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CCR7 Coordinates the Primary Immune Response by Establishing Functional Microenvironments in Secondary Lymphoid Organs

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Summary

The proper function of immune surveillance requires well-coordinated mechanisms in order to guide the patrolling immune cells through peripheral tissues and into secondary lymphoid organs. Analyzing gene-targeted mice, we identified the chemokine receptor CCR7 as an important organizer of the primary immune response. CCR7-deficient mice show severely delayed kinetics regarding the antibody response and lack contact sensitivity and delayed type hypersensitivity reactions. Due to the impaired migration of lymphocytes, these animals reveal profound morphological alterations in all secondary lymphoid organs. Upon activation, mature skin dendritic cells fail to migrate into the draining lymph nodes. Thus, in order to bring together lymphocytes and dendritic cells to form the characteristic microarchitecture of secondary lymphoid organs, CCR7 is required to rapidly initiate an adoptive immune response.

Introduction

Once being released from primary lymphoid organs in the bloodstream, the majority of lymphocytes continuously recirculate through almost all compartments of the body to bring the entire range of antigen-specific immune cells into close contact with invading pathogens. For this purpose, lymphocytes extravasate from blood vessels, migrate to lymphoid and nonlymphoid tissues, and from there travel back into the bloodstream (reviewed in Butcher and Picker, 1996). However, lymphocyte migration is not random. Early studies identified different recirculation pathways for naive and memory T cells by which they enter secondary lymphoid organs, such as lymph nodes (LNs) or Peyer's patches (PPs). After patrolling through peripheral tissue, memory T cells preferentially enter draining LNs by afferent lymphatics, whereas the majority of naive, antigen-inexperienced cells pass directly from the blood to secondary lymphoid tissue by emigrating through specialized high endothelium venules (HEVs) into the surrounding T cell (-rich) zone (Mackay et al., 1990). B cells take the same route as naive T cells, but after entering the T cell zone, they continue to move to adjacent follicles that are rich in B cells and follicular dendritic cells (Picker and Butcher, 1992). Once present in the T cell zone, naive T cells continue to migrate through this area, which enables them to screen dendritic cells (DCs) for presented peptides fitting into their antigen receptor. DCs found in the T cell zone of secondary lymphoid organs are derived from DCs of the skin, which have been activated and mobilized by antigen exposure and, as a consequence, migrated to the draining LNs by afferent lymphatics in order to present antigens to T cells (Steinman et al., 1997). Thus, the T cell areas of secondary lymphoid organs represent the microenvironments that allow extensive interactions of B cells, T cells, and DCs which seem to be required for initiating an adoptive immune response. The emigration of lymphocytes is tightly controlled at the level of lymphocyte–endothelial interactions and has been extensively studied on HEVs (reviewed in Springer, 1994). Here, two important control mechanisms regulate lymphocyte trafficking: first, the adhesion of lymphocytes to the endothelial cells and second, the transmigration through the endothelium into the surrounding tissue. Several families of adhesion molecules, such as selectins, integrins, and members of the immunoglobulin superfamily, have been shown to participate in both processes, but the exact molecular codes controlling selective lymphocyte trafficking are still poorly understood (Springer, 1994; Butcher and Picker, 1996).

Chemokines represent a family of small basic chemotactic proteins that mediate their effect by binding to seven transmembrane-spanning G protein–coupled receptors expressed on target cells. Chemokines have been identified originally by their properties to direct extravasation of inflammatory cells. However, recent data identified several chemokines that are expressed constitutively in lymphoid tissues (see below), indicating that these chemokines might have homeostatic functions by regulating lymphocyte trafficking to or within lymphoid organs (for recent reviews, Rollins, 1997; Baggiolini, 1998). Data derived from mice deficient in the cytokines lymphotoxin (LT) α/β and tumor necrosis factor (TNF) as well as their receptors show an impaired architecture of B cell follicles and T cell zones, suggesting that members of the TNF family might be involved in lymphocyte trafficking (reviewed in Chaplin and Fu, 1998). Interestingly, Ngo et al. (1999) demonstrated recently that mice deficient for various members of the TNF/LT family also have an aberrant expression of several chemokines. Reduced levels of chemokines might help to explain how the TNF/LT deficiency affects lymphocyte migration.

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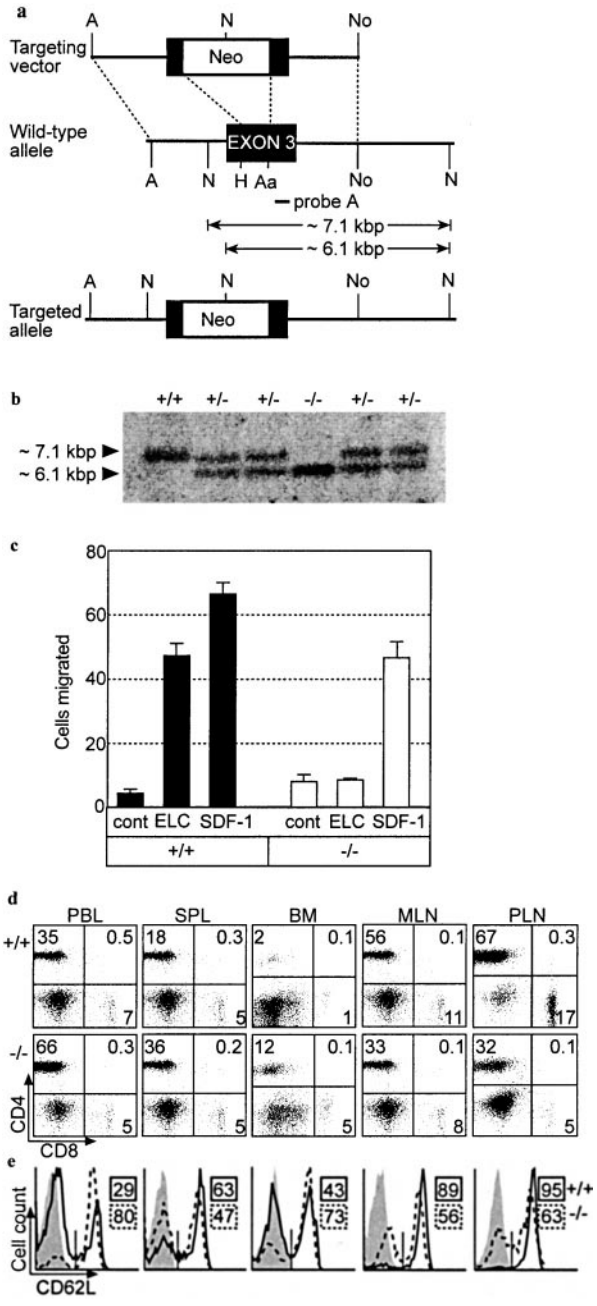

Figure 1. Targeted Disruption of the *CCR7* Gene by Homologous Recombination

(a) Cloning strategy. Targeting vector (top), wild-type *CCR7* allele (middle), and predicted mutant allele (bottom). The third exon of the gene is indicated as a filled box. Probe A is the probe used for Southern blot analysis. Restriction sites: AA, AatII; A, ApaI; H, HindIII; N, NcoI; and No, NotI.

(b) Representative Southern blot analysis of tail DNA from wild-type (+/+), heterozygous mutant (+/−), and homozygous mutant (−/−) littermates. The 7.1 kb wild-type and the 6.1 kb targeted allele NcoI-NcoI fragments identified by probe A are indicated.

(c) Lack of chemotaxis of *CCR7* mutant lymphocytes toward the chemokine ELC. Spleen cells of wild-type (+/+) or *CCR7* mutant (−/−) mice were used in transwell chemotaxis assays to assess their chemotactic properties toward medium alone (cont) or medium supplemented with 100 ng/ml of ELC or SDF-1.

(d and e) Organ-specific distribution of T cells in *CCR7*$^{-/-}$ mice. (d) Reduced numbers of T cells in LNs. Lymphocytes isolated from the blood and lymphoid organs of wild-type (+/+) or CCR7-deficient (−/−) littermates were stained with antibodies against CD8 (FITC) and CD4 (PE) and analyzed by flow cytometry. The percentages of cells with a particular cell surface expression phenotype are indicated within the appropriate quadrant. (e) The pool of naive T cells is expanded in the peripheral blood (PBL) and bone marrow (BM) but is reduced in secondary lymphoid organs. Lymphocytes were stained with antibodies against L-selectin (CD62L-FITC) and CD3 (PE). Gates were set on CD3$^+$ cells. The percentages of CD62L-positive T cells are indicated in boxes. Solid line, wild-type animals; dotted line, CCR7-deficient mice; shaded area, isotype staining. One of four experiments is shown. The percentage of CD62L$^+$ T cells is shown within the boxes. SPL, spleen; MLN, mesenteric lymph node; PLN, peripheral lymph node.

By generating mice deficient for the chemokine receptor Burkitt's lymphoma receptor 1 (BLR1, CXCR5), we provided essential evidence that the chemokine system is indeed involved in homeostatic lymphocyte trafficking to secondary lymphoid organs (Förster et al., 1996). CXCR5-deficient mice possess no or only few aberrantly developed PPs and show a severe impairment of B cell migration to B cell follicles of spleen and PPs. We and others identified BLR2/EBI-1, another member of the chemokine receptor family, which is now known as CCR7 (Schweickart et al., 1994; Burgstahler et al., 1995). CCR7 is expressed on B cells, T cells, and on activated mature DCs (Schweickart et al., 1994; Burgstahler et al., 1995; Dieu et al., 1998; Sallusto et al., 1998; Sozzani et al., 1998; Yanagihara et al., 1998).

To test the function of CCR7, we generated mice in which the *CCR7* locus has been disrupted by gene targeting. LNs of CCR7-deficient mice are devoid of naive T cells and DCs, whereas the T cell population is heavily expanded in the blood, the red pulp of the spleen, and in the bone marrow. Adoptive transfer experiments to wild-type recipients demonstrated that the migration of CCR7-deficient B cells and T cells into LNs and PPs, and of T cells into the splenic periarteriolar lymphoid sheath (PALS) was severely hampered. When compared to wild-type B cells, CCR7-deficient B cells rapidly left the outer PALS after being transferred into wild-type recipients, indicating that expression of CCR7 keeps B cells for a defined period of time in close contact with T cells to allow effective T cell–B cell interactions. Therefore, the overall disturbed microarchitecture of secondary lymphoid organs, caused by the impaired entry and retention of lymphocytes and antigen-presenting DCs, might explain why CCR7-deficient mice fail to mount a rapid primary B or T cell response.

Results

Generation of CCR7-Deficient Mice

Applying a murine *CCR7*-specific DNA fragment as a probe, we isolated a phage clone from a strain 129-Sv library harboring a genomic DNA insert containing exon 3 of *CCR7* (Figure 1a). We constructed a vector to target the *CCR7* gene by deleting large parts of exon 3 (Figure 1a). CCR7-deficient mice were generated by standard gene-targeting techniques. Embryo handling was done by blastocyst injection. As expected, about one-quarter of the offspring of the intercrosses of heterozygous animals carried the disrupted locus on both alleles (Figure

1b). To test whether the mutation of *CCR7* gene resulted in a null phenotype, we used spleen cells to investigate their migration potential in chemotaxis assays. Wild-type lymphocytes showed a strong chemotactic response toward the EBI-1 ligand chemokine (ELC) and toward the stroma derived factor (SDF)-1α, which are known ligands for the chemokine receptors CCR7 and CXCR4, respectively (Figure 1c). Although lymphocytes isolated from CCR7-deficient mice migrated toward SDF-1α in a manner comparable to wild-type cells, we could not observe any chemotactic response toward ELC (Figure 1c), suggesting that CCR7 is the sole receptor for this chemokine expressed on lymphocytes. Cross examinations of the lymphoid system demonstrated that LNs and PPs were smaller in *CCR7* mutant mice, whereas the spleens were usually 2- to 3-fold enlarged (data not shown; compare Figure 5e).

Organ-Specific Distribution of Naive and Memory T Cells in *CCR7*$^{-/-}$ Mice

Taking advantage of flow cytometry, we found a 2- to 6-fold increase of CD4-positive cells in the peripheral blood, the spleen, and the bone marrow (BM) of *CCR7*$^{-/-}$ mice (Figure 1d). In contrast, the amount of CD4^{+} cells was reduced to approximately 30%–50% of the wild-type level in mesenteric lymph nodes (MLNs) and peripheral lymph nodes (PLNs; Figure 1d), and to 75% in PPs (data not shown). Although L-selectin (CD62L) is expressed by some memory cells, the majority of CD62L^{+} T cells are naive, antigen-inexperienced T cells (reviewed in Dutton et al., 1998). Staining with an anti-CD62L monoclonal antibody (mAb) revealed that the population of naive T cells is expanded in blood and BM, whereas their portion is reduced in all secondary lymphoid organs in CCR7-deficient mice (Figure 1e). As we could not observe any differences during early stages of T cell development in the thymus (data not shown), our data indicate that naive T cells are preferentially excluded from entering LNs.

Morphological Alterations in Secondary Lymphoid Organs in *CCR7*$^{-/-}$ Mice

Immunohistology revealed that LNs of CCR7-deficient mice lacked the characteristic distribution of B cell follicles to the outer cortex as well as a paracortical T cell–rich area as observed in wild-type mice, but they displayed an irregular distribution of B and T cells within the paracortex (Figure 2a and upper part of Figure 2b). Furthermore in LNs of *CCR7*$^{-/-}$ mice, T cells located themselves toward the marginal sinus (arrows in Figure 2b). In addition, in some *CCR7*$^{-/-}$ LNs, prominent B cell follicles with considerably enlarged germinal centers could be identified in the paracortex (Figure 2b, lower part) in accordance with the observation of an increased pool of IgD^{-}IgM^{low} cells in these organs (see Figure 3). With regard to the presence and location of follicular dendritic cells, *CCR7*$^{-/-}$ mice did not differ from wild-type animals (data not shown). In *CCR7*$^{-/-}$ mice, T cell–rich zones are missing in PPs, but a small rim of T cells surrounding the B cell follicle can be observed that is not present in wild-type mice (Figures 2c and 2d). Likewise, T cells were dislocated in the spleen. In mutant mice these cells were not preferentially found in the

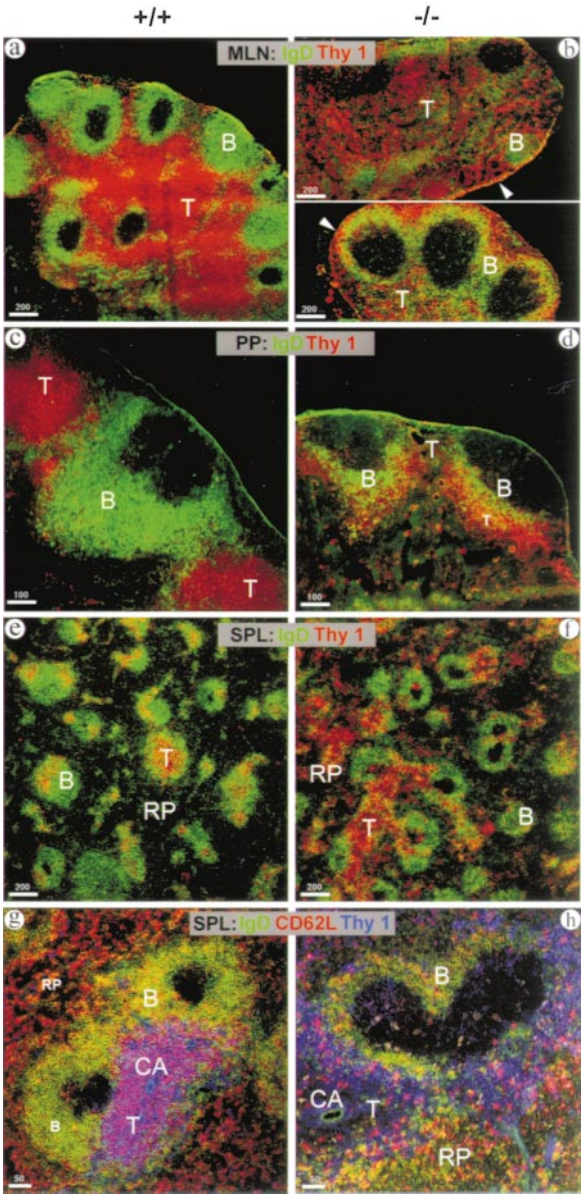

Figure 2. Altered Architecture of Secondary Lymphoid Organs in *CCR7*$^{-/-}$ Mice

Cryostat sections of lymphoid organs derived from wild-type (a, c, e, and g) or *CCR7*$^{-/-}$ (b, d, f, and h) mice were fixed with acetone stained with fluorescent antibodies as indicated and analyzed by confocal microscopy. The following combinations were used: (a–f) anti-IgD-FITC (green), anti-Thy1.2-Cy5 (red); (g and h) anti-IgD-FITC (green), CD62L-TRITC (red), and anti-Thy 1.2-Cy5 (blue). Staining of CD62L was amplified using the tyramide-biotin amplification system followed by staining with streptavidin-TRITC. B, B cell follicle; T, T cell zone; RP, red pulp; CA, central artery.

PALS, as observed in wild-type animals, but were spread throughout the marginal sinuses and the red pulp in large clusters (Figures 2e and 2f). Moreover, the distribution of naive CD62L^{+} T cells was profoundly disturbed in the spleen. In wild-type mice, naive T cells were primarily located in the T cell zone around the central artery (CA; Figure 2g). In contrast, in *CCR7*$^{-/-}$ mice very few T cells located themselves close to the CA

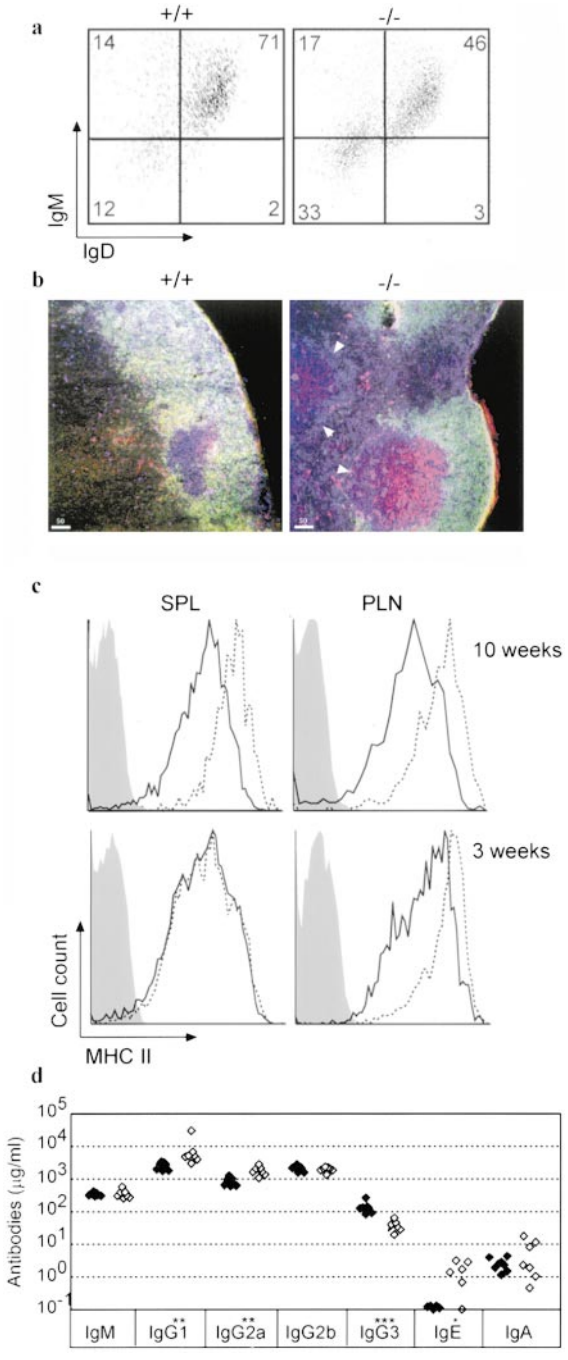

Figure 3. Activated B Cells in CCR7-Deficient Mice

(a) Lymphocytes of 10-week-old wild-type (+/+) or CCR7-deficient (−/−) mice were stained with antibodies against IgD (FITC), IgM (biotin/SA-PE), and B220 (Cychrome). B220-positive cells were analyzed for the expression of IgD and IgM. Numbers indicate the relative percentage of cells within a quadrant.

(b) Cryostat sections of PLNs of wild-type (+/+) or *CCR7*$^{-/-}$ (−/−) mice were stained with anti-IgD-FITC (green), anti-IgM-biotin/streptavidin TRITC (red), and anti B220-Cychrome (blue).

(c) Lymphocytes derived from wild-type (solid lines) and CCR7-deficient (dotted lines) mice either 10 weeks of age (upper panel) or 3 weeks of age (lower panel) were stained with antibodies specific for MHC II and B220. The staining patterns of B220-positive cells of one of two to five experiments are shown.

(d) Serum concentrations of immunoglobulin isotypes from wild-type and CCR7-deficient mice. Control mice (filled symbols) and *CCR7*$^{-/-}$ mice (open symbols) were bled from the retro-orbital plexus, and serum concentrations of the shown isotypes were determined by ELISA. Each circle represents the value obtained from one animal (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Mann-Whitney test).

and those T cells present in the PALS were of memory phenotype lacking CD62L expression. Naive T cells were found to reside outside the PALS in *CCR7*$^{-/-}$ mice (Figure 2h).

Activated B Cells in *CCR7*−/− Mice

Within the scope of our experiments, we could not observe any abnormalities during early stages of B cell development in *CCR7*$^{-/-}$ mice (data not shown). However, CCR7 deficiency seems to affect B cell activation. In B cells isolated from LNs, but not from spleen or peripheral blood, we identified an increased proportion of IgD^{-}IgM^{low} B cells (Figure 3a and data not shown). Because most of these cells represent activated germinal center (GC) B cells in normal individuals, we took advantage of confocal microscopy to delineate the location of B220^{+}IgD^{-}IgM^{low} cells. As shown in Figure 3b, the vast majority of these cells were located within the GC, suggesting that B cells are activated in CCR7-deficient mice. We, therefore, determined the expression levels of MHC II molecules. Analyzing 10-week-old animals, CCR7-deficient B cells isolated from blood and all secondary lymphoid organs expressed increased levels of MHC II molecules when compared to wild-type mice (Figure 3c, upper panel). However, mutant and wild-type B cells, derived from blood and spleen of 3-week-old animals, expressed equal amounts of MHC II, whereas levels of MHC II were increased on mutant B cells isolated from PLNs (Figure 3c, lower panel and data not shown). To test whether the B cell activation is also reflected by altered Ig production, we determined the concentration of antibodies of various isotypes in the sera of nonimmunized wild-type and *CCR7* mutant mice at the age of 8 weeks. We observed comparable levels of IgM and IgG2b antibodies, but we found increased levels of IgG1, IgG2a, and IgE in CCR7-deficient mice. In contrast, the amount of IgG3 antibodies in *CCR7*$^{-/-}$ mice was significantly reduced (Figure 3d). These data suggest that inappropriate activation of B cells is also reflected by altered isotype switching.

Impaired Migration of Activated Skin DCs into Draining LNs

Upon antigen uptake DCs are activated and, as a consequence, migrate to the T cell zone of draining LNs, where they present antigens to lymphocytes. As the expression of CCR7 is induced during DC maturation (Dieu et al., 1998; Sallusto et al., 1998; Sozzani et al., 1998; Yanagihara et al., 1998), this chemokine receptor might direct the migration of DCs to LNs. In order to detect DCs within epidermal sheaths and LNs, we applied anti-NLDC-145 mAb and anti-IA^{d} mAb immunohistologically. In epidermal sheaths, no differences in number of DCs between wild-type and *CCR7*$^{-/-}$ mice could be observed (data not shown). However, in LN sections, interdigitating DCs were readily present in the T cell zone of wild-type mice, but only few, morphologically altered

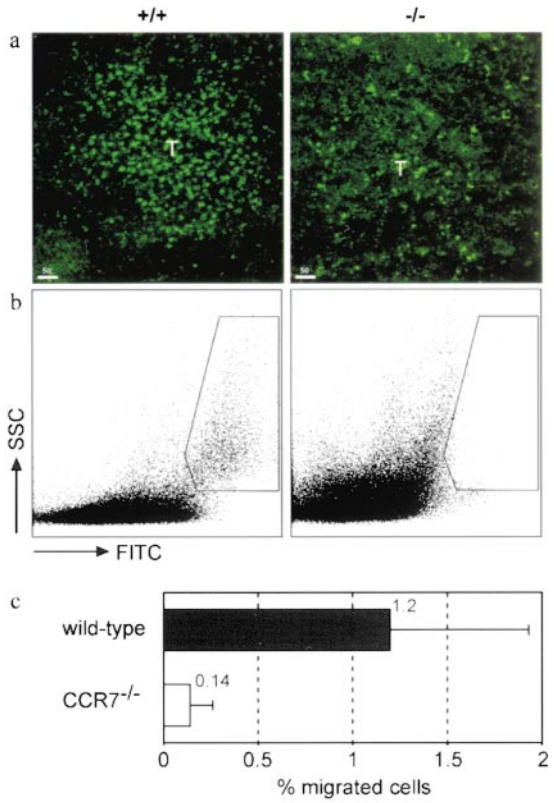

Figure 4. CCR7 Deficiency Affects DC Migration

(a) Reduced numbers and altered morphology of DCs in LN T cell areas. Cryostat sections of MLNs from wild-type (+/+) and *CCR7*$^{-/-}$ (−/−) were stained with anti-NLDC-145 mAb (green).

(b) Impaired migration of skin DCs to LNs following contact sensitization. Mice were painted with FITC for 24 hr before single-cell suspensions of draining LNs were analyzed by flow cytometry. Reduced numbers of *CCR7*$^{-/-}$ skin-derived DCs migrated to the LNs can be seen in the boxed areas (right). Cells located in the boxed area also express high levels of MHC II as determined by counter staining with anti-IA^{d}-PE mAb (data not shown).

(c) Same as (b). The average percentages of DCs identified in the boxed area are shown (mean ± SD, n = 18 LNs).

DCs could be identified in *CCR7*$^{-/-}$ mice (Figure 4a). Contact sensitization induced by FITC skin painting is known to induce DC maturation and mobilization (Hill et al., 1990). Consequently, a considerable amount of DCs residing in the LNs was found in wild-type mice following skin painting (Figures 4b and 4c), whereas only a statistically irrelevant number of DCs could be observed in the corresponding LNs of *CCR7*$^{-/-}$ mice (Figures 4b and 4c). These results could suggest that CCR7 plays a pivotal role in directing activated skin DCs to their draining LNs.

Impaired Migration of T Cells and B Cells to LNs and PPs

Next we transferred calcein-AM-labeled (green fluorescent) lymphocytes, either derived from wild-type or mutant mice, into recipients by retro-orbital injection. Five hours later, recipients were sacrificed and the frequency of donor B and T cells populating lymphoid organs of the recipients was determined. Slightly increased amounts of transferred T and B cells derived from mutant mice could be identified in the blood and the spleen of wild-type recipient animals (Figures 5a and 5b). However, *CCR7* mutant T cells entered the recipients' LNs and PPs only with a frequency of 5% to 25% of that observed after transfer of wild-type cells (Figure 5a). Although less pronounced, a similar pattern could be observed analyzing the migration of naive, IgD^{+} B cells. Compared to wild-type B cells, *CCR7*$^{-/-}$ B cells migrated into wild-type LNs and PPs with a frequency of 20%–50% (Figure 5b). To rule out the possibility that impaired B and T cell migration to lymphoid organ is also due to a defect on stroma cells, we performed similar experiments by transferring wild-type lymphocytes either into CCR7-deficient or into wild-type recipients. Both, wild-type B and T cells populate LNs and PPs of CCR7-deficient recipients even at an even higher frequency than they migrate to wild-type organs (Figures 5c and 5d). As this might be due to differences in size and architecture of secondary lymphoid organs of wild-type and mutant mice, we determined the number of B and T cells present in lymphoid organs. As demonstrated in Figure 5e, the amount of both CD4^{+} and CD8^{+} T cells is strongly reduced in LNs of *CCR7*$^{-/-}$ mice. In contrast, these animals show increased levels of T cells in blood and spleen. Taken together, these data support our hypothesis that the impaired migration of B and T cells is not caused by defective stroma cells but is due to the lack of CCR7 on lymphocytes.

CCR7 Retains B Cells in the PALS

Although CCR7-deficient lymphocytes migrated with high frequency to the spleen of wild-type recipients (compare Figure 5a and 5b), we were interested to see whether wild-type and mutant T and B cells migrate to the same microenvironments. Using MACS-purified donor cells, confocal microscopy revealed essential differences in the location of transferred cells depending on type (B cells versus T cells) and origin (wild-type versus mutant) of the donor cells. Upon 5 hr of transfer, the vast majority of T cells derived from wild-type animals had distributed uniformly to the PALS and only very few cells migrated to the B cell follicles (Figure 6a). In contrast, T cells isolated from *CCR7* mutant mice failed to migrate to the PALS but could be identified within the sinuses and the red pulp (Figure 6b). These data demonstrate that the aberrant location of T cells within the spleen is most likely due to impaired migration of T cells to the PALS. Furthermore, as expected, B cells, derived from wild-type donors, migrated to the outer PALS (see arrows in Figure 6c) and to the adjacent B cell follicles. However, the PALS was almost devoid of donor B cells of *CCR7*$^{-/-}$ origin, but a large amount of these cells could be detected in the follicles (Figure 6d). To test whether *CCR7*$^{-/-}$ B cells fail to migrate into the PALS of wild-type recipients or whether they rapidly leave this microenvironment due to lack of CCR7, we analyzed the location of these cells at earlier points of time after transfer. After 1 hr, both wild-type and deficient B cells could be identified in the recipients' PALS (Figures 6e and 6f), suggesting that CCR7 deficiency does not substantially interfere with the ability of B cells to migrate to this area. In order to determine the distribution of donor B cells quantitatively, we calculated the

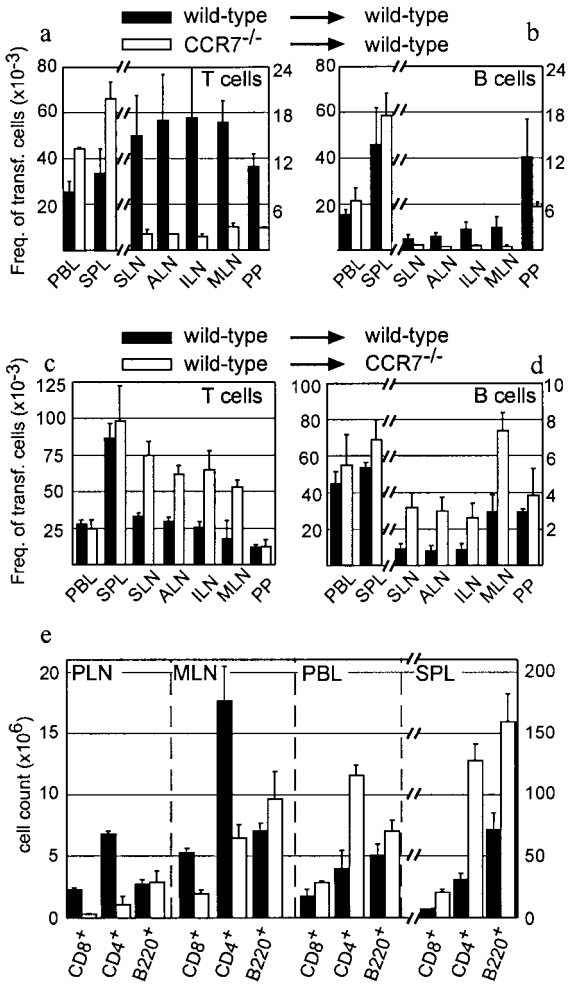

Figure 5. Impaired Migration of CCR7-Deficient Lymphocytes

(a and b) Impaired homing of *CCR7*$^{-/-}$ T (a) and B cells (b) into LNs and PPs. Calcein-AM-labeled lymphocytes (green fluorescent), either derived from wild-type (filled bars) or CCR7-deficient (open bars) mice were adjusted to contain equal amounts of IgD$^+$ or Thy1$^+$ cells. Approximately 7 $\times$ 10^7 cells were transferred into wild-type recipients by injection into the retro-orbital venous plexus. After 5 hr, cells were isolated from different lymphoid organs of sacrificed animals, stained with antibodies against Thy 1.2-Cy5 or IgD-biotin/SA-APC. At least 100,000 cells were examined and analyzed by flow cytometry. Shown are the frequencies of donor T (a) and IgD$^+$ B cells (b) identified within recipient organs (mean $\pm$ SD of data derived from three recipients; similar results were obtained from three to five further recipients).

(c and d) Unaffected migration of wild-type T (c) and B (d) cells in CCR7-deficient recipients. Using the same experimental procedure as described for (a) and (b), 1 $\times$ 10^8 wild-type lymphocytes were transferred either into wild-type recipients (filled bars) or CCR7-deficient mice (open bars; mean $\pm$ SD derived from three recipients of each group).

(e) Reduced numbers of T cells in CCR7-deficient LNs. Based on leukocyte counts and on their relative distribution, the number of CD8$^+$, CD4$^+$, and B220$^+$ cells present in various organs or per milliliter of blood of wild-type (filled columns) and *CCR7*$^{-/-}$ (open columns) has been determined. Data shown for PLNs derived from SLNs, but similar distributions were also obtained from brachial LNs, ALNs, and ILNs (mean $\pm$ SD; n = 3, 10-week-old female animals; PBL, peripheral blood leukocytes; SPL, spleen; SLN, submandibular lymph node; ALN, axilary lymph node; ILN, inguinal lymph node; MLN, mesenteric lymph node; PP, Peyer's patches).

relative distribution of these cells 5 hr after transfer (see Experimental Procedures). Analyzing several large follicles and the adjacent PALSs (such as shown in Figures 6c and 6d), we could not observe any difference with regard to the number of donor cells that had migrated to the white pulp (i.e., to follicle and PALS [wild-type donor: 40 cells/10,000 μm^2 $\pm$ 14; CCR7-deficient donor: 32 cells/10,000 μm^2 $\pm$ 11; mean $\pm$ SD, n = 14 and 16 follicles from two recipients of each group, p = 0.1811, Mann-Whitney test, data not shown]). However, we found significant differences with regard to the localization of donor cells. Sixty-seven percent of wild-type but only 19% of *CCR7* mutant B cells could be identified in the PALS (Figure 6g). Taken together, these data suggest that CCR7 expression is required to keep B cell for a defined period of time in the outer PALS.

CCR7-Deficient Mice Lack Primary T Cell Response

As CCR7 deficiency affects the migration of lymphocytes and DCs to lymphoid organs, we tested *CCR7*$^{-/-}$ mice for their ability to mount a primary T cell response. We used two models to address this question: contact sensitivity (CS; Figure 7a) and delayed type hypersensitivity (DTH; Figure 7b). In both models mice were exposed to a T cell–dependent antigen either by epicutaneous application (CS) or by s.c. injection (DTH). Four days later, the same antigen was applied to the right ear either by s.c. injection (DTH) or by epicutaneous application (CS). In both models wild-type mice exhibited a strong immune response after 24 hr, resulting in profound ear swelling (Figures 7a and 7b, filled symbols). In contrast, *CCR7* mutant mice completely failed to mount any primary T cell response lacking DTH and CS reactions (Figures 7a and 7b, open symbols). Similar results were obtained when assessing ear swelling after 48 hr (data not shown).

Impaired Primary Humoral Response in CCR7-Deficient Mice

We next tested whether mutant mice develop an appropriate humoral response toward the T-dependent antigen DNP-KLH. When analyzing the primary immune response 10 days after the first injection, wild-type mice produced approximately 100$\times$ more DNP-specific antibodies of IgG1, IgG2a, IgG2b, and IgG3 isotype, compared to CCR7-deficient mice (Figure 7c, rhomboid spheres). However, analyzing the same animals another 10 days later, *CCR7*$^{-/-}$ mice produced nearly as much DNP-specific antibodies as the control group (Figure 7c, squares). Furthermore, 14 days after the booster immunization (which was done at day 21), comparable levels of anti-DNP antibodies of IgG1 and IgG3 isotype were found in both groups, whereas CCR7-deficient mice produced on average even higher levels of IgG2a and IgG2b antibodies specific for DNP (Figure 7c, circles). Analyzing serum at day 86, we still observed a robust immune response in both groups, although levels of DNP-specific antibodies dropped slightly faster in

Please note that all panels except (c) have two different scales for the y axis. Numbers on the right-hand axis should be used for all data to the right of the -//- symbols.

CCR7$^{-/-}$ mice (Figure 7c, triangles). These data demonstrate that CCR7-deficient mice show a marked delay in IgG isotype switching after immunization with a T cell–dependent antigen.

Discussion

Secondary lymphoid organs are placed at strategic locations of the immune network to enable a rapid and effective immune response toward an antigenic challenge. It is believed that their distinguished microarchitecture which segregates both lymphoid and nonlymphoid cells into various characteristic areas in order to constitute follicles, GC, T cell–rich areas as well as different types of sinuses is required for the effective initiation and controlled maintenance of the immune reaction. Although the importance of chemokines and their receptors for the recruitment of inflammatory cells has been well established (reviewed in Rollins, 1997; Baggiolini, 1998), recent evidence suggests that the chemokine system might also trigger essential steps in lymphocyte trafficking and homeostasis. In accordance with this idea, a group of chemokines has been identified that are not induced following inflammation but that are expressed constitutively at high levels in lymphoid organs. Among others, this family includes ELC (Yoshida et al., 1997) and secondary lymphoid organ chemokine (SLC) (Nagira et al., 1997; Yoshida et al., 1998), both ligands for CCR7, as well as the B lymphocyte chemoattractant (BLC) (Gunn et al., 1998a) and its human homolog B cell–attracting chemokine 1 (BCA-1) (Legler et al., 1998). The expression of BLC, which is a ligand for CXCR5, has been identified within the B cell follicles, and it has been suggested that follicular dendritic cells (FDCs) might be a source of this factor. Interestingly, mice deficient for the chemokine receptor CXCR5/BLR1 show an altered architecture of B cell follicles in spleen and PPs that is caused by impaired migration of B cells to these microenvironments (Förster et al., 1996). Taken together, this data would fit into a model that a locally produced chemokine is required to attract certain cells to form a functional lymphoid microenvironment—the B cell follicle, in the particular case of CXCR5/BLC. A more general importance of chemokines and their receptors in establishing a functional microarchitecture of lymphoid organs is provided by the present study.

CCR7-deficient mice showed marked disturbances in the distribution of T cells, since expanded populations of naive T cells were present in the blood, but a severe deficiency of this cell population was encountered in the T cell areas of all secondary lymphoid organs (Figures 1, 2, and 6). Impaired T cell migration, being the most likely cause for the disturbed T cell distribution in *CCR7* mutant mice, could be confirmed by adoptive transfer experiments (Figures 5a and 6b). These data strongly suggest that CCR7 is a homing receptor for naive T cells. As outlined above, it is believed that naive T cells preferentially migrate into LNs and PPs via HEV. Interestingly, one of the ligands for CCR7, SLC, is expressed on HEV in PPs and LNs and on stroma cells within the T cell zones of the spleen, LNs, and PPs (Tanabe et al., 1997; Gunn et al., 1998b; Willimann et al., 1998). The expression of ELC, another ligand for CCR7, has been identified on interdigitating DCs in the T cell zone (Ngo et al., 1998). As described for SLC (Campbell et al., 1998a; Gunn et al., 1998b), ELC attracts B and T cells (Ngo et al., 1998) and mature DCs become responsive to both chemokines (Dieu et al., 1998; Sallusto et al., 1998; Sozzani et al., 1998; Yanagihara et al., 1998).

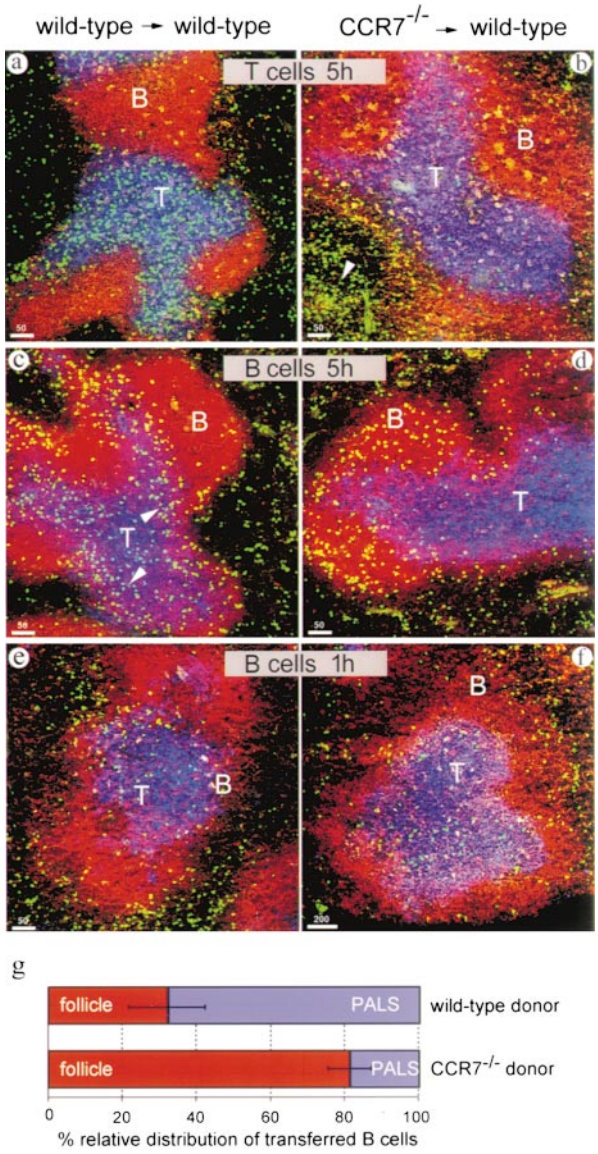

Figure 6. Impaired Retention of CCR7-Deficient B Cells in the PALS

T cells (a and b) or B cells (c–f) were isolated by MACS from LNs and spleen (a and b) or the spleen (c–f) of wild-type (a, c, and e) or *CCR7* mutant (b, d, and f) mice 25 days old. After labeling with Calcein-AM, cells were transferred to wild-type recipients for 5 hr (a–d) or for 1 hr (e and f). Cryostat sections of the spleen were stained with anti-B220-biotin/streptavidin-TRITC (red) and Thy1-Cy5 (blue) to determine B cell follicles and the PALS, respectively. Transferred cells could be identified by strong green fluorescence (B, B cell follicle; T, T cell area, PALS). (g) Five hours after transfer, the relative distribution (cells/unit of square measure) of donor B cells within the follicle and PALS was determined by image analysis; shown are percentages of cells within the follicle (red bars) and PALS (blue bars; data derived from 14 and 16 confocal sections of each two recipients; similar data were obtain from another experiment).

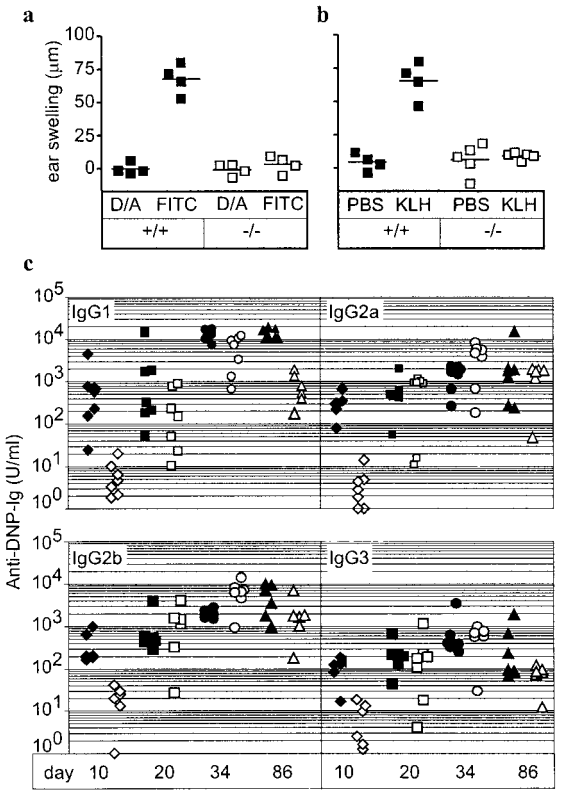

Figure 7. Impaired Primary T and B Cell Response in CCR7-Deficient Mice

(a and b) Lack of CS (a) and DTH (b). Wild-type mice (+/+, filled symbols) or *CCR7* mutant mice (−/−, open symbols) were sensitized by epicutaneous application of FITC in dibutylphtalate/acetone (D/A) (a) or by s.c. injection of KLH (b). Four days later, animals were reexposed to the same antigen either by s.c. injection of KLH or epicutaneous application of FITC on the right ear. The left ear of each animal received the carrier alone. Twenty-four hours later, the ear swelling was determined using a spring-driven dial gauge.

(c) Lack of primary IgG response toward T cell-dependent antigens. $CCR7^{+/+}$ mice (filled symbols) and $CCR7^{-/-}$ mice (open symbols) were immunized with 100 μg DNP-KLH and challenged with the same amount of antigen 3 weeks later. At the points of time indicated (days after the initial immunization), DNP-specific antibodies of the indicated isotypes present in sera were determined by ELISA.

Therefore, the expression pattern of both chemokines together with their capacity to induce chemotaxis of B cells and T cells renders SLC and ELC as potential candidates controlling the lymphocyte trafficking through HEV and the T cell zone. The data obtained from CCR7-deficient mice would support this hypothesis.

Nakano et al. (1998) reported a mutation in mice, paucity of lymph node T cells (*plt*), causing impaired homing of naive T cells to secondary lymphoid organs, thus resembling the phenotype of $CCR7^{-/-}$ mice with regard to T cell homing. Recently, it could be shown that *plt* mice lack expression of SLC, although the DNA sequence of SLC introns and exons is not affected in *plt* mice. In addition, these animals show reduced levels of ELC (Gunn et al., 1999). However, since the molecular basis of the *plt* mutation has not been identified, it might well be that this mutation affects other genes besides ELC and SLC.

In $CCR7^{-/-}$ mice, T cell migration is not only affected at the level of entry of naive cells through HEV but also at the level of positioning of those T cells that have managed to enter lymphoid organs. Most of these cells probably entered via afferent lymphatic but failed to populate those areas where they can be found in wild-type animals. Instead they settled close to the place of entry (i.e., the marginal sinuses of LNs and spleen [Figures 2b and 2h]). Because similar histological data were obtained from *plt* mice (Nakano et al., 1998), there is now strong evidence that CCR7 and its ligands are not only required for guiding naive T cells through HEV but also for directing T cells to their corresponding functional microenvironment once having entered via afferent lymphatics.

$CCR7^{-/-}$ mice also show several functional and morphological features that suggest an important role of this chemokine receptor concerning B cell migration and activation. The process of B cell activation is critically dependent on the proper action of T cells and DCs, both of which are severely affected in CCR7-deficient mice. Therefore, several alterations observed with B cells, especially those concerning the activation stage, cannot be distinguished as being a consequence of a B cell defect per se or being due to impaired T cell and DC function. As young mice (3 weeks of age) harbor activated B cells only in PLNs but not in blood or spleen, it seems likely that B cell activation is initiated to compensate impaired B cell help. As B cells, T cells, and DCs do not segregate into defined microenvironments in *CCR7* mutant mice, it seems equally possible that B cells might get inappropriately stimulated by dislocated T cells. The impaired activation of B cells is also reflected by changes in serum levels of IgG isotypes (Figure 3d).

Adoptive transfer experiments demonstrated that CCR7 deficiency directly interfered with B cell functions. CCR7-deficient B cells entered LNs and PPs only with a markedly reduced frequency (20%–50% of normal; Figure 5b). This observation is in agreement with previous findings demonstrating expression of CCR7 on B and chemotaxis of B cell toward ELC and SLC (Schweickart et al., 1994; Burgstahler et al., 1995; Campbell et al., 1998b; Gunn et al., 1998b; Ngo et al., 1998). As regular numbers of B cells are found in LNs of CCR7-deficient mice (Figure 5e), it seems that other mechanisms can compensate over time for the reduced migration of B cells we observed in our short-term migration experiments.

In addition to the defect in B and T cell homing, we also found that skin DCs are severely impaired in migrating to the draining LNs following activation (Figure 4). Responsiveness to ELC and SLC as well as the induction of CCR7 expression have been reported during final maturation of activated DCs (Dieu et al., 1998; Sallusto et al., 1998; Sozzani et al., 1998; Yanagihara et al., 1998). Interestingly, impaired migration of skin DCs into draining LNs has also been reported in *plt* mice (Gunn et al., 1999). Although we could not observe any difference in the number of skin DCs in $CCR7^{-/-}$ mice versus wild-type mice, it remains currently an open question whether the lack of mobilized $CCR7^{-/-}$ DCs in draining LNs is due to impaired mobilization of DCs to leave the skin or due to impaired migration into the regional LNs. Data

derived from *plt* mice would argue for the latter possibility because DCs can be mobilized in the skin but cannot adequately colonize the draining LNs in these animals (Gunn et al., 1999).

Data derived from homing experiments of purified CCR7-deficient B cells colonizing the spleen of wild-type recipients point to a novel function of the chemokine system: temporary retention and positioning of B cells in T cell areas. The outer PALS has been identified as a place of intensive interaction of various cell types including B cells, T cells, and activated DCs. This region seems to be essential for B differentiation, as it has been identified as an area where B cells undergo antigen-driven selection and activation as well as deletion (for review, Liu, 1997). It is currently unknown whether B cells circulate through this T cell–rich area at random, or whether molecular mechanisms are responsible for guiding and keeping B cells for a defined period of time in the outer PALS to allow an effective interaction with antigen-specific T cells. Our observation that adoptively transferred CCR7-deficient B cells are not retained in the outer PALS (compare Figure 6d) but rapidly migrate to the adjacent follicle suggests that CCR7 allows positioning and temporary retardation of B cells to this area. As ELC and SLC are produced by various cells in the T cell zone (Ngo et al., 1998), it is reasonable to propose that B cells, once having entered the marginal sinus, might follow these chemokine gradients which would keep them in the PALS. This would allow to establish an encounter of B and T cells specific for the same antigen. During this process, B cells would become increasingly unresponsive to ELC as binding of the chemokine induces desensitization of its receptor (Ngo et al., 1998). At this stage, B cells might follow the BLC/BCA-1 gradient, which would guide them into the B cell follicle. Our finding that CXCR5-deficient B cells accumulate in the outer PALS stands in accordance with this model (Förster et al., 1996).

The complex phenotype of CCR7-deficient mice is most apparently reflected by the lack of fast primary B and T cell responses. Following application of T-dependent antigens, *CCR7* mutant mice fail to produce specific antibodies of any IgG isotype within the first 10 days. In addition, *CCR7*$^{-/-}$ do not develop DTH and CS reactions (Figure 7). Although several steps can be imagined where CCR7 deficiency affects a T cell–dependent B cell response, we suggest that the impaired antigen presentation due to inadequate migration of naive T cells, B cells, and antigen-bearing DCs to secondary lymphoid organs is most likely the prime cause. The same defect seems to be responsible for the absence of a primary T cell response in *CCR7* mutant mice.

Thus, by bringing together T cells, B cells, and DCs to form functional microenvironments in secondary lymphoid organs, CCR7 has been identified as a major homing receptor and important regulator for initiating an antigen-specific immune response.

Experimental Procedures

Construction of *CCR7*$^{-/-}$ Mice

Gene targeting was done in accordance to established methods (Capecchi, 1989). A 0.5 kb genomic fragment of the third exon of *CCR7* encompassing Ser-139 to Asp-309 was disrupted by insertion of the neomycin resistance gene. The herpes simplex thymidine kinase gene was fused to the 5′ end. The construct was introduced into E14K ES cells by electroporation. Culturing of ES cells was done as described previously (Förster et al., 1996). A homologous recombinant line was injected into BALB/c blastocysts, which were transferred into pseudopregnant CD-1 females. Chimeric males were bred to BALB/c, and offspring were tested by Southern blot analysis using probe A as depicted in Figure 1a.

Antibodies

In addition to antibodies described elsewhere (Förster et al., 1996), the mAb directed against CD62L and MHCII (anti-IA^d) and CD44 were purchased from PharMingen and Sigma, respectively.

Immunohistology and Flow Cytometry

Mice were analyzed by flow cytometry and immunohistology as described earlier (Förster et al., 1994, 1996). In addition, immunohistology using CD62L mAb was done by enhancing signals with the tyramide signal amplification (NEN DuPont). Slides were analyzed by confocal microscopy (Leica, TCS, 4D-I).

Isotype and Antigen-Specific ELISAs

Eight-week-old animals were immunized subcutaneously at the level of the tail root with 100 μg DNP-KLH and boosted 3 weeks later with the same amount of antigen. DNP isotype–specific antibodies were determined by serial dilutions of serum on 96-well plates coated with DNP-BSA. Antibodies bound to the plates were revealed with peroxidase-conjugated isotype–specific antibodies (Amersham and PharMingen) and diaminobenzimidine (DAB, Sigma). Titers were calculated by nonlinear regression analysis. To determine serum-immunoglobulin-isotype levels, serum samples, as well as control monoclonal antibodies, were serially diluted on microplates, either coated with unlabeled anti–light chain antibodies or isotype-specific antibodies followed by incubation with peroxidase-conjugated isotype–specific antibodies.

Lymphocyte Migration

For transfer experiments, lymphocytes were isolated by mechanical disruption of the spleen and MLNs from wild-type or *CCR7* mutant mice. Following hypotonic lysis of erythrocytes, cells were labeled for 2 min at 37°C with 3 μM Calcein-AM (Molecular Probes). Washed cells were adjusted to contain equal numbers of IgD$^+$ or Thy1$^+$ cells and were injected into the retro-orbital venous plexus of recipient animals; 5 hr later, they were sacrificed and lymphocytes were isolated from blood, spleen, PPs, and LNs and stained with antibodies against IgD-biotin or B220 (Cychrome) or Thy1 (Cy5). Based on the bright green fluorescence of Calcein labeling and far red labeling of the antibodies, the frequency of transferred T and B cells was determined by flow cytometry (FACSCalibur, Becton Dickinson) and was expressed as the amount of double positive cells identified within 1000 lymphoid cells analyzed. To determine the microenvironmental homing properties of lymphocytes to the spleen, B and T cells were purified using MACS. The purity and viability of isolated cells were analyzed and always >92% and >95%, respectively. To determine the relative distribution of transferred B cells within the PALS and the follicle, we subjected confocal images of the spleen to image analysis. We applied ImageSpace software (Molecular Dynamics) to determine the size of both follicle and PALS and counted the absolute number of transferred cells within both areas. Based on this information, the relative distribution of donor cells (cells/unit of square measure) was calculated.

Chemotaxis Assay

Spleen cells (5×10^5) in 100 μl medium were loaded into collagen-coated transwells (Costar, 3 μm pore filter), which were placed to 24-well plates containing 450 μl medium supplemented with 100 ng/ml of ELC or SDF-1 (R&D Systems). After an incubation period of 2 hr at 37°C, cells that had migrated to the lower chamber were collected and counted.

Skin Painting

The clipped ventral abdomen and thorax were stained with 450 μl FITC (5 mg/ml dissolved in equal volumes of dibutylphtalate and

acetone; D/A). After 24 hr, cell suspensions of single LNs (inguinal, axillary, brachial) were stained with anti-IA^d-PE mAb to determine the MHC II expression on FITC-positive DCs.

Primary T Cell Response

To assess contact sensitivity reactions, mice were painted with FITC as described above. Four days later, 30 μl of FITC in D/A were applied to the right ear, whereas carrier alone was applied to the left ear. Ear thickness was determined before and 24 hr and 48 hr after painting using a spring-driven dial gauge. DTH against DNP-KLH was induced by immunizing mice subcutaneously (s.c.) with 100 μg DNP-KLH in CFA. After 4 days, 25 μg KLH in PBS was injected s.c. into the right ear and PBS into the left ear. Ear swelling was determined as above.

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