Early CCR6 expression on B cells modulates germinal centre kinetics and efficient antibody responses

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Running title: Role of CCR6 in the humoral response

Abstract

The CC-chemokine receptor 6 (CCR6) can be detected on naïve and activated B cells. Counterintuitively, its absence accelerates the appearance of germinal centres (GC) and increases the production of low-affinity antibodies. The detailed mechanism of CCR6 function during the humoral response has remained elusive but previously we identified a distinct CCR6^{high} B cell population *in vivo* early after antigenic challenge. In this study, we defined this population specifically as early, activated pre-GC B cells. In accordance, we show that CCR6 is up-regulated rapidly within hours on protein or mRNA level after activation *in vitro*. Additionally, only activated B cells migrated specifically towards CCL20, the specific ligand for CCR6. Lack of CCR6 increased the dark zone / light zone ratio of GC and led to decreased antigen-specific IgG1 and IgG2a antibody generation in a B cell intrinsic manner in mixed bone marrow chimeras. In contrast, antigen-specific IgM responses were normal. Hence, CCR6 negatively regulates entry of activated, antigen-specific pre-GC B cells into the GC reaction.

164 words

Introduction

An insight in the expression of chemokine receptors is one key to an understanding of the differentiation of B cells. Based on the dynamic expression of three chemokine receptors, the Epstein-Barr virus induced G-protein coupled receptor 2 (EBI2) and their ligands, B cells move after encounter of antigen and activation to the outer margins of the periarteriolar lymphoid sheath (PALS). There, they communicate with cognate T cells and receive help, and move subsequently to the emerging germinal centres (GC) where they undergo class switch recombination (CSR) and affinity maturation [1, 2]. In the GC, the interaction of light zone (LZ) B cells with T cells represents the rate-limiting step of B cell affinity selection [3]. Alternatively, B cells can remain outside the follicle to differentiate into short-lived plasma cells that represent a source of quickly available antibodies of low affinity [4, 5].

The functions of three chemokine receptors, CCR7, CXCR5 and CXCR4, and the receptor for oxysterols, EBI2, during the navigation of B cells within the follicle and the GC have been dissected in detail [2]. However, a fifth receptor, CCR6, has been implicated recently, to also contribute to B cell movement in the early stages of activation. Genetically modified mice which are deficient for CCR6 have a faster initiation of the GC response after immunization and display more low-affinity antibody secreting cells [6]. CCR6 can be detected on diverse cell populations such as immature dendritic cells [7], sub-populations of memory T cells [8], regulatory T cells [9] Th17 cells [10] and innate lymphoid cells [11], facilitating their migration to and their position within the small intestine via the CCR6/CCL20 axis [12, 13]. While many chemokine receptors are promiscuous in their ligand acceptance [14] CCR6 is an exception and has only one known chemokine ligand, CCL20 [15]. This ligand is

expressed in lung, liver, lymphatic and intestinal tissues; but is only found at an extremely low level in an unchallenged spleen [16].

Whereas CCR6 has been shown to control positioning of memory B cells [17], its role during a primary antibody response has not been analysed in detail. Therefore, we have analysed CCR6 expression *in vitro* and *ex vivo*, and the role of this chemokine receptor in GC formation. We find a strong antigen-specific as well as polyclonally induced activation-dependent expression on primary B cells *in vitro* and on pre GC B cells isolated after immunization. Loss of CCR6 increased formation of the dark zone of the GC but reduced the generation of antigen-specific IgG1a and IgG2a antibodies. These data suggest that one function of CCR6 during the early T cell dependent immune response is to balance GC formation with specific output of class-switched plasma cells.

Results

CCR6 upregulation *in vitro* after B cell activation.

CCR6 is expressed at a low level on naïve B cells in general. After NP-chicken gamma globulin immunization of anti-NP-BCR-transgenic mice the receptor is strongly up regulated [6] but the signals that induce this up-regulation, its kinetics and the exact B cell population expressing CCR6 are badly defined. To better understand the activation-induced expression of CCR6 on the surface of B cells we used HEL-specific activation of B cells isolated from the transgenic MD4 strain. Lymph node B cells were exposed to antigen for up to 72 hours and analysed after 4 hours, 16 hours, 40 hours and 72 hours in combination with the early activation antigen CD69. After 16 hours 38% of B cells reached peak expression after challenge with the specific antigen (Figure 1A). This expression level remained unchanged. Next we asked whether this up-regulation was specific for the BCR or whether CCR6 could also be up-regulated by a polyclonal stimulus. C57BL/6 lymph node B cells were activated by anti-BCR antibodies or with LPS for 72h, and CCR6 expression was correlated with generation number of the divided cells (Figure 1B, C). Whereas CCR6 was robustly up-regulated by BCR stimulation, LPS induced a more transient up-regulation that vanished as cells started to differentiate into plasma cells after 4-5 cell divisions (for review see [18]). These data reveal first, that the CCR6 up-regulation observed previously [6] is not due to the transgenic NP-specific BCR, second, that the up-regulation occurs rapidly and third, that CCR6 expression declines on differentiating B cells, i.e. LPS-stimulated B cells. The very early up-regulation was also confirmed at the mRNA level, indicating a rapid transcriptional up-regulation of *Ccr6* (Figure 2). At the mRNA level, the *Ccr6* expression after CD40 ligation and

LPS addition exceeded the *Ccr6* expression after BCR ligation in both the time to peak signal (4 hours versus 8 hours) and the total signal strength (Figure 2).

To test the impact of the CCR6-upregulation on the migration potential, we performed migration assays using B cells activated either with anti-BCR, or anti-CD40 with IL-4 or LPS (Figure 3). Every stimulus caused a significant and specific response in form of cell migration in response to CCL20 (Figure 3). The concurrent presence of CCL20 in the upper and the lower chamber abrogated migration. Recently, it has been reported that the expression of CXCR5 on CCR6-positive and -negative memory B cells remains unchanged [17]. To exclude an influence of a change in the CXCR5 expression on naïve B cells we tested the expression of CXCR5 on primary freshly isolated B cells. Both CCR6-positive and negative B cells showed an identical CXCR5 expression (Data not shown).

CCR6 expression *in vivo* on different B cell populations after activation

As we showed, upon activation, CCR6 is up-regulated functionally on B cells *in vitro* and *in vivo* but the exact B cell population expressing CCR6 in an ongoing T cell dependent immune response *in vivo* remains to be identified. To further analyse CCR6 expression *in vivo* we investigated CCR6 expression on different splenic B cell subsets seven days after immunization with NP-KLH by flow cytometry. Therefore, B cells were electronically gated for Plasma cells ($CD138^+$, $TACI^+$) [19], naïve B cells ($CD19^+$, CD95- PNA- GL7-), pre- (transient) GC B cells [6] ($CD19^+$, $CD95^+$ PNA- GL7-) and GC B cells ($CD19^+$, $CD95^+$ PNA^+ $GL7^+$) [20, 21] (Suppl. Figure 1).

A moderate expression of CCR6 could be detected on naïve B cells while the B cell population that downregulated IgD and upregulated CD95 (data not shown) and differentiated to GC B cells (pre- or transient GC B cells) were expressing CCR6 at a

significantly increased level as described before in BCR-transgenic B cells [6]. GC B cells and the majority of plasmablasts were negative for CCR6 surface expression. A small population of plasmablasts and GC B cells displayed CCR6 expression pointing to a heterogenous mixture of cells (Figure 4A). Consequently, CCR6 is specifically expressed at a high level on the transient population of pre GC B cells (Figure 4B).

Contribution of *CCR6*$^{-/-}$ B cells to GC structure and antibody production

The upregulation of CCR6 on transient, activated B cells and the observation that in the absence of CCR6, GC formation starts earlier and the number of GCs is increased, is an apparent conundrum [6]. However, taking into account that CCR6$^{-/-}$ mice develop antibodies of lower affinity, it could be possible that CCR6 influences GC dynamics and entry of B cells into the GC reaction. Therefore, we assessed GC structure in a time course by immunofluorescence in *CCR6*$^{-/-}$ (*CCR6*$^{eGFP/eGFP}$) and WT mice using antibodies against IgD (naïve follicular B cells), PNA (GC B cells) and CD35 (CR2; light zone FDC) [22] after immunization for 4, 7 and 14 days with NP-KLH and for 7 days with SRBC. Upon examination of spleen sections, more GC follicles were observed in *CCR6*$^{eGFP/eGFP}$ than WT mice [6] (Suppl. Figure 2). While both the size of the GC follicles and the size of the light zone within the GC seemed comparable, the PNA^{+} dark zone appeared slightly more prominent in the *CCR6*$^{eGFP/eGFP}$ mice. Therefore, we assessed the ratio of the area that was marked by PNA (activated B cells) and the area covered by the CD35^{+} FDC network (light zone) (Figure 5A). CD35^{+} marginal zone B cells were visually excluded from analyses. The result showed that the ratio of the area marked by PNA and that marked by CD35 was increased significantly at day 4 and day 7 after immunization in *CCR6*$^{eGFP/eGFP}$ mice (Figure 5A). This could reflect accelerated growth of the GC in size but delayed

transition of DZ cells to LZ cells in the absence of CCR6. Day 7 SRBC immunization also resulted in a significant difference (Figure 5B). At day 14 after immunization, the difference in the PNA/CD35 ratio had disappeared and the sizes had aligned (Figure 5A).

Using flow cytometry we re-assessed GC development and DZ/LZ ratio (Figure 6). At day 13 after immunization the frequency of WT GC B cells ($CD19^{+,}$ PNA^{+}) decreased but remained elevated in $CCR6^{-/-}$ mice (Figure 6A). DZ/LZ B cells were analysed using CD19, PNA, CXCR4 and CD86 (Figure 6B) [3]. A kinetic analysis of DZ and LZ B cells in the WT revealed a trend to an elevated DZ/LZ ratio at days 4-11 and significantly increased DZ B cells at d13 in $CCR6^{-/-}$ mice (Figure 6C). During the course of the experiment we also assessed plasma cells (not antigen-specific), revealing no significant difference in $CCR6^{-/-}$ mice.

To assess the contribution of *CCR6*$^{-/-}$ B cells to the formation of GC output we created mixed bone marrow chimeras. We combined B6.WT (CD45.1) and B6.*CCR6*$^{-/-}$ (CD45.2) and reconstituted lethally irradiated B6.WT (CD45.2) mice. An immunohistological analysis of the GC demonstrated that both genotypes contributed to the formation of the follicle as well as the GC (Suppl. Figure 3).

Since our histological analysis was only suitable to detect a gross deviation from an equilibrium of the contributing genotypes we analysed the CCR6-dependent production of different antigen-specific isotypes to determine an influence of CCR6 on the function of GC [6].

To be able to study the CCR6-dependant production of different antigen-specific isotypes we combined $Ly5.2^{+}$ bone marrow consisting of B6.WT (IgH_a) and B6.*CCR6*$^{-/-}$(IgH_b) and injected these cells into lethally irradiated $Ly5.1^{+}$ host animals

(Figure 7A). The proportion of the grafted donor B cells (Ly5.2^{+}) was tested in a blood sample two weeks after irradiation and mice positive for the graft were immunized four weeks after reconstitution (Data not shown). At day 7 (IgM) and day 21 (IgG1 and IgG2a) after immunisation, we analysed the contribution of the genotypes to antigen-specific IgM, IgG1 and IgG2a. While IgM at day 7 after immunization is most likely the result of the extrafollicular pathway, at day 21 IgG2a and IgG1 isotypes are typically secreted by B cells that have undergone maturation within a GC. Due to the different affinities of the detection antibodies used in this ELISA, only a comparison of the results of the same allotypes is possible. The significant reduction of these allotypes in the serum (Figure 7B) shows clearly a substantial disadvantage for the CCR6-negative B cells in the GC but not the extrafollicular pathways.

Discussion

Timing and position of the initial B-T cell contact during the humoral immune response is important to control the balance between high affinity and low affinity, class-switched and non-class switched antibodies [23]. Here we propose that CCR6 is a regulator of this early step and also has a subtle influence on GC development. One line of evidence for this proposal comes from our kinetic analyses of CCR6 expression during B cell activation *in vitro and in vivo*, revealing expression on early activated B cells *in vitro*, and a prominent expression solely on transient pre GC B cells *in vivo*. Both our *in vitro* and *in vivo* data confirmed the relative absence of CCR6 on plasma cells, assuming that LPS induces a robust plasmablast differentiation program *in vitro*, which is linked to cell division (for review see [18]). A second line of research comes from our previous report where we showed that *CCR6*$^{-}$

$^{-/-}$ mice develop antibodies of lower affinity and proposed that CCR6 influences GC dynamics and the entry of B cells into the GC reaction albeit these experiments had been performed in a transgenic background [6]. We provide a third line of evidence, showing that lack of CCR6 alters the DZ/LZ ratio of GC. Fourth, we reveal that CCR6 supports formation of hapten-specific class-switched antibodies. Therefore, we hypothesise that 1) CCR6 is involved in the early contact of activated pre GC B cells with activated T cells. 2) A second and not mutually exclusive possibility is that CCR6 supports B cell localisation in the LZ to enable B/T- cell contact in the LZ, which is the limiting step for affinity maturation in the GC [3]. Lack of CCR6 could therefore lead to early GC egress, or excessive LZ/DZ recycling without sufficient selection explaining increased DZ GC B cells in CCR6$^{-/-}$ mice. Indeed, CCR6 is expressed on light zone GC B cells [3]. It is tempting to speculate that this up-regulation is induced by the BCR signal that LZ GC B cells receive upon contact with antigen on follicular dendritic cells. Given a transient expression on early-activated B cells *in vivo* it could be possible that LZ B cells express CCR6 only transiently and moderately. Nevertheless, failure of CCR6 expression and the possibly resulting reduced size of the LZ B cell population could explain the secretion of low affinity IgG from GC derived CCR6$^{-/-}$ plasma cells as selection would be relaxed [6]. This concept is compatible with our data showing increased DZ/LZ ratios in the case of CCR6$^{-/-}$ GC. 3) Our data are compatible with a recent RNAseq analysis of sorted B cell populations confirming a progressive decline of *Ccr6* expression from naïve to plasma cells, which we also see in our experiments. Of note, in this analysis neither transient pre-GC B cells as described here nor BCR stimulated B cells or a refined kinetic analysis had been included [24]. Although we observed a robust CCR6 surface expression on freshly BCR activated cells, *ccr6* mRNA expression was higher on LPS

or anti CD40/IL-4 stimulated cells. These data suggest additional post-transcriptional mechanisms controlling CCR6 expression at an early phase of their response to antigen while they seek T cell help and before they acquire the full GC phenotype [23]. However, this result has been obtained in NP-carrier immunized mice of the NP-specific BCR-transgenic mouse model B1-8 [25] and the mechanism of up-regulation, i.e. whether it is the BCR, T cell help or an adjuvant effect has not been clear at all. Hence, our data extend these data to the wildtype situation and provide a mechanism. BCR, CD40, IL4R and TLR stimulation might provide consecutive stimuli regulating CCR6 expression. In our previous study we started to we analyse the kinetics of CCR6 expression on B cells *in vivo* in an adoptive transfer model using transgenic NP specific B cells [25]. Five days after immunization the donor B cells showed a large population with high CCR6 expression [6]. This B cell population was $CCR6^{high}$ but its expression of CD38, GL7, IgD and Fas was highly heterogeneous, thus resembling proliferating pre-GC cells described previously in the same transgenic transfer model [26]. Interestingly, in this study it had been shown that the up- or downregulation of signature molecules such as CD95 and IgD, respectively, on adoptively transferred B cells occurred at the perimeter of the follicle while they proliferated in the presence of dendritic cells and T cells [26]. Here, we show now clearly that transient B cells that still express CD38 but already GL7 reveal the highest CCR6 expression, arguing for a the proposed role of CCR6 in determining the fate of activated B cells by controlling their position. Importantly, this study was now performed with B cells expressing a wildtype repertoire of BCRs, excluding putative artefacts and biases elicited by transgenic BCR expression. Indeed, while loss of CCR6 promoted the formation of low-affinity antibodies [6] it hampered the production of isotype switched antigen specific antibodies in a competitive setting. Hence, we propose that CCR6 enables B

cells to enter the GC to allow for class switch recombination through acquisition of T cell help. This proposal is consistent with the notion that CCR6 has been associated with events such as B cell activation, T–B cell collaboration, early memory generation or the GC reaction [13]. In further agreement we show here a strong and early upregulation of CCR6 in an antigen-specific manner by the BCR but also by an anti CD40 antibody and IL-4. The latter stimulus is relevant for B cells awaiting or actively seeking T cell help in the pre GC phase. Along this line, the observation that there are no gross morphological differences in the architecture of the spleen in WT and CCR6-deficient mice is not surprising and indicates that CCR6 plays little role in the actual compartmentalisation of naïve leukocytes, but rather contributes to subtle interactions of early, activated B cells with yet undefined partners. It is worth noting that there have been observations pointing at roles other than chemotaxis for chemokine-receptor pairs [13, 14], and an emerging acceptance that CCR6 plays additional roles in the humoral immune response, such as maintaining activated B cells in a milieu where they find T cell help or in GC-dependent memory formation [17, 27] and the generation of extrafollicular memory B cells [21].

Methods

Mice

CCR6$^{-/-}$ mice on a C57BL/6 background (B6.*CCR6*$^{-/-}$) were generated as described [28] and have been backcrossed to the C57BL/6 background for more than 10 generations [29]. The *CCR6*$^{eGFP/eGFP}$ mice were purchased from Jackson Laboratory (Bar Harbour, Maine, USA) [30]. This mouse strain was used specifically in immunohistology. Furthermore, a B cell receptor transgenic mouse strain with specificity for hen-egg-lysozyme (HEL, MD4 strain [31]) was used. The strains C57BL/6 (B6.WT:CD45.2, IgH_b), C57BL/6.Ly5.1 (B6.Ly5.1:CD45.1, IgH_b) and C57BL/6.IgH_a (B6.IgH_a: CD45.2, IgH_a) were obtained from Jackson Laboratory (Bar Harbour)[32]. All animal experiments were approved by the local animal ethics committees of James Cook University and the Universities of Tasmania, Erlangen and Adelaide.

Antibodies

The following flow cytometry antibodies were obtained from BD Biosciences (BD Biosciences, Sydney, Australia) eBioscience (San Diego, CA, USA) or BioLegend (San Diego, CA, USA): Hamster anti-mouse TCRβ (FITC, clone H57-597); rat anti-mouse B220 (APC-Cy 7, clone RA3-6B2); rat anti-mouse GL7 (eFluor 450 or eFluor 660, clone GL-7); rat anti-mouse CD38 (PerCP-eFluor 710, clone 90); rat anti-mouse CD25 (APC or PE, clone PC61,); rat anti-mouse CD69 (Biotin or PE, clone HI2F3); rat anti-mouse CXCR5 (unlabelled, clone 2G8); rat anti-mouse CD19 (Brilliant Violet 421, clone 6D5); rat anti-mouse CD23 (PE, clone B3B4); rat anti-mouse CD86 (PE-Cy7, clone GL-1); rat anti-mouse CD21/35 (Biotin, clone 8D9); rat anti-mouse CD95 (PE-Cy7, clone Jo2); rat anti-mouse CXCR4 (PE, clone 2B11); rat anti-mouse CD138 (Biotin, clone 281.2); rat anti-mouse TACI (APC, clone ebio8F10-3); Peanut

Agglutinin (PNA) (FITC, Vector) and PerCP conjugated streptavidin as secondary antibody. Furthermore, we used mouse anti-mouse CD45.2 (APC, clone 104) and CD45.1 (PE, clone A20). A rat anti-mouse CCR6 mAb (unlabelled, FITC or PE, clone 29-2L17) was purchased from either R&D Systems (R&D Systems, Sydney, Australia) or BioLegend (Karrinyup, Australia). Streptavidin-V500 (BD Biosciences) or Cy5- and Dylight-643-conjugated mouse adsorbed goat anti-rat IgG (Jackson ImmunoResearch, West Grove, USA) were used for detection of biotinylated or unlabelled primary mAb.

For immunohistology and ELISA, we used the following unlabelled, fluorochrome - labelled or biotinylated mAbs: rat anti-mouse IgM (R6-60.2); mouse- anti-mouse CD45.1 (clone A20); mouse anti-mouse CD45.2 (clone 104); mouse anti-mouse IgM_a (clone DS-1); mouse anti-mouse IgM_b (clone AF6-78); mouse anti-mouse $IgG1_a$ (clone 10.9); mouse anti-mouse $IgG1_b$ (clone B68-2); mouse anti-mouse $IgG2a_a$ (clone 8-3); mouse anti-mouse $IgG2a_b$ (clone 5.7). Rat anti-mouse IgD (FITC, clone 11-26c.2a), rat anti-mouse CD35 (unlabelled,, clone 8C12) and Peanut Agglutinin (PNA) (BiotinVector, Abacus ASL, Brisbane, Australia) were used to analyse GC (all primary mAbs were obtained from BD Biosciences); the secondary antiserum goat anti-rat IgG (Alexa Fluor 488) and fluorochrome-labelled streptavidin (Alexa Fluor 488, 546 and 633) (Invitrogen, Sydney, Australia) were used to visualize the mAbs in immunofluorescence. The Strep/ABC (HRP) detection kit (Dako Australia, Victoria, Australia) was used to reveal NP-specific antibodies in ELISA.

Flow cytometry and immunohistology

Multicolour staining of activated or control HEL-specific B lymphocytes for surface antigens was performed as previously described [33]. Data were acquired either using a

Cyan ADP or a Gallios flow cytometer (Beckman Coulter, Fullerton, CA, USA). Analyses were performed using FlowJo version 9.4.10 (Tree Star Inc. Ashland, OR, USA) or Kaluza version 1.3 (Beckman Coulter, Brea CA, USA).

Fluorescence immunohistology was performed on frozen sections as described [32]. Sections were analysed using an Olympus BX51 immunofluorescence microscope (Olympus, Sydney, Australia) with high sensitivity gray scale digital camera (Olympus: Optotronics) and Magnifier software (Olympus) or a LSM 510 Meta Confocal Microscope (Carl Zeiss, Germany). The images were processed using ImageJ version 1.45s (ImageJ, Bethesda, U.S.A.).

Immunization and B cell activation

Mice were immunized with the T cell-dependent hapten antigen nitrophenyl (NP) coupled to keyhole limpet hemocyanin (KLH) by i.p. injection with 50 - 100 µg of immunogen (Biosearch Technologies, CA, USA) precipitated in alum (Sigma-Aldrich,NSW, Australia). Alternatively, Sheep Red Blood Cells (SRBC) were prepared and used as immunogen as described [34].

$CD43^-$ B cells were activated for the indicated time points with 10µg/ml HEL (Sigma-Aldrich, 10µg/ml), 10µg/ml anti-B cell receptor (µHC, clone b.7.6 [35]), with 10µg/ml anti-CD40 (clone FGK 45.5; kindly provided to DM by Dr. Fritz Melchers, Max-Planck-Institute for Infection Biology, Berlin [36]) and 0.1U/µl IL-4 (R&D Systems) or 10µg/ml LPS.

CFSE staining

To investigate the cell division dependent expression of CCR6 on proliferating inguinal lymph nodes B cells the same activating conditions were used as described above. Cells were subsequently labelled with 5µM carboxyfluorescein diacetate succinimidyl ester (CFSE; Life Technologies Australia, Soresby, Victoria, Australia)- according to manufactures instructions and analysed by flow cytometry [37].

PCR

Naïve splenic B cells were isolated and activated as described above. Cells were lysed in a volume of 500µl and RNA was isolated with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Primer: CCR6 fwd. primer: CCTGGGCAACATTATGGTGGT; CCR6 rev. primer: CAGAACGGTAGGGTGAGGACA; mHPRT fwd primer: TCAGTCAACGGGGGACTAAA; mHPRT rev. primer: GGGGCTGTACTGCTTAACCAG; The expression of *Ccr6* relative to murine *Hprt* (m*hprt*) (ΔCt) was determined using Absolute SYBR Green ROX-Mix (Thermo Scientific) in a 7300 Real Time PCR System (Applied Biosystems).

Transwell migration assay

For the transwell migration assay $CD43^-$ splenic B cells were either used freshly isolated or pre-activated for 48h at 37°C, 5% CO_2 ($5x10^5$ cells per transwell-insert) as described above. The lower part of the respective well contained medium with 20ng/mL of CCL20. As controls medium without any chemokine was used, or the chemokine was put into both the well and the insert at the same concentrations so that no gradient was established. The cells were incubated at 37°C in 5% CO_2. After four hours the migration was stopped by transferring the inserts into new wells containing

500µl cold stop medium. The specific migration rate was determined by flow cytometry using a defined number of counting beads in well and insert. For each mouse the transwell migration assay was performed in duplicates.

ELISA

NP-specific IgG1, IgG2a and IgM serum levels were determined by ELISA using NP17-BSA (20µg/ml), biotinylated anti-$IgG1_{a/b}$, biotinylated anti-$IgG2a_{a/b}$ and biotinylated anti-$IgM_{a/b}$ as isotype specific antibodies (BD Biosciences) and detected by Streptavidin-HRP (Dako Australia).

Generation of mixed bone marrow chimeras

The contribution of CCR6 to the generation of GC was investigated using mixed bone marrow chimeras. Lethally irradiated B6.CD45.2 host mice (after split dose of 2 x 450 rad within 48 hours as published [38]) received 2 x 10^7 bone marrow donor cells containing 50% $CD45.1^+$ and 50% $CD45.2^+$ *CCR6*$^{-/-}$ cells.

For isotype-specific ELISA of antigen-specific antibodies lethally irradiated B6.CD45.1 (IgH_b) mice received 2 x 10^7 bone marrow cells containing 50% IgH_a, $CD45.2^+$ B6.WT and 50% IgH_b $CD45.2^+$ B6.*CCR6*$^{-/-}$ cells. To establish control chimeras, the B6.Ly5.1 recipients received 2 x 10^7 bone marrow cells containing 50% IgH_a $CD45.2^+$ B6.WT and 50% IgH_b $CD45.2^+$ B6.WT mice. Twelve days after transfer, blood lymphocytes from the recipient mice were stained with fluorochrome conjugated antibodies against B220, CD45.1, CD45.2, or IgM_a and IgM_b, respectively, and analyzed by flow cytometry to reveal the extent of the reconstitution. The mice were immunized four to six weeks after reconstitution.

Conflict of interest:

The authors state that they had no conflict of interest.

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Figure legends

Figure 1: Regulation of CCR6 expression *in vitro*.

A) Flow cytometric time-course analysis of CCR6 and CD69 expression on naïve MD4-B cells isolated from spleen activated with HEL. B6.WT B cells isolated from inguinal lymph nodes were labelled *in vitro* with the proliferation dye CFSE and activated with either LPS (B) or anti-BCR antibody (C). 72 hours post activation CCR6 surface expression was determined by flow cytometry in correlation to cell proliferation (filled, grey histogram: CFSE at day 0; black line: cell division 0-7). Representative histograms are shown for generations 3 and 6 (black lines) for each stimulation (grey area: CCR6 expression at time point 0). Fold increase of CCR6 expression was calculated as the fold change in MFI comparing generation 0 with generation 3-7. (Standard error is shown as SD; n= 3; paired T-test; * p< 0.05, ** 0.001).

Figure 2: Analysis of *ccr6* mRNA expression.

Expression analysis of *ccr6* mRNA on naïve splenic B cells activated with anti-BCR, LPS and anti-CD40/IL-4 for the indicated timepoints (0h-24h) using qPCR (n= 4 in two independent experiments. The expression was determined in triplicates and is shown as SEM. Mann-Whitney- test; p< 0.05).

Figure 3: Effect of B cell activation on migration towards CCL20.

Transwell migration assays against CCL20 were performed using splenic B cells either activated with anti-BCR, anti-CD40/IL-4 or with LPS (n= 4 in two independent experiments. The specific migration rate towards CCL20 was normalized to the migration rate the cells had in overall presence of the chemokine. Migration was

determined in duplicates. (Standard error is shown as SEM. Mann-Whitney test; * p< 0.05, ** 0.001).

Figure 4: CCR6 expression on B cell subpopulations immunized with NP_{24}-KLH. Seven days after immunization with NP_{24}-KLH, splenic B cells were surface stained for CD19, CD95, GL7 and PNA to identify naïve ($CD19^+$ $CD95^-$ $GL7^-$ PNA^-), pre-GC B cells ($CD19^+$ $CD95^{int.}$ PNA^- $GL7^-$) and GC B cells ($CD19^+$ $CD95^{high}$ PNA^+ $GL7^+$). Moreover, splenic plasma cells were defined as $CD138^+$ $TACI^+$ (Gating strategy see Suppl. Figure 1). A) The CCR6 expression of these 4 subpopulations is depicted in histograms (white filled: CCR6 expression on $CCR6^{-/-}$ control B cells, grey filled: CCR6 expression on B6.WT B cells). B) The MFI of CCR6 expression is shown for the indicated B cell populations. (Standard error is shown as SD; n= 3; unpaired T-test; *** p< 0.001, **** 0.0001).

Figure 5: Structure of the GC in the CCR6 deficient GC reaction. B6.WT and $B6.CCR6^{eGFP/eGFP}$ mice were immunized with NP-KLH (A) or SRBC (B). After 4, 7 and 14 days the spleens were removed (SRBC only at day 7) and analysed by immunofluorescence microscopy (see Suppl. Figure 2). Spleen sections of immunized B6.WT and $B6.CCR6^{eGFP/eGFP}$ mice (4, 7 and 14 days post i.p. injection) were stained with antibodies against IgD, PNA and CD35. GC follicles were identified as IgD^- PNA^+ $CD35^+$ GC follicles. Samples were analysed using a confocal microscope (n=2-4 mice/genotype). For quantitative analysis the ratio of PNA^+ to $CD35^+$ area was determined. Data represent 2–4 mice per genotype and per immunogen (NP-KLH or SRBC) and 8-12 GC per individual mouse (Mann-Whitney- test; * p< 0.05, ** 0.001).

Figure 6: Flow cytometric quantification of GC dynamics in CCR6$^{-/-}$ mice.

B6.WT and B6.CCR6$^{-/-}$ mice were immunized with NP-KLH. On day 4, 7, 11 and 13 spleens were isolated and analysed for total GC B cells (A), dark zone (DZ) and light zone (LZ) B cells (B, C) (two independent experiments; n = 3-6 per genotype; unpaired T-test; * p< 0.05, ** 0.001).

Figure 7: Analysis of NP-specific IgM, IgG1 and IgG2a antibodies in allotype-specific ELISAs.

Mixed bone marrow chimeras to test allotype-specific immune responses from WT and CCR6$^{-/-}$ mice in a competitive setting were generated as indicated (A). Mice were immunized with NP-KLH and serum was analysed 7 and 21 after immunization using an allotype-specific ELISA in NP-BSA coated plates. (IgM serum dilution 1:1600; IgG1 serum dilution 1:6400; IgG2a serum dilution 1:6400) (B). Each dot represents one mouse (Mann-Whitney- test; * p< 0.05, ** 0.001).

Suppl. Figure 1: Gating strategy for B cell subpopulations.

Splenic B cells from mice immunized with NP-KLH were analysed 7 days after immunization. Four distinct populations of splenic B cells were defined: Naïve B cells (CD19^{+} CD95^{-} GL7^{-} PNA^{-}), pre-GC B cells (CD19^{+} CD95$^{int.}$ PNA^{-} GL7^{-}), GC B cells (CD19^{+} CD95^{high} PNA^{+} GL7^{+}) and plasma cells (CD138^{+} TACI^{+}).

Suppl. Figure 2: Structure of the GC in the CCR6 deficient GC reaction. B6.WT and B6.CCR6$^{eGFP/eGFP}$ mice were immunized with NP-KLH. After 4, 7 and 14 days

the spleens were removed and analysed by immunofluorescence microscopy. Spleen sections of immunized B6.WT and B6.CCR6$^{eGFP/eGFP}$ mice (4, 7 and 14 days post i.p. injection) were stained with antibodies against IgD (blue), PNA (green) and CD35 (red). GC follicles were identified as IgD^{-} PNA^{+} CD35^{+} GC follicles. Samples were analysed using a confocal microscope (n=2-4 mice/genotype) and representative images are shown. (Germinal centres (GC), B cell follicles (B) and T cell zones (T)). (Objective magnification: 20x; Scale bars are 100µm).

Suppl. Figure 3: Mixed bone marrow chimeras show a contribution of both genotypes to the formation of germinal centres.

B6.Ly5.2 host mice received 50% CD45.1^{+} and 50% CD45.2^{+} WT or CD45.2^{+} CCR6$^{-/-}$ bone marrow cells after lethal irradiation. The contribution of both genotypes was not quantified but comparable. The follicular B cells were stained with B220 (blue) and either anti-Ly5.1 or anti-Ly5.2 (red). (Objective magnification: 10x; Scale bars are 100µm).

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