CXCR5-Dependent Seeding of Follicular Niches by B and Th Cells Augments Antiviral B Cell Responses

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CXCR5-Dependent Seeding of Follicular Niches by B and Th Cells Augments Antiviral B Cell Responses

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The chemokine receptor CXCR5 and its ligand CXCL13 define the structure of B cell follicles within secondary lymphoid organs. Here, we examined the impact of CXCR5 on antiviral B cell responses in vivo. CXCR5−/− mice showed a normal production of IgM and IgG acutely after infection with vesicular stomatitis virus (VSV) and developed VSV-specific germinal centers. However, impaired Ig class switch and Ab production were observed under conditions of limited availability of Ag (i.e., after immunization with nonreplicating viral particles or soluble Ag). Adoptive transfer of CXCR5-deficient, VSV-specific B and Th cells demonstrated that CXCR5 expression on both B and Th cells is required for an efficient Ig class switch. These experiments revealed that CXCR5 is critical for the coordinated interaction of antiviral T and B cells through its impact on initial B cell expansion and the recruitment of Ag-specific B and Th cells to germinal centers.

A coordinated interaction of CD4+ Th and B cells is crucial for efficient Ig production (1). The collaboration of Ag-specific Th and B cells in secondary lymphoid organs depends on distinct chemokine-chemokine receptor interactions, such as CXCR5 and its ligand chemokine CXCL13 (2). CXCL13 is expressed in B cell follicles (3), and CXCR5 is expressed by naive B cells and a subpopulation of activated CD4+ helper T cells (4). After initial activation in the marginal zone of the spleen, B cells migrate to the B cell zone through down-regulation of S1P1 (5) and then to the border of T and B cell zones through up-regulation of CCR7 (6). After differentiation to plasma cells, B cells lose CCR7 and CXCR5 expression and thus leave secondary lymphoid organs (7). CD4+ T cells up-regulate CXCR5 during activation and localize near B cell follicles (8). These CD4+ CXCR5+ Th cells were shown to enhance the production of IgG and IgA and, to a lesser extent, of IgM in vitro (9, 10) and were thus termed “follicular helper T cells.” Contradictory findings in CXCR5-deficient mice, however, suggested that T-B collaboration is not affected by the lack of this chemokine receptor: CXCR5−/− mice produce normal levels of switched Ig isotypes in vivo after immunization with DNP-keyhole limpet hemocyanin in adjuvant (4), and B cells of CXCR5−/− mice underwent normal affinity maturation when 2-phenyl-oxazolone was used as an Ag (11).

We investigated this discrepancy in well characterized viral systems, by infecting CXCR5−/− mice with the cytopathic vesicular stomatitis virus (VSV), or with the non-cytopathic lymphocytic choriomeningitis virus (LCMV), or by immunizing CXCR5−/− mice with nonreplicating VSV-derived Ags (12). The production of neutralizing Abs in the acute phase of VSV infection that are known to be of high affinity was not impaired in CXCR5−/− mice. However, antiviral B cell responses were significantly reduced in CXCR5−/− mice under conditions of low antigenic doses. These impaired B cell responses were caused by the combined effect of CXCR5 deficiency on both Th and B cells. Our results from adoptive transfer experiments with CXCR5-deficient, VSV-specific Th and B cells indicate that CXCR5 increases the efficacy of T-dependent (TD) B cell responses by 1) fostering an efficient Ag-specific expansion of B cells in secondary lymphoid organs before the Ig class switch and 2) attracting Ag-specific B and Th cells to germinal centers (GCs) in the late phase of the immune response.

Materials and Methods

Mice

All mice were bred at the Institut für Laboratoriumsdiagnostik (University of Zürich, Zürich, Switzerland). B6PLT.1.1 and B6.Cg-H2b/H-Tg1/Cips1/Cri (H2b−/−) mice were obtained originally from The Jackson Laboratory. CXCR5−/− mice, nine times backcrossed to 129Sv, were PCR typed as described previously (4). Mice bearing a knock-in construct for the VSV-neutralizing Ab VI10 in their Ig locus (VI10) (13) and TCR-transgenic mice with the T cell epitope p8 of VSV (L7) (14) have been described previously. Activation-induced deaminase knockout mice (AID−/−) (15) were a gift from S. Fagarasan (Kyoto University, Kyoto, Japan). For adoptive transfer experiments, VI10 and L7 mice were crossed to CXCR5−/− mice that were backcrossed onto the C57BL/6 background for 11 generations.

Viruses and immunizations

The LCMV, WE strain, was obtained originally from Dr. F. Lehmann-Grube (Heinrich-Pette-Institut, Hamburg, Germany) and propagated as described previously (16). The VSV, Indiana strain (VSV-IND; Mudd-Summers isolate), was obtained originally from Prof. D. Kolakofsky

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Abs and flow cytometry

Abs for flow cytometry and fluorescence microscopy were purchased from BD Pharmingen, unless stated otherwise. Strepavidin-tetramethylrhodamine isothiocyanate (TRITC) was purchased from Southern Biotechnology Associates. Goat anti-mouse CXCL13 was obtained from R&D Systems, and donkey anti-goat IgG was obtained from Jackson ImmunoResearch Laboratories. The Ab 35.61 specific for a combination of heavy and light chains of the V110 Ab was produced as described previously (13). Aliquots of 5 × 10^6 cells or three drops of blood were stained in FACS buffer (PBS, 2% FCS, 20 mM EDTA, and 0.03% NaNO_3) at 4°C for 20 min. Before analysis of peripheral blood, erythrocytes were lysed with FACS Lysing Solution (BD Pharmingen). For intracellular staining of IgM, splenocytes were surface-stained with Abs against CD138 and B220, washed, fixed for 10 min at room temperature with 1% formalin in FACS buffer, incubated for 10 min at room temperature with permeabilization buffer (PB; FACS buffer containing 0.1% saponin (Sigma-Aldrich)), and incubated with anti-mouse IgM in PB. Cells were washed twice in PB and resuspended in FACS buffer. For staining of B cells, cells were analyzed by cotransfer of each 10^6 CXCR5-deficient or -competent, MACS-purified B_220^+ B cells (Milenyi Biotec) labeled with 0.1 μM and 1 μM CFSE (Molecular Probes). Eighteen hours after transfer, peripheral blood and spleen homogenates were stained with B220-APC and analyzed with a FACScalibur flow cytometer (BD Biosciences) using the CellQuest (BD Biosciences) software.

Detection of IgGs from adoptively transferred V110 B cells by serum ELISA

For the selective detection of VSV-specific IgM of the V110 Id, which was derived from adoptively transferred, purified B_220^+ cells of V110 mice, ELISA plates were coated overnight with 5 μg/ml of the 35.61 anti-idiotypic Ab (see above). After blocking with PBS and 5% BSA for 1 h at room temperature and washing, plates were incubated with serial 3-fold dilutions of sera in PBS, starting at a 1/50 dilution. After washing, HRP-coupled anti-mouse IgM (Sigma-Aldrich) was added for 1 h at room temperature. ELISAs were developed with 0.1 mg/ml ABTS (Roche) in 0.1 M phosphate buffer (pH 4.0), in the presence of 6%_H_2O_2. Plates were read at 405 nm.

For the detection of VSV-specific IgGAb or IgG_2b of the V110 Id, plates were coated overnight with 2 μg/ml VSV-IND particles, blocked, and incubated with 3-fold serum dilutions starting at a 1/30 dilution. After extensive washing, biotin-labeled rat anti-mouse IgG_2a or IgG_2b (BD Pharmingen) was added, followed by streptavidin-HRP (Jackson ImmunoResearch Laboratories) and ABTS substrate solution. The turning point of the sigmoid absorbance curves was determined as ELISA titer.

Virus neutralization test

VSV-neutralizing Ab titters of sera were determined as described previously (18). Sera were prediluted 40-fold in MEM and 2% FCS, and the highest dilution of the serum that reduced the number of plaques by 50% was taken as the neutralizing titer. To determine IgG titters, undiluted serum was incubated before the assay for 1 h at room temperature with an equal volume of 0.1 M 2-ME in PBS. LCMV-specific neutralizing activity was measured from serum of infected mice using a focus reduction assay (16).

Immunohistochemistry and fluorescence microscopy

Freshly removed organs were immersed in HBSS and snap frozen in liquid nitrogen. Five-micrometer tissue sections were air dried, fixed with acetone for 10 min, and stored at −70°C. Alkaline phosphatase was visualized using naphthol As-Bi phosphate and New Fuchsin (all from Sigma-Aldrich) as substrate.

UV-inactivated VSV and recombinant VSV-G are nonreplicating Ags, if administered i.v., induced a strong TI neutralizing IgM response, reflecting a functional direct B cell activation in CXCR5−/− mice (Fig. 1, Ji and Jii). In contrast, CXCR5−/− mice were not able to efficiently switch their Ig isotype to IgG after immunization with these preparations (Fig. 1, Ji and Jii). We thus concluded that CXCR5 deficiency influences the induction of TD B cell responses under conditions of limiting Ag availability. A similar impairment in the production of virus-neutralizing IgG was observed for CXCR5−/− mice at late time points after infection with the noncytopathic LCMV (Fig. 1Jii).

Role of CXCR5 deficiency on T vs B cells for TD B cell responses

The limited TD B cell responses under conditions of limited availability of Ag can be caused by three parameters: by the altered ability of Ag can be caused by three parameters: by the altered (University of Geneva, Geneva, Switzerland). For some experiments, inactivated VSV-IND was obtained using UV irradiation (7UV 15W; Philips). Recombinant VSV-glycoprotein (VSV-G) was obtained from a culture of Spodoptera frugiperda 9 cells after infection with a recombinant baculovirus (17).

Impaired Ig class switch in CXCR5−/− mice in response to limiting amounts of viral Ag

UV-inactivated VSV and recombinant VSV-G are nonreplicating VSV-derived Ag preparations with UV-inactivated VSV maintaining a high level of structural organization (12). Both Ags, if administered i.v., induced a strong TI neutralizing IgG response, reflecting a functional direct B cell activation in CXCR5−/− mice (Fig. 1, Ji and Jii). In contrast, CXCR5−/− mice were not able to efficiently switch their Ig isotype to IgG after immunization with these preparations (Fig. 1, Ji and Jii). We thus concluded that CXCR5 deficiency influences the induction of TD B cell responses under conditions of limiting Ag availability. A similar impairment in the production of virus-neutralizing IgG was observed for CXCR5−/− mice at late time points after infection with the noncytopathic LCMV (Fig. 1Jii).

Results

Antiviral B cell responses in CXCR5-deficient mice

To evaluate the consequences of CXCR5 deficiency for B cell responses, CXCR5−/− and 129Sv mice were i.v. infected with 2 × 10^6 PFU VSV-IND, and neutralizing Ab titters were assessed (Fig. 1A). TD VSV-neutralizing IgG responses were comparable during the acute phase in CXCR5−/− mice and controls (Fig. 1Aii). CXCR5−/− mice mounted a potent T-independent (TI) IgM response (Fig. 1Aii), and natural Ab titters were normal in CXCR5−/− mice (data not shown), despite reduced numbers of B-1 cells (19). During the memory phase (between days 50 and 220 after infection), VSV-specific neutralizing IgG titters declined more rapidly in CXCR5−/− mice, whereas 129Sv controls maintained ≥16-fold higher, stable IgG levels. After a secondary i.v. immunization with 2 × 10^6 VSV-IND, the recall IgG titters reached comparable levels. It is unlikely that the slower decline in IgG levels in 129Sv mice was caused by a prolonged virus persistence in 129Sv mice, because VSV-IND was cleared from both CXCR5−/− and 129Sv mice within the first 4 days after infection (data not shown). It is thus possible that the aberrant follicular structure of CXCR5−/− mice, or a defect in T-B collaboration, was responsible for the observed decline in the memory B cell response.

The TD phase of VSV infection is characterized by formation of VSV-specific GCs (Fig. 1, B, D, F, and H). On day 10 after infection with 2 × 10^6 PFU VSV-IND, CXCR5−/− mice formed peanut lectin (agglutinin)-positive GCs ectopically around the central arteriole (Fig. 1E) containing VSV-specific B cells (Fig. 1C), CD4^+ Th cells (Fig. 1F, arrow), and 4C11^+ follicular dendritic cells (Fig. 1G). These data confirm that Ag-specific GCs can develop in CXCR5-deficient mice (11).
lymphoid architecture in CXCR5−/− mice or by effects of CXCR5 deficiency on B or Th cells. We therefore devised an adoptive transfer system to dissect the role of CXCR5 on B vs Th cells for an optimal Ig class switch in an intact lymphoid environment.

In the first experimental setting, TCRβδ−/− mice served as recipients for VSV-G-specific TCR-transgenic Th cells, L7, or L7 × CXCR5−/− (Fig. 2A). TCRβδ−/− mice do not have a proper T cell zone yet still express wild-type levels of the T cell zone chemokines CCL19 and CCL21 in their spleens (20), potentially allowing for an undisturbed CCR7-dependent homing of naive Th cells. TCRβδ−/− mice were not able to mount neutralizing IgG responses after i.v. immunization with 20 μg of VSV-G (Fig. 2A). However, adoptive transfer of as few as 10⁵ sorted L7 CD4⁺ T cells potently facilitated the induction of VSV-specific IgG in TCRβδ−/− mice. One thousand sorted L7 × CXCR5−/− cells, however, induced only weak specific IgG titers, confirming that the expression of CXCR5 on Th cells plays an important role during Ig class switch.

AID−/− mice (15) that lack the capacity to form GC or switch their Ig isotopes were used as recipients for sorted VSV-specific B cells of VI10 or VI10xCXCR5−/− Ig-knock-in mice. AID−/− mice displayed a normal splenic morphology (data not shown), but their B cells failed to perform Ig class switch after VSV-G immunization (Fig. 2B). Adoptive transfer of only 10⁵ sorted VI10 B cells into these mice was sufficient to induce IgG in AID−/− mice. 10⁶ sorted VI10xCXCR5−/− B cells, however, did not lead to IgG induction in AID−/− recipients, indicating that CXCR5 expression on B cells plays an important role for the induction of IgG in this setting. Deficient expression of CXCR5 on Th and B cells uniformly impaired IgG production, even if higher cell numbers were transferred or if wild-type mice were used as recipients (see Fig. 5M). We therefore conclude that CXCR5 expression on both B and Th cells is necessary for an optimal Ig class switch in vivo.

Expansion of VSV-specific B cells but not Th cells in the spleen is governed by CXCR5 during the TI phase of an antiviral immune response

The frequency of primed B and Th cells in secondary lymphoid organs influences the efficiency of Ig class switch. We therefore examined whether CXCR5 affects the kinetics of B and Th cell expansion during the TI phase of the response through its impact on lymphocyte homing. Because the CXCR5 ligand CXCL13 is abundantly expressed in secondary lymphoid organs and CXCR5 is highly expressed on naive VSV-specific B cells (Fig. 3A), we first assessed whether CXCR5 increases the homing efficiency of naive B cells to the spleen. To this end, we simultaneously transferred equal amounts of CFSE-labeled, CXCR5-deficient (CFSElow) and -competent (CFSEhigh), VSV-specific B cells (Fig. 3B, top panel) into C57BL/6 mice and analyzed spleens (middle panel) and blood (bottom panel) of the recipients 18 h later. This analysis indicates that the distribution of B cells between the spleen and the circulation is not influenced by CXCR5. CXCR5 expression, however, affected the distribution of naive VI10 B cells between B cell follicles and the red pulp with only the CXCR5-positive B cells being attracted to the B cell zone (Fig. 3, C and D). To examine how CXCR5-dependent B cell localization affects early B cell expansion, we infected mice that had received similar numbers of sorted B cells from VI10 or VI10 × CXCR5−/− with VSV and
mice were transfused with $10^3$ MACS-purified B220+ T cells to the T cell zone (Fig. 4, A). In an adoptive transfer system of MACS-sorted, VSV-specific CD4+ cells, in spleens was not caused by an enhanced emigration of these cells but rather by causing a specific localization pattern of CXCR5 expression on T cells in splenic subcompartments that foster optimal B cell expansion, but rather by causing a specific localization pattern of CXCR5 expression on T cells in splenic subcompartments.

CXCR5 was most strongly induced on L7+ Th cells 7 days after infection (i.e., before Ig class switch) (Fig. 4). The strong expansion and localization of specific B cells in the TI phase. On day 2 after infection, V110+ B cells were frequently detected in B cell follicles and in foci at the bridging channels (Fig. 3F), whereas V110 × CXCR5−/− B cells were rare and localized almost exclusively to the bridging channels (Fig. 3F). The histological analysis was corroborated by cytometric analysis on day 4 after infection (i.e., at the end of the TI phase) (Fig. 3F). The expansion of adoptively transferred VSV-specific, B220+ B cells and of B220+CD138+ VSV-specific plasma cells in spleens was reduced by a factor of 3–4 in the absence of CXCR5. To demonstrate that the lower frequency of V110 × CXCR5−/− B cells in spleens was not caused by an enhanced emigration of these cells after an excessive differentiation to plasma cells, we determined serum IgM titers of the adoptively transferred V110 or V110 × CXCR5−/− B cells population and found significantly reduced titers if V110 × CXCR5−/− B cells were transferred (Fig. 3J). This supports the notion that CXCR5 helps localize B cells to splenic subcompartments that foster optimal B cell expansion early after infection and thus leads to an increased number of specific B cells that could potentially establish productive contacts with primed Th cells for class switch.

Th cells up-regulate CXCR5 after their activation in vitro (9, 10) or in vivo (8). In analogy to the situation observed for B cells, CXCR5 expression may cause an altered Th cell localization and expansion during T cell priming. As expected, sorted naive L7+ and L7+ × CXCR5−/− CD4+ T cells showed a comparable homing pattern to the T cell zone (Fig. 4, A and B), because naive Th cells do not express CXCR5 (data not shown). We next determined the activation-induced up-regulation of CXCR5 on VSV-specific Th cells. In an adoptive transfer system of MACS-sorted, VSV-specific CD4+ T cells (L7 × Thy1.1) into C57BL/6 recipients, CXCR5 was most strongly induced on L7 Th cells 7 days after infection with $2 \times 10^6$ PFU VSV (Fig. 4C). In absolute numbers, however, these cells were most frequent already at day 4 after infection (i.e., before Ig class switch) (Fig. 4D). The strong expansion of CXCR5+ Th cells did not lead to overall increased numbers of Ag-specific Th cells in spleens at this time point (Fig. 4E). It is therefore likely that CXCR5 expression enhanced T cell help for Ig class switch (Fig. 2A) not through an effect on Th cell expansion, but rather by causing a specific localization pattern of Th cells in splenic subcompartments.

**FIGURE 2.** Optimal production of serum IgG depends on CXCR5 on B and Th cells in a normal lymphoid environment. A, TCRβδ+ recipient mice were transfused with $10^3$ MACS-purified CD4+ splenocytes of L7 (■) or L7 × CXCR5−/− (□) mice or received no cells (○) and were immunized with 20 μg of VSV-G i.v. B, AID−/− recipient mice were transfused with $10^3$ MACS-purified B220+ splenocytes of V110 (■) or V110 × CXCR5−/− (□) mice or received no cells (○) and were immunized with 20 μg of VSV-G i.v. Data points represent means ± SD (n = 3–4).

**FIGURE 3.** Deficient expansion of CXCR5-deficient B cells before Ig class switch. A, FACS analysis of VSV-specific V110 or V110 × CXCR5−/− B cells for CXCR5. Histograms are gated on B220+35.61+ cells, and shaded histograms represent isotype controls. B, Adoptive co-transfer of $10^6$ CFSE-labeled, MACS-purified B220+ cells from V110 mice (CFSEhigh, right peak) or V110 × CXCR5−/− mice (CFSELow, left peak) into C57BL/6 recipients and analysis of the spleen and blood 18 h after transfer. Histograms were gated on B220+positive cell populations, and numbers represent percentages of CFSEhigh or CFSELow cells, within the gated population. One representative of two similar experiments is shown. C–H, Adoptive transfer of $8 \times 10^5$ B220+ cells of V110 or V110 × CXCR5−/− mice into C57BL/6 on day −1, followed by infection with $2 \times 10^6$ PFU VSV-IND on day 0. Detection of VSV-specific cells by fluorescence microscopy using the anti-idiotype marker 35.61 on the indicated day postinfection. I, Expansion of V110 (□) or V110 × CXCR5−/− (■) B cells in spleens of C57BL/6 mice on day 4 after immunization with $2 \times 10^6$ PFU VSV-IND i.v. Mice had received $10^6$ B220+ MACS-purified V110 or V110 × CXCR5−/− splenocytes on day −1. Bars represent the means ± SD (n = 3). J, ELISA of serum IgM of the V110 Id, in C57BL/6 mice, after adoptive transfer of $10^6$ V110 (□) or V110 × CXCR5−/− (■) MACS-purified B cells on day −1 and i.v. infection with $2 \times 10^6$ PFU VSV-IND on day 0. Points represent means ± SD of n = 3 mice. One of two similar experiments is shown.
CXCR5-dependent relative localization of VSV-specific T and B cells

We next wanted to examine in more detail how the CXCR5-dependent relative positioning of Th and B cells within secondary lymphoid organs influenced the development of efficient antiviral B cell responses. To this end, we transferred CXCR5-deficient or -competent, VSV-specific, purified CD4+ T cells (L7 and L7 × CXCR5−/−) and B220+ B cells (VI10 and VI10 × CXCR5−/−) into B6PLThy1.1 recipients and immunized these mice with VSV. On day 4 after immunization, at the time point of maximal induction of TI IgM in serum, we observed great numbers of VI10 B cells in the red pulp and interfollicular regions of the spleen (Fig. 5A). In contrast, VI10 × CXCR5−/− B cells mainly localized to the red pulp, and contacts with L7 × CXCR5−/− T cells in the bridging channels were rare (Fig. 5B, arrowhead). VI10 × CXCR5−/− B cells were detected in lower numbers compared with VI10 B cells, consistent with the cytfluorimetric analysis and the reduced serum IgM production (Fig. 3, I and J). At this time point, the majority of both CXCR5-competent or -deficient, VSV-specific Th cells, as detected by expression of a syngeneic Thy1 marker, localized to the T cell zone. In addition, CXCR5-competent Th cells were frequently present in B cell zones but had downregulated the Thy1 marker (Fig. 5A, inset). The presence of Th cells in the B cell zone is consistent with the maximal expansion of CXCR5+ Th cells at day 4 (Fig. 4D). L7 × CXCR5−/− cells, in turn, were very rarely seen in B cell zones (Fig. 5B). On day 7 after infection, frequent GCs were formed as indicated by prominent GL-7 staining in the B cell zone (Fig. 5, E and F, green). VI10 B cells had seeded these GCs (Fig. 5, C and E), whereas VI10 × CXCR5−/− B cells were efficiently excluded from GCs, and were only detectable at very low frequencies in the red pulp (Fig. 5, D and F). Also, L7 Th cells could be detected in VSV-specific GCs (Fig. 5C, arrowhead), whereas L7 × CXCR5−/− cells were exclusively detected within the T cell zone and interfollicular regions (Fig. 5D). Notably, the protein ligand for CXCR5, CXCL13, was confined to the B cell zone, even during infection (Fig. 5, G-O). Together, CXCR5 guides B and Th cells to B cell zones during an antiviral immune response and supports the formation of Ag-specific GCs.

An adoptive transfer of large numbers of Th and B cells was necessary to visualize the general homing behavior of CXCR5-deficient T and B cells by histology after infection, but this increase in the Ag-specific precursor frequency may have reconstituted the defective IgG class switch (Fig. 2, A and B). To control for this possibility, and to provide a functional correlate for our histological observations, we selectively determined IgG titers of VI10 or VI10 × CXCR5−/− B cells from the serum of mice that had received 5 × 10^6 VSV-specific, CXCR5-competent or -deficient B cells and Th cells. Igs of transferred VI10 B cells were detected in Thy1.1IgHa recipients by an ELISA using the IgHb marker. This assay allowed for the specific detection of Igs from the adoptively transferred B cells in the presence of a response by endogenous B cells of the host. On day 7 after infection with VSV, titers of both IgG2a and IgG2b were ~10-fold reduced when
Discussion

We have demonstrated here that CXCR5 expression augments the Ig class switch in antiviral B cell responses and that CXCR5 expression on both Th and B cells was necessary for this effect. In particular, CXCR5 confers a decisive advantage on Ag-specific B cells in their competition for follicular niches: it enhances B cell expansion during the TI phase of the response and fosters the entry of B and Th cells into GCs during the TD phase.

Our initial observation was that CXCR5<sup>−−</sup> mice did not show impaired antiviral neutralizing IgG production in response to live VSV infection. However, Ig class switch was significantly affected under conditions of limiting Ag availability (i.e., when nonreplicating VSV particles or protein preparations of VSV-G were used). Interestingly, the deficiency in the generation of antiviral neutralizing Abs could also be observed in long-term LCMV-infected CXCR5<sup>−−</sup> mice as well as late after VSV infection. To reduce the complexity of the viral system and to dissect the distinct impact of Th cells and B cells on Ig class switch, we established two adoptive transfer systems. Recipient mice had an undisturbed lymphoid stroma and selectively lacked either B cell or Th cell function, which was then complemented by transferring Ag-specific, CXCR5-competent or -deficient cells. TCRβ<sub>−−</sub> mice were chosen as recipients for CXCR5<sup>−−</sup>-competent or -deficient CD4<sup>+</sup> Th cells, because it was shown that mice lacking αβ T cells still mounted IgG after VSV infection (21). Although TCRβ<sub>−−</sub> lacks a T cell zone, they express wild-type levels of CCL19, CCL21, and CXCL13 (20), potentially allowing an undisturbed trafficking of adoptively transferred CD4<sup>+</sup> T cells. AID<sup>−−</sup> mice were ideal recipients for CXCR5-competent or -deficient, VSV-specific B cells, because they have an entirely normal lymphoid morphology (data not shown) but show a selective lack of somatic hypermutation, class switch (15), and GC formation.

The adoptive transfer experiments revealed the potential mechanisms of CXCR5-mediated enhancement of IgG responses. First, CXCR5-competent B and Th cells may gain better access to survival or proliferation signals in follicles than CXCR5-deficient cells. This seems particularly likely for B cells, because CXCR5-competent and -deficient B cells show differential expansion already very early during the TI phase of the response. This difference is probably not caused by differential probabilities of initial Ag encounter in the marginal zone (22), because CXCR5-competent and -deficient B cells express a similar set of integrins (19) and should therefore both be able to establish efficient contacts within the marginal zone (23). Immediately after Ag recognition, however, activated CXCR5-competent B cells acquire an enhanced responsiveness to CCR7 and thus localize to the T-B border (6). Absence of CXCR5 expression on activated B cells may cause the CCR7-dependent signals to prevail and disturb the rapid translocation of B cells to the T-B border after VSV infection (K. Fink, unpublished observations). The subsequent separation of activated B cells from the B cell zone, and emanating survival factors, may eventually limit their expansion. This interpretation is in line with a recent study that showed reduced expression levels of CXCR5 and follicular exclusion of autoreactive B cells at this stage of B cell activation (24). B cell survival factors within follicles may be supplied by a subset of dendritic cells (DCs) expressing a ligand for the cytokine-rich domain of the mannose receptor that translocate to the B cell zone in a CXCR5-dependent manner immediately after immunization (25). This subtype of DCs is capable of stimulating primary Ab responses (26), possibly by providing co-stimulation for B cells, for instance through BAFF (27).

A second mechanism how CXCR5 could enhance IgG production is that CXCR5 increases the frequency of cognate interactions between B cells and CXCR5<sup>+</sup> Th cells in or adjacent to B cell follicles. Indeed, primed CXCR5<sup>+</sup> Th cells express low levels of CCR7, have the propensity to localize to B cell follicles, and express, at least in the human system, elevated levels of CXCL13...
(28, 29). This mechanism may increase the local concentration of CXCL13 in B cell follicles facilitating the entry of other CXCR5+ Th and B cells and eventually results in increased frequencies of productive T-B interactions.

The positively stimulating environment of the follicle and the enhanced CXCR5-mediated recruitment of Th and B cells may synergistically enhance the formation of GCs and therefore the continuous output of IgG producing plasma cells in the memory phase. The limited GC formation of CXCR5-deficient, VSV-specific B cells is in part a consequence of the impaired initial B cell expansion, because even CXCR5-deficient B cells can enter GCs, if present in high enough numbers (30). Within GCs, however, CXCR5 expression is necessary for the attraction of Ag-specific B cells to the light zone of GCs (30) (i.e., to the site where Ag-dependent selection for high-affinity clones occurs). CXCR5-deficient cells may rapidly become excluded from the GC reaction in wild-type recipients due to a selective disadvantage over endogenous CXCR5-competent B cells in the intrafollicular competition for either follicular dendritic cell-bound Ag or other GC-derived survival factors. This CXCR5-dependent competition of B cells for survival factors or follicular niches is absent in CXCR5+/− mice, which may offer an explanation for the formation of phenotypically normal (11, 19) GCs in CXCR5−/− mice.

Although CXCR5−/− mice mounted normal acute IgG titers against VSV and developed VSV-specific GCs, we have observed long-term IgG titers to decline more rapidly than control titers. Furthermore, the production of neutralizing Abs against LCMV was impaired in the late phase of the response. It is unlikely that CXCR5 expression positively influenced the maintenance of memory IgG titers by directing plasma cells to particular bone marrow niches and thereby increasing their half-life. Plasma cells are CXCR5 negative, and their localization to the bone marrow mainly depends on CXCR4 (7). The effect of CXCR5 on memory IgG titers may thus rather be caused by an impact of CXCR5 on the maintenance of GCs and requires further investigation.

As discussed above, the initial experiments in CXCR5−/− mice revealed a particularly strong impairment of Ig class switch in situations of limiting Ag, but notably not in the acute phase of VSV infection. The slightly milder phenotype of CXCR5−/− mice, compared with adoptive transfers of CXCR5-deficient B and Th cells into wild-type hosts, may be caused by two effects. First, in CXCR5−/− mice, B and Th cells do not compete with CXCR5-competent B or Th cells for follicular niches or survival factors. Second, the altered lymphoid structure in CXCR5−/− mice may functionally compensate for the lack of CXCR5-dependent migration, in cases of abundant Ag. Only if Ag is limiting (i.e., late after virus clearance or after immunization with nonreplicating Ags) does CXCR5 become key for the induction or maintenance of IgG responses. There are other examples in which ectopic priming in an aberrant lymphoid environment can functionally compensate for migration defects, provided that the amount of Ag is not limiting. For example, plt/plt mice lacking lymphoid CCL19 and CCL21 (31) and CCR7−/− mice (32) that show defective DC and T cell migration in vivo are able to mount fully protective antiviral CTL responses through the interaction of DCs and CTL in the marginal zone.

In conclusion, this study shows that the CXCR5-dependent homing of B and Th cells to intact splenic microenvironments governs the continuous cross-talk of T and B cells during antiviral immune responses at two checkpoints: initial B cell expansion and GC formation. However, complete lack of this CXCR5-dependent cross-talk in CXCR5−/− mice still allows for the induction of acute neutralizing B cell responses against some viruses. It is thus most likely that the migration of lymphocytes to the constitutive chemokine CXCL13 is of minor relevance in antiviral B cell responses in a noncompetitive environment but is particularly important in situations in which the availability of Ag is limited.

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Disclosures
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