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J Immunol published online 12 December 2014
http://www.jimmunol.org/content/early/2014/12/12/jimmunol.1401553

Supplementary Material http://www.jimmunol.org/content/suppl/2014/12/12/jimmunol.1401553.3.DCSupplemental

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
CCR6-Dependent Positioning of Memory B Cells Is Essential for Their Ability To Mount a Recall Response to Antigen

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Chemokine-dependent localization of specific B cell subsets within the immune microarchitecture is essential to ensure successful cognate interactions. Although cognate interactions between T cells and memory B cells (Bmem) are essential for the secondary humoral immune responses, the chemokine response patterns of Bmem cells are largely unknown. In contrast to naive B cells, this study shows that Ag-specific Bmem cells have heightened expression of CCR6 and a selective chemotactic response to the CCR6 ligand, CCL20. Although CCR6 appears be nonessential for the initial clonal expansion and maintenance of Bmem, CCR6 is essential for the ability of Bmem to respond to a recall response to their cognate Ag. This dependency was deemed intrinsic by studies in CCR6-deficient mice and in bone marrow chimeric mice where CCR6 deficiency was limited to the B cell lineage. Finally, the mis-positioning of CCR6-deficient Bmem was revealed by immunohistological analysis with an altered distribution of CCR6-deficient Bmem from the marginal and parafollicular to the follicular/germinal center area. The Journal of Immunology, 2015, 194: 000–000.

Long-lived serological memory is the product of long-lived memory B cells (Bmem) and plasma cells (PC). Bmem cells are long-lived although their half life is as-yet undefined in both mouse and man (1). Bmem cells are quiescent, they can be IgM memory or express isotype-switched and somatically hypermutated membrane Ig (2, 3). IgM Bmem cells are produced independent of T cell help, differentiate in the absence of GC (4), produce natural Abs (3) and part of circulating marginal zone B cells (2). Upon exposure to their cognate Ag, Ig

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Materials and Methods

Mice and immunizations

These studies were approved and conducted in accredited facilities in accordance with the Institutional Animal Care and Use Committee of Dartmouth College (Lebanon, NH) and U.K. Animals (Scientific Procedures) Act 1986 (Home Office license number PPL 7007102). C57Bl/6 mice were purchased from the National Cancer Institute and Charles River Laboratories. CCR6+/−, Rosa YFPfl/fl, and μMT mice were purchased from The Jackson Laboratory. All animals were maintained in a pathogen-free facility at Dartmouth Medical School and King’s College London. For primary immunizations, 10 μg PE (Chromoprobe) adsorbed to prepared alum was injected i.p. in a volume of 200 μl. For secondary challenge, 10 μg PE in PBS in a volume of 200 μl was injected i.p.

Cell preparation

To sort memory B cells, single-cell suspensions of lymphocytes were prepared as described previously (1). Splenocytes were incubated with anti-IgD-biotin, anti-IgM-biotin, anti–CD3e-biotin, and anti–CD4-biotin, with these cells removed using the EasySep biotin Selection kit for mouse cells (Stem Cell Technologies).

Flow cytometry

Abs against the following Ags were used: B220 (clone 6B2), IgG1 (clone A85-1), IgD clone (11-26c), IgM (clone II4/1), CD4 (clone GK1.5), CD8 (clone 2.43), CD38 (clone 90), CD23 (clone B384), CCR6 (clone 29-2L17), and Sign-R1 (clone ERTR9). PE-binding cells were detected by staining with 1 μg/ml PE. Samples were acquired with a refurbished BD FACS-CAN running CellQuest software (BD Biosciences), and data were analyzed by FlowJo (Tree Star) software. Cell sorts were performed on a BD FACSaria with 10,000–100,000 cells sorted and postsort analysis indicating purities exceeding 98% (Flow Cytometry Facility at Dartmouth Medical School).

Real-time PCR

Total RNA was isolated from either purified memory or naive B cells using TRizol (Invitrogen) followed by secondary purification over RNeasy columns (Qiagen). Real-time PCR was performed with the SYBR Green PCR Core Kit (Applied Biosystems) on an iCycler iQ instrument (Bio-Rad Laboratories). Amplification conditions were 95˚C for 8 min, followed by 40 cycles of 94˚C for 15 s, 62˚C for 45 s, and 72˚C for 15 s. Primers for the control gene mouse β-actin were as follows: forward, 5′-CCAAATGTGTCATGTCA-TTT-3′, and reverse, 5′-CAATA-GTGATGACCTGGCCGT-3′. ΔΔCT = (cycle threshold [CT] gene of interest − CT β-actin in experimental sample) − (CT gene of interest − CT β-actin in a no-template control sample) (see the ΔΔCT method; TaqMan Bulletin 2; Applied Biosystems).

Chemotaxis assays

Chemotactic migration assays were performed, as previously described (15). Briefly, between 5 × 106 splenocytes were placed in the upper chamber of 24-well plates Transwell inserts (Corning Costar, Cambridge, MA), in contact with 600 μl medium (RPMI 1640 medium with 10% FBS) with or without chemokine. After 2 h, a fixed volume of counting beads (Polysciences, Warrington, PA) was added to each well for normalization, and the specific migration of Bmem or naive B cells was enumerated by flow cytometry after staining for B lymphocyte surface marker of interest. Mouse RANTES, CCL17, 1SIP, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CCL20, CCL21, and CCL22 were purchased from PeproTech.
FIGURE 1. CCR6 expression on memory B cells and their chemokine response profile. (A) PE-specific B_mem cells (B220^+CD38^+PE^+IgG1^+ and IgM/IgD^+/CD4/CD8^2) and naive B cells (NF B cells) (B220^+CD38^+CD23^+IgG1^2 and IgM/IgD^+) were sorted and mRNA expression levels of CCR6 were analyzed. (B) Flow cytometry analysis of CCR6 expression on naive B cells (left panel) and B_mem cells (right panel). Numbers indicate percentage of CCR6-positive cells. (C) Quantification for CCR6 expression in naive and B_mem cells by flow cytometry in (B) is shown. (D) Flow cytometry analysis of CCR6 expression on immature B cells (B220^+IgM^hiIgD^-), mature B cells (B220^+IgM^intIgD^+), pre-B cells (B220^+IgM^-IgD^-), and PCs (B220^-CD138^-IgD^-) from BM. (E) Percentage of CCR6-positive cells of each subset in D and NF B cells is shown. All data are representative of at least three independent experiments with at least three mice per group. (F) Quantification of chemokine profile response at 250 ng/ml B_mem cells from three independent experiments; each sample in duplicate. *p < 0.01, **p < 0.001.
B cells (Fig. 1B). CCR6 expression was also analyzed in immature B cells (B220+IgM+IgD−), mature B cells (B220+IgM−IgD+), pre-B cells (B220+IgM+IgD−), and PCs (B220+CD138+IgD−) from BM (21). Mature B cells expressed similar levels of CCR6 compared with naive B cells and CCR6 expression is increased during the development of B cells (Fig. 1D, 1E). Neither pre-B cells nor PCs express this chemokine receptor (Fig. 1D, 1E).

These results together establish that Bmem cells express increased levels of CCR6 mRNA and protein when compared with other B cell subsets, including PCs.

To evaluate whether CCR6 expression correlated with a heightened chemotactic response to CCR6 ligand, CCL20, in vitro migration assays were performed. Splenic B cell subsets were evaluated for their in vitro capability to migrate to different concentrations of a spectrum of chemokines. In vitro migration assays showed (Fig. 1F, Supplemental Fig. 1B, 1C) that Bmem preferentially migrated in response to CCL20, in a dose-dependent manner when compared with naive B cells. Bmem, like naive B cells were also able to migrate to CXCL12 and CXCL13. In addition, although naive B cells also express CCR6, they did not migrate in response to CCL20 (Supplemental Fig. 1B). These results established that Bmem and not naive B cells are able to migrate in response to CCL20.

**CCR6 is dispensable for the primary humoral immune response**

The expression of CCR6 by naive B cells potentially implicated CCR6 as playing a role in the primary humoral immune response (Fig. 1B). To evaluate the role of CCR6 in a primary B cell response, the ability of CCR6-deficient mice to mount a T-dependent primary Ab response was evaluated. The lack of CCR6 did not disrupt splenic architecture in PE-immune mice and there was no difference in the GC structure (Fig. 2A) or frequency of GC B cells, as defined by B220+CD95+GL−7+CD38−B cells (Fig. 2B–E) 14 d after immunization. Furthermore, there was no impact of CCR6 deficiency on anti–PE serum Ab titers or PE-specific IgG1 BM–Ab-secreting cell (ASC) at days 14 and 28 after immunization (Fig. 2F, 2G). These data show that CCR6 expression is not required for GC formation, the primary generation of Ag-specific ASC or enhanced serum Ag-specific Ab titers following primary immunization.

The heightened expression of CCR6 on Bmem suggested that it might play a role in the initial differentiation of Bmem during the primary response. To this end, CCR6-deficient and WT mice were immunized with PE, and after 4 mo, the percentage of Bmem was analyzed by flow cytometry. Bmem were defined as B220+PE+CD38+IgM−IgG1+, as shown in Fig. 2H, the frequency of Bmem cell in CCR6−/− mice and WT mice were indistinguishable between lymph nodes and spleen. These data established that CCR6 was dispensable for the differentiation of Bmem during the primary immune response.

**Involvement of CCR6 in the recall response of Bmem**

CCR6 is not involved in the development of the primary humoral immune response, and it was not in the early differentiation of Ag-specific Bmem or ASCs. Additional studies were designed to address the potential role of CCR6 in Bmem maintenance and recall responsiveness. As such, we analyzed the recall response in immune WT and CCR6-deficient mice. Immune CCR6-deficient and WT mice immunized 120 d previously were rechallenged with PE, and 5 d later, the number of ASCs was quantified. The results show that there was a marked reduction in the number of PE-specific IgG1 ASC in both spleen and BM of CCR6-deficient mice compared with WT mice (Fig. 3A, 3B). Also observed was a significant reduction in the serum titers of anti–PE IgG1 (Fig. 3C). These observations indicated that the recall capacity of Bmem is impaired in the absence of CCR6.

To determine whether the defect in Bmem function in CCR6-deficient mice is intrinsic to the B cell lineage, mixed BM chimeras were produced. Reconstitution of irradiated WT hosts with a mixture of μMT BM with either CCR6-deficient or WT BM resulted in chimeras in which the B cell compartment was derived from CCR6-deficient or WT BM precursors and all other compartments were predominantly of a WT genotype (Fig. 3D). After the reconstitution of the hematopoietic compartment, the mice that were immunized 60 d previously were rechallenged with PE, and 5 d later, the number of ASCs was analyzed by ELISPOT. The data showed that in both spleen and BM there were marked reductions in the number of ASCs in the chimeras where the B cells were derived from CCR6-deficient BM compared with those chimeras where the B cells were derived from WT BM (Fig. 3E, 3F). These results show that B lineage–restricted expression of CCR6 is critical for Bmem cells to respond to a secondary challenge by Ag.

**Involvement of CCR6 in the localization of Bmem**

The immunohistological tracking of Bmem is challenging given the lack of a definitive set of easily usable markers to identify them in situ (22–24). Nonetheless, it was important to determine whether the deficiency of CCR6 resulted in the altered anatomic localization of Bmem. As such, the distribution of B cells in WT- and CCR6-deficient mice was assessed. Tracking of Bmem was afforded by analysis using a mouse whereby Cre recombinase was inserted in the Cγ1 locus interbred with a mouse engineered with a conditional Rosa26-EYFP allele (in this paper, called RosaYFP) (25). In this strain, every post-GC B cell, including Bmem, expresses YFP. Flow cytometric analysis reveals that less than 1% of total B cells express YFP in the steady state (data not shown) (25). Subset analyses of the YFP+ cells in immune RosaYFP mice showed that the YFP+ population is composed of IgM/IgD+ and IgG+ activated B cells (Fig. 4A). On the basis of flow cytometric analysis, it was not evident that the YFP+ B cells that were IgM/IgD+ were Bmem and, as such, were independently scored for their distribution. The splenic localization of YFP+IgM−/IgD−/IgG1+ were quantified as a measure of Bmem distribution (Fig. 4B, 4F).

First, no difference were found in the percentage of activated or polyclonal Bmem cells in either the Cγ1-cre × RosaYFP × CCR6−/− and Cγ1-cre × RosaYFP mice (Fig. 4A, 4B), as expected from our analysis of the frequency of Bmem in CCR6−/− mice. Second, staining for YFP+ cells in WT mice revealed that the majority of the B220+, YFP+ cells were distributed widely outside of follicular areas. Note that double-positive cells (B220+, YFP+) did not appear yellow because the YFP stain, and the B220 stain associated with CD35 expression and PNA (Fig. 4G, 4H, Supplemental Fig. 2E), whereas in control mice RosaYFP Bmem
FIGURE 2. CCR6 expression on B cells does not affect the primary humoral response or memory B cell development. (A) CCR6KO (right panel) or WT (left panel) mice were immunized with 10 μg PE adsorbed in alum; after 7 d, the spleen were frozen and stained with PNA-FITC (green), B220–Alexa Fluor 647 (blue), and CD4-biotin plus streptavidin–Alexa Fluor 455 (Red) for germinal center analysis. A representative image of two independent experiments with three mice per group. Original magnification ×10. Scale bars, 80 μm. (B) Flow cytometry analysis of GC B cells (B220+CD38+CD95+GL-7+) in WT or CCR6KO mice, nonimmunized (NI; bottom panels) or immunized (top panels) with PE in alum 14 d before of the analysis. (C) Histogram of PE binding in GC B cells gated in B is shown. (D) Percentage of GC B cells of total B220+ cells at day 14 after immunization is shown. (E) Quantification of PE-specific GC B cells of total B220+ cells is shown. One representative test from two independent experiments with at least three mice per group. (F) CCR6KO or WT mice were immunized with 10 μg PE adsorbed in alum, nonimmunized mice (day 0), and 14 and 28 d after immunization, the sera were collected to analyze the production of anti-PE IgG1 titers by ELISA. (G) BMs were collected at the same time point than F to analyze number of PE+IgG1+BM-ASC by ELISPOT. Quantification from two independent experiments with at least three mice per group. (H) CCR6KO or WT mice were immunized with 10 μg PE in alum. One hundred twenty days afterward, the percentage of PE-specific Bmem cells (PE+IgG1+B220+CD38−IgM/IgD−) was evaluated in mesenteric (MLN), peripherical lymph (PLN) node, and spleen by flow cytometry. The graph shows the quantification of two independent experiments with three mice per group.
equally localize between perifollicular and marginal zone areas, colocalizing with monocyte and macrophage expression (Supplemental Fig. 2A, 2B).

The striking increase in follicular redistribution of virtually all YFP+ B cells in the CCR6<sup>-/-</sup> mice raised a number of additional questions. To determine whether an increase in GC numbers in the spleen of C<sup>g</sup>1-cre<sup>3</sup>RosaYFP<sup>3</sup>CCR6<sup>-/-</sup> mice in steady state could account for the redistribution of YFP+ cells, GC numbers were quantified. Although in CCR6<sup>-/-</sup> mice there is a tendency toward a higher percentage of GC B cells, this difference is not statistically significant in both percentage and absolute number (Fig. 4I, Supplemental Fig. 2C–E), suggesting that the accumulation of activated B cells in the follicular areas is not due to an increase in GC B cells. Perifollicular redistribution could also be the result of heightened expression of CXCR5 on Bmem relative to WT Bmem, and as such, the expression of CXCR5 was analyzed. CCR6<sup>-/-</sup> and WT mice express identical densities of CXCR5 (Supplemental Fig. 3). This indicates that the selective migration of CCR6<sup>-/-</sup> B<sub>mem</sub> is not due to heightened CXCR5 expression. Therefore, at this time, it is not clear why the loss of CCR6 results in the redistribution of B cells.

CCL20 is the ligand for CCR6, and as such, CCL20 gene expression in different splenic population of immune cells and whole spleen was evaluated in WT and CCR6<sup>-/-</sup> mice. Quantitative PCR for CCL20 gene indicated that CD4<sup>+</sup> T cells were the major producers of CCL20 in the spleen (Fig. 4J). Our results also show that the CCL20 abundance in total spleen is similar to that found in CD4<sup>+</sup> T cells, indicating that the CCL20 signal is truly enriched in CD4<sup>+</sup> T cells. Taken together, these results suggest that the lack of CCR6 expression on activated B cells prevents colocalization of B<sub>mem</sub> with CCL20-expressing CD4<sup>+</sup> T cell in the spleen, impacting on their ability to respond to Ag.

**Discussion**

Chemokine receptors play a crucial role for the appropriate localization of B<sub>mem</sub> in secondary lymphoid organs and impact on the ability of B<sub>mem</sub> to mount an effective secondary immune response. To our knowledge, the findings presented here are the first to es-
FIGURE 4. CCR6 affects the localization of B<sub>mem</sub> cells. (A) Quantification of splenic YFP<sup>+</sup> B cell composition from C<sub>γ</sub>1-cre × ROSAYFP × CCR6<sup>−/−</sup> or Cγ1-cre × ROSAYFP is shown. (B) Percentage of polyclonal B<sub>mem</sub> (B220<sup>+</sup>IgG1/YFP<sup>+</sup>CD38<sup>+</sup>CD80<sup>+</sup>) from spleens of Cγ1-cre × ROSAYFP × CCR6<sup>−/−</sup> or Cγ1-cre × ROSAYFP is shown. (C, E, and G) Histology of YFP expression (green) colocalizing with B220 (C), IgM/IgD (E), and CD35 (G) in red. (D, F, and H) Quantification of YFP<sup>+</sup>B220<sup>+</sup> (D), YFP<sup>+</sup>IgM/IgD<sup>+</sup> (F), and YFP<sup>+</sup>CD35<sup>+</sup> (H) on histological section of the spleen in the marginal zone and perifollicular area of Cγ1-cre × ROSAYFP × CCR6<sup>−/−</sup> or Cγ1-cre × ROSAYFP mice. (I) Percentage of GC B cells (CD95<sup>+</sup>GL-7<sup>+</sup>) of total YFP<sup>+</sup>B220<sup>+</sup> cells by flow cytometry is shown. (J) CCL20 gene expression on splenic CD4, CD8 T cells, B cells, and monocytes in addition to whole spleen and Peyer’s patch tissue. Graph represents two independent experiments with four mice per group. Scale bars represent 200 μm (C and E) and 50 μm (G). **p < 0.001.
establish a critical role of CCR6 in B<sub>mem</sub> function. The data shows that a high frequency of B<sub>mem</sub> cells express CCR6 and have a heightened chemotactic response to CCL20 compared with naive B cells. It is known that there is broad CCR6 expression across B cell subsets in the mouse and CCR6 is expressed at higher levels transiently during cognate activation of B cells (26, 27). CCR6-deficient mice have a faster GC response compared with littermate controls, resulting in an increase of low affinity Ag-specific plasmablasts (26). Interestingly though, like in this report, prior authors showed that there was heightened histological accumulation of activated B cells in the GC/follicle in the CCR6<sup>−/−</sup> mice. In studying the primary response to PE, we did not observe any difference in the primary immune response, with regard to GC formation, BM-PCs or Ab titers when compared with control mice. Our findings show that B<sub>mem</sub> retain high expression of CCR6 during the immunization period compared with that constitutively expressed on naive B cells. A significant impact of B cell-intrinsic CCR6 deficiency was a loss in the secondary recall response of B<sub>mem</sub>. Because chemokines are important in the anatomic migration and localization of cells within secondary lymphoid organs (28), it is proposed that the absence of CCR6 expression impacts on the localization of established B<sub>mem</sub> to receive the suitable signals to become PCs. Alternatively, CCR6 may play a role in PC survival; however, this is unlikely because our own data (Fig. 1E) and published studies report that PCs do not express CCR6 (29).

Loss of CCR6 appears to dramatically alter the histological distribution of activated B cells. Attempts to detect B<sub>mem</sub> in vivo to identify their location and migratory patterns have been met with limited success (22). It has been reported that there is preferential localization of B<sub>mem</sub> adjacent to contracted GCs in the spleen, which may be chemokine dependent, and which is different to the behavior of primary B cells that are responding to Ag (22). Furthermore, it has been argued that B<sub>mem</sub> localized in marginal zone area are IgM-B<sub>mem</sub> cells (22–24). In our model, using YFP expression under C<sub>Bmem</sub>, it was observed that polyclonal B<sub>mem</sub> cells are widely distributed across the marginal zone and peri follicular area, suggesting that it is not the exclusive location for Ag experienced IgM<sup>+</sup> B cells as was proposed previously (22–24). Most importantly, we observed an accumulation of B<sub>mem</sub> in the follicular areas in the absence of CCR6 expression. Why this redistribution of B<sub>mem</sub> impairs their ability to mount a recall response to the cognate Ag is currently under investigation.

Quantitative PCR analysis in immune mice revealed that CCL20 is expressed on CD4 T cells in the spleen and provides a reasonable basis for suggesting that CD4-primed CD4<sup>+</sup> T cell produce CCL20 and instructs the chemotaxis of CCR6-bearing B<sub>mem</sub> to facilitate their cognate Ag interactions in the secondary humoral response. CCL20 is expressed at low levels in splenic CD4<sup>+</sup> T cells at steady state compared with Peyer’s patches. However, it is significantly induced by proinflammatory signals via TRLs agonists or TNF-α (30). CCL20 is not the only ligand for CCR6; β-defensins and other nonchemokine proteins can also be ligands for CCR6. However, their affinity is much lower compared with CCL20 (31). CCR6 expression and the chemotactic response to CCL20 on B<sub>mem</sub> cells is consistent with CCR6 being an important molecule in the systemic migration B<sub>mem</sub> to mucosal and effector sites (18, 19), an event perhaps critical in the lifespan of B<sub>mem</sub>. Loss of CCR6 disorganized the colocalization of CCL20 to producing T cells and B<sub>mem</sub> that likely contributed to the impaired recall responses of B<sub>mem</sub> to their cognate Ag.

In conclusion, CCR6 is highly expressed on B<sub>mem</sub> and has an important role in the B<sub>mem</sub> function and localization in secondary lymphoid organs. Due to the profound role of CCR6 in the regulation of secondary humoral immune responses, this chemokine is an attractive target in diseases where the recall responses of B<sub>mem</sub> contribute significantly to disease pathogenesis.

Disclosures

The authors have no financial conflicts of interest.


Supplementary Fig. 1: (A) Stratigatic gate to analyze PE+ IgG1+ memory B cells. DUMP is IgM/IgD double negative. (B - C) Dose dependency of chemokine response profile of memory and naïve B cells: The migration capacity of PE-specific Bmem and NF B cells were evaluated to different chemokine ligands. Representative figure of chemotactic response of NF B cells (B) and Bmem (C) to different concentration of CCL20, CXCL12 and CXCL13.
Supplementary Fig. 2: CCR6 affects the localization of Bmem cells. (A) Histology of YFP expression (green) co-localizing with MOMA in red. Bar represent 100 μm. (B) Quantification of YFP+IgM/IgD+ on histological section of the spleen in the marginal zone and perifollicular area of Cγ1-cre x ROSAYFP x CCR6-/- or Cγ1-cre x ROSAYFP mice. (C) Histology of YFP expression (green) co-localizing with PNA (red) and DAPI(Blue). The original magnification is 40x. Bar represent 50 μm. For quantification of histology 10 slides from different mice were quantified. (D) Representative dot plot of GC B cells (B220+YFP+CD95+GL-7+) in wt or CCR6KO mice. (E) Absolute number of GC B cells in (D).
Supplementary Fig. 3: CCR6 deficiency does not affect CXCR5 expression on memory B cells. (A) CXCR5 expression was analyzed in PE+B220+IgG1+CD38+Dump- Bmem from spleens of wt or CCR6 deficient mice immunized with PE in alum 2 months before. (B) Percentage or (C) MFI of CXCR5 expression on splenic Bmem from wt or CCR6 deficient mice immunized with PE in alum 2 months before is shown. Data represent two independent experiments with at least 4 mice per group.