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CXCR4 Promotes B Cell Egress from Peyer's Patches

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CXCR4 Promotes B Cell Egress from Peyer’s Patches

by

Timothy Hunter Schmidt

Dissertation

Submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Biomedical Sciences

in the
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by

Timothy H. Schmidt
This dissertation is dedicated to my parents, Phoebe Gosho and Richard Schmidt, whose genes, parenting skills, and love were prerequisite for this endeavor.
Acknowledgments

With visiting students and postdocs, I often have the opportunity to field the question, “What do you like about Jason Cyster’s lab?” The first thing I always say is that the best thing about being in Jason’s lab is Jason. He loves the science we do so much that he has an unfathomable amount of energy to devote to it. As a graduate student in his lab, I have been a major beneficiary of that energy. Jason is always there to support me, scientifically and personally, through the trials and triumphs of graduate school. For this, I am deeply grateful. Though his thought process runs at a frequency at times hard to keep up with, I have noticed that he makes a point of pausing to emphasize teachable moments whenever they appear. From that, and other behaviors, it is clear that Jason not only is a great scientific teammate but also a true mentor. He has great expectations but his support is more than commensurate. This combination has helped me grow more than I could have hoped during my training.

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Contributions to the Presented Work

All work in this dissertation was done under the direct supervision and guidance of Professor Jason G. Cyster. Chapter 2 is published as Schmidt, T.H., O. Bannard, E.E. Gray, J.G. Cyster. 2013. CXCR4 promotes B cell egress from Peyer’s patches. *Journal of Experimental Medicine*. The manuscript is reproduced here in accordance with the policies of Rockefeller University Press. The research in this paper was designed and conceptualized by J.G. Cyster, O. Bannard, and me. O. Bannard made the initial observation that CXCR4 KO B cells accumulate in the Peyer’s patches of mixed bone marrow chimeras (Fig. 1A). He was also an important source of technical advice and mice for the other data in the paper. E.E. Gray developed the KikGR system used to photoconvert cells (Fig. 4) and was a critical source of technical advice for microscopic techniques (Figs 2 and 3). The Mouse Pathology Core at UCSF performed the H&E staining (Fig. 1C). B. Kovshilovskaya assisted with photography of the surgical procedure (Fig. 4A). S. Wu cloned the KikGR vector (Fig. 4F and G). J. Muppidi assisted me with the first bone marrow transduction experiment done for the paper (Fig. 4F). I wrote the paper in collaboration with J.G. Cyster. I assembled the data and generated the figures, in consultation with O. Bannard, E.E. Gray, and J.G. Cyster.
CXCR4 Promotes B Cell Egress from Peyer’s Patches

Abstract

Effective surveillance of the lymph-borne milieu of foreign antigens requires the recirculation of lymphocytes. Their patrol takes them from the blood, into secondary lymphoid organs, and into lymph, from which they ultimately return to the blood. Much has been discovered about how this process unfolds at the microanatomic and molecular levels. However, understanding of how the mechanisms involved differ for mucosal immune tissues continues to develop.

Peyer’s patches (PPs) play a central role in supporting B cell responses against intestinal antigens, yet the factors controlling B cell passage through these mucosal lymphoid tissues are incompletely understood. Here we report that, in mixed chimeras, CXCR4-deficient B cells accumulate in PPs compared to their representation in other lymphoid tissues. CXCR4-deficient B cells egress from PPs more slowly than wild-type cells while CXCR5-deficient cells egress more rapidly. The CXCR4 ligand, CXCL12, is expressed by cells adjacent to lymphatic endothelial cells in a zone that abuts but minimally overlaps with the CXCL13⁺ follicle. CXCR4-deficient B cells show reduced localization to these CXCL12⁺ peri-lymphatic zones whereas CXCR5-deficient B cells preferentially localize in these regions. By photoconverting KikGR-expressing cells within surgically exposed PPs, we provide evidence that naïve B cells transit PPs with an approximate residency half-life of 10 h. When CXCR4 is lacking, KikGR⁺ B cells show a delay in PP egress. In summary, we identify a CXCL12hi peri-lymphatic zone in PPs that
plays a role in overcoming CXCL13-mediated retention to promote B cell egress from these gut-associated lymphoid tissues.
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Chapter 1: Introduction
One of the central conceptual challenges of adaptive immunity is to understand how rare, antigen-specific lymphocytes find their cognate antigens. The active process by which dendritic cells (DCs) acquire antigen and travel to secondary lymphoid organs (SLOs) is an important part of the solution. However, without the underlying lymphatic circulatory system, efficient surveillance could not be achieved. This is because the lymphatic system plays two key roles in this process: It funnels antigen-rich, DC-laden lymph into nodes and it provides an accessible network through which lymphocytes can circulate. By providing antigen-concentrating hubs easily accessible for lymphocytes, the lymphatic system provides the anatomical solution to the lymphocyte-cognate antigen problem.

Of growing interest is the role of mucosal lymphoid tissues in immunity. The differences in the mechanisms by which lymphocytes gain access to, transit through, and egress from these structures continue to be elucidated. Peyer’s patches (PPs) are an important hub of mucosal immunity in the small intestine. Knowledge of these patches, including their processes of matching antigen to lymphocyte, continues to expand. Yet more must be learned in order to place PPs in the context of the entire immune system.

Peyer’s Patches

History

In 1645, Marco Aurelio Severino described aggregated lymphoid follicles (Jung et al., 2010) and in 1677, Johann Conrad Peyer published his observations of “glands aggregates, which resemble small grapes” in the intestines of mammals (Griebel and Hein, 1996). He believed that his patches were mucous-producing glands, likely because he observed “small pores or tiny openings and [that] a viscous fluid is expressed when
the glands are pressed between the fingers.” In fact, the follicle associated epithelium (FAE) over the PP likely produces less mucous that normal intestinal epithelium because it lacks a high density of goblet cells.

Kenzaburo Kumagai, in 1922, found that *Mycobacterium tuberculosis*, when fed to rabbits, was taken up into PPs (Owen, 1999). However, he did not speculate on the possible functional ramifications of this because he also found that PPs could take up inert antigenic material – sheep red blood cells (SRBCs) and heat-killed *M. tuberculosis*. Fifty years later, Robert L. Owen and colleagues used scanning electron microscopy to look more closely at the FAE and discovered the unique morphological features of microfold (M) cells. This sparked a new interest in the role of PPs in immunity and tolerance to gut antigens.

**Development**

PPs develop in a manner similar to lymph nodes (LNs), but with some important differences (reviewed by Coles et al., 2010). Interestingly, RORγt+ lymphoid tissue inducer (LTi) cells are necessary – as they are for LNs – but not sufficient for PPs (Veiga-Fernandes et al., 2007; Eberl et al., 2004). PP development also includes rearranged during transfection (RET) tyrosine kinase-dependent lymphoid tissue initiator cells (LTins), which are distinct from LTis and work with lymphoid tissue organizer (LTo) stromal cells prior to the LTi-dependent phase.

A behavior of LTis unique to PP development has recently been described (Nakagawa et al., 2013). It was observed that LTis pass through a developmental transition during which they downregulate the chemokine receptors, CXCR5 and CCR7, and upregulate CXCR4. This change in chemokine receptor expression was necessary for
the relocation of LTis from their prenatal, follicular location to their postnatal, perifollicular location. This unique role for CXCR4 – and its ligand, CXCL12 – is accompanied by a unique requirement for CXCR5 on LTis for PP development (Finke et al., 2002). CXCR5 deficient LTis are inefficient at inducing PPs due to a decrease in CXCR5-mediated activation of α5β1 integrin. Indeed, mice lacking either CXCR5 or CXCL13 do not develop many PPs (Ansel et al., 2000; Förster et al., 1996). The specialized roles of CXCL12 and CXCL13 in the developing PP suggests that these chemokines may play a unique role in regulating the movement of cells within mature PPs.

**Tolerance**

One interesting aspect of PPs is that they are SLOs situated in an area where foreign antigenic stimulation – food and flora – is the norm rather than the exception. This suggests that they may serve as sentinels primarily of tolerance rather than immunity. However, PPs’ criticality for tolerance has proven difficult to pin down (Mowat, 2003). Some data suggest that a lack of PPs, caused by interfering with LTα1β2 signaling during development, can break tolerance to experimental protein antigens (Fujihashi et al., 2001). The same group has also suggested that aging PPs are less able to induce oral tolerance (Kato et al., 2003). Also, in a mouse model of food-mediated anaphylaxis, transferred cells from mesenteric LNs (MLNs) appeared to have a greater capacity to induce atopic antibodies than cells from PPs do (Eigenmann et al., 2004). However, a number of other studies have failed to show any effect of genetic perturbations that impair the development of PPs on tolerance (Spahn et al., 2001, 2002; Alpan et al., 2001). Indeed, even surgical removal of rat PPs and oral administration of
SRBCs or ovalbumin (OVA) administration into PP-deficient bowel loops fails to break tolerance (Enders et al., 1986; Kraus et al., 2005). In contrast, MLNs do appear to be an important tolerogenic barrier in the absence of PPs (Spahn et al., 2002). However, the results generated in genetically perturbed systems are somewhat hard to interpret as they may be confounded by other effects on the immune system that could dampen effective mucosal immune responses.

The above studies focused on specific antigens rather than the antigens derived from the commensal microbiome. The role of PPs in the tolerance to and regulation of flora is another important area of investigation. Commensal species are important to the functioning of PPs, which are much smaller in germ free mice (Umesaki and Setoyama, 2000; Macpherson et al., 2000). While it has been suggested that PPs may play an important role in the loss of tolerance to commensals in inflammatory bowel disease (IBD), a clear mechanistic link has yet to be found (Van Kruiningen et al., 1997). Nuclear oligomerization domain (NOD)2 recognizes the pathogen associated molecule, muramyl dipeptide, and plays an important role in promoting intestinal immunity (Kobayashi et al., 2005). In addition, mutations in NOD2 in humans confers susceptibility to Crohn’s disease (Ogura et al., 2001; Hugot et al., 2001). Interestingly, NOD2 has been shown to promote PP homeostasis by regulating FAE permeability (Barreau et al., 2007, 2010). These data suggest that PPs may play an important role in surveilling and promoting tolerance to intestinal microbiota. Loss of this normal, tolerogenic function may contribute to inflammatory diseases such as Crohn’s disease.
Immunity

If data suggest that PPs can regulate commensals, then it might follow that PPs can control pathogens. However, as with tolerance to food antigens, the magnitude of the contribution that PPs make to active immunity is unclear. PPs have been shown to be important for the generation of protective IgA titers in a model of Salmonella infection (Hashizume et al., 2008). Indeed, a specific mechanism in which DCs rely on CCR6 to migrate to the subepithelial dome (SED) in order to receive antigen and promote a protective T cell response has been described (Salazar-Gonzalez et al., 2006). Interfering with PP development by inhibiting LTβ signaling has been shown to reduce IgA levels (Spahn et al., 2001; Enders et al., 1986). However, the use of the mucosal adjuvant, cholera toxin, is able to eliminate the defects in CD4 responses locally and systemically otherwise observed in these mice (Yamamoto et al., 2000). Furthermore, selective IgA deficiency in humans (living in populations amenable to academic study) is rarely severe, resulting in generally a minor increase in upper respiratory and gastrointestinal infection rates (Latiff and Kerr, 2007).

In addition to PPs, the small intestine contains isolated lymphoid follicles, structures analogous to a single follicle of a PP, and cryptopatches, clusters of intraepithelial T cell progenitors found in the lamina propria of the intestinal crypt (Hamada et al., 2002; Saito et al., 1998). PPs’ role in this complex system is likely complementary and overlapping with the roles of these other structures and of the MLNs. Together, they must provide tolerance to food antigen, protection from pathogens, and regulation of commensals. Greater understanding of the molecular cues that guide lymphocytes through PPs will help us better understand their function in mucosal immunity.
Extravasation into mucosal sites

The course that lymphocytes must navigate – from blood, to SLO, to lymph, and back – involves a number of migratory steps that must be precisely orchestrated, a collaboration between the tissues’ microanatomy and the cells themselves. The first step in this process, extravasation from blood, is one of the conceptual pillars of immunology (reviewed in Butcher and Picker, 1996 and Johnston and Butcher, 2002). This process involves the canonical steps of rolling, tight adhesion, and transmigration out of high endothelial venules (HEVs). Importantly, the determinants of efficient extravasation differ among SLOs. For example, while L-selectin is necessary to promote leukocyte rolling in the HEVs of lymph nodes (LNs), it is not entirely necessary for extravasation into PPs and MLNs (Bargatze et al., 1995). In these mucosal SLOs, HEV expression of mucosal vascular addressin cell adhesion molecule-1 (MAd-CAM-1) promotes both rolling and adhesion via interaction with leukocyte α4β7 integrin (Berlin et al., 1993). Another example of how homing differs between mucosal and non-mucosal sites is that, in PPs, T and B cells preferentially arrest in different segments of HEVs (Warnock et al., 2000). These PP HEVs are often found within the follicle, in contrast to their general exclusion from the follicle in LNs (Warnock et al., 2000; Miura et al., 1995; Okada et al., 2002). Finally, the chemokines CXCL12 and CXCL13 demonstrate unique roles in promoting adhesion triggering in PPs (Okada et al., 2002; Förster et al., 1996). These differences suggest that there may also be microanatomic differences between mucosal and non-mucosal SLOs that create different mechanisms of lymphocyte transit after extravasation has occurred.
**Mucosal lymphoid organization**

Once lymphocytes entered into the parenchyma of an SLO, they assort into a spatial arrangement that facilitates the cell-cell interactions necessary for adaptive immunity (Förster et al., 1999). Though different SLO organizations vary, one of the central organizational principals is the separation of the T zone and the B cell follicle (Cyster, 1999). These zones are defined by the expression of chemokines: CCL19 and 21 in the T zone and CXCL13 in the B cell follicle. Lymphocytes and myeloid cells use CCR7 to migrate toward the T zone chemokines and CXCR5 to migrate toward the B cell follicle.

Another important organizer in SLOs is the chemokine/receptor pair, CXCR4/CXCL12. This pair plays an important role in organizing the germinal center (GC) as well as guiding antibody-secreting cells to the medullary cords of lymph nodes and bone marrow (Cyster, 2005; Kunkel and Butcher, 2003). CXCR4 has a number of other essential functions, including roles in hematopoiesis, vascular development, cancer, HIV, and neurobiology (Sugiyama et al., 2006; Gelmini et al., 2008; Alkhatib, 2009; Tiveron and Cremer, 2008).

PPs demonstrate many of the same patterns of organization as the spleen and LNs do. However, the functional ramifications of their differences in organization from other SLOs have yet to be fully described. PPs have follicles that are separated by perifollicular regions, which tend to be rich in T cells, HEVs, and lymphatics. The follicles extend out toward the serosa, creating a macroscopic bump on the antimesenteric side of the small intestine. The follicles are situated in close proximity to the FAE on the luminal side of the PP. Here, the specialized epithelial cells, M cells, deliver antigen from the intestinal...
lumen into the PP, thus serving a function analogous to the afferent lymphatics of LNs, which PPs lack (Neutra et al., 2001). These microfold cells differentiate from enterocyte precursors under the influence of lymphotoxin (LT)α1β2 provided by B cells. Extending between the FAE and follicular areas are subepithelial domes (SEDs), which are composed of a mix of stromal, myeloid, and lymphocytic cells. The precise role that these domes play in mucosal immunity has yet to be fully elucidated but is likely related to their proximity to the source of luminal antigen. In the mouse, there is substantial variation in the numbers and sizes of PPs in the small intestine. Their numbers can range from 5 to 13. They can have diameters of just less than one mm up to several mm. The PPs tend to be at higher frequencies in the duodenal and ileal ends than in the jejunal segment. During dissection, they can be macroscopically identified not only by their protrusion into the serosal surface but also by their color – a whiter shade than the surrounding intestine – and the coronal arrangement of vessels extending from the mesenteric side of the intestine. It is known that PPs exhibit chronic GC activity (Griebel and Hein, 1996), but both this and the LN-like organization of B and T cells within PPs are not fully ablated in germ-free mice (Crabbé et al., 1970).

Egress

The last step in a lymphocyte’s recirculatory path is egress from an SLO into lymph, from which it can return to blood and travel to a new SLO. To do so, a lymphocyte must travel from its site of entry (i.e. an HEV) to a lymphatic, be competent to cross its endothelial barrier, and egress out with the efferent lymph. In LNs, the cortical sinuses are major sites of lymphocyte egress (Pham et al., 2008; Grigorova et al., 2009; Sinha et al., 2009), though medullary sinuses may also accept some egressing cells.
These sinuses are located just deep to the follicles and are thus situated so that both B and T cells can access them. They often demonstrate fluid flow (Grigorova et al., 2009). From these sinuses, lymphocytes flow to the macrophage-rich medullary region and can egress from the LN.

To egress, however, lymphocytes must travel to the lymphatic. Mathematical modeling has suggested that, in LNs, stromally-guided, random walk motion would be sufficient to explain the observed egress rates after entry blockade of T cells (Grigorova et al., 2010). This model assumes that lymphocytes do not change in their chemotactic sensitivities. It also assumes that the functionality of S1PR1 – a GPCR that promotes chemotaxis toward the lipid, sphingosine-1-phosphate (S1P), and egress into S1P-rich lymph (Matloubian et al., 2004) – in its promotion of transmigration into lymphatics does not change outside the first 30 minutes. These assumptions may not be correct, as some data suggest that expression of regulators of G protein signaling (RGSs), which are capable of desensitizing chemokine receptors and other GPCRs (Han et al., 2006; Kehrl, 2006), may change over the duration of a lymphocyte’s stay in a LN (Sinha et al., 2009). More data is needed to determine whether and in which contexts a timed, “clock” model of lymphatic access is appropriate.

A lymphocyte’s capacity to transmigrate across the endothelial wall of a lymphatic is influenced by type I interferon (IFN) (Shiow et al., 2006). IFN-α/β upregulates expression of CD69, which desensitizes a cell to sphingosine-1-phosphate receptor 1 (S1PR1)-mediated chemotaxis toward S1P. S1PR1 is necessary for efficient transmigration from the S1P-low environment of LN parenchyma to the S1P-high lymph (Grigorova et al., 2009). In addition, antigen receptor signaling can also downmodulate
S1PR1 expression and function (Graeler and Goetzl, 2002). These mechanisms are also at play in PPs, where lymphatic generation of S1P is critical for efficient T cell egress (Pham et al., 2010).

PPs, due to their unique microanatomy and function, may differ in their process of promoting efficient lymphocyte transit. Their relatively inflammatory milieu may promote differences in egress mechanism. For example, CD69 appears to be expressed on the surface of lymphocytes in PPs to a greater degree than on the surface of lymphocytes in LNs (unpublished data). Also, because of their distinct anatomy, PPs do not have cortical sinuses. Indeed, the relevant exit structures for lymphocytes and plasma cells appear to exist near the serosa of the PP (Azzali, 2003; Azzali and Arcari, 2000; Gohda et al., 2008). Microanatomical and environmental differences between LNs and PPs may necessitate different models of lymphocyte egress for each site.
Chapter 2: CXCR4 Promotes B Cell Egress from Peyer’s Patches

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Abstract

Peyer’s patches (PPs) play a central role in supporting B cell responses against intestinal antigens, yet the factors controlling B cell passage through these mucosal lymphoid tissues are incompletely understood. Here we report that, in mixed chimeras, CXCR4-deficient B cells accumulate in PPs compared to their representation in other lymphoid tissues. CXCR4-deficient B cells egress from PPs more slowly than wild-type cells while CXCR5-deficient cells egress more rapidly. The CXCR4 ligand, CXCL12, is expressed by cells adjacent to lymphatic endothelial cells in a zone that abuts but minimally overlaps with the CXCL13⁺ follicle. CXCR4-deficient B cells show reduced localization to these CXCL12⁺ peri-lymphatic zones whereas CXCR5-deficient B cells preferentially localize in these regions. By photoconverting KikGR-expressing cells within surgically exposed PPs, we provide evidence that naïve B cells transit PPs with an approximate residency half-life of 10 h. When CXCR4 is lacking, KikGR⁺ B cells show a delay in PP egress. In summary, we identify a CXCL12⁺ peri-lymphatic zone in PPs that plays a role in overcoming CXCL13-mediated retention to promote B cell egress from these gut-associated lymphoid tissues.

Introduction

Humans have over 100 Peyer’s patches (PPs) (Cornes, 1965) and there are typically 6-12 in mice (Azzali, 2003; Sobhon, 1971). These mucosal lymphoid tissues play an important role in supporting B cell responses against gut antigens, both commensal and pathogen-derived (Macpherson et al., 2005). Consistent with their role in fostering B cell responses, PPs have a higher frequency of B cells (~80%) than do lymph nodes (LNs, ~30%). B cell entry to both PPs and mucosal LNs requires α4β7 integrin
and mucosal addressin cell adhesion molecule-1 (Berlin et al., 1993; Bargatze et al.,
1995), but PPs uniquely demonstrate a substantial CXCR5-CXCL13 contribution to the
adhesion triggering step (Warnock et al., 2000; Okada et al., 2002). So far, the
requirements for lymphocyte egress from PPs have appeared similar to LNs, including a
strong dependence on sphingosine-1-phosphate receptor-1 (S1PR1) and on lymphatic
endothelial cell-produced S1P (Pham et al., 2010). However, the anatomy of PPs is quite
distinct from LNs: In LNs, lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1)
expressing cortical sinuses extend into the interface of B cell follicles and T cell zones
and contribute to lymphocyte exit (Pham et al., 2010; Grigorova et al., 2009; Sinha et al.,
2009). In PPs, lymphatic sinuses have been described near the serosal surface and in
interfollicular regions (Ohtani and Murakami, 1990; Gohda et al., 2008; Azzali and
Arcari, 2000) but whether these are the main sites of lymphocyte egress has not been
determined. The chemokine receptor CXCR4 is abundant on naïve lymphocytes and it
can promote cell entry to LNs though this function is largely redundant with that of the
dominant entry receptor, CCR7 (Okada et al., 2002). In vitro, naïve B cells migrate
robustly to even low doses of CXCL12 (Bleul et al., 1996; Nie et al., 2004). Yet, in
contrast to the prominent roles of this chemokine-receptor system in developing B cells,
germinal center B cells and plasma cells (Nagasawa et al., 1996; Nie et al., 2004;
Hargreaves et al., 2001; Pereira et al., 2009; Allen et al., 2004), CXCR4 and CXCL12
have no well-defined function in guiding naïve B cell movements within lymphoid
tissues. Mice lacking CXCR4 in all B cells were reported to have aberrant PP follicle
morphology, but the basis for or significance of this effect was unknown (Nie et al.,
2004).
Here we report a unique role for CXCR4 in mediating B cell access to PP lymphatic sinuses and in promoting egress from PPs into lymph. CXCR5 plays an opposing role, limiting B cell access to these sinuses and promoting B cell retention in PPs. Using a mouse transgenic for a photoconvertible protein, we confirm the PP-egress promoting role of CXCR4 and provide evidence that B cells have an approximately 10 h residency time in PPs before travelling to mesenteric LNs (MLNs) and then returning to circulation.

Results and Discussion

To test the possible role of CXCR4 in B lymphocyte recirculation through lymphoid organs we generated CXCR4<sup>-/-</sup>Mb1-Cre<sup>+</sup> (CXCR4 KO) CD45.2<sup>+</sup>:wild-type (WT) CD45.1<sup>+</sup> mixed bone marrow (BM) chimeras and examined B cell distribution in lymphoid tissues. Compared to their frequencies in spleen and MLNs, PPs showed a marked accumulation of KO over WT naive B cells (Fig. 1A). This accumulation was not seen in control CXCR4<sup>+/+</sup>Mb1-Cre<sup>+</sup> CD45.2<sup>+</sup>:WT CD45.1<sup>+</sup> mixed BM chimeras (Fig. 1A). The lower frequency of CD45.2<sup>+</sup> B cells in spleen and MLNs of CXCR4 KO mixed BM chimeras than in the control mixed BM chimeras is a consequence of the reduced B lymphopoiesis supported by CXCR4 KO hematopoietic cells (Zou et al., 1998; Sugiyama et al., 2006; Nie et al., 2004).

To explore whether the accumulation of CXCR4-deficient cells in PPs may be a consequence of reduced egress, we performed short-term transfer experiments. CXCR4 KO and WT cells were cotransferred into WT hosts and, after a 2 day equilibration, further lymphocyte entry to PPs was blocked by treatment with a combination of α4 and αL integrin neutralizing antibodies for 18 h (Lo et al., 2003). Compared to their
frequency prior to entry blockade, CXCR4 KO cells became over represented selectively in PPs (Fig. 1B). By contrast, they became underrepresented in LNs (Fig. 1B). Similar cotransfer experiments were performed using mixtures of CXCR5 KO and WT B cells to test for a possible role of the CXCR5-CXCL13 chemokine receptor-chemokine pair in B cell transit through lymphoid organs. Based on the previous finding that CCR7-CCL21 promote T cell retention in LNs (Pham et al., 2008), we had anticipated CXCR5 may have a similar retention-promoting role for B cells. However, CXCR5-deficiency did not produce a statistically significant effect on the rate of B cell transit through LNs (Fig. 1B). In contrast, there was a significantly greater reduction in CXCR5 KO than WT B cells in PPs after 18 h of entry blockade, revealing a retention-promoting role for this chemokine receptor in PPs (Fig. 1B). These data suggested unique roles for CXCR4 and CXCR5 in promoting PP B cell egress and retention, respectively.
Figure 1

CXCR4 promotes and CXCR5 inhibits B cell transit through PPs.
(A) Flow cytometric analysis of naïve B cell distributions in CXCR4 mixed BM chimeras. Percentages of congenically marked (CD45.2+) CXCR4+/+Mb1-Cre+ (Control, black, filled circles) or CXCR4+/− Mb1-Cre− (KO, blue, open circles) versus WT (CD45.1+) naïve B cells (B220−IgD−/−CD95−) in lymphoid organs. Spl, Spleen. Represents 7 control and 17 KO mixed BM chimeric mice. (B) Flow cytometric analysis of the distribution of co-transferred control or KO vs. WT naïve B cells after entry blockade. Ratios of the percentages of control (solid circles) or KO (open circles) B cells among total transferred B cells 18 h after blockade of entry with α4 and αL integrin neutralizing antibodies (t18h) versus immediately before entry blockade (t0h) are plotted. The CXCR4 (blue, upper) and CXCR5 (red, lower) data consist of 4 and 3 experiments, respectively, with each point for a given organ representing a t18h/t0h pair of mice (8 and 6 pairs for each group, respectively). * P<0.05, ** P<0.01, *** P<0.001. **** P<0.0001 P-values were obtained with Student’s unpaired T tests comparing the mean ratios of independent experiments. (C) H&E staining of a representative fixed and paraffinized PP. The inset shows a magnified view of a subserosal lymphatic with its endothelium indicated by a green arrow. Empty space near the lymphatic is likely an artifact of sectioning. The follicle (F, dashed outline) was identified by its high density of nuclear staining. IF regions were approximated as flanking the follicle. S, serosal surface. Scale bar represents 100 µm.
Lymphatic sinuses are present in both subserosal and interfollicular regions of PPs (Ohtani and Murakami, 1990; Gohda et al., 2008; Azzali and Arcari, 2000) and hematoxylin and eosin (H&E) staining of fixed and paraffin embedded PP tissue showed that these sinuses were often filled with lymphocytes (Fig. 1C). We next examined the distribution of CXCL12-producing cells in PPs in relation to the lymphatic sinuses, using anti-LYVE-1 to identify lymphatic endothelial cells. LYVE-1+ sinuses were routinely detectable in T cell-rich interfollicular regions and in many sections large sinuses were found coursing beneath the serosal lining (Fig. 2, A and B). The distribution of CXCL12-producing cells was determined by staining for GFP in CXCL12-GFP reporter mice (Ara et al., 2003). CXCL12-GFP+ cells were abundant in interfollicular and subserosal regions, including cells in close association with LYVE-1+ sinuses (Fig. 2B). Notably, the CXCL12-GFP+ areas were adjacent to but largely non-overlapping with the follicular CXCL13 protein distribution and CXCL12-GFP+ cells were often closely juxtaposed to LYVE-1+ endothelial cells (Fig. 2, A). Additionally, the CXCL13-CXCL12 transitional, often perilymphatic, margins of the follicles appeared to have lower densities of naïve, IgD+ B cells than the centers of the follicles (Fig. 2A, Fig. 3A and data not shown). Analysis of peripheral LNs from CXCL12-GFP reporter mice showed the expected (Hargreaves et al., 2001) high CXCL12 expression in medullary cords and lower expression throughout the T zone (Fig. 2C). In PPs, the closely juxtaposed yet separate distributions of CXCL12 and CXCL13, along with their potentially opposing roles in dictating B cell transit time, suggested that CXCR4 and CXCR5 might dictate access to lymphatic sinuses in these mucosal structures.
CXCL12 and CXCL13 are disparately distributed relative to lymphatics in PPs.

Immunofluorescent detection of CXCL12 expression in GFP reporter mice in frozen sections. (A) PP follicle, oriented with its serosa on top, stained to detect GFP (reporter of CXCL12 expression, green), CXCL13 (red), and LYVE-1 (blue). The inset shows a serial section stained for IgD (white) and LYVE-1 (blue). Scale bars represent 200 µm. (B) Magnified view of the subserosal lymphatics in A. The inset shows a similar area from a GFP mouse, also stained for GFP and LYVE-1. Scale bars represent 100 µm. (C) PLN stained equivalently as the PP in A. F, follicle; TZ, T cell zone; M, medullary cords. Scale bar represents 200 µm. Images are representative of >8 PPs or PLNs.
To determine how CXCL12-CXCR4 influenced B cell positioning in PPs we immunohistochemically examined the distribution of transferred KO (Igh\textsuperscript{b+}) and WT (HEL-binding, B cell receptor transgenic) B cells in WT, Igh\textsuperscript{a+} hosts. In control experiments the Igh\textsuperscript{b+} and HEL\textsuperscript{+} B cells were codistributed in follicles and peri-lymphatic areas (Fig. 3A), establishing that the Ig\textsuperscript{HEL} transgene did not alter B cell distribution in PPs. By contrast, in CXCR4 KO:WT cotransfers, the KO B cells were localized within follicles and were markedly underrepresented in peri-lymphatic regions (Fig. 3A). Reciprocally, CXCR5 KO B cells were excluded from PP follicles, as expected (Förster et al., 1996), and were enriched in areas adjacent to lymphatic vessels (Fig. 3A). Similar observations were made when sections were examined by three-color immunofluorescence microscopy to better distinguish LYVE-1\textsuperscript{+} lymphatics and transferred cells (Fig. 3B). Enumeration of cells within 200 µm of subserosal lymphatics established that CXCR4 KO cells were significantly underrepresented in these areas, while CXCR5 KO cells were overrepresented, compared to their frequencies in the center of the follicle (Fig. 3C). Similarly, enumeration of cells overlapping with or internal to the LYVE-1\textsuperscript{+} lymphatic endothelial staining showed a paucity of CXCR4 KO cells and an enrichment of CXCR5 KO cells (Fig. 3D). These observations are consistent with a role for CXCR4 in promoting B cell access to PP egress sites and for CXCR5 in antagonizing such access. In peripheral LNs, cell distributions were more variable but CXCR4 KO cells were on average underrepresented in medullary regions (Fig. 3E). Despite advances in our knowledge of lymphocyte egress from LNs, the contribution of medullary regions to this process has been difficult to define due to their complex...
anatomy and high content of LYVE-1\(^+\) macrophages (Wei et al., 2005; Grigorova et al., 2009, 2010; Sinha et al., 2009).
Figure 3

CXCR4 promotes and CXCR5 inhibits B cell access to PP lymphatics.

(A) Immunohistochemical staining of frozen sections of PP follicles from mice that had received CXCR4+/+ Mb1-Cre+ control (Ai), CXCR4fl/fl Mb1-Cre+ (KO) (Aii), or CXCR5 KO (Aiii) Ig^b^ splenocyte, cotransferred with splenocytes WT Ig^HEL^-transgenic (Igh^b^). Slides were stained with anti-IgD^b^+IgM^b^ to detect Ig^b^ cells (blue) and with HEL to detect Ig^HEL^ B cells (brown). Lymphatics were detected with anti-LYVE-1 (also in brown). Endogenous phosphatase activity in the epithelium is responsible for its blue staining. The PPs were oriented with their serosae on top. Similar observations were
made in PPs from CXCR4 KO:WT and CXCR5 KO:WT mixed BM chimeras (3 mice per condition, not shown). Scale bars represent 100 µm. Black arrows indicate some of the subserosal lymphatics. (B) Definition of follicular and perilymphatic areas in immunofluorescent micrographs of PPs from mice that had received mixed splenocyte transfers as in A. The 200x200 µm squares define the centers of the follicles. Perilymphatic areas were defined by 200 µm vectors extending from subserosal lymphatics toward the center of the follicle. Scale bars represent 200 µm. (C) Quantification of the cell densities in the zones defined in B. Plotted are the frequencies of the indicated cell type in the perilymphatic zones relative to the center of the follicle. (D) Quantification of the frequencies of transferred cells found within or overlapping with the LYVE-1⁺ endothelia in immunofluorescent micrographs like those in B. (E) PLN quantification of the frequencies of transferred cells in 200x200 µm areas in the medullary cords relative to the same area in the centers of follicles in mice that had received mixed splenocyte transfers, as in B. Micrographs represent more than 10 PPs from 3 mice examined in 3 experiments, per condition for both the immunohistochemical and immunofluorescence studies. Horizontal bars in C-E indicate means. ** P<0.01, **** P<0.0001. P-values were obtained with Mann Whitney T tests.
As an independent approach to measure lymphocyte egress rates from PPs, we adapted the photoconversion procedure of ‘time stamping’ cells expressing a photoconvertible protein, Kaede, in LNs (Tomura et al., 2008; Tomura et al., 2010) and skin (Tomura et al., 2010) to PPs. We used transgenic mice ubiquitously expressing the green-to-red, irreversibly photoconvertible protein, KikGR (Tsutsui et al., 2005; Nowotschin and Hadjantonakis, 2009). PPs were surgically exposed through a ~1.5 cm incision in the midline just below the costal margin and exposed to violet light for 3 min while protecting the surrounding tissue from light exposure using sterile foil (Fig. 4A). The PP was then returned to the abdomen and a second PP accessed and violet light exposed. Typically 4-7 PPs were converted per mouse, leaving 2-3 PPs unexposed to serve as internal controls. When PPs were isolated immediately after the procedure and analyzed for naïve IgD<sup>hi</sup>B<sup>220</sup><sup>+</sup> B cells (Fig. 4B), 99.4% (SD=0.3%, n=8 PPs in 2 mice) of the cells in violet light exposed PPs had undergone the green to red conversion (Fig. 4C, ii) whereas very few cells in the control PPs (Fig. 4B, i) were converted (0.25±0.25%, n=12 PPs in 2 mice). The spleen and peripheral LN (PLN) contained a very small frequency (<0.5%) of partially converted cells at 0 h after surgery (not shown), which may have been cells partially photoconverted within blood vessels or early PP emigrants. When mice were examined 6 h after photoconversion, the frequency of converted naïve B cells in individual PPs was reduced to 77.7% (SD=6.2%, n=27 PPs in 5 mice) (Fig. 4C, iii) while converted cells were now detected in circulation (not shown) and MLNs (Fig. 4C, vi) at rates higher than observed immediately after PP conversion (Fig. 4C, v). Analysis of PPs at 40 h showed that the red fluorescence intensity of converted naïve B cells had decayed only slightly compared to the earlier time points, consistent with other
data (Tomura et al., 2008), and showed that most of the converted cells had exited the PPs by this time (Fig. 4C, vii). In contrast, germinal center B cells showed much greater loss of red fluorescence even after 18 h, consistent with dilution of the photoconverted protein by cell division (not shown).
**Figure 4**

**Kinetics of lymphocyte egress from PPs analyzed using photoconversion of KikGR-transgenic PPs and effect of B cell CXCR4-deficiency.**

(A) PP violet light exposure procedure. (i) Mouse abdomen showing incision and exteriorized segment of small intestine. Arrow indicates a PP. (ii) Sterile foil is placed over the surgical site to isolate the PP. (iii) PP is exposed to violet light from a 415 nm LED source for 3 minutes before replacement into the peritoneal cavity. (B) Representative flow cytometry plot showing gating for naïve, B220$^+$IgD$^+$ B cells. (C) FACS plots of naïve B cells in PPs (i-iv, vii) and MLNs (v-vii) from KikGR transgenic (i-vii) or transduced (viii) mice. KikG (unconverted) and KikR (converted) profiles at 0 h (i, ii, v), 6 h (iii, vii), 18 h (viii), and 40 h (iv) after surgery. 0 h plots represent 12 and
8 PPs for unconverted and converted, respectively, and 2 MLNs, from 2 mice. MLN at 6 h represents 5 mice. 40 h plot represents 3 mice. (vii) MLN from mouse treated with FTY720 for 16 h prior to PP photoconversion. (viii) PP from a chimera reconstituted with KikGR-transduced BM. Lower KikGR expression in the transduced cells results in diminished signal as compared to that of the transgenic mice. (D) Converted naïve B cell PP retention. PPs from KikGR transgenic mice were photoconverted, after which the mice were rested for 6, 18, and 40 h. Mean (±SD), recirculation-corrected percentages (see Methods) of converted, KikR naïve B cells remaining in individual, exposed PPs are fit to a single-order exponential decay curve. 6 h represents 27 PP from 5 mice; 18 h, 25 PP from 5 mice; 40 h, 21 PP from 3 mice. Figure represents 7 independent experiments. (E) Naïve B cell residency half-life values were calculated (see Methods) for each exposed PP (from C) from mice rested for 18 and 40 h based on their recirculation-corrected frequencies of KikR+ cells. (F, G) Frequencies of KikR+ naïve B cells remaining after 18 h in the exposed PPs of KikGR-transduced CXCR4+/+ Mb1-Cre+ (Control) and CXCR4flu/Mb1-Cre+ (KO) BM chimeras (F) and wild-type mice transferred with splenocytes from those BM chimeras (G). Represent 3 and 4 pairs of mice from 3 and 4 experiments, respectively. Horizontal bars in E-G indicate means. * P<0.05. P-values were obtained with Student’s unpaired T tests of the mean % KikR values obtained from each experiment.
Importantly, at both 6 and 18 h, converted cells were present at a higher frequency in MLNs than in other LNs, consistent with movement of PP cells to MLNs via the mesenteric lymphatic (Table 1). When mice were treated with FTY720 to block lymphocyte S1PR1 function (Matloubian et al., 2004) prior to PP photoconversion, very few photoconverted cells were found in MLNs or other lymphoid organs after 6 h (Fig. 4C, vii and not shown). These data demonstrate the specificity of the photoconversion and confirm the crucial role of S1PR1 and S1P in lymphocyte egress from PPs (Pham et al., 2010). Plotting the frequencies of photoconverted naïve B cells remaining in individual PPs after 6, 18 and 40 h revealed a single order decay curve with a half-life of 11.5 h (Fig. 4C). Calculation of the apparent half-lives of naïve B cells within individual PPs at the later time points – thereby allowing at least 18 h for recovery from surgical stress – revealed mean naïve B cell half-lives of ~10 h (Fig. 4D). An assessment of all photoconverted cells remaining in PPs at 18 h showed that there was a greater exchange of naïve CD4 and CD8 T cells than B cells, indicating that naïve T cells exit PPs more rapidly than B cells (Table 1). Effector CD4 and CD8 T cells in PPs were replaced by newly arriving cells considerably less rapidly than naïve cells (Table 1). The low rate of effector CD4 T cell egress is consistent with many of these cells being follicular helper T cells that are known to have little S1PR1 expression and to reside within B cell follicles (Rasheed et al., 2006; Crotty, 2011).
<table>
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</tbody>
</table>

**Table 1**

The percentages, ±SD, of KikR⁺ lymphocytes in lymphoid organs and blood of KikGR-transgenic mice, 18 h after surgery to photoconvert their accessible PPs.

Naïve B cells were defined as B220⁺IgD⁺. Photoconverted germinal center B cells (defined as B220⁺IgD⁺CD95⁺ or B220⁺IgD⁺CD95⁺GL7⁺) in PPs decreased in KikR over 18 h, but remained at high frequency (91.2±2.5%). Naïve T cells were defined as CD44⁻CD62L⁺ and effector T cells as CD44⁺CD62L⁻. The data represent 5 mice.
Having established a KikGR photoconversion ‘time stamping’ procedure for PPs, we next applied this approach to test the egress-promoting role of CXCR4 in naïve B cells. PP cells in KikGR transduced CXCR4£-Mb1-Cre+ and CXCR4+/+Mb1-Cre+ BM chimeras were time stamped and analyzed 18 h later, gating on cells expressing the CD90.1 (Thy1.1) reporter and then determining the frequency of photoconverted (red) cells remaining in the PP (Fig. 4C, viii). Summarizing data from a number of animals revealed that a greater fraction of CXCR4-deficient than control B cells remained converted in PPs at 18 h, indicating a slower egress rate (Fig. 4F). To control for possible indirect effects on PP organization of CXCR4-deficiency in all B cells, we performed similar time stamping experiments in wild-type mice that had received transfers of cells from KikGR transduced CXCR4£-Mb1-Cre+ and CXCR4+/+Mb1-Cre+ BM chimeras. Again, when the frequency of red cells remaining in photoconverted PPs was analyzed after 18 h, a significantly greater fraction of CXCR4-deficient than control B cells were retained (Fig. 4F).

Concluding Remarks

The above findings suggest a model where, following CXCR5-CXCL13 mediated entry to PP follicles, B cell access to subserosal and interfollicular lymphatic vessels is promoted by CXCR4-CXCL12 and antagonized by CXCR5-CXCL13. It is notable that the egress-promoting role of CXCR4 appears to be restricted to PPs. This might reflect PPs’ unique anatomical organization, requiring an additional factor to facilitate B cell access to lymphatic sinuses. Consistent with this notion, a recent study identified an essential role for lymphoid tissue inducer (LTI) cells in promoting organization of postnatal PPs but not LNs through sequential movement from a CXCL13+ zone to an
adjacent CXCL12 zone (Nakagawa et al., 2013). The ~10 h half-life of B cell residency in PPs is shorter than the ~16 h time reported for LNs (Tomura et al., 2008). While we cannot exclude the possibility that differences in the surgical procedures between studies contributes to the different rates, we suggest that the egress-promoting role of CXCR4 in PPs contributes to the more rapid transit from PPs. It is also possible that differences in B cell entry pathways are involved. In PPs, B cells enter through high endothelial venules (HEVs) that often course through the follicle (Warnock et al., 2000; Miura et al., 1995; Okada et al., 2002). In contrast, HEVs in LNs are situated in extrafollicular regions (von Andrian and Mempel, 2003) and recent B cell immigrants to LNs dwell in the perivenular space near the HEVs for a few hours before migrating to the follicle (Park et al., 2012). Given the presence of CXCL12 in association with LN HEVs (Okada et al., 2002), it seems possible that this distinct organization not only contributes to the longer B cell transit time through LNs but also accounts for why CXCR4 had a slight B cell retention effect in LNs, a possibility that warrants future investigation. In addition, T cells can migrate from medullary sinuses into the medulla (Braun et al., 2011) and egressing lymphocytes have been observed to move back from lymphatic sinuses to the LN parenchyma (Sinha et al., 2009; Grigorova et al., 2009). The reduced representation of CXCR4-deficient B cells in medullary regions might indicate that B cells passing from cortical to medullary sinuses occasionally return to the tissue parenchyma in a CXCR4-dependent manner, promoting increased residence time in the LN. Finally, our findings suggest a finely tuned balance of CXCR4 and CXCR5 function in PP B cells. The opposing roles of CXCR4 and CXCR5 raise the possibility that small changes in the abundance or function of either receptor might alter the egress rate. Thus, it will be
important in future work to determine whether CXCR4 or CXCR5 function changes over time (for example, due to desensitization by ligand exposure (Park et al., 2012)) after B cell entry into PPs. Studies on LNs have shown that S1PR1 is an important point of egress regulation (Matloubian et al., 2004; Shiow et al., 2006). It would be interesting to determine whether B cell CXCR4 serves as a point of PP egress regulation during mucosal immune responses.

Materials and Methods

Mice and retroviral transduction

C57BL/6 (01C55, C57BL/6NCr) and CD45.1+ congenic (01B96, B6-LY5.2/Cr) mice were obtained from the National Cancer Institute. These mice were used as cotransfer donors and recipients (Fig. 1). We received mice with conditional and deleted CXCR4 alleles from Y.R. Zou (The Feinstein Institute for Medical Research, Manhasset, NY) and D. Littman (New York University, New York, NY). CXCR4+/fl mice (Nie et al., 2004) were crossed to CXCR4+/− mice (Zou et al., 1998) heterozygous for MbICre (Cd79a^{tm1(cre)Reth}, MGI:3687451), provided by M. Reth (Max-Planck Institute of Immunobiology, Freiburg, Germany) (Hobeika et al., 2006). CXCR5−/− mice (Fürster et al., 1996) were received from R. Forster (Hannover Medical School, Hannover, Germany) and M. Lipp (Max Delbrück Center, Berlin, Germany) and backcrossed at least 10 generations to B6. CXCL12-GFP knock-in mice (Ara et al., 2003), B6 crossed to CD1 one generation, were generously provided by Samuel Pleasure’s lab (University of California San Francisco (UCSF), San Francisco, California), which received the mice
originally from the Nagasawa lab (Kyoto University, Kyoto, Japan). For the CXCR4 and CXCR5 localization transfer recipients (Fig. 3), Igh\(^a\) (Jackson, 001317, B6.Cg-Igha Thy1a Gpi1a/J) mice and Ig\(^{HEL}\) (MD4) transgenic mice were bred in our colony. 

\(CAG::KikGR^{Tg/+}\) (013753, Tg(CAG-KikGR)33Hadj/J) (Nowotschin and Hadjantonakis, 2009) mice were purchased from Jackson Laboratory. \(CAG::KikGR^{Tg/+}\) mice were received on an outbred ICR strain background and backcrossed to C57BL/6 for 2-4 generations before use. BM was transduced as previously described (Green et al., 2011) with virus produced by Platinum E cells (Cell Biolabs) transfected with the MSCV CD90.1 (Thy1.1) retroviral vector carrying CoralHueTN KikGR1 (MBL, AM-V0081). The control and CXCR4 KO transduced chimeras had 70-80% and 50-80%, respectively, B220\(^+\) lymphocytes in their PPs. BM chimeras were produced by lethally irradiating host mice with a split dose of 1100-1300 rads, followed by IV transfer of BM. Chimeras were analyzed at least 7 weeks after irradiation. For PP egress blockade, mice were treated with 1 mg/kg FTY720 (Cayman Chemicals) in PBS for 18 h prior to surgery. Animals were housed in a specific pathogen–free environment in the Laboratory Animal Research Center at UCSF, and all animal protocols were approved by the UCSF Institutional Animal Care and Use Committee.

Short-term transfers and entry blockade

RBC-lysed, CD45 congenically-distinct mutant or matched control and WT splenocytes were mixed and labeled with 500 nM CFSE in 1% FBS in RPMI for 8 minutes. Splenocytes were mixed such that control and CXCR4 KO naïve B cells represented ~50% of transferred B cells and CXCR5 KO naïve B cells represented ~50-80% of
transferred B cells. >50% CXCR5 KO B cell input proportions were used in 2 experiments in order to achieve closer to 50% ratios in the PPs. CXCR5 KO B cell proportions at 0 h were relatively low in PPs as compared to other lymphoid organs because of an expected PP-specific entry defect (Okada et al., 2002). After 2 days equilibration, lymphoid entry blockade with a combination of α4 (Bio X-Cell, clone PS/2, rat IgG2b) and αL (Bio Express, clone M17/4, rat IgG2a) integrin neutralizing antibodies for 18 h was performed as described (Lo et al., 2003).

Histology, immunohistochemistry, and immunofluorescence

During harvest, care was taken not to put any pressure on the PPs in order to preserve their lymphatic contents. To that end, the small intestine was cut into 3-4 sections and flushed with PBS through a 19 G needle to remove the intestinal contents. Then, the PPs were excised, still attached to a ring of intestine, with scissors. For paraffinization and H&E staining, PPs were fixed in 4% PFA in PBS for 4 h at 4°C. They were then washed 5 times for 10 min each in PBS at 4°C. The tissues were then dehydrated stepwise, with 1 h of 30% ethanol, followed by 1 h of 50%, and then 70% overnight. For GFP fixation, PPs were fixed as above. They were then dehydrated stepwise, starting with 30 min of 10% sucrose in PBS, followed by 30 min of 20%, and finally 30% overnight. The sucrose solution was “rinsed” in OCT and the tissues were frozen as normal. 7 µm frozen sections were incubated with primary antibodies for 2-4 h at room temperature; secondary and tertiary antibodies were applied for 1 h. To detect IgHEL (MD4) B cells, the sections were pre-incubated with 100 ng/ml HEL for 20 min. The primary antibodies used and their concentrations were: goat CXCL13 (R&D, AF470, 4 µg/ml), rabbit GFP (Invitrogen,
A11122, 8 \mu g/ml), goat IgD (Cederlane, GAM/IGD(FC)/7S, 20 \mu g/ml), rat LYVE-1 (R&D, MAB2125 3.2 \mu g/ml), FITC CD3 (BD, 553062, 5 \mu g/ml), FITC IgDb (BD, 553510, 5 \mu g/ml), FITC IgMb (BD, 553520, 5 \mu g/ml), biotinylated rabbit \alpha HE L (Rockland, 200-4672, 100 \mu g/ml). Otherwise, immunohistochemistry and immunofluorescence were done as previously described (Allen et al., 2004; Pham et al., 2010). Immunohistochemical slides were mounted with AquaMount (Thermo) while immunofluorescence slides were mounted with Fluoromount-G (Southern Biotech). Slides were imaged at room temperature with Axiovision version 4.8 using an Axio Observer Z1 microscope (Carl Zeiss), equipped with an AxioCam Icc3 camera, an ORCA-ER camera (Hamamatsu), an X-cite Series 120 lamp (Lumen Dynamics), and a plan apochromat 20X objective with a 0.8 NA.

Surgery, photoconversion, and flow cytometry

Survival surgeries were performed with proper anesthetic, sterile technique, and analgesia. They were approved by and in accordance with the University of California, San Francisco’s IACUC guidelines. A mouse was anesthetized with isoflurane, shaved, and antiseptically prepped with 0.02% chlorhexidine gluconate. The mouse was then draped and a \sim 1.5 cm skin incision was made anteriorly at the midline, below the costal margin. The abdominal wall was next incised. The cecum was identified and gently externalized for localization of the small intestine. The cecum was replaced and the small intestine was systematically searched for PPs. Each PP was sequentially externalized (Fig. 4Ai), with care taken not to directly manipulate the PP or damage the mesentery.
For each PP, the surgical site was covered by a piece of sterile foil with a ~4 mm hole punched in it to leave only the PP exposed (Fig. 4Aii).

A Silver LED 415 (Prizmatix), set to maximum intensity, with a high numerical aperture polymer optical fiber (core diameter, 1.5 mm) light guide and fiber collimator, was used as a 415 nm violet light source. Each PP was exposed for 3 minutes (Fig. 4Aiii) and immediately replaced into the peritoneal cavity to avoid drying. Exposures were not possible proximal to the ligament of Treitz. The abdominal cavity was closed with 4-0 absorbable suture and the skin was closed with an Autoclip (Fisher). The entire surgery took ~35-45 minutes, depending on the number of PPs discovered. The count of exposed PPs was recorded for later analysis. Doses of ~0.1 mg/kg buprenorphine in PBS, given subcutaneously over the left shoulder, were administered immediately before and after surgery, and every 4-12 h as needed thereafter. The mice were closely monitored for signs of pain. They were rested for 0, 6, 18, and 40 h from the time of wound closure. For the KikGR-transduced splenocyte transfer experiments, cell suspensions from single spleens were transferred into CD45 congenically-distinguishable WT mice, which were then allowed to equilibrate for 48 hours prior to photoconversion surgery.

Cell suspensions were stained for 20 min on ice in FACS buffer (PBS with 0.1% sodium azide, 2% FBS, and 1μM EDTA). KikG was excited by a 50 mW 488 nm laser and detected through LP 505, BP 525/50, filters. KikR was excited by a 50 mW 561 nm laser and detected through a BP 582/15 filter. To correct for recirculating, converted B cells in mice rested for 18 or 40 h, the percentages of converted cells arriving in unexposed PPs at those time points were measured. Unexposed PPs were identified by their low frequencies of KikR⁺ effector CD4 T cells relative to the frequencies of these cells in
exposed PPs (~1-3% vs. ~80% at 40 h). 4.8%, (SE=0.9%) and 55.3% (SE=6.3%) of converted naïve B cells in 18 and 40 h PPs, respectively, had recirculated. Thus, a correction for recirculating KikR⁺ cell frequency was applied by subtracting the above mean recirculating backgrounds for the given time points from the observed PP KikR⁺ frequencies to obtain retained converted cell frequencies (Fig. 4, C and D).

Statistical analysis and half-life calculation

Least-squares regression, as well as unpaired Student’s and Mann Whitney T tests, were performed using Prism software (Graphpad). The least-squares regression to the single-order exponential decay curve included the constraints that \( y(0)=100 \) and that it asymptotically approaches 0. To calculate half-lives, the equation

\[
\lambda = \frac{(t \cdot \ln(2))}{(\ln(100 / \%R^+) )}
\]

was used, where \( t \) is the number of post-operative hours and \( \%R^+ \) is the recirculation-corrected percentage of retained naïve B cells.

Acknowledgements

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Chapter 3: Conclusion
Model

We have shown that efficient B cell egress from PPs uniquely requires CXCR4. PP B cell follicles are chemoattractive centers, rich in CXCL13, which organizes the PP and decreases B cell access to lymphatics, often lying just outside the follicle proper. Though some B cells likely move directly from interfollicular HEVs to perifollicular lymphatics, as has been observed for T cells in LNs (Grigorova et al., 2009), most home to the follicle. While there, they have access to antigen displayed on follicular dendritic cells (FDCs) and T cell help. However, if they do not encounter their cognate antigen, they must leave in order to provide surveillance in other SLOs. To do so, they need access to the perifollicular lymphatics that drain the PP.

This is where CXCR4 expression on B cells interacts with the unique microanatomy of PPs to provide B cells an exit. CXCL12 is expressed – besides in the dark zones of GCs – around the follicle in a pattern that does not greatly overlap with CXCL13 expression (Fig. 2). Thus, CXCR4 expression on B cells increases their probability of escaping the follicle and encountering a lymphatic exit site. From there, as evidenced by the fact that the S1PR1 antagonist, FTY720, can inhibit egress from PPs (Fig. 4 and Pham et al., 2010), the B cell likely probes the lymphatic (Grigorova et al., 2009) and exits (Supp. Fig. 1).
**Figure 5**

**Model of CXCL13- and CXCL12-constrained B cell egress from PPs.**

Blue indicates CXCL13 expression. Red indicates CXCL12 expression. Yellow indicates lymphatics. Stromal cells (blue, red) and B cells (green) are shown in the magnified area. Rounded B cells fill the lymphatic. S, serosa; IF, interfollicular area; F, follicle.
This unique role for CXCR4 in B cell egress from PPs may partly account for the rapid rate with which they transit PPs relative to LNs (Tomura et al., 2008). Also, we observed higher CD69 expression on B cells in PPs as compared to those in LNs (unpublished data), perhaps due to the inflammatory milieu of the PP. This anti-egress characteristic of PPs may need to be balanced by the pro-egress effect of CXCR4 to achieve efficient B cell transit.

**CXCR4 Regulation in Peyer’s Patch B Cells**

Based on our work, CXCR4 could potentially serve as a tunable, pro-egress chemokine receptor. Individual B cells’ likelihood of achieving egress may be modified by changing their surface expression of CXCR4. CXCR4 has been reported to be regulated in B cells in a number of ways. Hypoxia can induce CXCR4 expression in B cells (Piovan et al., 2007). B cell receptor engagement can decrease B cells’ migratory sensitivity toward CXCL12 (Guinamard et al., 1999; Vlad et al., 2009), though there is some disagreement about this in the literature (Wang et al., 2009). Also, stimulation with CD40L appears to upregulate B cell CXCR4 expression (Moir et al., 1999). Finally, lipopolysaccharide (LPS) stimulation of peritoneal B-1 B cells causes an upregulation of CXCR4 (Moon et al., 2012).

While the data do not consistently point to a link between B cell activation in PPs and inhibition of egress, they motivate future work to test whether modulating the bacterial contents of the small intestine could change B cell transit time through PPs. For example, antibiotic treatment or infection may have varying effects on CXCR4 expression in PP B cells.
CXCR4 is upregulated on GC B cells compared to their naïve precursors (Allen et al., 2004). GC B cells use CXCR4 to access the dark zone of the GC. Of note, in PPs, the GC dark zone is closer to the serosa than is the light zone. This is in contrast to the dark zone of a LN GC, which is positioned further from the capsule – and its subcapsular sinus – than is the light zone. These distinct orientations make teleological sense as they position the light zones, with their antigen-bearing FDC, closer to the source of antigen: the subcapsular sinus in the LN and the intestinal lumen in the PP. Thus, the distinctive distribution of CXCL12 near the serosa of the PP may have evolved to promote a more efficient orientation of the GC. This, in turn, created a unique role for CXCR4 in allowing B cells to access the serosal lymphatics.

Understanding how B cell transit occurs in PPs is an important part of understanding the role of B cells in the function and homeostasis of gut mucosa. Regulatory B cells may play an important role in regulating intestinal inflammation (Li et al., 2011). In addition, IgA production modifies the intestinal microbiome – for example, its segmented filamentous bacterial population – in ways whose significance, in mice and humans, remains to be explored (Suzuki et al., 2004). The fact that CXCR4 appears to play a unique role in B cell egress from PPs means that it may represent an important point of leverage for pharmacological modulation of B cells’ effects on intestinal homeostasis.

**CXCR4 and B Cell Egress from Lymph Nodes**

An interesting observation from our study is that CXCR4 plays a retentive, rather than egress-promoting, role for transferred B cells in LNs. A possible explanation is differing LN anatomy, that the expression pattern of CXCL12 in LNs is such that
CXCR4 pulls B cells away from or out of lymphatics (Grigorova et al., 2009; Sinha et al., 2009; Braun et al., 2011, Fig. 3E). CXCL12 may also play a role in detaining recently immigrated B cells near HEVs – which in LNs are associated with CXCL12 expression (Okada et al., 2002).

Though we were unable to detect any gross changes in follicular positioning of CXCR4-deficient B cells in LNs, we did note that they were found less frequently in the medulla, indicating that WT B cells may be detained there during their egress (Fig. 3E). Interestingly, a mouse model of warts, hypogammaglobulinemia, infections, myelokathexis (WHIM) syndrome – which is caused by a CXCR4 allele that fails to desensitize – shows an increase in number of B cells in LNs (Balabanian et al., 2012). However, in this model, LN B cell follicles appear much smaller and the medullary areas appear packed with B cells. The PPs of this mouse do not appear disturbed. This may be due, however, to PPs’ lower frequencies of T cells, which caused much of the homeostatic perturbation in other SLOs in this model.

The hypothesis that CXCR4 plays an independently important role for B cell egress from LNs is not supported by the limited amount of data from CXCR4 mixed chimeras (Fig. 1A). Here, the statistically insignificant difference between the proportions of CXCR4 KO B cells in the spleen versus those in the MLNs does not mirror the apparently larger difference in retention after integrin blockade (Fig. 1B). However, the proportions of CXCR4 KO B cells in the SLOs of these mice are low and may not provide enough sensitivity to detect a difference. Also, compensatory changes may be possible in the homeostatic, mixed-chimeric setting that are not possible in the transfer
and entry blockade experiment. Further exploration of the possible role of CXCR4 in B cell egress from PPs is warranted.

Control of Secondary Lymphoid Organ Size

Balanced Migration

In order to achieve leukocyte number homeostasis, SLOs must balance the equation,

\[
\frac{dN}{dt} = \frac{dI}{dt} + \frac{dR}{dt} - \frac{dE}{dt} - \frac{dD}{dt} = 0,
\]

where \( t \) is time, \( N \) is the total number of leukocytes in a SLO, \( I \) is the number ingressed (entered), \( R \) is the number replicated, \( E \) is the number egressed (exited), and \( D \) is the number dead. If one assumes that the rates at which cells replicate and die are equivalent, then the rates at which cells enter and exit must also match.

Given that SLOs tend to maintain a steady size in homeostasis and are reasonably stereotyped with respect to size between mice, what regulates the rate at which cells enter and exit (\( \frac{dI}{dt} \) and \( \frac{dE}{dt} \)) to maintain this consistency? The most intuitive speculation is that one or both of these rates is directly tied to lymph node size. Thus, if the lymph node gets too small, the rate of ingress could increase or the rate of egress could decrease, or both. The corollary could occur if the lymph node were to become too big. However, what could control those rates and how would that control be linked to lymph node size?

Of course, there is one obvious example where both ingress and egress are actively modulated to change an SLO’s size: inflammation of a LN. Egress is inhibited by IFN- mediated upregulation of CD69, which decreases cells’ migratory sensitivity to S1P (Shiow et al., 2006). In addition, inflammatory cytokines cause upregulation of P- and E-selectins as well as deposition of chemokines, CCL2 and CXCL9, induced by
peripheral inflammation on HEV (Miyasaka and Tanaka, 2004; von Andrian and Mempel, 2003). These changes in the HEV create a rapider influx of leukocytes. Thus, in the setting of inflammation, the rate of ingress increases, the rate of egress decreases, and thus the rate of change of LN size becomes positive. Also, inflammation is often associated with rapid proliferation of lymphocytes, contributing to the increase in LN size as well.

**Dendritic Cell Support of Lymph Node Homeostasis and Growth**

Another factor important to the size of LNs are DCs. DCs have been shown to be important supporters of HEVs, in both inflammatory and homeostatic settings, through their expression of vascular endothelial growth factor (VEGF) (Webster et al., 2006; Wendland et al., 2011). Semimature steady state dendritic cells promote HEV growth, thus maintaining the available surface area over which cells can enter. They also promote the expression and local capture of CCL21 on the T zone fibroblastic reticular cells (FRCs), which helps retain T cells in the LN (much as CXCL13 retains B cells in the PP). This mechanism by which DCs support a normal flux of lymphocytes through LNs suggests that DC number may be an important constraint on LN size. Indeed, increasing the number of DCs in a LN through transfer does increase the size of the LN (Wendland et al., 2011). In addition, the authors postulate that DCs may affect egress as well as entry through their effect on CCL21 expression. They propose that increased CCR7 engagement could lead to increased egress via random, haptokinetic encounter with cortical or medullary lymphatics.

It is possible then that the limited number of DCs and their intrinsic rate of turnover limits LN size. For example, when a LN enlarges due to an inflammatory
stimulus, it can return to its normal size once the stimulus has abated because the supply of DCs to support its expanded HEV network becomes limiting. However, one might also hypothesize that the supply of DCs from the blood is not limiting, even for a larger HEV network. In this case, some other mechanism to contract the network would have to be invoked because homeostatic contraction due to insufficient DC support would not be possible. Another potential mechanism to return an enlarged LN to its homeostatic size would be to increase the rate of lymphocyte egress. Inflammatory lymphangiogenesis is a phenomenon that occurs in enlarging LNs that could achieve accelerated egress. Lymphatics expand upon inflammation and B cells are critical to this process (Webster et al., 2006; Angeli et al., 2006). An expanded network of lymphatics, with increased surface area, would putatively promote a higher rate of lymphocyte egress. After the inflammatory stimulation has subsided, a CD11c<sup>hi</sup> set of DCs has been reported to be important for reestablishing vascular normalcy (Tzeng et al., 2010). These data suggest that DCs are central to LN size, both homeostatically and during inflammation.

It will be important to elucidate whether PPs may similarly rely on DCs for their homeostasis. The role and nature of DCs in PP-related immunity are unique. DC-T cell mixtures from PPs have been known to preferentially induce IgA production in co-cultured B cells, indicating a unique role for the PP itself in promoting mucosal antibody defense (Spalding et al., 1984). Indeed, there are distinct subsets of DCs associated with PP residency, including CD11b<sup>+</sup> DCs in the SED, CD8α<sup>+</sup> DCs in the interfollicular area, and DCs negative for both of these markers in both areas (Iwasaki and Kelsall, 2000). These subsets appear to have distinct cytokine production capacities (Iwasaki and Kelsall, 2001). CCR6 is particularly important for the function of DCs that promote immunity to
intestinal pathogens as it allows DCs to access the subepithelial dome (Iwasaki and
Kelsall, 2000; Salazar-Gonzalez et al., 2006). PP DCs may even play a role in acquiring
antigen directly from the intestinal lumen (Lelouard et al., 2012) in a manner analogous
to CX3CR1+ DC sampling of antigen from the lamina propria (Rescigno et al., 2001;
Niess et al., 2005).

However, whether DCs (perhaps those that are CD8α+CD11b− or CD8α−CD11b−)
regulate HEV maturity and fibroblastic reticular cell (FRC) function in PPs warrants
further study. PP HEVs are qualitatively different from LN HEVs (Bargatze et al., 1995;
Miura et al., 1995; Warnock et al., 2000; Okada et al., 2002), so it might be expected that
the mechanisms of their regulation would differ as well. The KikGR photoconvertible
system we used in our study of B cell egress from PPs could be used to identify newly
arrived, unconverted DCs as well as the rates at which they arrive and turnover. Thus, the
connection between DC function and PP migratory homeostasis could be the subject of
future work.

Lymphocyte Transit and Temporal Constraint

Clock Model of Lymphocyte Transit

It is possible that CXCR4 is a tunable variable that can control the pace at which
B cells egress from PPs in the setting of infection. Also possible, though, is that CXCR4
expression is regulated based on the amount of time that a B cells spends within the
parenchyma of a PP in a homeostatic setting. That is, CXCR4 surface expression or
function could be low on lymphocytes that have recently entered the PP but then
gradually rise, increasing the probability of egress for cells that have already spent some
time in the PP.
This egress clock model is appealing because it would ensure that B cells that enter a PP would have a certain amount of time for surveillance before they return to circulation. However, it may not be necessary. A model of T cell egress based on stromally-guided random walk behavior was able to recapitulate experimentally observed dwell times (Grigorova et al., 2010). Intravenously transferred T cells have been observed in cortical sinuses, to which they presumably egressed, within 20 minutes, though there were no observations of direct transmigration from HEV to cortical sinus (Grigorova et al., 2010). Thus, a clock model would not necessitate a threshold of time before which lymphocytes are unable to egress. Instead, it would incorporate some continuous, time-dependent variable that affects a B cell’s likelihood of egress.

Chemokine Receptor Sensitivity

Chemoattractant sensitivity is one possible method by which B cell egress could be temporally controlled. Indeed, relatively higher expression of RGSs has been observed in LN B cells remaining after entry blockade by treatment with a CD62L (L-selectin)-blocking antibody (Park et al., 2012). It was noted in the same study that B cells more recently transferred are more chemokine sensitive than those remaining in a LN after a longer period of transfer. In addition, B cells sampled from the blood are more sensitive to chemokines than their LN counterparts. The authors suggest that high CXCR5 sensitivity of B cells entering a LN from the blood decreases their likelihood of egress due to their attraction to the CXCL13-rich follicle. Continuous, RGS-mediated desensitization would cause the cells to stray further from the follicle, until they eventually egress. This ultimate egress requires resensitization of S1PR1 after its GRK2-mediated downregulation in the blood (Arnon et al., 2011). Thus, any time-dependent
desensitization of CXCR5 would have to be somewhat specific to this receptor in order to allow S1PR1 to upregulate anew.

The experiments demonstrating varying B cell chemokine sensitivity after entry blockade or transfer are only able to observe cells that have been in a LN for a given amount of time (Park et al., 2012). The cells that are observed have either avoided egress during entry blockade or have been retained after transfer. This means that they cannot distinguish whether the migratory properties of the B cells they analyze are due to their retention in the LN or vice versa. With the assumption that B cells persist in the LN after entry blockade or transfer because they have lower chemokine sensitivity, a different model arises. This model would suggest that B cells actively downregulate their chemokine sensitivity upon entry and then gradually upregulate their sensitivity to specific chemokines as time passes. This description is an oversimplification because some chemokine receptors (e.g. CXCR5) will be desensitized due to high follicular ligand concentration, while others will be resensitized (e.g. S1PR1) due to low parenchymal ligand concentration. However in the case of the PP, where the main part of the B cell follicle is CXCL12-low, a progression of CXCR4 desensitization followed by resensitization would provide a mechanism for an egress clock. Essentially, desensitization could occur quickly after entry, followed by gradual, egress-promoting resensitization.

The KikGR photoconvertible system that we have used in our study of B cell egress from PPs could be effectively applied to study this problem. It allows for the identification of both converted cells that have be retained in an SLO for a given amount of time from conversion and unconverted cells that have recently immigrated. These two
types of cells could be analyzed for differences in migratory sensitivity, RGS expression, and transcriptomes. Whether chemokine receptors sensitize or desensitize over time in a LN, it will be important to understand how this desensitization occurs. If RGSs are major regulators of the process, the types of RGSs that act, their receptor-specificities, and the signals that regulate their activity will need to be dissected. By studying how naïve B cells’ chemokine sensitivities change over the course of their stay in a LN, evidence for or against temporal regulation of their migration and egress could be developed.

Summary

Here we have provided evidence for a previously unknown and unique role of CXCR4 in promoting B cell egress from PPs. This mechanism appears to rely on the specific microanatomy of the PP, which demonstrates CXCL12 expression near the lymphatics that serve as sites of egress. We have also further developed the KikGR photoconversion system by applying it to the study of lymphocyte migration kinetics in PPs. Using this novel method, we have generated data on the transit rate of lymphocytes through PPs, which may be rapider than that previously measured for LNs. This tool can be further used to study frontier topics in lymphocyte migration, including homeostatic control of SLO size by DCs and modulation of chemokine receptor sensitivity in SLO-resident lymphocytes.
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