety of chemokines, but neither receptor is thought to transduce intracellular signals, such as calcium flux, or a chemotactic response upon agonist binding (reviewed in [52] and by Rot et al. in this issue). D6 is expressed at high levels in lymphatic, but not venular, endothelial cells primarily in the skin and the intestinal tract [53]. Recent evidence indicates that engagement of D6 leads to agonist internalization and degradation, but not transcytosis of chemokines in apical-to-basal or basal-to-apical directions [54]. Therefore, D6 might primarily act as a lymphatic scavenging receptor for chemokines, preventing the sapping of draining PLN with bioactive molecules during the course of inflammation.

On the other hand, DARC has been implicated in chemokine uptake and transport, because it is constitutively expressed by postcapillary venular endothelial cells, including HEV, where it localizes in caveolae as well as the plasma membrane. DARC has the capacity to bind numerous inflammatory CC and CXC chemokines [55,56]. It is also expressed on erythrocytes, where it acts as a receptor for Plasmodium vivax [57]. Lee et al. recently demonstrated that DARC, when transfected into endothelial cells, facilitates the transport of the chemokine CXCL1 (growth-related oncogene-a or gro-a) across endothelial monolayers [58]. This also led to enhanced migration of neutrophils toward CXCL1 across DARC expressing endothelial monolayers. Whereas DARC-deficient mice have no apparent developmental alterations, and are healthy during life, at least under physiopatho-free vivarial conditions, neutrophil recruitment to lungs in response to CXCL8 (interleukin-8) was attenuated [58,59]. Therefore, DARC may be involved in the regulation of inflammatory cell trafficking by controlling the availability of chemokines of the surface of endothelial cells at sites of inflammation.

Another group of molecules that is important for chemokine-endothelial interactions are GAGs (reviewed in [51]). The highly basic C-terminus of chemokines interacts with negatively-charged GAGs present on cell surfaces, in exocytic granules, and in the extracellular matrix. These interactions are thought to be critical for the function of chemokines [51]. For example, it has been proposed that GAGs immobilize chemokines on the surface of endothelial cells, thus forming solid-phase-stabilized concentration gradients which are presented to rolling leukocytes [61]. Binding of some chemokines, such as CXCL8 and CXCL4 (PF4), to GAGs has been shown to involve the C-terminal a-helix, which is distinct from chemokine receptor binding domains [50,62,63]. C-terminal truncation of CXCL8 (IL-8) results in impaired heparan sulfate binding, but retains similar neutrophil activation characteristics in vitro as compared to native CXCL8. In vivo, transcytosis of truncated CXCL8 to the luminal surface of endothelial cells was markedly reduced. Functionally, this was reflected by decreased recruitment of neutrophils in response to the mutant as compared to the native form [63].

We have reported that intradermal injection of recombinant truncated CCL21 generated in Escherichia coli (CCL21ΔCT, truncation of the 41 C-terminal amino acids) led to decreased accumulation in draining PLN as compared to full-length CCL21 [33]. More recently, we have repeated these experiments with CCL21ΔCT generated in yeast. Interestingly, the yeast-derived material, unlike the E. coli-generated CCL21ΔCT, was readily transported to draining PLN, similar to full-length E. coli-derived CCL21 (unpublished data). Thus, chemokine transport from skin to PLN not only depends on C-terminal (GAG-binding) recognition elements, but may also be influenced by host cell-specific post-translational modifications. This issue is further complicated by apparent tissue-specific differences in chemokine transport. Yeast-derived CCL21ΔCT was readily translocated from the skin to HEV, but upon injection into subcutaneous air pouches in mice it was unable to attract naive T cells, whereas full-length CCL21 attracted such cell when injected at equimolar concentrations. In contrast, the potential of CCL21ΔCT to chemottractant naive T cells in vitro was ~50% lower than that of native CCL21 (Fig. 2). We speculate that, in analogy to CXCL8 [63], GAG binding of the truncated molecule may be impaired, which could result in insufficient transcytosis and presentation in vivo.

3.1. Transport of chemokines from the periphery and presentation on HEV: a remote control mechanism for leukocyte recruitment in inflammation

What might be the functional relevance of chemokine transport from the periphery to draining PLN? As efficient as the lymphatic system is in transporting interstitial fluid under physiologic conditions, it carries the inherent danger that intact and/or cell-associated pathogens reach regional PLN via the same route, which eventually could lead to systemic spreading of such organisms. Thus, it might be of advantage to recruit inflammatory cells and phagocytes as early as possible to draining PLN in order to provide a second line of defense at this site. For example, monocytes and macrophages are normally sparse in the T cell areas of PLN,
but their numbers increase markedly during inflammation [48]. We have recently demonstrated that CCL2 (MCP-1/JE) mRNA and protein are rapidly induced in inflamed skin, and CCL2 protein was readily detectable in draining PLN several hours before mRNA induction [48]. Furthermore, similar to CCR7 ligands, CCL2 protein injected intradermally accumulated rapidly in draining PLN, and localized to the luminal surface of HEV, where it induced blood-borne monocyte recruitment [48]. These studies suggest that peripheral inflamed tissues modify the profile of chemokines displayed on HEV in draining PLN, thus remotely controlling the composition of leukocyte populations that access these organs from the blood stream.

4. Involvement of CCL21 in lymphoid neogenesis and autoimmunity

Under certain chronic inflammatory conditions, primarily in autoimmune and some infectious diseases, the ectopic formation of lymph node-like tissue can be observed in adult peripheral organs. This phenomenon is called lymphoid neogenesis and is thought to play a causative/perpetuating role in the pathogenesis of the disorders in which it occurs (reviewed in [64] and by Cupedo and Mebius and by Ohl et al. in this issue). Consequently, there is considerable interest in the molecular mechanisms that trigger lymphoid neogenesis. Experimental evidence suggests that some signals involved in normal lymph organogenesis might also play a role in lymphoid neogenesis. During development, a conspicuous population of fetal-liver derived progenitor cells, defined as CD3−CD4+CD45+IL-7Ra+CXCR5+ cells, colonizes the PP and PLN anlagen (reviewed in [65] and in Cupedo and Mebius in this volume). Together with resident mesenchymal cells, these cells establish a positive cytokine/chemokine feedback loop, which involves lympho-toxin (LT) family members (in particular membrane-bound LTα1β2) expressed by the homed leukocytes, and the mesenchymal chemokine CXCL13 [65,66]. Expression of LT or CXCL13 under the rat insulin II promoter (RIP11) leads to the formation of ectopic lymph node-like tissue in islets [67,68], and it has been suggested that under these conditions CD3−CD4+CD45+ cells might be recruited to the pancreas where they induce lymphoid neogenesis. Other studies, however, indicate that there may be distinct cues in the formation of lymphoid tissue during embryonic development compared to adult organims. For example, transgenic expression of CCL21 under RIP11 (Ins-TCA-4 mice) is sufficient to elicit lymphoid neogenesis in pancreatic islets [36,69,70]. However, both CCR7−/− and pho/pho mice have PLN and PP, which are smaller and disorganized. Thus, tissue expression of CCL21 (or CCL19) is sufficient for lymphoid neogenesis, but not essential for LN formation. Strikingly, the formation of ectopic lymphoid tissue depends on the presence of T cells, but not B cells, in Ins-TCA-4 mice [69]; when these mice were backcrossed to RAG-1−/− mice, which lack all lymphocytes, but still have small lymph nodes, no lymphoid tissue developed in islets. In contrast, when Ins-TCA-4 mice were crossed to Ikaros−/− mice, in which only B cells are absent, lymphoid tissue formed in the pancreas. Of note, CD3−CD4+CD45+ cells do not arise in Ikaros−/− mice, and these animals lack all LN and PP [65].
The accumulation of T cells and DC preceded by several weeks the occurrence of B cells in the islets of Ins-TCA-4 mice, which further suggests that T cells are important for lymphoid neogenesis. If this assumption is correct, the question arises as to how these cells enter non-lymphoid organs, since naïve T cells express only CXCR4 and CCR7. Despite the fact that the ligand for CXCR4, CXCL12, is constitutively expressed in many tissues, and is even up-regulated upon inflammation, naïve T cells are usually excluded from peripheral organs and sites of acute inflammation, thus making CXCL12 an unlikely player in lymphoid neogenesis [71–73]. This argument is further corroborated by recent data showing that transgenic expression of CXCL12 in pancreatic islands induced considerably smaller infiltrates than CCL21. The CXCL12-induced infiltrates contained very few T cells, but were enriched for DC [70]. In lymphoid neogenesis CCL21 is expressed, together with PNAd and/or MAdCAM-1, on HEV-like vessels, which therefore are probable ports of entry for naïve T cells [64]. However, it remains unclear at what time point HEV develop in the course of lymphoid neogenesis, and whether they are the cause or the consequence of this process.

How then do naïve T cells enter non-lymphoid organs? A possible explanation comes from the recent identification of CCL21 expression in venular endothelial cells in human and murine autoimmune diseases, such as rheumatoid arthritis (RA), ulcerative colitis (UC), certain skin and liver diseases, and experimental autoimmune encephalitis [74–78]. Both CCL21 mRNA and protein were clearly detectable in PNAd+ non-lymphatic endothelial cells within and also outside organized of lymphoid tissue in RA synovial membrane, and the colon in UC, and naïve T cells were commonly found in close vicinity to such CCL21+ vessels [78]. Moreover, using IVM we recently demonstrated that naïve T cells can roll in inflamed mouse cremaster muscle venules in an l-selectin-dependent manner, but they do not arrest [78]. However, exogenous application of CCL21 induced the accumulation of naïve T cells in cremaster muscle venules as well as in subcutaneous air pouches in vivo, despite the absence of PNAd in these vascular beds [78]. In contrast, CXCL12 did not attract naïve T cells to this model. These results strongly indicate that CCL21 represents the bottleneck for naïve T cell migration to non-lymphoid tissues.

Although naïve T cells express low amounts of membrane LTα1β2, it is up-regulated by their activation via the TCR, certain cytokines, such as IL-4 and IL-7, and, interestingly, CCL21 and CCL19 [70,79,80]. Whereas CD3−CD4+CD45+ progenitor cells also express CCR7 and respond to CCL19 in chemotaxis assays in vitro [81–83], it is unclear whether these cells exist in the adult organisms, and if so, whether they are recruited to sites of inflammation. Taken together, the ectopic expression of CCL21 on

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**Fig. 3. Proposed role of CCL21 and T cells in lymphoid neogenesis in autoimmune diseases.** The formation of lymph node-like tissue at peripheral sites of (chronic) inflammation, for example in the synovial membrane in rheumatoid arthritis, is proposed as a sequential multistep process. (I) Blood vessel endothelial cells at sites of inflammation up-regulate the expression of CCL21 both at the mRNA and protein level. This leads to firm adhesion of CCR7 expressing naïve (grey) or central memory (Tcm, green) T cells. (II) After extravasation, T cells are attracted into the interstitium by chemotactic mediators released by stromal cells or other leukocytes. (III) Mature extravascular CCR7+ dendritic cells (DC, blue), which carry autoantigens or microbial peptides, also migrate towards CCL21, and can stimulate auto-reactive T cells and/or T cells that recognize microbial antigens. (IV) This leads to T cell activation, proliferation, and differentiation into effector cells. Activated T cells also up-regulate membrane lymphotoxin (LT, mainly LTα1β2) expression. (V) LTs then induce the expression of CXCL13 in stromal cells, which is chemotactic for B cells and also plays a role in the formation of HEV. (VI) Finally, the inflammatory tissue is organized into lymph node-like structure.
blood vessels is likely to play a role early in the course of lymphoid neogenesis by recruiting naïve and central memory T cells to sites of inflammation and by inducing LT family members on lymphocytes (Fig. 3).

5. Positive and negative regulators of naïve T cell homing to PLN

Naïve T cell traffic to PLN has always been considered to occur at a constant rate under steady-state conditions. However, there is evidence for the existence of factors that negatively or positively regulate T cell–HEV interactions in vivo. One example are findings of a negative regulatory effect on naïve T cell migration to PLN by CD43, a large, negatively-charged sialoglycoprotein, which attenuates t-selectin-mediated adhesion [84]. Conversely, inflammatory signals in peripheral tissues cause an increase in lymphocyte numbers in draining LN by two mechanisms: first, inflammatory signals trigger a rapid, but transient shutdown of lymphocyte egress into efferent lymph; second, T cell recruitment via HEV is several-fold increased during the following days [85]. Very recently an entirely new avenue of research has been launched by the discovery that a novel immunosuppressant, FTY720, sequesters T cells in PLN and PP (reviewed in [11]).

FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride) is a synthetic derivative of a product isolated from the ascomycete Isaria sinclairii [11]. Upon administration to animals or exposure to blood plasma, FTY720 becomes rapidly phosphorylated (FTY720-P). FTY720-P is a structural homologue of SIP [86,87], a pleiotropic lysophospholipid that binds to a family of five G protein-coupled receptors (S1P1–5; formerly known as endothelial differentiation genes (edg)-1, -5, -3, -6, -8, respectively; reviewed in [88]). FTY720-P interacts with all of these receptors, with the exception of S1P6, which are down-regulated after activation, whereas S1P1 and S1P2 are found on endothelial cells [88–90]. SIP is released from activated platelets, mast cells, and monocytes, and is found at up to micromolar levels in plasma [88]. Arguably, the most prominent and pharmacologically useful biologic effect of FTY720 is the potent inhibition of allograft rejection [11]. Immunosuppressive doses of FTY720 and other SIP-agonists induce a rapid disappearance (within a few hours) of mononuclear cells, especially T cells, from peripheral blood, thoracic duct, and spleen, and a parallel increase in T cell numbers in PLN, MLN, and PP [86,91]. Adoptive transfer of naïve lymphocytes into FTY720-treated recipient animals suggested accelerated homing to the PLN, MLN, and PP [91], which was reversible after cessation of the drug [91,92]. Apoptotic effects of FTY720 have been suggested to be involved in the clearance of T cells from the circulation. However, the plasma levels of FTY720 reached with therapeutic doses in vivo are far below the concentrations necessary to induce T cell apoptosis in vitro [91]. Importantly, FTY720 does not interfere with the generation of effector and memory T cells or the production of antibodies in mice with systemic viral infections [92]. However, the drug causes a shift in the distribution of virus-specific effector cells from spleen and blood to PLN [92]. In response to percutaneous immunization with non-infectious antigen, the draining PLN of FTY720-treated mice contained reduced numbers of antigen-activated CD4+ T cells, while the numbers of effector CD8+ T cells in peripheral blood were reduced [93]. This was interpreted as a consequence of reduced recirculation of naïve precursor T cells resulting in decreased access to antigen-loaded APC [93]. By contrast, circulating levels of adoptively transferred in vitro-generated Th1 cells were not affected by FTY720 treatment.

Thus, a reasonable hypothesis for the mode of action of FTY720 is that naïve and effector T cell populations are sequestered and trapped in PLN and PP, hence limiting their access to APC and effector sites.

What are the molecular mechanisms behind the sequestration of T cells into PLN and PP by FTY720? A first clue came from a study by Chen et al. who demonstrated that FTY720-mediated lymphopenia was PTX-sensitive, and that treatment of lymphocytes with FTY720 increased their in vitro chemotactic response towards chemokines, including CCL5, CCL19, CCL21, and CXCL13 [94]. Honig et al. found that FTY720 enhances the activity of the lipid transporter Abcb1 (MDR-1, P-glycoprotein) and the leukotriene C4 transporter Abc1 (MRP-1) [95]. Blocking either of these channels with pharmacologic inhibitors or using T cells from Abcb1 or Abc1-deficient mice abrogated the FTY720-induced increased chemotaxis toward CCL19. Also, in vivo chemotaxis towards CCL19 and in vivo homing depended on the 5-lipoxygenase (5-LO)-mediated synthesis of cysteinyl leukotrienes. Thus, a model of sequential engagement of Abcb1, S1P receptors, 5-LO, and Abc1 by FTY720 resulting in increased response to PLN homing chemokines was proposed [95]. Henning et al. demonstrated that the severe homing defect of CCR7-deficient T cells to PLN could be alleviated by FTY720 treatment in a PTX-sensitive manner [96]. Of note, the reduction of circulating lymphocyte numbers in CCR7−/− mice followed delayed kinetics, and was less complete than in wild-type mice. These results indicate both a CCR7-dependent (early) and a CCR7-independent (later) component of FTY720-mediated homing to PLN.

This hypothetical mode of action could also explain some discrepant results obtained in FTY720-treated plt/plt mice. One study found normal circulating lymphocyte numbers 6 h after FTY720 application in plt/plt mice, whereas two other papers describe a decrease in circulating T cells of plt/plt mice after 24 h or later [94–96]. Of note, although FTY720 treatment depleted T cells from peripheral blood and spleen in plt/plt mice, T cells did not accumulate in PLN of these animals [95]. This raises the question whether T cell sequestration in SLO is the sole mechanism of the
lymphopenia induced by S1P agonists, and, if not, in which organ(s) the cells were sequestered.

Besides increasing T cell homing to PLN, S1P agonists inhibit lymphocyte egress from these organs [86]. As early as 3 h after application of such reagents, the subcapsular sinus of PLN is depleted of lymphocytes, which accumulate on the abluminal side of the sinus-lining endothelium [86]. This explains the previously noticed disappearance of T cells from thoracic duct lymph. However, it remains to be determined whether this observation is due to effects of FTY720 on lymphocytes, endothelial cells, or both.

Another level of complexity is added by observations that (presumably) non-phosphorylated FTY720 can exert effects on T cells in vitro [89]. Moreover, there is disagreement whether its effects are due to agonism of S1P receptors or inhibition of S1P binding [89]. Furthermore, recent findings suggest that the effects of S1P receptor ligands on T cell trafficking vary dependent on the dose [99]. At concentrations below those found in plasma (∼0.1 μM), S1P enhanced the chemotactic response of naïve T cells to CCL21. In contrast, at 0.3–1.0 μM (which is similar to plasma concentrations), S1P inhibited CCL21-mediated chemotaxis by up to 70%. Thus, it has been proposed that S1P might regulate the threshold of naïve T cell responsiveness to chemotractions. Besides C345, which acts on the rolling step in HEV, these data are suggestive of the possibility that S1P and its receptors may represent a molecular counterbalance at the chemokine level. However, further work will be needed to elucidate the mechanisms behind the physiologic actions of S1P at the molecular level.

6. Compartmental navigation on naïve T cells and DC

Once naïve T cells have managed to cross the HEV barrier in the T cell zone of PLN, they must screen as many APC as possible for the presence of cognate antigen. It has been proposed that extravascular leukocytes, including T cells, sense chemotactic gradients, which provide guidance to reach their interstitial target [6]. As discussed above, CCL21 and CCL19 are abundantly expressed by stromal cells throughout the T cell zone. These chemokines are clearly involved in the organization of specific anatomic compartments within SLO, as evidenced by the disturbed architecture of SLO in pE/pt and CCR7+/− animals. Thus, CCL21 and CCL19 are attractive candidates for the guidance of interstitial T cell migration; indeed, it has been suggested that CCL21 expressing stromal cells might establish migrational corridors that bring together naïve T cells and mature CCR7-expressing APC [37]. However, this question is difficult to address experimentally in vivo.

Until recently, most of our knowledge about interstitial migration has been based on immunohistologic examination of lymphocyte and DC localization within SLO compartments. A drawback of such studies is that they can only provide static snapshots of these dynamic processes. Nevertheless, these studies were instrumental in establishing the concept that inter-compartmental migration of lymphocytes depends on precisely orchestrated chemokine gradients and chemokine receptor expression (Fig. 4). This is best exemplified in the redistribution of lymphocytes in T cell-dependent antibody responses (reviewed in [97]). Shortly after immunization, antigen-specific B cells move to the border between B follicles and the T cell area [98–100]. After first proliferating in the T cell zone, antigen-specific CD4+ T cells migrate towards the edge of follicles, where they physically interact with B cells. Activation of CD4+ T cells in vivo leads to a reprogramming of their chemokine receptor expression profile, including down-regulation of CCR7 and up-regulation of CXCR5 [101]. Thus, the cells gain the ability to respond to CXCL13 (the ligand for CXCR5), which is expressed by stromal cells in B cell follicles, while reducing their sensitivity towards CCL21 and CCL19. However, many helper T cells will soon leave SLO by mechanisms that are still incompletely understood. These cells are equipped with molecules that allow for trafficking to sites of inflammation, such as P/E-selectin ligands and inflammatory chemokine receptors. It is currently unclear, which signals induce this differential chemokine receptor expression, although accumulation of CD4+CXCR5+ T cells in follicles depends on signals through CD28 and OX40 [102,103]. In the case of B cells, Reif et al. recently demonstrated reciprocal regulation of CXCR5 and CCR7 after antigen activation, which facilitates their positioning at the follicle border [104].

Given the high expression of CCR7 ligands in the T zones of SLO, the prediction would be that, in the absence of antigen, naïve T cells (as a population) reveal a relatively organized movement away from HEV into the T zones, and eventually into the marginal sinus leading to their egress. However, it has been shown recently that such a choreographed motion pattern does not occur in vivo. The availability of novel microscopy techniques, in particular 2P microscopy, has provided new insights from deep within the parenchyma of living organs, including PLN (reviewed in [105]). Two-photon microscopy has several advantages over "conventional" confocal microscopy: the longer excitation wavelength of the laser (near infrared light) allows for deeper penetration into tissues (up to several hundred μm; confocal: <80 μm); and, as excitation of fluorescent dyes is confined to a narrow focal plane, phototoxic effects in surrounding tissues are reduced, and photobleaching minimized (reviewed in [105]). Two-photon microscopy enables analysis of dynamic processes in six dimensions: space, time, color, and fluorescence intensity [106].

Using 2P microscopy, Miller et al. examined the locomotion of fluorescently-labeled naïve T cells in the cortex of PLN, both in intact isolated organs and also in PLN in vivo, with similar results [8,9]. Strikingly, they found that naïve T cells possess an autonomous migratory program. Peak velocities of naïve T cells within the parenchyma reached >25 μm/min, with an average of ∼11 μm/min, which was nearly twice that of B cells within follicles (∼6 μm/min).
Fig. 4. Compartmental navigation of lymphocytes and DC in PLN. (A) Under homeostatic conditions, CCR7+ naïve T cells and B cells enter PLN from the bloodstream through HEV. DC precursors and plasmacytoid DC are also thought to enter PLN via HEV, but the mechanism is not understood. Naïve T cells reveal completely random non-directional “high-speed” movement in the interstitium after transmigration. This behavior may enable T cells to screen as many as possible DC in the T cell areas of SLO. CXCR5 hi B cells migrate into B cell follicles, the place of highest CXCL13 expression in the PLN. Within follicles, B cells also show random motion, albeit at a lower speed as compared to T cells. Under steady-state conditions, immature DC in the T cell areas, that may either arrive via HEV or afferent lymph, may be important for the induction/maintenance of T cell tolerance. In the absence of cognate antigen, naïve T cells leave the LN via efferent lymphatics. (B) In the case of an immune response, for example against an invading pathogen, regional PLN get inflamed, which leads to recruitment of lymphocytes from the blood, as well as the influx of mature CCR7+ DC from the periphery via afferent lymph vessels. Mature DC express high levels of costimulatory and MHC molecules on their surface. This enables them to activate antigen-specific naïve T cells. Activation not only induces differentiation of T cells into effector cells, but also a re-programming of their traffic molecules. In the case of CD4+ T cells, CCR7 is down-regulated, whereas CXCR5 is up-regulated. Consequently, activated CD4+ T cells are attracted towards the border of B cell follicles. The opposite happens after activation of B cells, which down-regulate CXCR5 and up-regulate CCR7 expression. Thus, activated B cells move towards the follicle border, where they interact with antigen-specific T cells. Activated CD4+ T cells will also be equipped with molecules, such as selectin-ligands, integrins, CD44, and inflammatory chemokine receptors, that enable their migration to peripheral sites of inflammation. These cells leave the PLN via efferent lymphatics.

This “high-speed” behavior is surprising given how densely packed lymphocytes are in the cortex of PLN. Naïve T cells migrated randomly in all directions (xyz axis), with each individual cell taking an independent traffic path. Such migration behavior might facilitate the ability of naïve T cells to screen the maximal number of antigen-bearing DC within the shortest possible time. Nevertheless, the apparently non-directional and seemingly chaotic motion of naïve T cells within the PLN cortex also calls into question the role of chemokine gradients. So why then are CCL21, CCL19, and CXCL12 so highly expressed by stromal cells in the T cell zones of SLO under homeostatic conditions? One possibility is that these chemokines keep T and B cells segregated from each other. It has been reported that, at high concentrations, CXCL12 mediates the repulsion of a subset of T cells, a phenomenon referred to as chemotaxis [107]. Also, it is conceivable that CCR7 ligands are important for recruiting APC that continuously arrive via afferent lymphatics to the T cell zone (reviewed in [108] and in Gunn in this issue).

In the future, the availability of conditionally gene-targeted mice (for example CCR7 in T cells) in combination with 2P microscopy should make it possible to study these questions in detail.

The locomotion of T cells in the cortex of PLN dramatically changes in the presence of antigen. Using TCR transgenic CD4+ T cells, Miller et al. showed that in antigen-challenged mice a minority of the adoptively transferred T cells form tight clusters of relatively immobile cells with enlarged diameters [8]. Although APC were not visualized in these experiments, it can be speculated that T cells clustered around antigen-loaded APC. Other T cells grouped in swarms, which were proposed to form after cluster formation, but before cell division. After several divisions, as visualized by dilution of CFSE, the antigen-primed T cells regained their individual motility, resembling naïve T cells.
It is apparent from previous in vitro studies that naive T cells engage in stable contacts with antigen-presenting DC (reviewed in [109]). Using a confocal microscopy approach in isolated PLN, Stoll et al. recently demonstrated that naive T cells preferentially associated with antigen-bearing DC, when both antigen-pulsed and antigen-free DC were present in the same PLN [110]. Antigen-specific T cells were primarily found in a one-to-one association with APC, which was stable for many hours. This study also showed that the large antiadhesive CD43 was excluded from the contact zone between T cells and DC, a phenomenon that is consistent with the formation of the so-called immunological synapse or supramolecular activation cluster [110–112]. The kinetics of T cell and DC behavior observed by Stoll et al. are somewhat in contrast to the report by Miller et al., who found several T cells clustered in the presence of antigen, although DC were not visualized [8]. Possible explanations for this discrepancy could be the technical differences in the experimental approaches used in these studies; for example, physiologic oxygen tension appears to be essential for the autonomous movement of T cells within the explanted PLN [115]. The ultimate approach to test these questions will be to directly visualize DC-T cell interactions in intact PLN in situ, which circumvents the problems of oxygen and nutrients deprivation of organs analyzed ex vivo.

Finally, the question remains as to which signals induce the immobilization of naive T cells upon encounter of antigen-bearing APC. Mature DC in the T cell zones of SLO produce a variety of chemokines, such as CCL19, CCL17 (TARC), CCL22 (MDC), and CCL18 (DC-CX1/PARC), all of which have the potential to attract T cells (reviewed in [113]). Not only could these chemokines be important in bringing together DC and T cells, but some may also be involved in the formation of the immunological synapse [114].

7. Future prospects

During the past decade we have learned a great deal about the molecular cues that facilitate naive T cell and APC entry into and migration within SLO. CCR7 and its ligands have proved to be important players in the guidance of these cells to particular microenvironments. Due to both their constitutive expression and their role in lymphocyte recirculation, CCL21 and CCL19 have been considered homeostatic chemokines. The finding that these mediators can be turned on in blood vascular endothelium under certain inflammatory conditions, and may therefore contribute to the pathogenesis of common autoimmune diseases, such as RA and UC, poses questions as to the regulatory elements that control these events. It will be interesting to study the role of CCR7 in experimental models of lymphoid neogenesis and autoimmunity, for example, by employing small molecular antagonists, blocking antibodies, or gene-targeted animals. Furthermore, it will be important to dissect the relative contributions of the isoforms of CCL21 and CCL19 in the control of T cell recirculation by generating knockout mice for each of these genes.

The unprecedented finding that lipid mediators, such as S1P, and structurally similar pharmacological agents, profoundly influence T cell migration during homeostasis has opened a new field of research with important clinical implications. Moreover, it will be necessary to dissect the contribution of the individual S1P receptors both from the endothelial and lymphocyte side.

Arguably the most exciting future prospects are based on the novel glimpses into living tissues provided by 2P microscopy. We are just beginning to appreciate the complexity of cell-cell interaction within intact organs, both on the single-cell and subcellular level. Visualization of T cell–APC interactions; arrangement of interacting molecules at the T cell–APC interface; and interactions of APC and T cells with microbes or tumors are just a few of the nearly unlimited questions that can be addressed with this new technology.

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