Published May 5, 2003

Brief Definitive Report

Cooperating Mechanisms of CXCR5 and CCR7 in Development and Organization of Secondary Lymphoid Organs

Lars Ohl,[1] Golo Henning,[1] Stefan Krautwald,[2] Martin Lipp,[3] Svenja Hardtke,[1] Günter Bernhardt,[1] Oliver Pabst,[1] and Reinhold Förster[1]

[1]*Institute of Immunology, Hannover Medical School, 30625 Hannover, Germany*
[2]*Department of Nephrology, University of Kiel, 24105 Kiel, Germany*
[3]*Max-Delbruck Center for Molecular Medicine, 13092 Berlin, Germany*

Abstract

Homeostatic chemokines participate in the development of secondary lymphoid organs and later on in the functional organization of these tissues. The development of lymph nodes (LNs) and Peyer's patches depends on the recruitment of $CD3^-$ $CD4^+$ interleukin (IL)-$7R\alpha^{hi}$ cells to sites of future organ development. $CD3^-$ $CD4^+$ IL-$7R\alpha^{hi}$ cells express the chemokine receptor CXCR5 and might be attracted by its ligand CXCL13, which is secreted by mesenchymal cells. Mesenchymal cells also secrete CCL19, a ligand for CCR7, yet it is not clear whether CCR7 and CCL19 are important for secondary lymphoid organ development. Analyzing $CXCR5^{-/-}$ $CCR7^{-/-}$ double deficient mice we now show that these mice lack all examined peripheral LNs suggesting a profound role for both receptors in secondary lymphoid organ development. We demonstrate that $CD3^-$ $CD4^+$ IL-$7R\alpha^{hi}$ cells express CXCR5 as well as CCR7 indicating that both receptors cooperate during an early step of secondary lymphoid organ development. Furthermore, $CXCR5^{-/-}$ $CCR7^{-/-}$ mice display a severely disturbed architecture of mesenteric LN and spleen. Due to an impaired migration of B cells into the white pulp, $CXCR5^{-/-}$ $CCR7^{-/-}$ mice fail to develop B cell follicles but show small clusters of unorganized lymphocytes in the spleen. These data demonstrate a cooperative function of CXCR5 and CCR7 in lymphoid organ organogenesis and organization.

Key words: chemokine receptors • development • B cells • lymphoid organs • migration

Introduction

Chemokines are a family of small chemotactic cytokines that are known to be involved in various aspects of the immune response including the functional organization of lymphoid organs. We could show that the chemokine receptor CCR7 plays a pivotal role in the recruitment of naive T cells and antigen-activated dendritic cells to the T cell–rich areas of secondary lymphoid organs (1). In contrast, CXCR5 and its ligand, CXCL13, control B cell migration and thus the organization of B cell follicles (2, 3). Furthermore, recent studies suggested that CXCL13 is capable of recruiting hematopoietic precursor cells ($CD3^-$ $CD4^+$), thereby fixing sites of future development of LNs and Peyer's patches (PP; references 4 and 5). Apart from CXCR5, these precursor cells are known to express $\alpha4\beta7$ integrin, IL-$7R\alpha$, and lymphotoxin (LT)$\alpha1\beta2$ (6). It is assumed that for a short period during embryonic development, expression of the mucosal addressin cell adhesion molecule-1 by postcapillary high endothelial venules enables $CD3^-$ $CD4^+$ cells initially to enter the sites of prospective development of LNs and PP. Once there, these precursors might stimulate mesenchymal $LT\beta R^+$ cells to produce the chemokines CXCL13 and CCL19 as well as the adhesion molecules vascular cell adhesion molecule-1 and intracellular adhesion molecule-1 (4). In addition to the interaction of mucosal addressin cell adhesion molecule-1 with $\alpha4\beta7$ integrin, the interaction of vascular cell adhesion molecule-1 with $\alpha4\beta1$ integrin expressed on $CD3^-$ $CD4^+$ cells possibly contributes to the establishment of developing LN and PP (5).

The vital function of CXCR5 expression on these founder cells and the presence of its ligand at the site of LN development has been recently suggested (3, 5). Mice lacking either the receptor or the ligand miss most PP and lack

Address correspondence to Reinhold Förster, Institute of Immunology, Hannover Medical School, Feodor-Lynen-Str. 25, 30625 Hannover, Germany. Phone: 49-511-5329721; Fax: 49-511-5329722; E-mail: foerster.reinhold@mh-hannover.de

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2003/05/1199/6 $8.00
Volume 197, Number 9, May 5, 2003 1199–1204
http://www.jem.org/cgi/doi/10.1084/jem.20030169

Published May 5, 2003

several defined LNs such as inguinal, axillary, and parathymic LNs whereas the mesenteric LNs (MLNs) and certain peripheral LNs are always present (2, 3). The underlying mechanisms that selectively support or suppress the development of defined LNs and PP in CXCR5- or CXCL13-deficient mice are not understood.

It has been recently demonstrated that mesenchymal cells at the site of LN development express CCL19, a ligand for CCR7 (4). Because CCR7-deficient mice revealed no overt abnormality of lymphoid organ (1) development it is currently unknown whether CCR7 and its ligands participate in LN development. Given that ligands for both CXCR5 and CCR7 are expressed at the site of lymphoid organ development, and the importance of these chemokines in lymphocyte migration (7), we were interested whether CCR7 and CXCR5 have overlapping functions in lymphoid organ organogenesis and organization.

We provide evidence that the function of CXCR5 on the hematopoietic $CD3^-$ $CD4^+$ IL-$7R\alpha^{hi}$ cells in LN development and on B follicle organization is complemented by CCR7. Mice lacking both chemokine receptors fail to develop any peripheral LNs whereas MLNs and spleen, which are always present in double deficient mice, lack lymphoid follicles due to an impaired migration of B cells.

Materials and Methods

Detection of LNs. Animals were bred at the central animal facility of Hannover Medical School under specific pathogen-free conditions. CXCR5/CCR7 double deficient mice were generated by intercrossing $CXCR5^{-/-}$ mice (six times backcrossed to 129SV; reference 2) with $CCR7^{-/-}$ mice (on a mixed BALB/c 129SV background; reference 1). Littermates of double deficient mice lacking none or one of both receptors were also bred as reference animals and used in this study. LNs were addressed as previously described (8).

Flow Cytometry. Newborn mice were killed at day 0.5 by decapitation. Adult mice were killed by CO_2 inhalation. To obtain single cell suspensions of spleen and MLNs, organs were minced through a nylon mesh and washed with PBS supplemented with 3% FCS. Erythrocytes of the spleen were removed by hypotonic lysis with NH_4Cl buffer and then stained as previously described (9). $CD4^+$ IL-$7R\alpha^+$ cells in neonatal organs were identified with anti–IL-7R (clone A7R34) followed by a mouse anti–rat Cy5 conjugate (Jackson ImmunoResearch Laboratories), anti–CD3-PE (Caltag), and anti–CD4-PerCP (BD Biosciences). CXCR5 was stained with biotinylated anti-CXCR5 (clone 2G8) and streptavidin-conjugated Alexa488 (Molecular Probes). CCR7 expression was monitored by staining cells with a CCL19–$hIgG_1$ fusion protein followed by goat anti–human IgG_1 biotin and streptavidin Alexa488. Before staining with the fusion protein, 10^6/ml cells were incubated in RMPI with 10% FCS for 20 min at 37°C. Subsequent staining was performed on ice. Analysis was performed using a FACSCalibur® (BD Biosciences).

Immunohistochemistry. Immunohistological analysis of adult spleen and MLNs was performed as previously described (2) using a motorized Axiovert M200 microscope (Carl Zeiss MicroImaging, Inc.). Cryosections of spleen and MLNs were blocked with rat serum and stained with antibodies against the indicated markers. Overviews of spleen sections shown in Fig. 3 were achieved using automated image assembly applying the KS300 MosaiX software (Carl Zeiss MicroImaging, Inc.). The relative distribution of transferred cells to red pulp, marginal zone (MZ), and white pulp (WP) was enumerated by image analysis of assembled micrographs.

Adoptive Transfer of MACS-purified $B220^+$ Cells. Adoptive transfer of B cells was performed as previously described (1). In brief, B cells were isolated from the spleen by positive selection with B220 biotin/streptavidin microbeads on MACS LS columns (Miltenyi Biotec). Purified B cells were labeled with fluorescein diacetate succinimidyl ester (CFSE) as previously described (10). Labeled cells were transferred by intravenous injection into 8-wk-old wild-type recipients, which were killed 4 h after transfer.

Results and Discussion

Lack of Peripheral LNs in $CXCR5^{-/-}$ $CCR7^{-/-}$ Mice. Previous studies demonstrated that LN development in mice deficient for CXCR5 or CXCL13 is characterized by the lack of distinct peripheral LNs and reduced numbers of PP whereas spleen and MLN are present (2, 3). In contrast, CCR7-deficient mice showed no obvious phenotype regarding LN development (1). Confirming previous observations, CXCR5-deficient mice consistently developed superficial cervical and facial LNs, possessed reduced numbers of PP per individual, but always completely lacked axillary, inguinal, parathymic, mediastinal, and iliac LNs (Table I; reference 3). Some other LNs such as popliteal and renal LNs were found to develop sporadically, a phenomenon that might relate to the genetic background of the animals under investigation as demonstrated earlier. In those studies, popliteal LNs were always missing in CXCR5-deficient mice of a mixed B6/129 strain background whereas CXCR5-deficient mice of a 129 strain background (six times backcrossed) regularly developed these LNs (3). The effect of the strain background on LN development could be further confirmed in this study, where all animals analyzed possessed a BALB/c/129 strain background. CXCR5-deficient mice of this background always developed brachial LNs, which was rather infrequently present in the animals of the study by Ansel et al. (Table I; reference 3). CCR7 deficiency resulted in a sporadic unilateral or bilateral absence of inguinal LNs (Table I). Most notably, mice deficient for both CXCR5 and CCR7 suffered from an enhanced defect in LN development. Although the MLN was present in these animals, they consistently lacked all other investigated LNs including those that are regularly observed in CXCR5-deficient mice (Table I; reference 3), demonstrating a cooperative function of CXCR5 and CCR7 during LN development. Therefore, it can be assumed that the development of PP and MLNs on the one hand and other LNs on the other hand obeys different signaling pathways suggesting a differential requirement of CXCR5 and CCR7 in both processes. The number of PP per animal was also reported to be influenced by the genetic background in CXCR5-deficient mice (3), thereby offering one possible explanation as to why mice lacking both chemokine receptors on average possess slightly more PP than their CXCR5-deficient

Published May 5, 2003

Table I. *Development of LNs and PP in Wild-type, CXCR5$^{-/-}$, CCR7$^{-/-}$, and CCR7$^{-/-}$ CXCR5$^{-/-}$ Mice*

Genotype	Superficial Cervical	Facial	Axillary	Brachial	Inguinal	Popliteal	Parathymic	Mediastinal	Mesenteric	Renal	Iliac	PP 0	PP 1–2	PP 3–6	PP >6
Wild-type	8(0)/8	8(0)/8	8(0)/8	8(0)/8	8(0)/8	8(0)/8	8(0)/8	8(0)/8	8/8	8(0)/8	8(0)/8	0	0	0	8
CCR7$^{-/-}$	12(0)/12	12(0)/12	12(0)/12	12(0)/12	3(7)/12	10(2)/12	11(1)/12	12(0)/12	12/12	12(0)/12	12(0)/12	0	0	2	10
CXCR5$^{-/-}$	11(0)/11	11(0)/11	0(0)/11	11(0)/11	0(0)/11	1(1)/11	0(0)/11	0(0)/11	11/11	1(3)/11	0(0)/11	2	4	2	3
CCR7$^{-/-}$ CXCR5$^{-/-}$	0(0)/9	0(0)/9	0(0)/9	0(0)/9	0(0)/9	0(0)/9	0(0)/9	0(0)/9	9/9	0(0)/9	0(0)/9	0	0	5	4

The presence of the indicated LNs was analyzed by microscopic examination of mice of each indicated genotype. Numbers to the right indicate the number of mice and the numbers to the left indicate the number of animals with the full complement of that LN type. Numbers in parentheses indicate the number of mice with unilateral LNs.

counterparts. However, this also implies that the combined absence of both CXCR5 and CCR7 does not hamper PP development beyond the defects observed in the CXCR5 single mutant. Remarkably, PP anlagen but not LN anlagen remain functional postnatally in CXCR5-deficient mice (5). This improves the likelihood that PP come into existence considerably. Therefore, PP development might be less dependent on combined signaling mediated by CXCR5 and CCR7.

Hematopoietic CD3$^-$ CD4$^+$ IL-7Rα^{hi} Cells Coexpress CXCR5 and CCR7. In search for the event causing the disturbed LN development in double deficient mice, hematopoietic CD3$^-$ CD4$^+$ IL-7Rα^{hi} cells, known to be involved in LN organogenesis, were analyzed for the expression of CXCR5 and CCR7. To this end, cells were stained with a CCL19–hIgG1 fusion protein (unpublished data). As shown in Fig. 1, CD3$^-$ CD4$^+$ IL-7Rα^{hi} cells isolated from the MLN or spleen of newborn mice express both chemokine receptors.

This finding further supports the notion that CXCR5 and CCR7 cooperate during lymphoid organ development. During this process, CD3$^-$ CD4$^+$ IL-7Rα^{hi} CXCR5$^+$ cells settle in LN anlagen participating in organ development (6). As outlined above, only distinct LNs are missing in CXCR5- and CXCL13-deficient mice, indicating that the migration of CD3$^-$ CD4$^+$ cells to some but not all places of prospective LN development depends on this part of the chemokine/chemokine receptor system. It seems conceivable that the establishment of other LNs is driven by receptors that can partially compensate for CXCR5 and CXCL13 deficiency. Several lines of evidence suggest that CCR7 and its ligands CCL19 and CCL21 are involved in LN organogenesis: (a) ectopic expression of CCL19 or CCL21 in pancreatic islets induces development of lymphoid tissue (11, 12), (b) in addition to CXCL13, mesenchymal cells in PP anlagen produce CCL19 upon activation by LTα1β2 (4), and (c) CD3$^-$ CD4$^+$ IL-7Rα^{hi} cells show chemotaxis toward CCR7 ligands (4).

Defects in various parts of the LT/LT receptor system resemble in their final consequence, the lack of lymphoid organs, the CXCR5/CCR7 double deficient mice reported here (13–15). This might in principle be explained either by a consecutive requirement of the chemokine and the LT system but might also involve the interaction of both signaling pathways. Most notably it has been recently shown that the organization of B cell follicles is driven by a LT/chemokine feedback loop. Upon activation by CXCL13, B cells express LTα1β2, which induces expression of CXCL13 by follicular dendritic cells. This in turn attracts additional B cells to the follicle thereby amplifying the corresponding signals (3, 16). A similar mechanism may

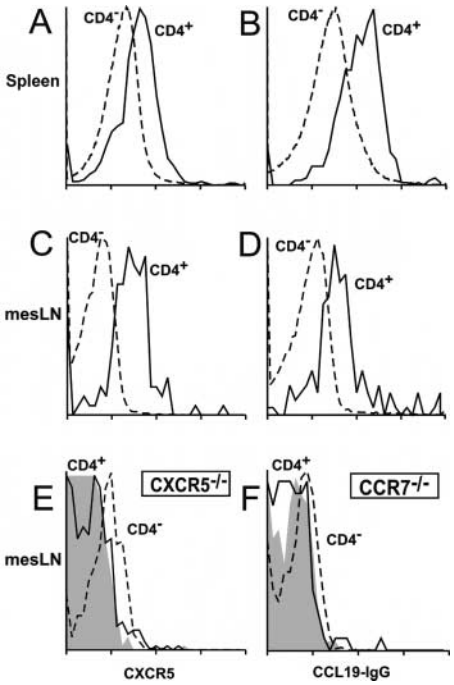

Figure 1. Coexpression of CCR7 and CXCR5 on CD3$^-$ CD4$^+$ IL-7R^{hi} cells. Cells from day 0.5 animals were isolated from the spleen (A and B) and MLNs (C–F). Wild-type (A–D), CXCR5$^{-/-}$ (E), and CCR7$^{-/-}$ (F) mice. Cells were stained with anti-CD3, anti-CD4, and anti–IL-7Rα followed by either anti-CXCR5 (A, C, and E) or CCL19-IgG (B, D, and F). CXCR5 and CCR7 staining is shown for CD3$^-$ CD4$^+$ IL-7Rα^{hi} cells (solid lines) and for CD3$^-$ CD4$^-$ cells (dashed lines). Shaded areas in E and F represent isotype staining of CD3$^-$ CD4$^+$ cells derived from CXCR5$^{-/-}$ or CCR7$^{-/-}$ mice, respectively.

Published May 5, 2003

regulate LN development where $CD3^-$ $CD4^+$ IL-$7R\alpha^{hi}$ cells activated by IL-7–mediated signaling secrete $LT\alpha1\beta2$ that stimulates mesenchymal cells to produce CXCL13 and CCL19, thus recruiting additional $CD3^-$ $CD4^+$ IL-$7R\alpha^{hi}$ $CXCR5^+$ $CCR7^+$ cells.

The latter cell population has been initially identified in PP anlagen. As described above, the development of PP and MLNs is affected to a similar extent in $CXCR5^{-/-}$ and $CXCR5^{-/-}$ $CCR7^{-/-}$ mice. This suggests that CCR7 expression on $CD3^-$ $CD4^+$ IL-$7R\alpha^{hi}$ cells is not the compensating factor in the development of these organs. It is currently unknown whether different progenitor cells populate these sites or whether environmental factors other than those generated by CXCR5, CCR7, and their ligands are responsible for the recruitment of common progenitor cells. In any case, further development of LNs and PP depends on signals provided by LT and IL-7.

$CXCR5^{-/-}$ $CCR7^{-/-}$ Mice Lack Organized B and T Cell Compartments in Spleen and MLNs. Apart from few PP, spleen and MLNs are the only secondary lymphoid organs consistently found in $CXCR5^{-/-}$ $CCR7^{-/-}$ mice. Analyzing the total white blood cell count of spleen and MLNs in these mice revealed that double deficient mice have a phenotype similar to that of CCR7-deficient animals, i.e., enlarged spleen with increased numbers of lymphocytes and reduced lymphocyte counts in MLNs (unpublished data). B cells express CXCR5 as well as CCR7 (reference 3 and unpublished data). Therefore, we further characterized B cells originating from double deficient mice. No obvious discrepancy concerning the percentage of follicular B cells ($CD19^+$ IgD^+) in spleen (Fig. 2 A) and MLNs (unpublished data) as well as splenic MZ B cells ($CD19^+$ $CD21^+$ $CD23^-$) could be observed between wild-type, $CCR7^{-/-}$, $CXCR5^{-/-}$, and $CCR7^{-/-}$ $CXCR5^{-/-}$ mice (Fig. 2 A). However, immunohistology revealed dramatic differences between the four strains. Although wild-type spleen showed the typical pattern of the splenic WP divided in the T cell–rich periarteriolar lymphoid sheath (PALS) and B cell follicles (Fig. 2 B, first panel), spleens of CCR7-deficient mice were characterized by the accumulation of T cells within the red pulp (Fig. 2 B, second panel). As described above, CXCR5 deficiency leads to distorted B cell follicles in the spleen (Fig. 2 B, third panel). In contrast, $CXCR5^{-/-}$ $CCR7^{-/-}$ mice fail to develop regular lymphoid follicles at all. Only small clusters of B and T cells, assembled in an unorganized manner, could be observed in the spleen (Fig. 2 B, fourth panel). Although in wild-type and single deficient mice follicles are largely free of reticular fibroblasts and fibers (Fig. 2 C, first three panels, respectively), lymphocyte aggregates in $CXCR5^{-/-}$ $CCR7^{-/-}$ mice locate within the reticular network (Fig. 2 C, fourth panel), substantiating the disorganization of lymphoid structures.

Analysis of the MLNs revealed a similar picture. In wild-type animals the characteristic segregation of B cells to the follicle-rich region of the cortex and of T cells to the paracortex is apparent (Fig. 2 D, first panel) whereas CCR7 deficiency is characterized by reduced numbers of T cells within this area (Fig. 2 D, second panel). Although primary follicular dendritic cell–containing B cell follicles are missing in MLNs of CXCR5-deficient mice, the overall architecture of this LN is not affected (Fig. 2 D, third panel). In contrast, mice lacking both CXCR5 and CCR7 fail to develop any organized B or T cell areas inside their MLNs (Fig. 2 D, fourth panel) whereas high endothelial venules are abundantly present (unpublished data). Taken together,

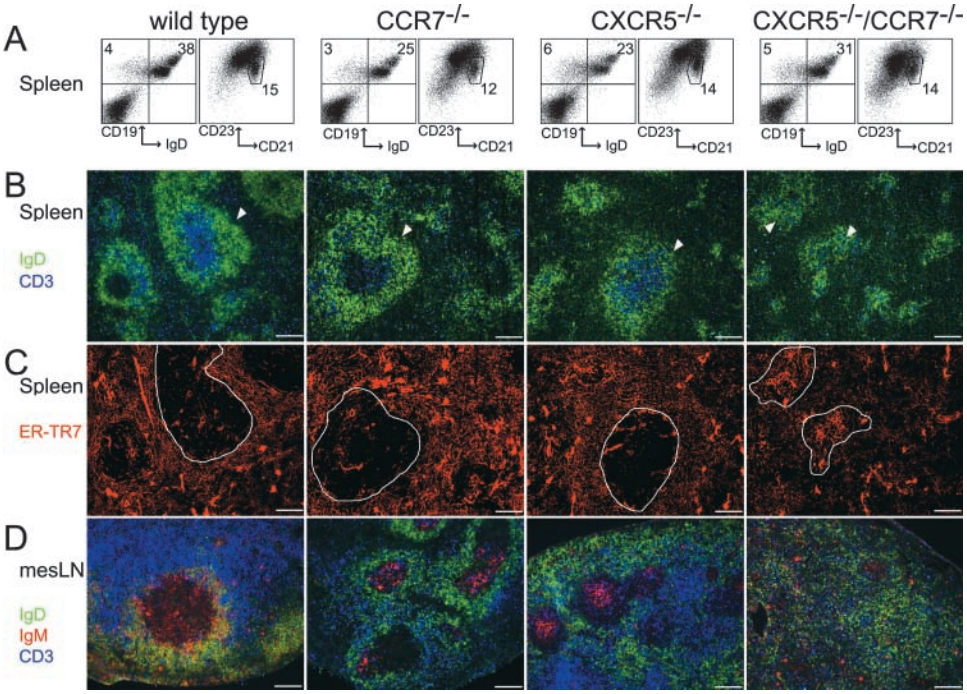

Figure 2. Disturbed architecture of spleen and MLNs in $CXCR5^{-/-}$ $CCR7^{-/-}$ mice. (A). Flow cytometric analysis of spleen cells. The left histogram of each panel shows the percentages of B cells ($CD19^+$ IgD^+ and $CD19^+$ IgD^-) and the percentage of MZ B cells ($CD23^-$ $CD21^+$) within the $CD19^+$ population is shown in the right panel for each indicated genotype. Immunohistology of cryostat sections from spleens (B and C) and MLNs (D). (B) Spleen sections were stained with anti-IgD (green) and anti-CD3 (blue). (C) Fibroblasts and fibers of the same sections shown in panel B stained with ER-TR7 (red). White line–surrounded areas shown in C indicate the position of the lymphocyte-rich areas indicated by arrows in B. (D) Cryostat sections of MLNs stained with anti-IgD (green), anti-IgM (red), and anti-CD3 (blue). Bars, 100 μm.

Published May 5, 2003

these data indicate that CXCR5 and CCR7 exert synergistic functions in the organization of lymphoid organs.

Deficiency of CXCR5 and CCR7 Abrogates B Cell Migration to WP of the Spleen. To elucidate the cause for the disturbed architecture of the WP in CXCR5$^{-/-}$ CCR7$^{-/-}$ mice, B220^{+} cells were isolated from the spleens of wild-type, single deficient, or CXCR5$^{-/-}$ CCR7$^{-/-}$ mice. After labeling with CFSE, these cells were adoptively transferred to wild-type recipients to study their distribution within the recipient's spleen. B cells of wild-type donors entered the WP and were found in the T cell–rich PALS as well as in the follicle 4 h after adoptive transfer (Fig. 3 A). Although CCR7-deficient B cells still were able to enter the WP (unpublished data; compare with reference 1), CXCR5$^{-/-}$ donor B cells entered the T cell area but failed to migrate further into the B cell follicle as demonstrated earlier (unpublished data; compare with reference 2). In contrast, B cells isolated from CXCR5$^{-/-}$ CCR7$^{-/-}$ animals were virtually completely impaired in migrating to the WP. Instead, they were retained in the red pulp and only on occasion transferred cells locate to the borders between B cell areas and red pulp within the MZ (Fig. 3 B). To further address more accurately the relative distribution of transferred B cells, cryo sections were stained with anti–IgD-Cy5 and anti–B220-Cy3. MZ B cells were identified by bright B220 staining and negative to dim IgD staining (not depicted). Image analysis revealed equal numbers of transferred B cells within the recipient's spleen independent of the donor's genotype (wild-type donor: 346 ± 16 cells/mm^2, CXCR5$^{-/-}$ CCR7$^{-/-}$ donor: 353 ± 14 cells/mm^2; mean ± SD). 4 h after transfer, B cells derived from wild-type donors were located with a frequency of 43% to the red pulp, 11% to the MZ, and 46.0% to the WP. In contrast, 88% of CXCR5$^{-/-}$ CCR7$^{-/-}$ B cells were found within the red pulp and 9.2% in the MZ whereas 2.8% were located in the WP (Fig. 3 C; data derived from 31 WP/MZ representing 34.7% of the analyzed area [wild-type B cells] and 28 WP/MZ representing 33.3% of the analyzed area [CXCR5$^{-/-}$ CCR7$^{-/-}$ B cells]).

CCR7-dependent migration of B cells has been demonstrated for antigen-activated follicular B cells in the spleen (7). In this model, B cells up-regulate CCR7 after antigen encounter and subsequently migrate to the T-B boundary where help is provided by T helper cells specific for the same antigen. B cells are known to enter the WP passing the marginal sinus through the bridging channels into the T cell–rich area from where they migrate into the B cell follicle. Data derived from gene-targeted mice revealed that the migration of B cells to the T cell–rich PALS is independent of CXCR5. However, B cells require this receptor to migrate further on into the B cell follicle (2). In previous adoptive transfer experiments we have observed that although CCR7$^{-/-}$ B cells are compromised in migrating to the PALS, some cells were found in this location in the first hour after transfer. Nevertheless, migration of B cell to the follicle was not hampered in transfer experiments (1). Consequently, it had been assumed that another chemokine–receptor interaction might guide B cells into the PALS. However, data shown here indicate that B cells use either CXCR5 or CCR7 to locate into the T cell area. Once both receptors are missing, B cells are largely excluded from the WP. As it has been shown that B cells influence splenic T cell area development by providing signals that induce the accumulation of both dendritic cells and T cells (17), the severely disrupted architecture of lymphoid organs observed in CXCR5$^{-/-}$ CCR7$^{-/-}$ mice can at least in part

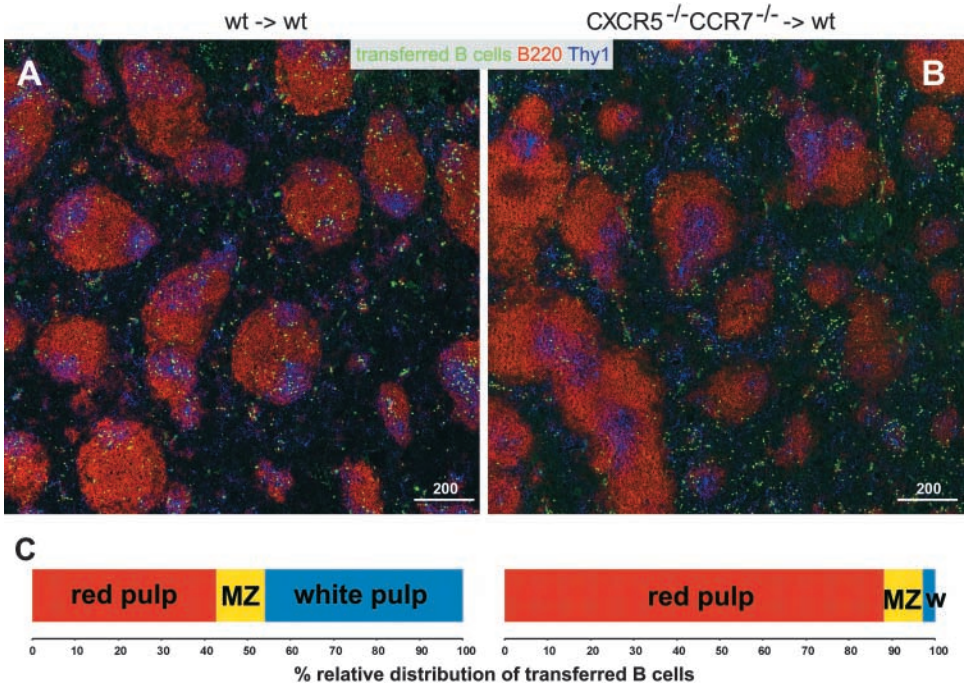

Figure 3. Impaired migration of CCR7$^{-/-}$ CXCR5$^{-/-}$ B cells into the splenic WP. B220^{+} cells were isolated by MACS from the spleen of wild-type or CCR7$^{-/-}$ CXCR5$^{-/-}$ mice. After labeling with CFSE, cells were retro-orbitally injected into wild-type recipients. 4 h later, mice were killed and the distribution of wild-type (A) and CCR7$^{-/-}$ CXCR5$^{-/-}$ (B) donor cells was analyzed by immunohistology of spleen sections. Green, transferred cells; red, anti-B220; blue, anti-Thy1. Each picture is composed of nine individual micrographs using automated assembly of the KS300 MosaiX software (Carl Zeiss MicroImaging, Inc.). (C) Additional sections were stained with anti–B220-Cy3 and anti–IgD-Cy5 to identify the MZ (not depicted) and were used to further analyze the distribution of transferred B cells within the spleen. The relative distribution of transferred B cells to the red pulp, the MZ, and the WP are shown for wild-type (left) and CCR7$^{-/-}$ CXCR5$^{-/-}$ (right) donor B cells.

Published May 5, 2003

be explained by the combination of migration defects of chemokine receptor–deficient B cells.

Development of lymphoid organs falls into two parts: an initiation step and further events accomplishing development. The former is equivalent to the task to convert the organ anlage into a structure recognized by future "inhabitants" homing there, and the latter into the challenge to organize the growing organ into substructures such as T and B cell–rich areas. There is compelling evidence that CXCR5 and CCR7 and their ligands provide a network to fulfill in large parts the requirements for both steps mentioned above. Because the network of receptor cooperation is the key feature, only the lack of both receptors in our animals revealed the importance of this system in lymphoid organogenesis. The first step in organogenesis, colonization of LN anlagen with $CD3^-$ $CD4^+$ progenitors, might be controlled by a mixture of signals provided by CXCL13, CCL19, and probably others. The organogenesis of distinct LNs (axillary, inguinal) relies predominantly on a CXCR5 signal as opposed to others where a missing CXCR5 signal can be substituted by CCR7. If such signaling is not provided at the appropriate time during embryogenesis, development of the corresponding LNs ceases. PP are exceptional in this context because their anlagen remain active after birth. It is currently not possible to dissect whether this is an inborn trait or a consequence of a persistent survival signal provided by activated $CD3^-$ $CD4^+$ cells present in the gut but no where else. However, in the case of the spleen and MLNs, another molecule may act as the dominant attractant recruiting $CD3^-$ $CD4^+$ precursors in initiating organogenesis. Yet, subsequent steps cannot be performed properly once CXCR5/CCR7 are missing, leading to the severe disorganization of these secondary lymphoid organs as observed in the double deficient mice.

We would like to thank Eva Stüwe for excellent technical assistance and Sandra Wilkening and Mahimaidos Manoharan for maintaining the mouse facility.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG Fo334/1-1) to R. Förster.

Submitted: 31 January 2003
Revised: 25 February 2003
Accepted: 13 March 2003

References

1. Förster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Müller, E. Wolf, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell.* 99:23–33.
2. Förster, R., E.A. Mattis, E. Kremmer, E. Wolf, G. Brem, and M. Lipp. 1996. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell.* 87:1037–1047.
3. Ansel, K.M., V.N. Ngo, P.L. Hyman, S.A. Luther, R. Forster, J.D. Sedgwick, J.L. Browning, M. Lipp, and J.G. Cyster. 2000. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature.* 406:309–314.
4. Honda, K., H. Nakano, H. Yoshida, S. Nishikawa, P. Rennert, K. Ikuta, M. Tamechika, K. Yamaguchi, T. Fukumoto, T. Chiba, et al. 2001. Molecular basis for hematopoietic/mesenchymal interaction during initiation of Peyer's patch organogenesis. *J. Exp. Med.* 193:621–630.
5. Finke, D., H. Acha-Orbea, A. Mattis, M. Lipp, and J. Kraehenbuhl. 2002. CD4+CD3− cells induce Peyer's patch development: role of alpha4beta1 integrin activation by CXCR5. *Immunity.* 17:363–373.
6. Mebius, R.E., P. Rennert, and I.L. Weissman. 1997. Developing lymph nodes collect CD4+CD3− LTbeta+ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. *Immunity.* 7:493–504.
7. Reif, K., E.H. Ekland, L. Ohl, H. Nakano, M. Lipp, R. Forster, and J.G. Cyster. 2002. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature.* 416:94–99.
8. Tilney, N.L. 1971. Patterns of lymphatic drainage in the adult laboratory rat. *J. Anat.* 109:369–383.
9. Förster, R., T. Emrich, E. Kremmer, and M. Lipp. 1994. Expression of the G-protein-coupled receptor BLR1 defines mature recirculating B cells and a subset of T memory helper cells. *Blood.* 84:830–840.
10. Lyons, A.B., and C.R. Parish. 1994. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods.* 171:131–137.
11. Chen, S.C., G. Vassileva, D. Kinsley, S. Holzmann, D. Manfra, M.T. Wiekowski, N. Romani, and S.A. Lira. 2002. Ectopic expression of the murine chemokines CCL21a and CCL21b induces the formation of lymph node-like structures in pancreas, but not skin, of transgenic mice. *J. Immunol.* 168: 1001–1008.
12. Luther, S.A., A. Bidgol, D.C. Hargreaves, A. Schmidt, Y. Xu, J. Paniyadi, M. Matloubian, and J.G. Cyster. 2002. Differing activities of homeostatic chemokines CCL19, CCL21, and CXCL12 in lymphocyte and dendritic cell recruitment and lymphoid neogenesis. *J. Immunol.* 169:424–433.
13. Alimzhanov, M.B., D.V. Kuprash, M.H. Kosco-Vilbois, A. Luz, R.L. Turetskaya, A. Tarakhovsky, K. Rajewsky, S.A. Nedospasov, and K. Pfeffer. 1997. Abnormal development of secondary lymphoid tissues in lymphotoxin beta-deficient mice. *Proc. Natl. Acad. Sci. USA.* 94:9302–9307.
14. De Togni, P., J. Goellner, N.H. Ruddle, P.R. Streeter, A. Fick, S. Mariathasan, S.C. Smith, R. Carlson, L.P. Shornick, J. Strauss-Schoenberger, et al. 1994. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science.* 264:703–707.
15. Koni, P.A., R. Sacca, P. Lawton, J.L. Browning, N.H. Ruddle, and R.A. Flavell. 1997. Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta-deficient mice. *Immunity.* 6:491–500.
16. Ngo, V.N., H. Korner, M.D. Gunn, K.N. Schmidt, D.S. Riminton, M.D. Cooper, J.L. Browning, J.D. Sedgwick, and J.G. Cyster. 1999. Lymphotoxin α/β and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J. Exp. Med.* 189: 403–412.
17. Ngo, V.N., R.J. Cornall, and J.G. Cyster. 2001. Splenic T zone development is B cell dependent. *J. Exp. Med.* 194: 1649–1660.