CXCR4 promotes B cell egress from Peyer’s patches

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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. We report that, in mixed chimeras, CXCR4-deficient B cells accumulate in PPs compared with their representation in other lymphoid tissues. CXCR4-deficient B cells egress from PPs more slowly than wild-type cells, whereas 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+ perilymphatic zones, whereas CXCR5-deficient B cells preferentially localize in these regions. By photoconverting KikGR-expressing cells within surgically exposed PPs, we provide evidence that naive 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 perilymphatic zone in PPs that plays a role in overcoming CXCL13-mediated retention to promote B cell egress from these gut-associated lymphoid tissues.
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 ∼10 h residency time in PPs before traveling 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>Mbl-Cre<sup>+</sup> (CXCR4 KO) CD45.2<sup>+</sup>:WT CD45.1<sup>+</sup> mixed BM chimeras and examined B cell distribution in lymphoid tissues. Compared with their frequencies in spleen and MLNs, PPs showed a marked accumulation of KO versus WT naive B cells (B220<sup>+</sup>IgM<sup>hi</sup>Cd95<sup>−</sup>) in lymphoid organs. Spl, spleen. The graph represents 7 control and 17 KO mixed BM chimeric mice. (B) Flow cytometric analysis of the distribution of co-transferred control or KO versus WT naive 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 (t<sub>18h</sub>) versus immediately before entry blockade (t<sub>0h</sub>) are plotted. The CXCR4 (blue, top) and CXCR5 (red, bottom) data consist of four and three experiments, respectively, with each pair for a given organ representing a t<sub>18h</sub>/t<sub>0h</sub> pair of mice (eight and six 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. Bar, 100 µm.
CXCR4 and CXCR5 in promoting PP B cell egress and retention, respectively.

Lymphatic sinuses are present in both subserosal and interfollicular regions of PPs (Ohtani and Murakami, 1990; Azzali and Arcari, 2000; Gohda et al., 2008) and hematoxylin and eosin (H&E) staining of fixed and paraffin-embedded PP tissue showed that these sinuses were often filled with lymphocytes (Fig. 1 C). 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. 2 B). Notably, the CXCL12-GFP+ areas by treatment with a combination of α4 and αL integrin neutralizing antibodies for 18 h (Lo et al., 2003). Compared with their frequency before entry blockade, CXCR4 KO cells became overrepresented selectively in PPs (Fig. 1 B). In contrast, they became underrepresented in LNs (Fig. 1 B). 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 promotes T cell retention in LNs (Pham et al., 2008), we had anticipated that 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. 1 B). 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. 1 B). These data suggested unique roles for CXCR4 and CXCR5 in promoting PP B cell egress and retention, respectively.

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were adjacent to but largely not 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. 2 A, Fig. 3 A, and not depicted). Analysis of peripheral LNs (PLNs) 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. 2 C). In PPs, the closely juxtaposed yet separate distributions of CXCL12 and CXCL13, along with...
three-color immunofluorescence microscopy to better distinguish LYVE-1+ lymphatics and transferred cells (Fig. 3 B). Enumeration of cells within 200 µm of subserosal lymphatics established that CXCR4 KO cells were significantly underrepresented in these areas, whereas CXCR5 KO cells were overrepresented, compared with their frequencies in the center of the follicle (Fig. 3 C). Similarly, enumeration of cells overlapping with or internal to the LYVE-1+ lymphatic endothelial staining showed a paucity of CXCR4 KO cells and an enrichment of CXCR5 KO cells (Fig. 3 D). 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 PLNs, cell distributions were more variable but CXCR4 KO cells were, on average, underrepresented in medullary regions (Fig. 3 E). Despite advances in our knowledge of lymphocyte egress from LNs, the contribution of medullary regions to this process has been difficult to define as a result of 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.

To determine how CXCL12-CXCR4 influenced B cell positioning in PPs, we immunohistochemically examined the distribution of transferred KO (Ighb+) and WT (hen egg lysozyme [HEL]-binding, B cell receptor transgenic) B cells in WT, Igha+ hosts. In control experiments, the Ighb+ and HEL+ B cells were codistributed in follicles and perilymphatic areas (Fig. 3 A), establishing that the IgHEL transgene did not alter B cell distribution in PPs. In contrast, in CXCR4 KO:WT cotransfers, the KO B cells were localized within follicles and were markedly underrepresented in perilymphatic regions (Fig. 3 A). Reciprocally, CXCR5 KO B cells were excluded from PP follicles, as expected (Fürster et al., 1996), and were enriched in areas adjacent to lymphatic vessels (Fig. 3 A). Similar observations were made when sections were examined by three-color immunofluorescence microscopy to better distinguish LYVE-1+ lymphatics and transferred cells (Fig. 3 B).

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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 PP

| Tissue | Retention or arrival | Naive B | Naive CD4 | Naive CD8 | Effector CD4 | Effector CD8 |
|--------|---------------------|---------|-----------|-----------|--------------|--------------|
| PP     | Retention           | 30.1 ± 5.9 | 7.4 ± 2.5 | 5.2 ± 1.7 | 85.1 ± 7.4 | 58.6 ± 16.0 |
| MLN    | Arrival             | 10.0 ± 4.2 | 3.8 ± 1.0 | 3.5 ± 1.0 | 2.7 ± 0.5 | 2.3 ± 0.5 |
| PLN    | Arrival             | 1.7 ± 0.5  | 1.9 ± 0.3 | 1.4 ± 0.3 | 0.2 ± 0.2 | 0.3 ± 0.3  |
| Spleen | Arrival             | 3.5 ± 0.6  | 2.3 ± 0.4 | 1.6 ± 0.3 | 1.9 ± 0.5 | 0.4 ± 0.1  |
| Blood  | Arrival             | 7.4 ± 0.6  | 2.3 ± 0.4 | 1.4 ± 0.3 | 0.5 ± 0.1 | 0.4 ± 0.1  |

Naive 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 CD4⁺CD62L⁺ and effector T cells as CD4⁺CD62L⁻. The data represent five mice.

anatomy and high content of LYVE-1⁺ macrophages (Weig et al., 2005; Grigorova et al., 2009, 2010; Sinha et al., 2009).

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, 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 an ~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. 4 A). The PP was then returned to the abdomen and a second PP accessed and violet light exposed. Typically, four to seven PPs were converted per mouse, leaving two to three PPs unexposed to serve as internal controls. When PPs were isolated immediately after the procedure and analyzed for naive IgD⁺B220⁺ B cells (Fig. 4 B), 99.4% (SD = 0.3%, n = 8 PPs in two mice) of the cells in violet light exposed PPs had undergone the green to red conversion (Fig. 4 C, i), whereas very few cells in the control PPs (Fig. 4 C, i) were converted (0.25 ± 0.25%, n = 12 PPs in two mice). The spleen and PLN contained a very small frequency (<0.5%) of partially converted cells at 0 h after surgery (unpublished data), 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 naive B cells in individual PPs was reduced to 77.7% (SD = 6.2%, n = 27 PPs in five mice; Fig. 4 C, iii), whereas converted cells were now detected in circulation (not depicted) and MLNs (Fig. 4 C, vi) at rates higher than those observed immediately after PP conversion (Fig. 4 C, v). Analysis of PPs at 40 h showed that the red fluorescence intensity of converted naive B cells had decayed only slightly compared with 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. 4 C, 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 (unpublished data).

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) before PP photoconversion, very few photoconverted cells were found in MLNs or other lymphoid organs after 6 h (Fig. 4 C, vii; and not depicted). 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 naive 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. 4 C). Calculation of the apparent half-lives of naive B cells within individual PPs at the later time points—thereby allowing at least 18 h for recovery from surgical stress—revealed mean naive B cell half-lives of ~10 h (Fig. 4 D). An assessment of all photoconverted cells remaining in PPs at 18 h showed that there was a greater exchange of naive CD4 and CD8 T cells than B cells, indicating that naive 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 naive 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).

Having established a KikGR photoconversion time stamping procedure for PPs, we next applied this approach to test the egress-promoting role of CXCR4 in naive 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. 4 C, viii). Summarizing data from several 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. 4 F). To control for possible indirect effects on PP organization of CXCR4 deficiency in all B cells, we performed similar time stamping experiments in WT 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. 4 F).
Concluding Remarks
The above findings suggest a model where, after CXCR5-CXCL13-mediated entry to PP follicles, B cell access to subepithelial 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). Although 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 (Miura et al., 1995; Warnock et al., 2000; 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 periluminal 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 which warrants future investigation. In addition, T cells can migrate from medullary 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, as a result of 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; Shioy 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 V.R. Zou (The Feinstein Institute for Medical Research, Manhasset, NY) and D. Littman (New York University, New York, NY). CXCR4KO mice (Nie et al., 2004) were crossed to CXCR4F/F mice (Zou et al., 1998) heterozygous for Mb1Cre (Cd79atm1(cre)RobR, MGI:3687451) provided by M. Roth (Max-Planck Institute of Immunobiology, Freiburg, Germany; Hobeka et al., 2006). CXCR5F/F mice ( Förster et al., 1996) were received from R. Förster (Hannover Medical School, Hannover, Germany) and M. Lipp (Max Delbrück Center, Berlin, Germany) and backcrossed at least 10 generations to B6. CXCL12-GFP knockin mice (Ara et al., 2003; B6 crossed to CD1 one generation, were provided by S. Pleasure’s laboratory (University of California, San Francisco [UCSF]), which received the mice originally from the Nagasawa laboratory (Kyoto University, Kyoto, Japan). For the CXCR4 and CXCR5 localization transfer recipients (Fig. 3), Igh<sup>+</sup> (The Jackson Laboratory; 001317, B6.Cg-Ighg1 Thylta1Gpafj/L) mice and Igh<sup>-</sup> mice (MD4) transgenic mice were bred in our colony. CAG::KikGR<sup>+</sup> (013753, Tg(CAG-KikGR)33Hadj/J; Novotschn and Hadjiantanoukas, 2009) mice were purchased from The Jackson Laboratory. CAG::KikGR<sup>+</sup> mice were received on an outbred ICR strain background and backcrossed to C57BL/6 for two to four 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 (Thyl1.1) retroviral vector carrying CoralHucTN KikGR1 (MBL, AM-V0081). The control and CXCR4 KO transduced chimeras had 70–80% and 50–80% B220<sup>+</sup> lymphocytes in their PPs, respectively. BM chimeras were produced by lethally irradiating host mice with a split dose of 1,100–1,300 rad, followed by i.v. transfer of BM. Chimeras were analyzed at least 7 wk after irradiation. For PP egress blockade, mice were treated with 1 mg/kg FTY720 (Cayman Chemicals) in PBS for 18 h before 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% PBS in RPMI for 8 min. 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 two experiments to achieve closer to 50% ratios in the PPs. CXCR5 KO B cell proportions at 0 h were relatively low in PPs as compared with other lymphoid organs because of an expected PP-specific entry defect (Okada et al., 2002). After 2 d of 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 previously described (Lo et al., 2003).

Histology, immunohistochemistry, and immunofluorescence. During harvest, care was taken not to put any pressure on the PPs to preserve their lymphatic contents. To that end, the small intestine was cut into three to four 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, sections and flushed with PBS through a 19 G needle to remove the intesti

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Brief Definitive Report
Surgery, photoconversion, and flow cytometry. Survival surgeries were performed with proper anesthesia, sterile technique, and analgesia. They were approved by and in accordance with UCSF IACUC guidelines. A mouse was anesthetized with isoflurane, shaved, and antisепtically prepared with 0.02% chlorhexidine gluconate. The mouse was then draped and a ~1.5 cm skin incision was made anteriorly at the midline, below the costal margin. The abdominal wall was then 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. 4A, left), 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. 4A, middle).

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 min (Fig. 4A, right) and immediately replaced into the peritoneal cavity to avoid drying. Exposures were not possible proximal to the ligament of Treitz. The intestinal wall was next incised. The cecum was identified and gently externalized for 48 h before photoconversion surgery.

Photoconversion surgery. Photoconversion surgery was used, where \( t \) is the number of post-operative hours and \( R \) that it asymptotically approaches 0. To calculate half-lives, the equation
\[
\ln \left[ \frac{\lambda \times \ln(2)}{\ln[100 / 5\% R]} \right]
\]
was used, where \( \lambda \) is the number of post-operative hours and \( 5\% R \) is the recirculation-corrected percentage of retained naive B cells.

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