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CXCL13 Is Required for B1 Cell Homing, Natural Antibody Production, and Body Cavity Immunity

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Summary

B1 cells are a predominant cell type in body cavities and an important source of natural antibody. Here we report that in mice lacking the chemokine, CXCL13, B1 cells are deficient in peritoneal and pleural cavities but not in spleen. CXCL13 is produced by cells in the omentum and by peritoneal macrophages, and in adoptive transfers, B1 cells home to the omentum and the peritoneal cavity in a CXCL13-dependent manner. CXCL13$^{-/-}$ mice are deficient in preexisting phosphorylcholine (PC)-specific antibodies and in their ability to mount an anti-PC response to peritoneal streptococcal antigen. These findings provide insight into the mechanism of B1 cell homing and establish a critical role for B1 cell compartmentalization in the production of natural antibodies and for body cavity immunity.

Introduction

B1 cells are specialized B cells that are distinguished from the major recirculating B cell population (B2 cells) by their unique tissue distribution, distinct cell surface phenotype, capacity for self-renewal, and prominent role in natural antibody production (Hardy and Hayakawa, 2001; Kantor and Herzenberg, 1993; Martin and Kearney, 2001; Wortis and Berland, 2001). B1 cells express high levels of surface IgM and low levels of IgD, CD23, and B220, and peritoneal B1 cells express Mac-1. A major fraction of B1 cells express CD5 and are known as B1a cells, while the remaining CD5$^{lo/-}$ B1 cells are designated B1b. In wild-type mice, B1a cells develop predominantly from fetal hematopoietic stem cells, whereas B1b cells develop from both fetal and adult stem cells (Hardy and Hayakawa, 2001). The development of B1 cells is strongly influenced by antigen receptor specificity (Hardy and Hayakawa, 2001). Among the known B1-associated antigen receptors are several that react with autoantigens and others that bind conserved epitopes present on common pathogens (Hardy and Hayakawa, 2001). One well-characterized example is the rearrangement of the germline V_H1/V_K22 genes, which encode T15 idiotype-containing antibodies that bind phosphorylcholine (PC), a hapten present on the surface of many pathogenic bacteria (Benedict and Kearney, 1999; Masmoudi et al., 1990).

Although B1 cells constitute only a few percent of all B cells, they are a significant source of serum antibody, and they make a dominant contribution to low-affinity IgM antibodies that are present in serum of unimmunized mice, known as natural antibodies (Avrameas and Ternynck, 1995; Kantor and Herzenberg, 1993), including antibodies that bind PC (Masmoudi et al., 1990) and phosphatidylcholine (Hayakawa et al., 1984). Studies in mice deficient in natural antibodies have established their critical role in providing early protection from a variety of pathogens (Baumgarth et al., 2000; Boes et al., 1998; Ochsenbein et al., 1999; Pecquet et al., 1992). An especially prominent role has been demonstrated for PC binding antibodies in protection from bacterial infections (Benedict and Kearney, 1999; Briles et al., 1981).

Despite the importance of the B1 cell population, relatively little is known about how the cells enter or accumulate in the body cavities. Early studies indicated that the B cells that could be released from the peritoneum by peritoneal lavage might be related to B cells present in the omentum (reviewed in Williams and White, 1986). The omentum is a bilayered sheet of mesothelial cells connecting the spleen, pancreas, stomach, and transverse colon, terminating in an apron-like structure that contains numerous adipocytes (Williams and White, 1986). The omentum has long been appreciated for its role in abdominal wound repair and particle capture (Williams and White, 1986). Aggregates of macrophages and lymphocytes, and smaller numbers of mast cells and plasma cells, are present in both the thin mesothelial sheets and the fat pads of the omentum. These structures were first described by Ranvier in the 19th century when they were given the name “milky spots” (reviewed in Williams and White, 1986).

Recently, chemokines and chemokine receptors have been shown to play a critical role in lymphocyte homing to subcompartments of lymphoid organs. B cell homing to lymphoid follicles is directed by the chemokine CXCL13 (formerly BLC or BCA1), a chemokine made in a lymphotoxin (LT)-dependent manner by follicular stromal cells (Cyster et al., 2000; Gunn et al., 1998), and mice lacking CXCL13 or the CXCL13 receptor, CXCR5, fail to form lymphoid follicles (Ansel et al., 2000; Förster et al., 1996).

Here we demonstrate that CXCL13 is constitutively expressed by cells in the peritoneal and pleural cavities, including expression in macrophages and in the omentum. We find that mice lacking CXCL13 have a severe paucity of B cells in the peritoneal and pleural lavages and within the omentum. Using a short-term transfer approach, B1 cell homing to the omentum and peritoneal cavity is found to depend strongly on CXCL13. By parabiosing pairs of adult mice differing in their Ly5 alleles, we demonstrate that B1a and B1b cells circulate to the peritoneum in adult mice. Furthermore, CXCL13-directed accumulation of cells in the peritoneum appears to be important for promotion of natural antibody

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production and for local immunity, as CXCL13-deficient mice have markedly reduced PC-specific natural antibody levels and diminished responses to intraperitoneally injected bacterial antigens.

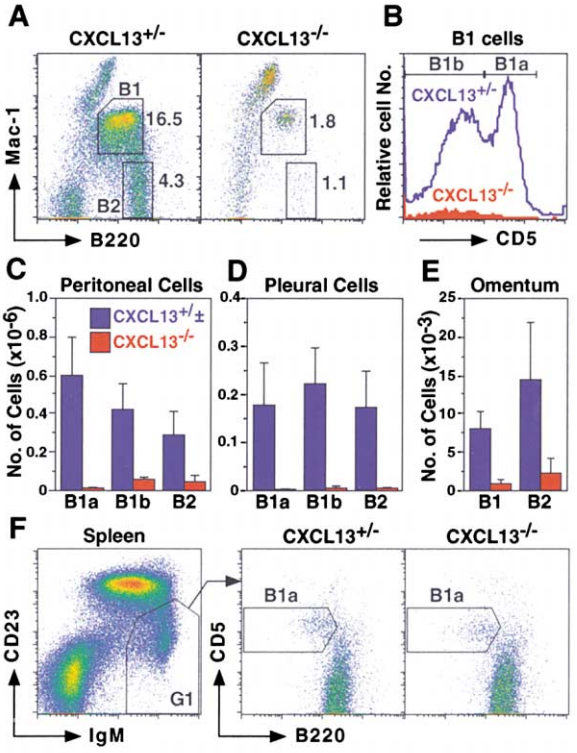
Results

Decreased Body Cavity B1 Cell Numbers in CXCL13$^{-/-}$ Mice

Flow cytometric analysis of peritoneal lavage cells (PerC) revealed a dramatic reduction in the frequency of both B1 and B2 cells in CXCL13$^{-/-}$ mice (Figure 1A). As expected, B1a cells predominated in the PerC of control mice, but of the few B1 cells detected in the PerC of CXCL13$^{-/-}$ mice, most were of the CD5$^{lo/-}$ B1b subset (Figure 1B). Enumeration of PerC revealed that CXCL13$^{-/-}$ mice had 50-fold fewer B1a, 8-fold fewer B1b, and 6-fold fewer B2 cells than heterozygous and wild-type controls (Figure 1C), while peritoneal macrophage numbers were unaffected (data not shown). B cell numbers were also markedly decreased in the pleural cavity of CXCL13$^{-/-}$ mice (Figure 1D). Analysis of the lymphocytes present in wild-type omentum revealed a similar population to that of the peritoneal and pleural cavities, including a prominent Mac1^{+} B1 cell population (Figure 1E). B1 cells constituted approximately one-third of the omental B cell population, and this tissue also exhibited a strong requirement for CXCL13 in B cell accumulation (Figure 1E). In contrast with these deficiencies, the number of B cells in spleen was not reduced in CXCL13$^{-/-}$ mice (Figures 1F and 1G). The approach used to identify B1 cells in the body cavities and the omentum was unsuccessful for the spleen due to the large excess of B2 cells and the reduced Mac1 expression of B1 cells in the spleen (Martin et al., 2001). However, we were able to enumerate splenic B1a cells by identifying cells with an IgM^{hi}CD23$^{lo/-}$B220^{lo}CD5^{+} surface phenotype (Figure 1F). In contrast to findings for the PerC, the number of splenic B1a cells in CXCL13$^{-/-}$ animals was not reduced compared to controls (Figure 1G). Interestingly, the frequency of B1a cells in the blood was increased (Figure 1G), suggesting that CXCL13 may influence B1 cell exit from the bloodstream.

Chemokine Responses of Peritoneal B Cells

In agreement with previous findings (Bowman et al., 2000; Cyster et al., 1999; Ishikawa et al., 2001), B1 cells express CXCR5, with B1a and B1b cells both expressing approximately 2-fold higher amounts than peritoneal B2 cells (Figure 2A). B1a and B1b cells showed robust chemotactic responses to CXCL13 in in vitro assays, although the total fraction of responding cells was less than for B2 cells (Figure 2B). B1 cells also responded to CXCL12, but less strongly than to CXCL13, and they showed only very weak chemotactic responses to CCL19 and CCL21 (Figure 2B). Calcium flux analysis showed a similar chemokine response profile, with B1 cells responding strongly to CXCL13 and weakly to CXCL12, CCL19, and CCL21 (Figure 2C). The calcium flux analysis also revealed that all B1 cells were responsive to CXCL13, consistent with the CXCR5 expression profile (Figures 2A and 2C). Therefore, the finding that fewer B1 cells than B2 cells migrate to CXCL13 may reflect



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Genotype	PerC B1a (x10^{-4})	Spleen B1a (x10^{-4})	Spleen B2 (x10^{-6})	Blood B1 (%)
CXCL13+/-	19±7	31±16	38±11	0.11±0.05
CXCL13-/-	0.2±0.2	36±20	43±15	0.40±0.17

Figure 1. Reduced B Cell Numbers in the Peritoneal and Pleural Cavities and the Omentum of CXCL13$^{-/-}$ Mice

(A) Flow cytometric analysis of Mac-1 and B220 expression by size-gated PerC. Boxes demarcate Mac-1^{+}B220^{lo} B1 and Mac-1^{-}B220^{hi} B2 subsets, and numbers represent their mean percentage of total PerC cells from 20 CXCL13$^{+/\pm}$ (wild-type or heterozygous) mice (left) and 20 CXCL13$^{-/-}$ littermates (right).

(B) CD5 expression on peritoneal B1 cells from CXCL13$^{+/-}$ (blue) and CXCL13$^{-/-}$ (red) mice. Brackets demarcate the CD5^{+} B1a and CD5^{-} B1b subsets.

(C–E) Mean number of B cell subsets in PerC (C), pleural lavage cells (D), and omentum (E) of CXCL13$^{+/\pm}$ (blue bars) and CXCL13$^{-/-}$ (red bars) mice. Error bars indicate 95% confidence intervals ($n \geq 24$ for PerC, $n \geq 7$ for pleural cells, $n \geq 8$ for omentum). Differences in numbers of B cells between CXCL13$^{+/\pm}$ and CXCL13$^{-/-}$ mice are statistically significant for all subsets in all tissues ($p < 0.005$). No differences in PerC B cell numbers were detected between wild-type and heterozygous mice.

(F) Four-color flow cytometric analysis to detect B1a cells in the spleen. Analysis of IgM and CD23 expression by size-gated splenocytes (left panel). IgM^{hi}CD23$^{lo/-}$ cells demarcated by the box labeled G1 (left panel) were analyzed for expression of B220 and CD5 (center and right panels). B220^{lo}CD5^{+} B1a cells (demarcated by boxes) are detected in both CXCL13$^{+/-}$ (center) and CXCL13$^{-/-}$ (right) mice.

(G) Four-color flow cytometric analysis as in (F) was used to identify IgM^{hi}CD23$^{lo/-}$B220^{lo}CD5^{+} B1a and IgM^{+}CD23^{+}B220^{+}CD5^{-} B2 cells in PerC, spleen, and blood of CXCL13$^{+/-}$ mice and their CXCL13$^{-/-}$ littermates. Numbers represent mean ± standard deviation for absolute cell numbers from eight mice each (PerC and spleen) or percentage of total cells from seven mice each (blood). The number of PerC B1a cells was significantly reduced and the number of blood B1a cells was significantly increased in CXCL13$^{-/-}$ compared to CXCL13$^{+/-}$ mice ($p < 0.005$; unpaired Student's t test).

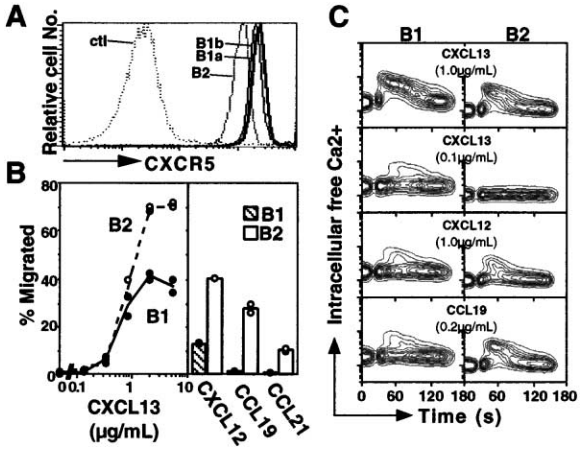

Figure 2. Heightened CXCR5 Expression and CXCL13 Sensitivity of B1 Cells

(A) CXCR5 expression on PerC B1a (bold line), B1b (solid line), and B2 (dashed line) cells. Background staining with nonspecific rabbit IgG on B1a, B1b, and B2 cells was equivalent, and B1a cells are shown as a representative control (ctl, dotted line).

(B) Chemotaxis of PerC B1 cells (solid line, hatched bars) and B2 cells (dashed line, open bars) in response to the indicated chemokines (CXCL12, 0.3 μg/ml; CCL19, 0.2 μg/ml; CCL21, 0.8 μg/ml). Data are representative of four experiments.

(C) Calcium flux of fluo-3-loaded $B220^{lo}Mac1^{+}$ B1 cells (left) and $B220^{hi}Mac1^{-}$ B2 cells (right) in response to the indicated chemokines added 20 s after the start of data collection. Chemokine concentrations are shown in parentheses. Splenic B2 cells responded equally to PerC B2 cells in chemotaxis and calcium flux assays (data not shown).

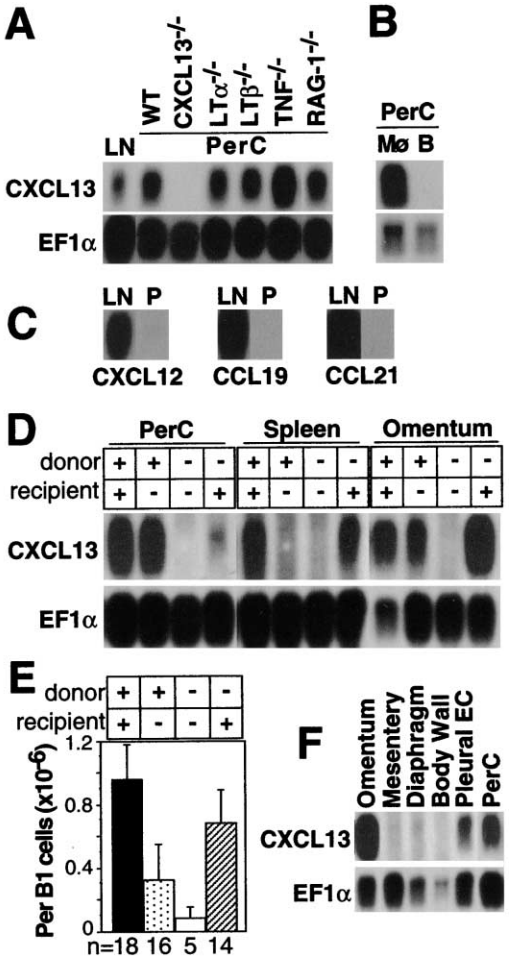

Figure 3. Two Sources of CXCL13 Contribute to Peritoneal B1 Cell Accumulation

(A) Northern blot analysis of total RNA from lymph node (LN) and peritoneal lavage cells (PerC) of wild-type mice and the indicated mutants probed to detect CXCL13 mRNA. Elongation-factor-1α (EF-1α) hybridization indicates amount of RNA in each lane. WT, wild-type; LT, lymphotoxin; TNF, tumor necrosis factor; and RAG, recombinase activating gene.

(B) Total RNA from FACS-sorted peritoneal macrophages (Mø) and B cells (B) was probed to detect CXCL13 and EF-1α mRNA.

(C) Northern blot of wild-type lymph node and PerC (P) RNA shown in (A), reprobed to detect expression of CXCL12/SDF-1, CCL19/ELC, and CCL21/SLC.

(D) Total RNA from PerC, spleen, and omentum of bone marrow chimeric mice was probed to detect CXCL13 and EF-1α mRNA. The CXCL13 genotypes of donor and recipient mice are indicated above each lane (+, $CXCL13^{+/\pm}$; −, $CXCL13^{-/-}$).

(E) Mean number of B1 cells in PerC of bone marrow chimeras counted and analyzed as in Figure 1. Genotypes of donor and recipient mice are indicated as in (D). Error bars, 95% confidence intervals. The number (n) of each type of chimera analyzed is indicated.

(F) Total RNA from the indicated tissues or cells of a wild-type mouse were probed to detect CXCL13 and EF-1α mRNA.

differing abilities of the cells to undergo chemotaxis in the in vitro assay rather than a lower ability of this population of cells to respond to the chemokine. In fact, the sensitivity of B1 cells to CXCL13 is greater than that of B2 cells. Analysis of the dose response curves revealed that B1 cells reached their maximal chemotactic response at lower CXCL13 concentrations than B2 cells (Figure 2B and Ishikawa et al., 2001) and that CXCL13 induces calcium flux in B1 cells at a low concentration that does not induce a response in B2 cells (Figure 2C).

Two Sources of CXCL13 within the Peritoneal Cavity

The deficiency of peritoneal and pleural cavity B cells in $CXCL13^{-/-}$ mice suggested that CXCL13 might function locally in the body cavities. Northern blot analysis revealed CXCL13 expression in total peritoneal and pleural lavage cells (Figure 3A) and in purified peritoneal macrophages (Figure 3B). In contrast to the requirements for CXCL13 expression in spleen (Ngo et al., 1999), CXCL13 expression by peritoneal macrophages was independent of B cells and of LTα, LTβ, and TNF (Figure 3A). Comparing CXCL13 levels in different organs of wild-type mice revealed a stronger signal in RNA prepared from PerC than from lymph nodes and spleen (Figures 3A and 3D). By contrast, CXCL12, CCL19, and CCL21 were expressed at very low to undetectable levels in PerC (Figure 3C).

To characterize the contribution of macrophage-derived CXCL13 to B cell accumulation in the peritoneum, lethally irradiated $CXCL13^{-/-}$ $Ly5^{b}$ mice were reconstituted with wild-type $Ly5^{a}$ bone marrow. Analysis of chimeric animals after 8 weeks of reconstitution showed that more than 95% of the macrophages in the peritoneal cavity were replaced by donor-derived $Ly5^{a+}$ cells (data not shown). Analysis of PerC from such bone marrow

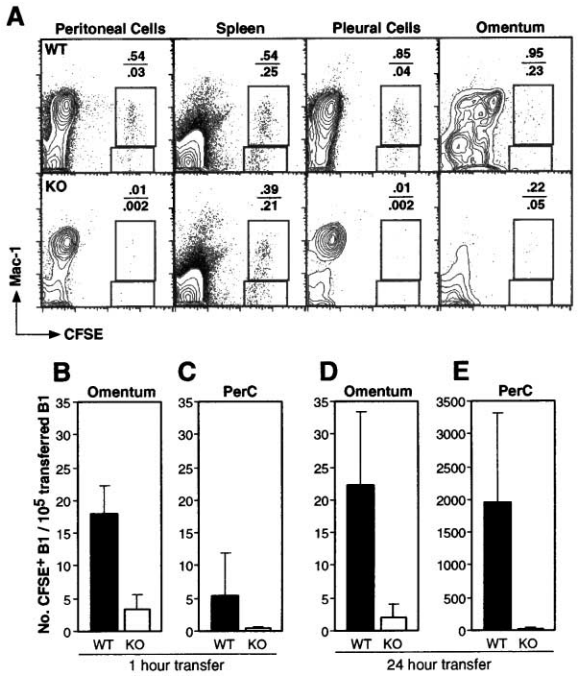

Figure 4. CXCL13-Dependent Selective Recruitment of Adoptively Transferred B1 Cells to the Peritoneal and Pleural Cavities and the Omentum

(A) Representative flow cytometric analyses of CFSE fluorescence and Mac-1 expression of IgM^+ size-gated cells from peritoneal lavage, spleen, pleural lavage, and omentum of wild-type (WT, upper panels) and $CXCL13^{-/-}$ (KO, lower panels) recipients of CFSE-labeled wild-type PerC, 24 hr after transfer. Boxes demarcate the positions of large Mac-1^+IgM^{hi} donor-derived B1 cells and smaller Mac-1^-IgM^+ donor-derived B2 cells, and numbers in each panel indicate the mean percentage of total cells that were donor B1 (upper) or donor B2 (lower) cells for at least three independent experiments for each tissue.

(B–E) Enumeration of donor B1 cells recovered from omentum (B and D) or PerC (C and E) of wild-type (filled bars) and $CXCL13^{-/-}$ (open bars) recipients one hour (B and C) or ~24 hr (D and E) after transfer. To normalize differences in the number of B1 cells injected in each transfer experiment, data are expressed as the number of CFSE-labeled B1 cells recovered in each recipient per 10^5 B1 cells intravenously transferred into that recipient. Bars and error bars represent the mean and 95% confidence intervals, respectively. Note the increased scale for PerC at 24 hr after transfer (E). Data are representative of at least three independent experiments per recipient tissue.

chimeras revealed significant restoration of CXCL13 expression (Figure 3D) and showed that hematopoietically derived CXCL13 was sufficient to promote B1 cell accumulation (Figure 3E). Unexpectedly, however, the reconstitution of B1 cells was limited to 30% of the number found in reconstituted wild-type animals (Figure 3E). These findings suggested that CXCL13 was also produced by radiation-resistant cells within the peritoneum. Consistent with this, analysis of RNA prepared from the omentum revealed strong CXCL13 expression (Figure 3F). CXCL13 mRNA was also sometimes detectable in the diaphragm, although at much lower levels, whereas little or no signal could be detected in RNA prepared from mesentery or peritoneal wall (Figure 3F and data not shown). Analysis of omentum RNA prepared from bone marrow chimeric mice indicated that a fraction of omental CXCL13 emanates from bone marrow-derived cells, most likely omentum-associated macrophages (Figure 3D). However, when wild-type mice were irradiated and reconstituted with $CXCL13^{-/-}$ bone marrow to eliminate hematopoietic sources of CXCL13, there was a marked loss of CXCL13 expression in the PerC, but little effect on expression by the omentum (Figure 3D). Therefore, a substantial fraction of omental CXCL13 is expressed by radiation-resistant cells. Consistent with an important role for CXCL13 produced by radiation-resistant cells, B1 cells accumulated in animals reconstituted with $CXCL13^{-/-}$ bone marrow to 75% of the number found in mice reconstituted with wild-type bone marrow (Figure 3E).

B1 Cells Home to the Omentum and Peritoneal Cavity in a CXCL13-Dependent Manner

Numerous studies have shown that intravenous transfer of fetal liver, bone marrow, or peritoneal cells to irradiated recipients allows reconstitution of the peritoneal B1 cell compartment over a period of weeks (Figure 3E and Hardy and Hayakawa, 2001; Kantor and Herzenberg, 1993). However, it has not been reported whether mature B1 cells are able to home to the peritoneum. To visualize B1 cell trafficking, we established a short-term adoptive transfer system using CFSE-labeled PerC (Figure 4). By this approach, we found that a notable fraction of transferred B1 cells home to the peritoneal compartment within 24 hr of transfer (Figures 4A and 4E). Peritoneal B2 cells home to spleen but were found at a very low frequency in the PerC (Figure 4A and data not shown). In recipients that lack CXCL13, both B1 and B2 cells failed to migrate to the peritoneum, while the spleen contained both populations at frequencies similar to wild-type controls (Figure 4A). The frequency of transferred B1 and B2 cells recovered from the pleural cavity was similar to that of the PerC, and again homing was defective in $CXCL13^{-/-}$ recipients (Figure 4A). Thus, mature B1 cells are selectively recruited to body cavities in a CXCL13-dependent manner.

Transferred B1 cells were also enriched in the omentum, though donor B2 cells were detected at appreciable numbers in this tissue as well (Figure 4A). As observed for the PerC, B cell homing to the omentum was CXCL13 dependent (Figure 4A). To test whether the omentum may function as a portal of B1 cell entry to the peritoneum, we repeated the intravenous transfer of PerC cells and analyzed recipient mice 1 hr after transfer, a time point when transferred cells are likely to be concentrated at points of compartmental entry. Consistent with a role for the omentum in B1 cell recruitment to the peritoneal cavity, transferred B1 cells were readily detected 1 hr after transfer in the omentum of wild-type, but not $CXCL13^{-/-}$ animals (Figure 4B). The proportion of B1 cells reaching the peritoneal cavity (assessed by recovery in the PerC) at this early time point was minimal, and in some cases undetectable (Figure 4C). Strikingly, while B1 cell homing to the omentum increased little between 1 and 24 hr (Figure 4D), the proportion of transferred B1 cells found in the PerC increased more than

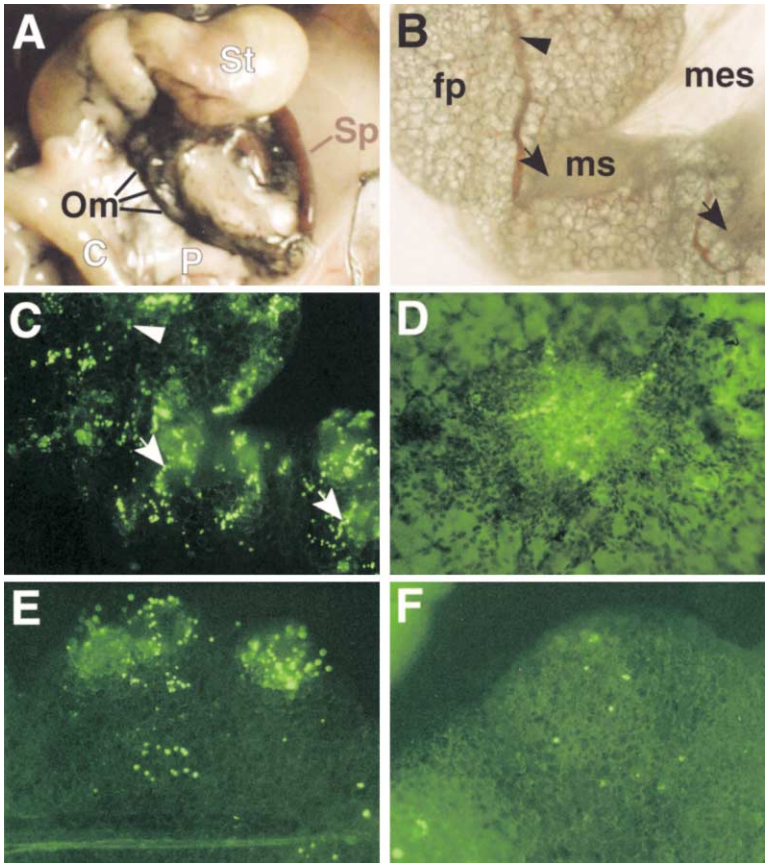

Figure 5. B Cell Homing within the Omentum

(A) A photograph of the mouse omentum (Om) in situ 4 hr after intraperitoneal injection of carbon particles (India ink). The efficient uptake of particulate antigens by the omentum is illustrated by the concentration of black staining in the omental fat pads. The transparent mesothelial sheet of the omentum connects these fat pads to the spleen (Sp), stomach (St), pancreas (P), and colon (C).

(B–E) Whole-mount microscopic images of the omentum of recipients of CFSE-labeled (green fluorescent) peritoneal lavage cells. (B) Light transmission and (C) fluorescent images of an omental fat pad (fp) and a small portion of the mesothelial sheet (mes) of a wild-type recipient 1 hr after transfer. Arrows indicate transferred cells in vessels associated with milky spots (ms). Arrowheads indicate infrequent cells in large vessels of the omentum. Arrows and arrowheads are located in identical positions in (B) and (C) for alignment of the images. (D) Higher magnification of a milky spot with vessel-associated transferred cells 1 hr after transfer. The omentum was stained with hematoxylin to highlight nuclei (black). (E) The omentum of a wild-type recipient 24 hr after cell transfer. (F) The omentum of a CXCL13$^{-/-}$ recipient 24 hr after cell transfer. Data are representative of at least three recipients at each time point.

300-fold (Figure 4E). These findings suggest that B1 cells home from the bloodstream to the omentum and then pass from the omentum into the peritoneal cavity.

B1 Cell Homing within the Omentum

To examine the pathway of B1 cell homing into the omentum (Figure 5A), we performed whole-mount light and fluorescence microscopy on tissue isolated from recipients of CFSE-labeled PerC. One hour after transfer, CFSE^{+} cells were found proximal to or within vessels located in omental milky spots (Figures 5B and 5C). Although we were unable to stain the transferred cells for B cell markers in situ, analysis of mechanically dispersed omental cells showed that more than 80% of the CFSE^{+} cells were B cells (Figure 4 and data not shown). CFSE^{+} cells were highly enriched in the omentum compared to the adjacent pancreas (Figure 5D). Twenty-four hours after transfer, CFSE-labeled cells were observed to have exited vessels, but remained clustered within milky spots (Figure 5E). In CXCL13$^{-/-}$ recipients, transferred cells were found in decreased numbers within the omentum, mainly associated with vessels scattered throughout the tissue (data not shown). Even after 24 hr in CXCL13$^{-/-}$ mice, the cells failed to move out to the periphery of the omentum or to form clusters, instead remaining scattered in a distribution similar to that seen at 1 hr after transfer (Figure 5F). Fewer preexisting milky spots were evident in CXCL13$^{-/-}$ omentum, although whether this reflected a lower number of total aggregates or the near absence of lymphocytes is presently unclear.

Parabiosis Reveals B1 Cell Homing to Body Cavities in Adult Mice

To examine the extent of peritoneal B1 cell recirculation in adult animals, pairs of wild-type C57BL/6 (Ly5^{b}) and congenic C57BL/6 Ly5^{a} mice were surgically joined in parabiosis. In previous studies, parabiosed mice have been shown to undergo complete blood exchange approximately ten times per day (Harris et al., 1997). Analysis 8 weeks later revealed that the proportion of Ly5^{a+} B and T cells in lymph nodes was equal in each partner, indicating that these recirculating lymphocytes had reached an equilibrium distribution in the parabiosed mice (Figure 6A), consistent with published results (Suzuki et al., 1998). By contrast, more than 90% of the peritoneal macrophages in each partner remained of host origin (Figure 6B), indicating that peritoneal macrophages do not recirculate frequently and are not rapidly replaced from cells outside of the peritoneum. Compared to the macrophages, peritoneal B1 cells had undergone a significant degree of mixing between partners, although not to the extent observed for peritoneal B2 cells or T cells (Figure 6B). Similar results were obtained for pleural lavage cells (data not shown). Importantly, a similar degree of mixing was observed for the B1a and B1b subsets (Figure 6B). As B1a cells are predominantly generated during fetal and neonatal life and

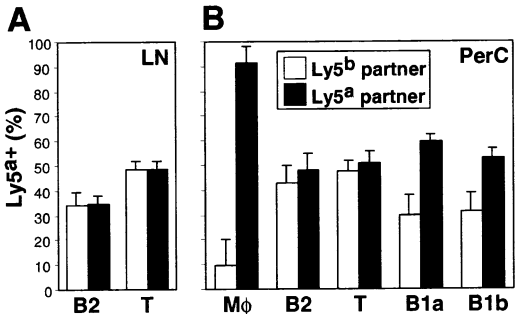

Figure 6. Mixing of B1 Cells between Parabiotic Mice with Shared Blood Circulation

Five parabiotic pairs, each consisting of one C57BL/6 Ly5^b-expressing mouse and one congenic C57BL/6 Ly5^a-expressing mouse, were analyzed 8 weeks after surgical anastomosis. For each cell type, the mean percentage of Ly5^{a+} cells in the Ly5^b partner (open bars) and in the Ly5^a partner (filled bars) is shown. Error bars represent 95% confidence intervals. Data are representative of two independent experiments.

(A) Equilibrium distribution of Ly5^{a+} lymph node (LN) B2 and T cells in parabiotic mice.

(B) Distribution of Ly5^{a+} macrophages (Mø), B2, T, B1a, and B1b cells in peritoneal lavage cells (PerC) of parabiotic mice.

are not efficiently generated by adult bone marrow (Hardy and Hayakawa, 2001; Kantor and Herzenberg, 1993), this finding supports the interpretation that B1 cells are able to leave and reenter the peritoneal and pleural cavities. While the degree of mixing was not complete, indicating that B1 cells are not freely recirculating, the findings establish that B1 cell homing to the peritoneal cavity occurs in adult animals.

Defective Natural Antibody Production and Body Cavity Immunity in CXCL13$^{-/-}$ Mice

To examine the impact of peritoneal and pleural B1 cell deficiency on natural antibody production, we measured levels of PC binding antibody in serum of unimmunized CXCL13$^{-/-}$ mice. As antibodies containing the T15 idiotype bind PC and are produced exclusively by B1 cells (Masmoudi et al., 1990), we also measured T15-containing antibodies. Unimmunized CXCL13$^{-/-}$ mice had detectable but significantly reduced levels of PC binding antibodies (Figure 7A) and T15-containing antibodies (Figure 7B) despite normal total IgM levels (Figure 7C). CXCL13$^{-/-}$ mice also had normal serum concentrations of IgG and IgA (Figure 7D). These data establish a critical role for CXCL13 in natural antibody production. To further test B1 cell function in the absence of CXCL13, mice were immunized with the R36A streptococcal vaccine. In wild-type C57BL/6 mice, intravenous injection of the R36A vaccine induces a T-independent (TI) antibody response against PC. This response originates in the spleen and is mediated by both T15-expressing B1 cells and a smaller number of marginal zone B cells (Martin et al., 2001). In contrast, only B1 cells respond to a low dose of intraperitoneally administered vaccine, presumably due to efficient antigen capture within the peritoneum. Consistent with a defect in peritoneal B1 cell function, CXCL13$^{-/-}$ mice had a 5-fold reduction in PC-specific and a 10-fold reduction in T15-containing anti-

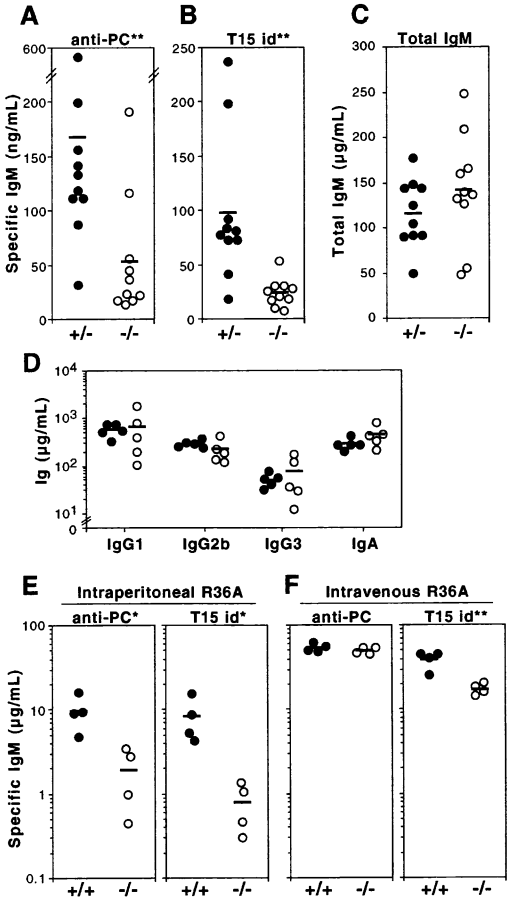

Figure 7. Defective Natural Antibody Production and Body Cavity Immunity in CXCL13$^{-/-}$ Mice

ELISA measurements of serum immunoglobulin (Ig) levels in CXCL13$^{+/\pm}$ (filled circles) and CXCL13$^{-/-}$ (open circles) mice. Lines indicate the mean value for each data set. Statistically significant differences between CXCL13$^{+/\pm}$ and CXCL13$^{-/-}$ antibody levels are indicated by asterisks (*, $p < 0.05$; **, $p < 0.005$; Student's t test). Note the change in units between parts (A) and (B) (ng/ml) and parts (C)–(F) (μg/ml).

(A–D) Sera from unimmunized mice were analyzed for phosphorylcholine-specific IgM (anti-PC) (A), T15 idiotype-containing IgM (T15 id) (B), and total IgM (C) in parallel. Total Ig of the indicated isotypes was measured in separate experiments (D).

(E and F) Sera from mice immunized 5 days previously by intraperitoneal (E) or intravenous (F) injection of R36A streptococcal vaccine were analyzed for PC-specific IgM (left panels) and T15-containing IgM (right panels).

body responses to intraperitoneal injection of the R36A vaccine (Figure 7E). However, CXCL13$^{-/-}$ mice were able to mount a relatively normal response to intravenously injected vaccine, indicating that B1 cells are functional in the spleen of CXCL13$^{-/-}$ mice (Figure 7F and data not shown). Therefore, effective anti-PC responses against intraperitoneally introduced bacterial antigen depends on CXCL13-mediated B1 cell accumulation in the peritoneum.

Discussion

Chemokines have emerged as key regulators of basal lymphocyte trafficking, directing different cell types to

microenvironments that maximize their immune surveillance function. Here we have characterized the mechanism of B1 cell homing and accumulation in body cavities and revealed a critical role for CXCL13 in these processes. Our studies indicate that B1 cells can migrate from blood vessels into lymphoid aggregates within the omentum. We propose that B1 cells then move from the omentum into the peritoneal cavity, and we suggest that B1 cells home to pleural and pericardial cavities by similar pathways. Our findings are also consistent with CXCL13 functioning to help retain the self-replenishing B1 cell population within the body cavities. In addition to a localization defect, CXCL13-deficient mice have defective B1 cell function, with reduced natural antibody levels and reduced production of antibody in response to intraperitoneal immunization. Our observations support the conclusions that B1 compartmentalization in body cavities serves to promote natural antibody production and to ensure the rapid antibody responses needed for protection from low-grade but potentially life-threatening infections of the body cavities.

The observation that macrophages can act as a physiologically important source of CXCL13 in the peritoneum raises the possibility that macrophages and related cells, such as DCs, contribute to CXCL13 expression and B cell recruitment at sites of inflammation. Several reports have established that CXCL13 can become upregulated at sites of chronic inflammation, and in one case, the cells expressing CXCL13 were shown to include DCs (Ishikawa et al., 2001). Reciprocally, our studies strengthen the previous evidence (Cyster et al., 2000; Gunn et al., 1998) that stromal cells, rather than hematopoietic cells, are the predominant source of CXCL13 in secondary lymphoid tissues, as CXCL13 expression was not restored in CXCL13$^{-/-}$ spleen or mesenteric lymph nodes after reconstitution with wild-type bone marrow (see Figure 3D). The nature of the radiation-resistant cell type(s) in the peritoneum that produces CXCL13 has been difficult to define as we have so far been unable to detect CXCL13 in peritoneal tissues by immunohistochemistry (data not shown). Mesothelial cells appear a likely source as they constitutively express CXCL12/SDF1 and they upregulate several chemokines, including CCL8/IL-8 and CCL2/MCP1, during peritoneal inflammation (Foussat et al., 2001; Topley et al., 1996).

Early microscopy studies of the omentum of many species, including humans, identified the universal presence of lymphoid aggregates or milky spots (Williams and White, 1986). Our findings indicate that B1 cells can enter the omentum directly from the blood, entering most efficiently across vessels within the milky spots. Electron microscopic analysis provided evidence that capillaries present in the lymphoid aggregates are points of cellular traffic (Hodel, 1970), and monocytes and neutrophils have been suggested to traverse these vessels (Beelen et al., 1980; Doherty et al., 1995). The delayed accumulation of transferred B1 cells in the peritoneal lavage compared to their appearance in the omentum (see Figure 4) favors the notion that B1 cells enter the peritoneal cavity from the omentum. During inflammation, macrophages and neutrophils may also follow this route into the peritoneal cavity (Doherty et al., 1995). Microscopy studies have shown that cellular passage between milky spots and the surface of the omentum occurs via fenestrations in the mesothelium overlying the aggregates (Williams and White, 1986). Taking all the findings together, we conclude that B1 cells use the omentum as a portal of entry to the peritoneal cavity. Milky spots have also been observed in the mediastinal pleura and the pericardium (Doherty et al., 1995; Nakatani et al., 1988; Williams and White, 1986). As we found that transferred B1 cells appear in the pleural cavity with similar kinetics to their appearance in the peritoneal cavity, all the findings are consistent with the possibility that mesothelial surfaces of the mediastinal pleura, the pericardium, as well as the omentum, provide passage of B1 cells into the respective body cavities.

Homing to body cavities was strikingly selective for B1 versus B2 cells (see Figure 4). The greater sensitivity of B1 cells to CXCL13 rather than B2 cells, together with their reduced responsiveness to CCL19 and CCL21 (see Figure 2), may contribute to their selective tropism for body cavities. However, analysis of B cells recruited to a site of ectopic CXCL13 expression did not show evidence for selective recruitment of B1 cells (Luther et al., 2000 and our unpublished data), indicating that additional mechanisms must contribute to homing specificity. These may include differential selectin- or integrin-mediated adhesion. Our experiments are also consistent with a critical role for CXCL13 in helping retain B1 cells within the body cavities. The importance of chemokine-mediated cell retention has been established by the finding that CXCL12 is critical for precursor cell retention in the bone marrow (reviewed in Ansel and Cyster, 2001). Interestingly, treatment with neutralizing antibodies to CXCL12 was recently found to reduce B cell numbers in the peritoneum by 2-fold (Foussat et al., 2001). However, this effect was suggested to occur at the level of B1 cell survival rather than homing. It will be interesting to determine whether the few remaining B1 cells that accumulate in the peritoneal cavity of CXCL13$^{-/-}$ mice do so in response to CXCL12.

From studies showing development of B1 cells in in vitro fetal liver and bone marrow cultures (Chumley et al., 2000; Hardy and Hayakawa, 1991) and, under some conditions, from B2 cells (Qian et al., 2001; Wortis and Berland, 2001), it appears likely that B1 cells can acquire many of their phenotypic characteristics outside the body cavities and then home to the cavities in a CXCL13-dependent manner. The ability of B1 cells to localize in the peritoneal cavity of partner mice during parabiosis confirms that B1 cells migrate to the peritoneum in adult animals. Furthermore, the similar mixing of B1a and B1b cells between parabiotic partners, despite the lower production of B1a cells by adult bone marrow (Hardy and Hayakawa, 2001), suggests that cells are exiting and reentering the peritoneum in a pattern of recirculation. A well-developed network of lymphatics exists within the peritoneum, including vessels in the omentum and in the diaphragm (Williams and White, 1986), providing a possible exit route for B1 cells. In addition to entry from other sites, some B1 cells may develop within the peritoneum itself as the fetal omentum contains transferable B1 cell precursors (Solvason et al., 1991). Furthermore, once an initial population is established, B1 cells can maintain themselves by self-replenishment. The purpose of B1 cell circulation is not yet clear, but the

recent demonstration that B1 cells work together with marginal zone cells in T-independent responses to systemic bacteria (Martin et al., 2001) suggests that recirculation allows the unique B1 repertoire to be available for responses against infections not involving the body cavities.

B1 cells are well characterized as a source of natural antibodies that are important for defense against infections by bacteria (Boes et al., 1998; Briles et al., 1981; Pecquet et al., 1992) and viruses (Baumgarth et al., 2000; Ochsenbein et al., 1999). PC-specific natural antibody levels were substantially reduced in CXCL13$^{-/-}$ mice, despite normal B1 cell numbers and function in the spleen (Figures 1G and 7E). Thus, B1 cell homing to body cavities appears to be critical for their role in innate immunity, and factors peculiar to the body cavity microenvironments are presumably necessary for stimulating natural antibody production. These factors may be represented by a specialized local cytokine milieu (Hardy and Hayakawa, 2001) and low-level exposure to commensal bacterial flora. Consistent with the latter possibility, germ-free BALB/c mice have markedly reduced levels of serum anti-PC antibodies compared to conventionally reared animals (Gearhart et al., 1975; Lieberman et al., 1974). Antibodies against human blood group antigens and related carbohydrates found in lower mammals, but not humans, are responsible for acute responses against mismatched transfusions and xenotransplanted organs. These clinically important natural antibodies are also believed to be derived from peritoneal B1 cells that are stimulated by commensal bacteria (Ohdan et al., 2000). Peritoneal localization may also function to sequester B1 cells away from circulating autoantigens that might otherwise lead to their deletion (Okamoto et al., 1992). Although our studies support the view that B1 cells recirculate via the bloodstream, this occurs at a lower rate than for B2 cells, and it seems possible that only a fraction of B1 cells undergo recirculation. This may therefore permit B cells that recognize circulating autoantigens to persist in the peritoneal compartment for longer periods than is the case for cells recirculating between secondary lymphoid organs. Strategies to inhibit localization of B cells within body cavities may therefore be clinically relevant in both transplantation and autoimmunity.

Finally, the unique compartmentalization of B1 cells places them in a position to intercept infectious agents that enter the body cavities. Intraperitoneally or intrapleurally injected antigens are rapidly captured within the omentum or mediastinal pleura, respectively, and these tissues likely contribute to the efficiency of body cavity immunity by concentrating antigens in sites of B1 cell traffic. Intraperitoneally injected streptococcal vaccine may mimic intestinal injury or leakage that would result in the release of bacteria into the peritoneal cavity, and the defective antibody response of CXCL13$^{-/-}$ mice demonstrates the importance of B1 cell homing for effective body cavity immunity. It is interesting to speculate that the earliest function of CXCL13 may have been to recruit primitive B lymphocytes to body cavities for T-independent responses, prior to its involvement in the more complexly organized secondary lymphoid tissues that support T-dependent antibody responses.

Experimental Procedures

Mice and Parabiosis

Wild-type, RAG-1$^{-/-}$, TNF$^{-/-}$, and LT$\beta^{-/-}$ mice were from a C57BL/6 (B6) colony. Additional wild-type B6, B6-CD45.1 (Ly5a^{+}), and LT$\alpha^{-/-}$ mice on a mixed B6×129 background were purchased from Jackson Laboratories (Bar Harbor, ME). CXCL13$^{-/-}$ mice were generated as described (Ansel et al., 2000) and maintained on a B6×129 strain background, or backcrossed ten times to B6. Compared to B6×129 mice, B6 mice have increased numbers of PerC B2 cells, but no strain differences were observed for the CXCL13 dependence of B1 and B2 cell homing, accumulation in body cavities, or antibody production. IgH$^{b/b}$ mice were used for all serum antibody measurements. Bone marrow chimeric mice were generated as described (Hargreaves et al., 2001). The donor origin of lymphocytes in the chimeric mice was confirmed using mismatched allelic markers (Ly5a/b or Ly9.1). Consistent with previous reports, bone marrow transplantation reconstituted peritoneal B1b cells, but few B1a cells (Kantor and Herzenberg, 1993). CXCL13$^{-/-}$ fetal liver cells reconstituted B1a and B1b cells equally in wild-type recipients (data not shown). All mice were maintained in specific pathogen-free conditions at the University of California, San Francisco, except parabiotic mice, which were housed at the University of Georgia (Athens, GA). Six-week-old male B6 and B6-Ly5^{a} mice were matched for body weight and surgically joined by parabiosis as described (Harris et al., 1997). Blood exchange was confirmed for all pairs 2 weeks postsurgery by injection of Evan's blue dye as described (Harris et al., 1997).

Cell Preparation and Flow Cytometry

Peritoneal lavage cells were isolated by flushing the peritoneal cavity with 4–6 ml of PBS supplemented with 0.5% w/v bovine serum albumin (BSA). For pleural lavage cells, 1–2 ml of PBS/BSA was injected through the diaphragm. Cell suspensions were prepared from mouse omentum, spleen, and lymph nodes by gentle mechanical disruption and passed through a 70 μm nylon mesh. Care was taken to avoid spleen contamination in omental preparations and vice versa. Blood was collected from the inferior vena cava, and red blood cells were lysed. Cells were resuspended in media (RPMI, 10 mM HEPES, 50 IU/ml penicillin, and 50 μg/ml streptomycin) with 2% fetal calf serum (FCS) and counted using a haemocytometer (PerC, pleural cells, omentum) or Coulter counter (spleen, lymph node, and blood), and surface stained for flow cytometry using mAbs to CD4, F4/80, B220, Mac1 (Caltag Laboratories, Burlingame, CA); IgM (Jackson Immunoresearch Inc, West Grove, PA); CD5, CD23, IgD, Ly5^{a} (clone A20), CD8, rabbit IgG (BD Pharmingen, San Diego, CA); and polyclonal rabbit anti-mouse CXCR5. Stained cells were analyzed on a FACSCalibur (Becton Dickinson, Mountain View, CA), and files were plotted using FlowJo software (TreeStar Inc., San Carlos, CA). For cell sorting, PerC were stained with IgM-FITC, IgD-FITC, F4/80-PE, and Mac1-biotin/SA-APC, and Mac1^{+}F4/80^{+}IgM/D^{-} macrophages and IgM/D^{+}F4/80^{-} B cells were sorted to >90% purity using a MoFlo fluorescence-activated cell sorter (Cytomation, Fort Collins, CO).

Chemotaxis and Calcium Flux Assays

Chemotaxis assays with PerC were performed as described for spleen cells (Hargreaves et al., 2001). To determine what subsets of cells transmigrated, a fraction of the cells in the lower chamber were stained with anti-CD5-bio/SA-APC, anti-B220-TC, and anti-Mac1-PE and analyzed on the FACSCalibur. To detect calcium signaling, PerC were loaded with 1 μg/ml fluo-3 (Molecular Probes, Eugene, OR) during 30 min at 37°C in media/5% FCS. After the first 20 min, anti-B220-APC and anti-Mac1-PE were added. Cells were washed twice and resuspended in media/5% FCS at ~5×10^{6} cells/ml and analyzed on a FACSCalibur. CXCL13 and CCL19 (R&D Systems, Minneapolis, MN), and CXCL12 (Peprotech, Rocky Hill, NJ) were purchased. HIS_6-tagged CCL21 was produced and purified as described (Hargreaves et al., 2001).

Northern Blot Analysis

To produce intact RNA from the omentum, the omental fat pads and mesothelial sheet were carefully dissected, discarding all pancreatic

tissue. PerC, pleural cells, and sorted PerC populations were pelleted by centrifugation (6 min, 300 × g). Total RNA was prepared and Northern blot analysis performed as described using a CXCL13 exon 2 probe (Ansel et al., 2000; Ngo et al., 1999).

Adoptive Transfers and Whole-Mount Microscopy

Donor PerC were fluorescently labeled by incubating cells for 10–15 min at 37°C in media/2%FCS and 0.2 μM (for flow cytometry studies) or 10 μM (for microscopy studies) carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) and washed with media/2%FCS. Donor cells were analyzed by flow cytometry to calculate the percentage of B1 cells, and 0.5–3 × 10^7 total PerC were injected into each recipient via the lateral tail vein. For whole-mount microscopy, omenta were dissected from recipient mice, placed on a slide, and covered with PBS for viewing. In some experiments, the omentum was soaked for 5–10 min in hematoxylin to stain nuclei and washed with PBS. No gross differences were observed in the size or structure of omenta from CXCL13$^{-/-}$ mice and controls.

Immunizations and ELISAs

For responses against streptococcal antigen, 12- to 15-week-old male mice were immunized i.v. or i.p. with 10^7 heat-killed pepsin-treated *S. pneumoniae* strain R36A (Martin et al., 2001) (gift of John Kearney, University of Alabama, Birmingham), and serum was prepared from blood collected from the tail vein 5 days later. For natural antibody measurement, serum was prepared from unimmunized mice. For PC-specific and T15-containing antibody detection, microtiter plates were coated overnight at 4°C with PC-BSA (5 μg/ml) (Biosearch Technologies Inc., Novato, CA) or the anti-T15 idiotype monoclonal antibody AB1-2 (1 μg/ml) (gift of J. Kearney) diluted in carbonate buffer (pH 9.6). Washing was with PBST (0.01% Tween-20), blocking was with PBST/5% BSA, and serum and antibodies were diluted in PBST/1% BSA. Serum dilutions were incubated in the coated wells for 1–2 hr, and bound antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL) and development with ABTS (Southern). The PC-specific T15-containing monoclonal IgM antibody, BH8 (a gift of J. Kearney), was used as a standard for quantitation. For unimmunized mice, high concentrations of serum (1:5, 1:25, 1:125, 1:625 dilution) were used. RAG-1$^{-/-}$ serum diluted 1:5 produced no signal above buffer-only controls. For detection of total serum Igs, plates were coated with goat anti-mouse Ig(M+G+A) (10μg/ml) (Southern), and bound antibodies were detected with HRP-conjugated purified antisera for IgM, IgG1, IgG2b, IgG3, or IgA (Southern). Purified mouse Igs (Southern) were used as standards for quantitation. Standard curves and serum antibody concentrations were calculated using Excel (Microsoft Corporation, Redmond, WA), and statistical analyses were done with Statview software (v5.0; SAS Institute Inc., Cary, NC).

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