The Role of CXCR4 in Maintaining Peripheral B Cell Compartments and Humoral Immunity

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Abstract

The chemokine receptor CXCR4 is expressed in B cells at multiple stages of their development. CXCR4 function in humoral immunity has not been fully investigated. We have generated gene-targeted mice in which CXCR4 can be selectively inactivated in B cells and have shown that it is required for retention of B cell precursors in the bone marrow. CXCR4-deficient B cell precursors that migrated prematurely became localized in splenic follicles despite their unresponsiveness to CXCL13. Concomitantly, mature B cell populations were reduced in the splenic marginal zone and primary follicles, and in the peritoneal cavity in the mutant animals, as were T-independent antibody responses. In addition, aberrant B cell follicles formed ectopically in intestinal lamina propria around Peyer’s patches. These findings establish an important role for CXCR4 in regulating homeostasis of B cell compartmentalization and humoral immunity.

Key words: chemokine receptor • B lymphocytes • migration • plasma cell • Peyer’s patches

Introduction

B lymphocytes at different stages of development and with diverse functions reside in distinct anatomic locations. After their generation in the bone marrow, newly formed B cells migrate into the spleen, where, as transitional B cells, they are subjected to further selection and become fully mature (1). In the spleen, mature B cells are found in primary follicles and in the marginal zone (MZ). MZ B cells are confined to the spleen, participate mainly in the T-independent (TI) antibody responses, and mount critical humoral responses against blood-borne pathogens (2, 3). Follicular B cells circulate between lymphoid organs and upon antigen stimulation and cognate interaction with T cells, they form germinal centers (GCs) within secondary lymphoid organs such as lymph nodes, the spleen, and Peyer’s patches. Within the GC, they differentiate further into plasma cells and memory B cells that express high affinity, class-switched antibodies (4, 5). Although memory B cells recirculate in lymphoid organs, plasma cells home preferentially to the bone marrow (6–9). A third subset of mature B cells, the B1 cells that are predominantly localized in body cavities, is the main source of natural antibodies (10, 11).

Genetic studies have established that discrete sets of chemokines in the respective niches and their cognate chemokine receptors expressed on B cells cooperatively determine the positioning of B cell subsets within lymphoid organs. Several chemokine receptors, including CCR7, CXCR4, and CXCR5, are known to regulate trafficking and retention of B cells (12). CXCR5 is a prominent chemokine receptor that is specifically expressed on mature B cells (13, 14). The interaction of CXCR5 and its ligand CXCL13 plays a critical role in recruiting mature B cells into primary follicles in the peripheral lymphoid organs and in guiding B1 cell homing to body cavities (15–19). Activated B cells, on the other hand, up-regulate CCR7 expression and thus acquire the capacity to migrate into the T cell zone and to follicles in Peyer’s patches, where the CCR7 ligands, CCL19 and CCL21, are highly expressed (18, 20).

CXCR4 is expressed by all subsets of B cells throughout B cell ontogeny. Its ligand, CXCL12, is broadly distributed in many tissues. In the immune system, CXCL12 mRNA has been detected in bone marrow stroma, high endothelial venules, medullary cords in lymph nodes, red pulp and MZ
bridging channels of the spleen, and peritoneal mesothelial cells (18, 21–24). Genetic studies have provided evidence for a role for CXCL12 and CXCR4 in B cell development and function. Mice deficient in CXCL12 or CXCR4 lack B lymphopoiesis. This has been ascribed to failure in colonization of hematopoietic progenitors in the bone marrow (25–28). In mice reconstituted with CXCR4−/− fetal liver cells, the number of plasma cells in the bone marrow was reduced (23). Although CXCR4 deficiency alone did not affect B cell homing to the peripheral lymphoid organs (29), B cells homing to Peyer’s patches were impaired when their responsiveness to CXCL12, CCL19, and CCL21 was simultaneously abolished (18).

Thorough studies of the role for CXCR4 in B cell maturation, trafficking, and in humoral immunity using Cxcr4−/− fetal liver chimeras were limited by the poor generation of B cells due to the deficiency of Cxcr4−/− hematopoietic progenitors homing into the bone marrow. To directly study the function of CXCR4 in B cell development, we have generated a mouse strain in which CXCR4 can be selectively inactivated in B lineage cells. We found that the CXCR4-deficient B lineage precursors escaped from the bone marrow prematurely and homed into the splenic follicles despite lacking responsiveness to CXCL13. The premature migration of these precursors was accompanied by a reduction of the mature B cell compartments in the spleen. Inactivation of CXCR4 also resulted in decreased numbers of peritoneal B cells and defective TI responses. Furthermore, additional B cell follicles were formed in the small intestine in the absence of CXCR4. These findings establish an important role for CXCR4 in regulating homeostasis of B cell compartments and humoral immunity.

Materials and Methods

Mice. Genomic DNA fragments used for constructing the targeting vector were isolated from the previous CXCR4 targeting vector (27). We inserted two loxP sites along with a neomycin cassette into the Cxcr4 locus to flank exon 2. Chimeric mice carrying the targeted allele were crossed to EIIa-cre mice to generate progeny with the neo gene–deleted floxed Cxcr4 allele. The following PCR primers were used to genotype wild-type or the loxP-inserted Cxcr4 allele: 5′ primer (5′-CACATCGCAGTCGAAATG-3′) and 3′ primer (5′-GTGTGGCTGGTGATGC-3′). Heterozygous mice carrying the floxed Cxcr4 allele were then bred to Cd19−/− mice. We used 6–12 wk-old littermates for all experiments, unless otherwise specified. All mice were maintained under specific pathogen-free conditions. The mouse protocol was approved by Columbia University’s animal committee.

Flow Cytometry and Magnetic Cell Sorting (MACS). Cells from the bone marrow, spleen, lymph nodes, and peritoneal cavity were harvested. The following antibodies were used: FITC-conjugated anti-CD3, anti-CD11b, and anti-CD21; PE-conjugated anti-CD5, anti-CD23, and anti-IgM; allophycocyanin (APC)-Cy7–conjugated anti-B220; streptavidin–PE-Cy7 (all from eBioscience); PE-conjugated anti-CD138; and biotinylated anti-CXCR4 and anti-CXCR5 (BD Biosciences). Analysis was performed with an LSR II (Becton Dickinson). To purify splenic B cells for Southern analysis and chemotaxis assay, splenocytes isolated from the mutant and wild-type mice were labeled with biotinylated anti-B220, and then bound to streptavidin-conjugated magnetic beads. Labeled cells were sorted with the use of MACS (Miltenyi Biotec). Statistical analysis was performed on Microsoft Excel software.

Annexin V Assay. Splenocytes were labeled with biotinylated annexin V in the annexin V–binding buffer (BD Biosciences). Cells were then stained with PE anti-B220 and FITC anti-IgM (BD Biosciences). Biotin–annexin V was visualized by streptavidin–conjugated APC. Cells were analyzed on an LSR II (Becton Dickinson).

Chemotaxis Assay. MACS-purified B cells were resuspended in DMEM, 10% FCS at 10⁷/ml. 100 µl B cells were loaded into the upper chamber of a 5-µM pore-size transwell (Corning Costar). The lower chambers were filled with 600 µl of medium either with or without 500 ng/ml CXCL12 (R&D Systems). Cells that migrated into the lower chambers were harvested after 3 h of incubation and counted.

Immunohistology. Mouse spleens were snap frozen in liquid nitrogen and embedded in OCT embedding medium (Sakura Finetek). 8–µM sections were air-dried and fixed with acetone. Staining was performed with the use of the following reagents: anti-B220–Alexa 488 or anti-B220–Alexa 568 (Molecular Probes), anti–MOMA-1–biotin (BMA Biomedical), anti–IgM–FITC (BD Biosciences), anti–IgG–FITC (Southern Biotechnology Associates, Inc.), anti–PNA–biotin (Vector Laboratories), anti–CD3–biotin (BD Biosciences), streptavidin–Alexa 633, and streptavidin–Alexa 568 (Molecular Probes).

Immunization, ELISA, and ELISPOT. 8–10-wk-old mice were immunized by intraperitoneal injection of 25 µg of 4-hydroxy-3-nitrophenylacyl (NP) coupled to Ficoll (NP–Ficoll) in PBS or 50 µg NP conjugated to KLH (NP–KLH from Biosearch Technology) mixed with alum (Pierce Chemical Co.). Mice were boosted with NP–KLH in PBS at day 21 after the first immunization. Sera were collected on days 0 and 9 for the NP–Ficoll responses, on days 0, 7, 14, and 21 after the first NP–KLH immunization, and on days 7 and 90 after NP–KLH boosting. Serum Ig isotopes and antigen–specific Ig isotopes were determined by ELISA as described previously (30). Relative affinities of anti-NP IgG1 were measured using a plate-binding assay that is based on the direct correlation of antibody affinity and the ratio of antibody binding to NP–BSA conjugates at low (NP₃) and high (NP₅) hapten density (both from Biosearch Technology; reference 31). To determine the number of plasma cells by ELISPOT, cells were isolated freshly from the spleen, mesenteric lymph nodes, and bone marrow, and plated into NP–KLH–precoated plates (Biosearch Technology). IgM and IgG were detected with alkaline phosphatase–conjugated anti–mouse IgM or IgG (Southern Biotechnology Associates, Inc.).

Results

Generation of Mice with CXCR4-deficient B Cells. Exon 2 of Cxcr4 encodes 98% of the CXCR4 molecule. Cre recombinase–mediated deletion of exon 2 will therefore abolish CXCR4 function. We inserted loxP sites 791 bp upstream and 221 bp downstream of exon 2 of Cxcr4 by homologous recombination in mouse embryonic stem (ES) cells (Fig. 1 A). Chimeric mice derived from the targeted ES clones were crossed to EIIa-cre transgenic mice to generate loxP-flanked (floxed) Cxcr4f/f mice.
To selectively inactivate CXCR4 in B cells, Cxcr4f/f mice were crossed to Cd19-Cre mice, which can induce efficient deletion of floxed target sequences specifically in B cells (32). Southern blot analysis of DNA of purified splenic T and B cells from Cxcr4f/fCd19/H11001/Cd19/Cre mice showed that deletion of the Cxcr4 gene occurred in 95% of alleles in B, but not in T, cells (Fig. 1B). Splenic B cells purified from Cxcr4f/fCd19/H11001/Cd19/Cre mice were subjected to CXCL12-mediated chemotaxis in a transwell. B cells from Cxcr4f/fCd19/H11001/Cd19/Cre mice failed to migrate toward CXCL12, confirming the loss of CXCR4 function in the Cxcr4f/fCd19/Cd19/Cre B cells (Fig. 1C).

CXCR4 Prevents Premature Migration of B Cell Precursors into the Periphery. Development and localization of T cell and myeloid populations in Cxcr4f/fCd19/Cd19/Cre mice were unaffected by B lineage–specific inactivation of CXCR4 (not depicted). Generation of pro–/pre– (CD19+ B220lo IgM+CD4−CD8−CD11b−CD11c−) and immature (CD19+ B220+ IgM+) B cells was also normal in the Cxcr4f/fCd19/Cd19/Cre bone marrow (Fig. 2B). The efficient generation of B cells in the bone marrow of Cxcr4f/fCd19/Cd19/Cre mice was not surprising, as it is known that the CD19 promoter activity is increased with progression of B cell development, resulting in incomplete Cre-mediated deletion in B precursors (33). We observed only ~40% deletion of Cxcr4 in the B220+ IgM+ population (not depicted).

B cells that have completed their maturation process in the bone marrow express a high level of IgM, lose their tropism for bone marrow stroma, and are released into the circulation. Given the important role of CXCR4 in retaining hematopoietic progenitors in the bone marrow, it has been extrapolated that the bone marrow tropism of B cell precursors also involves CXCR4 signaling. Consistent with this prediction, we observed that CXCR4 was expressed at its highest level in B cell precursors (B220lo) and down-regulated in mature B cells (B220hi) that became capable of bone marrow egress (Fig. 2A, top). In Cxcr4f/fCd19/Cd19/Cre mice, CXCR4 was absent from the surface of mature B cells in the bone marrow and spleen, as well as from the surface of a large fraction of B cell precursors (B220lo) and down-regulated in mature B cells (B220hi) that became capable of bone marrow egress (Fig. 2A). Correlated with the inactivation of CXCR4, a substantial fraction of cells in the blood of Cxcr4f/fCd19/Cd19/Cre mice exhibited characteristics of B cell precursors (CD19+B220lo IgM−CD4−CD8−CD11b−CD11c−; Fig. 2B and not depicted). This finding extends previous observations that CXCR4 is critical for retaining hematopoietic progenitors in the bone marrow.
CXCL12 is the bone marrow tropic factor for B cell precursors. The CXCL12-independent homing of CXCR4-deficient B cell precursors to splenic follicles. The CXCR4-deficient CD19<sup>+</sup> B220<sup>lo</sup> IgM<sup>-</sup> cells were found only in the spleen. They were absent from lymph nodes, the peritoneal cavity, and Peyer’s patches (not depicted). They were of lymphoid but not dendritic cell lineage, as they were small in size and express a B lineage-specific marker, CD19, but not CD4, CD8, CD11b, and CD11c (not depicted). These B cells were not PNA<sup>+</sup> GC cells, but likely to be B cell precursors, as they expressed low levels of B220 and surface Ig (Fig. 3 A, right, and not depicted). To examine the localization of the CXCR4-deficient B cell precursors within the spleen, splenic sections were stained to simultaneously visualize B cell follicles (B220<sup>-</sup> IgM<sup>-</sup> IgD<sup>-</sup>) and B cell precursors (B220<sup>+</sup> IgM<sup>-</sup> IgD<sup>-</sup>). The overall splenic structure was indistinguishable between wild-type and mutant mice (Fig. 3 A, left). We observed that the majority of CXCR4-deficient B cell precursors were localized in the splenic follicles in Cxcr<sup>4</sup>f/fCd19<sup>+</sup>/Cre mice (Fig. 3 A, middle).

B cell trafficking into primary lymphoid follicles depends on CXCR5 signaling (16). Next, we examined whether homing of the CXCR4-deficient B cell precursors into splenic B cell follicles was mediated by CXCR5. Flow cytometry analysis of splenic B cells from both wild-type and mutant mice revealed that CXCR5 expression was barely detectable on B cell precursors and increased with progression of B cell maturation (Fig. 3 B). When CXCL13-mediated chemotaxis was measured by a transwell assay, we observed that the responsiveness to CXCL13 was concordant with the CXCR5 level on different B cell subsets (Fig. 3 C). Together, these data indicate that the recruitment of CXCR4-deficient B cell precursors into splenic follicles is a process that is independent of CXCL13 and CXCR5, and may involve other chemokines and their receptors.

Altered B Cell Compartments in Lymphoid Organs in Cxcr<sup>4</sup>f/fCd19<sup>+</sup>/Cre Mice. Cxcr<sup>4</sup>f/fCd19<sup>+</sup>/Cre mice had comparable numbers of total splenocytes and CD19<sup>+</sup> cells as wild-type mice (total cell number: 1.5 × 10<sup>8</sup> ± 0.5 in controls and 1.2 × 10<sup>8</sup> ± 0.3 in mutants; CD19<sup>+</sup> cell numbers: 4.8 × 10<sup>7</sup> ± 0.9 in controls and 4.1 × 10<sup>7</sup> ± 0.3; n = 7). However, premature homing of the CXCR4-deficient B cell precursors to the spleen led to a significant expansion of the immature B cell pool (B220<sup>+</sup> CD21<sup>-</sup> CD23<sup>-</sup>) in the mutant mice (Fig. 4 A). Consequently, the total cellularity of
Figure 3. Localization of B cell precursors in Cxcr4<sup>f/f</sup> Cd19<sup>+/Cre</sup> mice. (A) Immunofluorescent staining of splenic sections with FITC anti-IgM, FITC anti-IgD, and Alexa 568–anti-B220 antibodies (left, a magnification of 10; middle, a magnification of 40). On the right, flow cytometry shows that B cells in the periphery in normal animals express B220 and IgM or IgD, and that B cell precursors in mutants can be detected as they express only B220, but not IgM or IgD. (B) CXCR5 expression in the bone marrow and splenic B cell subsets. Bone marrow cells and splenocytes were isolated from Cxcr4<sup>f/f</sup>Cd19<sup>+/Cre</sup> and Cxcr4<sup>f/f</sup>Cd19<sup>+/Cre</sup> mice and stained with anti-CXCR5, anti-B220, and anti-IgM. B cell subsets of mature B220<sup>hi</sup>, immature B220<sup>lo</sup>IgM<sup>+</sup> B cells, and B cell precursors B220<sup>lo</sup>IgM<sup>−</sup> were gated. Histograms show the CXCR5 expression level of these subsets. Solid lines represent CXCR5 level on wild-type B cells and dashed lines denote that on the CXCR4-deficient B cells. The CXCR5 level on wild-type splenic B220<sup>hi</sup> cells, shown as the black solid line in both histograms, stands for a positive indicator. Background staining with nonspecific rat IgG on B220<sup>hi</sup> cells is shown as shaded histograms. (C) Splenocytes of Cxcr4<sup>f/f</sup>Cd19<sup>+/Cre</sup> mice were analyzed for their CXCL13 responsiveness by a transwell assay with an optimal concentration of 1 μg/ml CXCL13. Input and migrated cells were stained with PE anti-CD19, FITC anti-IgM, and APC anti-B220. The composition of input and migrated cell populations was determined by flow cytometry. The percentages of migration of splenic B cells at different developmental stages are shown. Data are from three experiments performed in duplicates.
the mature B cell compartment comprised of MZ B cells (CD21hi CD23lo) and follicular B cells (CD21lo CD23hi) was reduced to ~65% of that observed in the wild-type mice (Fig. 4A). The shrinkage of the mature B cell compartment in the Cxcr4f/fCd19cre spleen was not likely due to the dislodgment of MZ and follicular B cells by B cell precursors, because the number of IgDhi IgMlo mature B cells was not increased in the splenic red pulp or the peripheral blood. To exclude the possibility that the decrease in mature B cells was caused by enhanced cell death due to a competition between B cell precursors and mature B cells for the supportive niches in the spleen, we examined apoptosis in each B cell populations by annexin V staining. We detected a sizable pool of apoptotic cells (annexin V+) in the B220lo IgM+ population, but cell death of mature B cells was not increased compared with that of the corresponding B cell population in wild-type mice (Fig. 4B). Interestingly, the proportion of the CXCR4-deficient immature B cells (B220lo IgM-) undergoing apoptosis in the spleen was four times larger than that of the wild-type immature B cells in the bone marrow, reflecting that the spleen is a less accommodating environment for supporting cell death.

Figure 4. Altered B cell compartments in the peripheral lymphoid organs of Cxcr4f/fCd19cre mice. (A) Splenocytes were stained with antibodies against B220, CD21, and CD23. The expression of CD21 and CD23 in the B220 gated population is shown. Immature B cells (CD21+ CD23+), follicular B cells (CD21lo CD23hi), and MZ B cells (CD21hi CD23lo) are shown in polygons with the mean percentages of each gated population (n = 5). (B) Cell death of splenic B cell subsets was examined by FACS analysis. Bone marrow cells (BM) and splenocytes (Spl) from wild-type controls and Cxcr4f/fCd19cre mice were stained with anti-B220, anti-IgM, and annexin V. Apoptotic (Annexin V+) B cells are boxed and shown with mean percentages (n = 4). (C) Representative sections of small intestine show the structure of Peyer’s patches in control (n = 3) and mutant mice (n = 5). B cells were stained by anti-B220 and T cells were labeled with anti-CD3. Arrows indicate enlarged T cell area or ectopic B cell follicles in the mutant mice (a magnification of 10). (D) Peritoneal cells were isolated from 3-wk-old or 6-mo-old mice. Cells within the lymphocyte gate are displayed by staining with anti-B220, anti-IgM, anti-CD5, and anti-CD11b. B2 cells (B220hi CD5-), B1 cells (B220hi CD5+), and T cells (B220lo CD5hi) are shown in marked gates with percentages. (E) Absolute numbers of B2, B1a, and B1b cells in the peritoneal cavity of wild-type (closed bars, n = 5) and mutant mice (open bars, n = 7).
and GC formation in responses against NP with similar kinetics and magnitude when challenged with TD antigen NP-KLH, mounted redistribution of Peyer’s patches along the small intestine found in lymph nodes (not depicted). The number and distribution of Peyer’s patches along the small intestine appeared to be normal in Cxcr4f/fCd19+/Cre mice. Mature CXCR4-deficient B cells can home efficiently into the primary follicles in the Peyer’s patches. However, the B cell follicles became more dispersed. Among a total of 20 Peyer’s patches examined in 5 mutant mice, 18 had aberrant B cell follicles deep in the lamina propria of the Cxcr4f/fCd19+/Cre gut, of which 6 displayed enlarged T cell zones (Fig. 4 C).

A discernible change in the peritoneal B cell compartment was also observed in Cxcr4f/fCd19+/Cre mice. The peritoneal B cells can be divided into two main populations. Although the conventional B2 population (CD5− CD11b− B220+ IgM+ IgD+) in body cavities is replenished by mature recirculating B cells, the B1 cell pool (CD5+ CD11b+ B220− IgM− IgD−) develops mostly from fetal liver precursors and is maintained by constant self-renewal (34). In young Cxcr4f/fCd19+/Cre mice, there were normal numbers of B2 cells in the peritoneal cavity, whereas the B1 cell population was slightly reduced compared with that of the wild-type control (Fig. 4 D). In aged Cxcr4f/fCd19+/Cre mice, a marked reduction was observed not only in the B1 (2.4-fold), but also in the B2 peritoneal subset (threefold; Fig. 4 E).

Humoral Immunity in Cxcr4f/fCd19+/Cre Mice. In Cxcr4f/fCd19+/Cre mice, the basal levels of serum Ig of all isotypes were moderately decreased compared with wild-type controls (Fig. 5 A). To assess to what extent the loss of CXCR4 function in B cells would affect humoral responses, mice were immunized with NP coupled to the TI-2 antigen Ficoll or the T-dependent (TD) antigen KLH. In the response to NP-Ficoll, we observed a sevenfold decrease of ASCs in the bone marrow at this time point was accompanied by an increase of splenic ASCs, reflecting impaired bone marrow homing of ASCs as previously documented (Fig. 6 C).

Long-lived plasma cells have been shown to reside in the bone marrow, where it is thought that they receive survival signals from stromal cytokines and chemokines (35). These long-lived plasma cells contribute to >80% of the antibodies in immune serum and are critical for maintaining long-term humoral responses (7, 36, 37). Because CXCL12 signals from stromal cytokines and chemokines (35). These long-lived plasma cells contribute to >80% of the antibodies in immune serum and are critical for maintaining long-term humoral responses (7, 36, 37). Because CXCL12 appeared to be critical for recruiting plasma cells to the bone marrow, long-lived plasma cells and long-term humoral responses may not persist in the absence of CXCR4. Contrary to this expectation, we observed that long-term antibody production was not impaired in Cxcr4f/fCd19+/Cre mice (Fig. 6 A). It is possible that CXCR4-deficient plasma cells were affected (Fig. 6 B and not depicted). Analysis of NP-specific plasma cells at day 9 of the primary response revealed that the frequency of antibody-secreting cells (ASCs) was reduced in the Cxcr4f/fCd19+/Cre bone marrow. The decrease of ASCs in the Cxcr4f/fCd19+/Cre bone marrow at this time point was accompanied by an increase of splenic ASCs, reflecting impaired bone marrow homing of ASCs as previously documented (Fig. 6 C).

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CXCR4-deficient B cells are able to elicit normal TD responses. (A) Serum levels of NP-specific Ig isotypes of CXCR4 conditional mutant (○) and control (●) mice at the indicated time points after NP-KLH immunization was measured by ELISA. (B) Relative affinities of IgG1 of CXCR4 conditional mutant (○) and control (●) mice were determined at the indicated time point after antigen challenge. Each symbol represents the value obtained from one mouse. (C) Numbers of NP-specific ASCs in the spleen (Spl) and bone marrow (BM) isolated from control (solid bars) and CXCR4 conditional mutant mice (gray bars) 9 d after first immunization. (D) Frequencies of NP-specific ASCs in the spleen (Spl) and bone marrow (BM) from control (●) and CXCR4 conditional mutant (○) mice 90 d after second immunization, and from 8-mo-old nonimmunized mice (▲). (E) Analysis of CXCR4 expression in plasma cells. Bone marrow cells and splenocytes were isolated from wild-type and mutant mice at day 90 after secondary immunization. Plasma cells are identified as B220⁻CD138⁺ blast (B220/forward scatter, B220/FSC) with low surface lineage markers (CD3, CD11b, and CD61/FSC). Quadrants that separate CXCR4⁺ cells from CXCR4⁻ cells are set by the position of CXCR4-deficient B cells in the dot plots. The percentages of cells in the given quadrants are indicated. The level of CXCR4 expression on CD138⁺ cells from the spleen and bone marrow is shown in the histograms along with the isotype control (green shaded histograms).
cells that failed to reside in bone marrow could survive in other organs (38–40). On the other hand, long-lived plasma cells could home to bone marrow independently of CXCR4. To distinguish between these possibilities, we examined the ASC frequency 90 d after secondary immunization. ELISPOT analysis revealed a normal number of NP-specific ASCs in the spleen and lymph nodes (not depicted), and a slightly reduced number of NP-specific ASCs in the bone marrow of mutant mice (Fig. 6 D). To exclude the possibility that the few plasma cells in Cxcr4f/fCd19+/+ mice had escaped deletion of the Cxcr4 gene, we examined CXCR4 expression on these cells. Our flow cytometry data showed that the majority of plasma cells in Cxcr4f/fCd19+/+ mice had escaped deletion of the Cxcr4 gene, indicating that CXCR4 deletion was efficient and that plasma cells could survive without CXCR4 signaling (Fig. 6 E). Consistent with previous findings, we also detected a substantial number of CXCR4− plasma cells in the immunized wild-type mice (41). Interestingly, the proportion of CXCR4− plasma cells within the total plasma cell population was similar in both bone marrow and spleen, reflecting a lack of selection of CXCR4+ plasma cells by the bone marrow environment (Fig. 6 E). Together, these data indicate that long-lived plasma cells can populate the bone marrow independently of CXCR4.

Discussion

We have shown that in the absence of CXCR4, B cell precursors are dislocated from bone marrow to peripheral blood and the spleen. The finding of premature release of B cell precursors into the periphery is consistent with earlier observations of displacement of other hematopoietic precursors in the absence of either CXCR4 or its ligand, CXCL12. By using a system in which CXCR4 can be deleted as B cells undergo maturation in the bone marrow, we also were able to demonstrate that the displaced splenic immature B cells were localized in follicles, despite lacking expression of CXCR5 and responsiveness to CXCL13. In addition, we found in these mice aberrant B cell clusters in lamina propria adjacent to the B cell follicles of Peyer’s patches. We also observe substantial numbers of long-lived plasma cells that develop in the bone marrow even in the absence of CXCR4.

It has been shown that B cells enter the spleen independently of Gi protein signaling (42). However, their homing into the splenic B cell follicles requires CXCR5 (16). CXCR5 expression is absent in the bone marrow B cell precursors and becomes progressively elevated during B cell maturation in the periphery (13). Our data show that the CXCR4-defective splenic B cell precursors, which express low levels of CXCR5, correspondingly respond poorly to CXCL13. It is therefore surprising to find that CXCR4-deficient B cell precursors reside exclusively in the splenic B cell follicles. This suggests that other chemoattractants are involved in migration of these cells into splenic follicles. It is unclear if this represents an adaptive induction of an alternative migration program in the absence of CXCR4, or if immature B cells normally have this capacity, but are constrained from exercising it because of CXCR4-mediated retention in bone marrow.

A striking phenotype in mice lacking CXCR4 in B cells was the appearance of B cell clusters deep in the intestinal mucosa independent of Peyer’s patches. This phenotype establishes a novel role for CXCR4 in B cell compartmentalization. The dislocated B cell colonies in the small intestine of Cxcr4−/−Cd19+/+ mice are reminiscent of misplaced clusters of neurons and germ cells in Cxcr4−/− mice and zebrafish (27, 43–46). Like the CXCR4−deficient B cells described in this paper, the Cxcr4−/− neurons and germ cells retain their motile behavior. Some are able to arrive at the appropriate destination, but a number of these cells lose the migratory direction and form ectopic cell clusters at the wrong sites. These phenotypes together illustrate a conserved function of CXCR4 signaling in prohibiting alternative migratory routes.

The existence of alternative migratory routes suggests that the migrating cells express multiple chemokine receptors and are responding to different directional cues. Cells are guided to their target sites by a balanced force mediated by chemoattractants, chemorepellants, as well as retention factors. Although previous findings demonstrate that CXCR4 signaling can promote lymphocyte homing into Peyer’s patches, the fact that CXCR4-deficient B cells can form normal B cell follicles in this organ clearly indicates that CXCL12 is not the only chemoattractant (18 and our results). Other chemokines, including CXCL13 and CCL21, have been shown to function as dominant tropic factors for Peyer’s patches (18). It is unlikely that CXCL12 serves as a chemorepellent because CXCL12-expressing high endothelial venules in Peyer’s patches are found to be surrounded by B cells (18). Therefore, we propose that CXCL12-expressing cells in the developing Peyer’s patches define the territory of the B cell zone, and CXCR4 is responsible for confining B cells in this area during Peyer’s patches development.

The progression of B cell development is accompanied by changes in the microanatomic localization of the developing cells. The coordinated spatial changes of developing B cells are believed to influence the functionality and longevity of these cells. This notion is supported by our finding that misplaced CXCR4-deficient B cell precursors undergo extensive cell death and do not contribute efficiently to the mature B cell pool. These data indicate that the spleen is an inappropriate microenvironment to support the survival of B cell precursors and subsequent B cell maturation. Different survival factors counteract apoptosis of B cells at different developmental stages. The prominent survival factor for B cell precursors is IL-7 (47). Mature B cells up-regulate BAFF receptor and exhibit increasing dependence on BAFF to survive (48–50). These differences, along with differences in the expression levels of these cytokines in the spleen and bone marrow, may account for the death of misplaced B cell precursors in the spleen of mutant mice. It is also possible that the high level of
CXCL12 in the bone marrow sustains the survival or promotes proliferation of B cell precursors. CXCL12 was originally identified as a growth-promoting factor for B cell precursors, as it can stimulate pre–B cell proliferation in vitro (22). It has been shown that CXCL12 prevents apoptosis of peritoneal B cells in an in vitro system. Furthermore, administration of neutralizing antibody against CXCL12 significantly reduced both B1 and B2 populations in the peritoneal cavity, indicating that CXCL12 is involved in maintaining the peritoneal B cell compartment (51). Consistent with this finding, we demonstrated that the loss of both B1 and B2 cells in the peritoneal cavity increased as mutant mice aged. The reduction of B1 cells was already obvious when mutant mice were only 3 wk old, although the peritoneal B2 population was unaffected by the CXCR4 defect at this age. This result indicates that although CXCR4 is involved both in generating and maintaining the B1 pool, it is redundant in establishing the B2 compartment, but critical for maintaining it.

The biological consequence of changed B cell compartments in Cxcr4<sup>f/f</sup>/Cd19<sup>+/Cre</sup> mice has been examined by measuring TI and TD humoral responses. Consistent with the decrease in B1 and MZ B cell compartments in Cxcr4<sup>f/f</sup>/Cd19<sup>+/Cre</sup> mice, the antibody response against NP-Ficoll was reduced, confirming the importance of the B1 and MZ B cell compartments for efficient TI responses (52, 53). It was previously shown that accumulation of donor-derived Cxcr4<sup>−/−</sup> plasma cells in bone marrow of mice receiving fetal liver transplants was compromised shortly after antigen challenge (23). We also found a profound reduction in the number of bone marrow IgG ASCs after primary stimulation in Cxcr4<sup>f/f</sup>/Cd19<sup>+/Cre</sup> mice. By using conditional inactivation of CXCR4, we have been able to extend the analysis to examine long-lived responses that surprisingly turned out to be normal despite reduced numbers of splenic follicular B cells. It was noted earlier that some donor plasma cells were present in bone marrow even when mice were transplanted with pertussis toxin–treated B cells (9). Thus, over an extended period, long-lived plasma cells may eventually accumulate in the bone marrow without a stringent chemokine requirement.

B cells in Cxcr4<sup>f/f</sup>/Cd19<sup>+/Cre</sup> mice exhibit phenotypes similar to those found in the VCAM conditional knockout mice. Both mutant mouse strains showed impaired retention of B cells in the bone marrow, but normal numbers of IgG ASCs in old animals (54). An explanation for this intriguing phenotype could be that ASCs generated at different phases of immune responses use different mechanisms to home to bone marrow. As reported previously, ASCs in the bone marrow arise from at least two distinct populations. One contains plasma cells that have been fully differentiated in the spleen and lymph nodes within several days after immunization. The other is composed of plasma cell precursors that undergo further differentiation in the bone marrow 2–4 wk after antigen stimulation (55). We propose that the migration to the bone marrow of plasma cells that rapidly arise in the peripheral lymphoid organs at the early phase of responses is CXCR4 dependent, whereas plasma cell precursors home into bone marrow independently of CXCR4 and give rise to long-lived ASCs.

In summary, we have shown that B cell–specific inactivation of CXCR4 affects the structure of B cell compartments in the bone marrow, spleen, peritoneal cavity, and Peyers patches. This study underscores the central role of CXCR4 in confining migrating B cells to the proper target sites.

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