Cannabinoid receptor 2 positions and retains marginal zone B cells within the splenic marginal zone

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Specialized B cells residing in the splenic marginal zone (MZ) continuously survey the blood for antigens and are important for immunity to systemic infections. However, the cues that uniquely attract cells to the MZ have not been defined. Previous work demonstrated that mice deficient in cannabinoid receptor 2 (CB2) have decreased numbers of MZ B cells but it has been unclear whether CB2 regulates MZ B cell development or positioning. We show that MZ B cells are highly responsive to the CB2 ligand 2-arachidonylglycerol (2-AG) and that CB2 antagonism rapidly displaces small numbers of MZ B cells to the blood. Antagonism for longer durations depletes MZ B cells from the spleen. In mice deficient in sphingosine-1-phosphate receptor function, CB2 antagonism causes MZ B cell displacement into follicles. Moreover, CB2 overexpression is sufficient to position B cells to the splenic MZ. These findings establish a role for CB2 in guiding B cells to the MZ and in preventing their loss to the blood. As a consequence of their MZ B cell deficiency, CB2-deficient mice have reduced numbers of CD1d-high B cells. We show that CB2 deficiency results in diminished humoral responses to a CD1d-restricted systemic antigen.

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Abbreviations used: 2-AG, 2-arachidonylglycerol; CB2, cannabinoid receptor 2; DTR, diphtheria toxin receptor; MZ, marginal zone; NP, nitrophenyl; PTX, pertussis toxin; S1P, sphingosine-1-phosphate.

The splenic marginal zone (MZ) is located at the border of the white pulp and red pulp. The arterial circulation of the spleen terminates in a porous vascular sinus, the marginal sinus, which lies in the MZ. Blood from the marginal sinus then transits through the MZ and into the red pulp (Mebius and Kraal, 2005). The MZ contains specialized macrophages, B cells, and dendritic cells. Cells in the MZ are continuously exposed to antigens carried in the blood (Mebius and Kraal, 2005).

MZ B cells differ from follicular B cells in several ways. Murine MZ B cells do not recirculate; they have a partially activated phenotype that allows for quick and vigorous antibody responses to blood-borne antigens and they are able to self-renew (Martin and Kearney, 2002). Additionally, MZ B cells differ from follicular B cells immunophenotypically by high surface expression of IgM, the complement receptor CD21, and the nonclassical major histocompatibility complex I molecule CD1d that allows for presentation of lipid antigens (Pillai and Cariappa, 2009). In vitro experiments have shown that MZ B cells can present CD1d-restricted lipid antigens to invariant (i) NKT cells, although their in vivo contribution to CD1d-restricted antibody responses has not been determined (Barral et al., 2008; Leadbetter et al., 2008).

It is thought that positioning of MZ B cells is dependent on signaling through various G protein–coupled receptors specifically through receptors coupled to Gαi, as treatment of mice with pertussis toxin (PTX), which inhibits all Gαi signaling, leads to a selective loss of B cells from the MZ (Guinamard et al., 2000). Positioning of MZ B cells is promoted by sphingosine-1-phosphate (S1P), which signals primarily through S1P receptor 1 (S1P1) and, to a lesser extent, through S1P receptor 3 (S1P3) to overcome the follicular attracting activity of the

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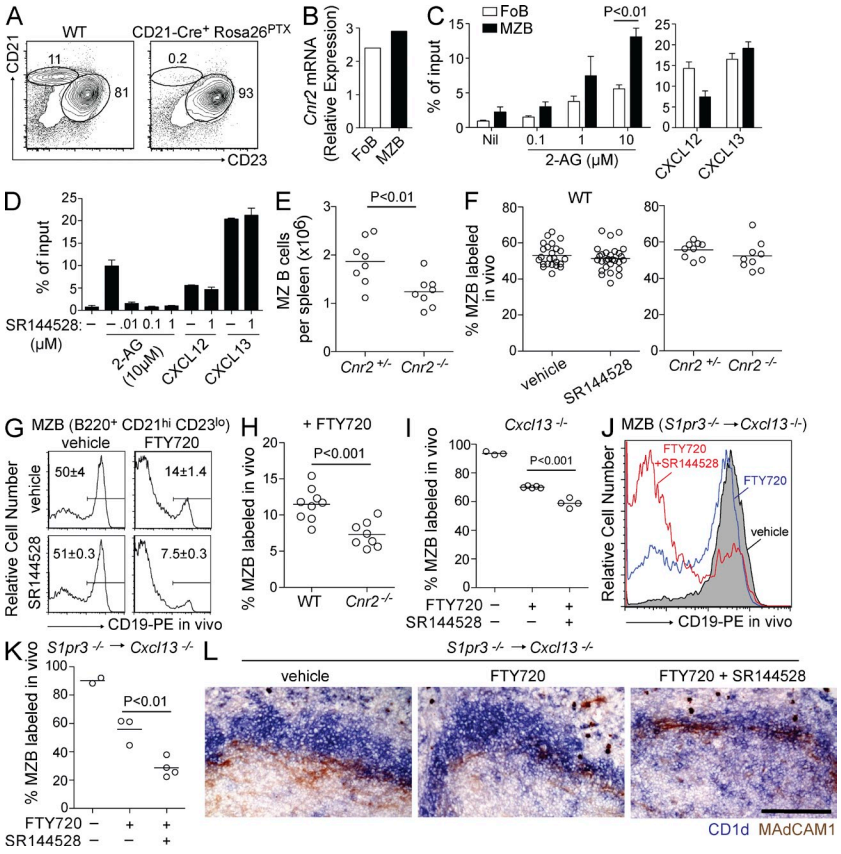

Figure 1. CB2 acts as a positional cue for MZ B cells. (A) Flow cytometry of splenic B cells from irradiated mice reconstituted with BM from either WT or CD21-Cre^{+} ROSA26^{PTX} mice. Dot plots are gated on CD19^{+} cells. Numbers indicate percentage of B cells in the outlined gates. Data are representative of more than five mice of each type. (B) Quantitative PCR analysis of *Cnr2* transcripts in sorted follicular B cells (FoB) and MZ B cells (MZB), presented relative to *Hprt1* expression. One of at least four independent experiments is shown. (C) Chemotactic responses of MZ B cells (CD21^{hi}CD23^{lo}B220^{+}) and follicular B cells (CD21^{int}CD23^{hi}B220^{+}) toward 2-AG, 100 ng/ml CXCL12, or 1 µg/ml CXCL13. One of six independent experiments is shown. (D) Chemotactic responses of MZ B cells toward 10 µM 2-AG, 100 ng/ml CXCL12, or 1 µg/ml CXCL13 in the absence or presence of the indicated concentrations of the CB2 antagonist SR144528. One of three independent experiments is shown. Error bars in C and D represent SEM. (E) MZ B cells per spleen (gated as in A) in CB2 heterozygous (*Cnr2*$^{+/-}$) or CB2-deficient animals (*Cnr2*$^{-/-}$). Data shown are pooled data from three independent experiments with two to four mice per group. (F) Percentage of MZ B cells labeled 5 min after i.v. injection of anti–CD19-PE in mice treated with SR144528 or vehicle for 3 h (left) or *Cnr2*$^{+/-}$ or *Cnr2*$^{-/-}$ mice (right). Data shown are pooled from three or more independent experiments with greater than two mice per group. (G) Flow cytometry of the binding of CD19-PE antibody 5 min after i.v. injection by splenic MZ B cells in mice treated with vehicle or FTY720 for 1.5 h, followed by vehicle or SR144528 for a further 3 h. Numbers indicate fraction of cells in the indicated gate, shown as mean ± SD (n = 3 mice). P < 0.01. Histograms are gated on B220^{+}CD21^{hi}CD23^{lo} MZ B cells. Data shown are one of two independent experiments with at least two mice per group. (H) Percentage of MZ B cells labeled 5 min after i.v. injection of anti–CD19-PE in WT or CB2-deficient chimeric mice treated with FTY720 for 4.5 h. Shown are pooled data from two independent experiments representative of three with at least four mice per group. (I) Percentage of MZ B cells labeled 5 min after i.v. injection of anti–CD19-PE in *Cxcl13*$^{-/-}$ mice treated as in G. (J and K) Flow cytometry (J) or percentage of MZ B cells labeled with CD19-PE antibody 5 min after injection (K) in *Cxcl13*$^{-/-}$ mice reconstituted with S1P3-deficient (*S1pr3*$^{-/-}$) BM treated as in F and G. Data in K are pooled data from two independent experiments with one to two mice per group. (L) Immunohistochemistry of spleen sections from mice in J stained for CD1d (blue) and MAdCAM1 (brown). Data are representative of at least three mice of each type. Bar, 100 µm. In all dot plots, dots indicate individual mice and lines indicate means.

chemokine CXCL13 signaling through its receptor CXCR5 on MZ B cells (Cinamon et al., 2004, 2008). However, in the absence of signaling through both S1P1 and CXCR5, MZ B cells remain positioned within the MZ, in contrast to the loss of MZ B cells after PTX treatment, suggesting that there are additional inputs through receptors coupled to Gαi that mediate positioning of MZ B cells.

The Gαi-coupled cannabinoid receptor 2 (CB2) is expressed in several immune cell types including B cells (Galiègue et al., 1995). The endocannabinoid 2-arachidonylglycerol (2-AG) is present within the spleen (Sugiura et al., 2006) and can act as a chemoattractant for mature B cells in vitro (Tanikawa et al., 2007). Mice deficient for CB2 have fewer MZ B cells than WT mice (Ziring et al., 2006). However, it is currently unclear whether CB2 deficiency results in defects in MZ B cell development, retention, positioning, or function. Recently it was shown that CB2 promotes retention of immature B cells within BM sinusoids (Pereira et al., 2009a), raising the question of whether CB2 could also promote cell positioning in the spleen, a possibility which we explored here.

RESULTS AND DISCUSSION

CB2 can act as a positional cue for MZ B cells

Previous work demonstrated that PTX treatment of mice caused a rapid loss of MZ B cells from the spleen (Guinamard et al., 2000). To determine whether this outcome reflected a B cell–intrinsic requirement of Gαi signaling for normal accumulation of MZ B cells, we crossed CD21-Cre^{+} mice (Kraus et al., 2004) to mice in which the ADP-ribosylating subunit of PTX has been introduced into the *ROSA26* locus but whose expression was prevented by a premature stop codon flanked by loxP sites (ROSA26^{PTX} mice; Regard et al., 2007). We found there was a striking loss of MZ B cells in CD21-Cre^{+} ROSA26^{PTX} mice compared with WT controls (Fig. 1 A), whereas follicular B cell numbers were preserved

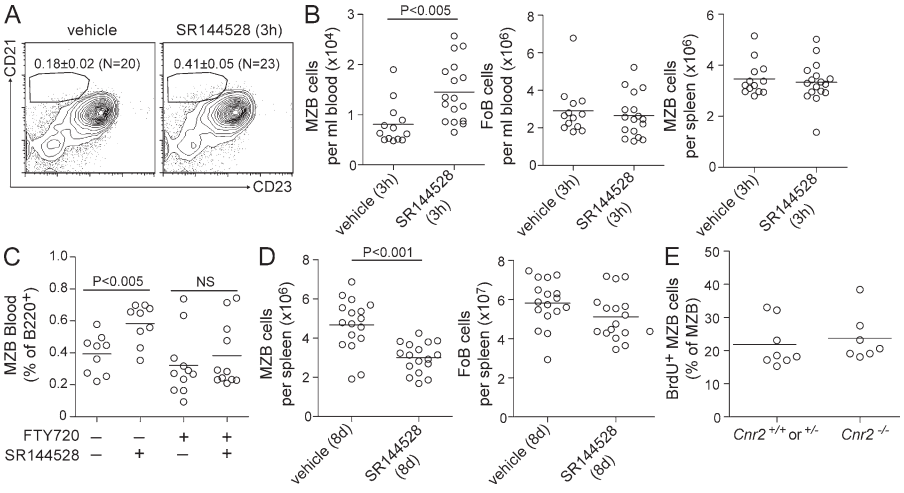

Figure 2. CB2 retains MZ B cells within the spleen. (A) Flow cytometry of blood from mice treated with vehicle or the CB2 antagonist SR144528 for 3 h. Dot plots are gated on B220⁺ cells. Numbers are means (±SD) of percentage of B cells in this gate for the indicated number of mice. $P < 0.001$. Data are representative of at least four independent experiments with at least four mice per group. (B) MZ B (MZB) cell and follicular B (FoB) cell counts from blood and spleen from mice treated as in A. Data are pooled from three independent experiments with four to six mice per group. (C) MZ B cells as percent of B220⁺ cells in blood from mice treated with vehicle or FTY720 for 1.5 h followed by vehicle or SR144528 for 3 h. Data are pooled from three experiments with two to five mice per group. (D) MZB and FoB cell counts per spleen from mice treated twice daily with SR144528 for 8 d. Data are pooled from four experiments with two to five mice per group. (E) BrdU-positive cells as percent of MZ B cells from *Cnr2*$^{+/+}$, *Cnr2*$^{+/-}$, or *Cnr2*$^{-/-}$ mice treated with BrdU in drinking water for 8 d. Data are pooled from three experiments with two to four mice per group. In all dot plots, dots indicate individual mice and lines indicate means.

(not depicted), confirming that there is a critical intrinsic requirement for Gαi signaling to establish or maintain the MZ B cell population.

Transcripts encoding the Gαi-coupled receptor CB2 are abundant in both MZ and follicular B cells (Fig. 1 B). In migration assays, MZ B cells showed significantly greater chemotactic responses toward 2-AG than follicular B cells (Fig. 1 C). 2-AG–induced migration was blocked by the highly selective CB2 antagonist SR144528 (Rinaldi-Carmona et al., 1998), whereas migration to CXCL13 and CXCL12 was not affected by SR144528 (Fig. 1 D), establishing that the compound was not having nonspecific effects on MZ B cell migration. In agreement with previously published data (Ziring et al., 2006), mice deficient in CB2 (encoded by *Cnr2*) had decreased MZ B cells compared with heterozygous littermate controls (Fig. 1 E).

To assess MZ B cell positioning in vivo in mice deficient for CB2 or mice treated for 3 h with the CB2 antagonist SR144528, we used a previously described antibody pulse-labeling technique in which PE-labeled CD19 antibody is administered i.v. for 5 min to preferentially label B cells within vascular spaces such as the MZ (Cinamon et al., 2008; Pereira et al., 2009a). In WT C57BL/6 mice, 50–60% of MZ B cells are located in the blood-exposed MZ compartment at any given moment and the remainder are sheltered within the adjacent follicles (Cinamon et al., 2008). We found that the proportion of MZ B cells localized within the MZ compartment was not altered in WT mice treated with SR144528 or in CB2-deficient mice as determined by in vivo labeling as well as immunohistochemistry (Fig. 1 F and not depicted). We then asked whether the contribution of CB2 to MZ B cell positioning might be revealed by removing other Gαi inputs known to affect positioning of these cells. In mice given FTY720 to functionally antagonize S1P1 signaling (Brinkmann et al., 2010), only a small percentage of MZ B cells were CD19-PE antibody labeled in vivo (Fig. 1 G) as a result of relocalization of most cells into follicles (Cinamon et al., 2004). Importantly, this fraction of labeled MZ B cells was further reduced in mice treated with SR144528 in addition to FTY720 (Fig. 1 G), indicating a further displacement of cells from the MZ into follicles. FTY720 treatment of CB2-deficient mice also caused a larger reduction in the fraction of labeled MZ B cells than occurred after treatment of WT mice (Fig. 1 H). In mice deficient for CXCL13, nearly all MZ B cells are positioned in the MZ as assessed by both in vivo labeling and immunohistochemistry (Fig. 1 I; Cinamon et al., 2004). After treatment with FTY720, the fraction of MZ B cells labeled in vivo in CXCL13-deficient mice was reduced to ∼70% (Fig. 1 I). The fraction of MZ B cells labeled in vivo was further reduced when CXCL13-deficient mice were treated with SR144528 in addition to FTY720 (Fig. 1 I). This small reduction in cell positioning in the MZ was not easily visualized by immunohistochemistry (unpublished data). In addition to S1P1, S1P3 (encoded by *S1pr3*) contributes to positioning MZ B cells within the MZ (Cinamon et al., 2004). S1P3 is not down-regulated after exposure to the active form of FTY720 (Sensken et al., 2008). To remove this additional Gαi-coupled receptor, we generated radiation chimeras using S1P3-deficient BM to reconstitute CXCL13-deficient hosts. When these chimeras were treated with FTY720 to functionally antagonize S1P1, 50–60% of the cells remained blood exposed (Fig. 1, J–L, middle). Administration of CB2 antagonist in addition to FTY720 now resulted in a large change in the distribution of MZ B cells, leaving only ∼25% of the cells in the blood-exposed MZ (Fig. 1, J and K). By immunohistochemical analysis of spleen sections, using CD1d to detect MZ B cells and MAdCAM1 to detect marginal sinus lining cells that delineate the boundary of MZ and white pulp, we found a redistribution of MZ B cells into the white pulp in mice treated with CB2 antagonist (Fig. 1 L). Accumulation of these multiple receptor–deficient MZ B cells within the white pulp may be mediated by CCR7 and/or EBI2 signaling

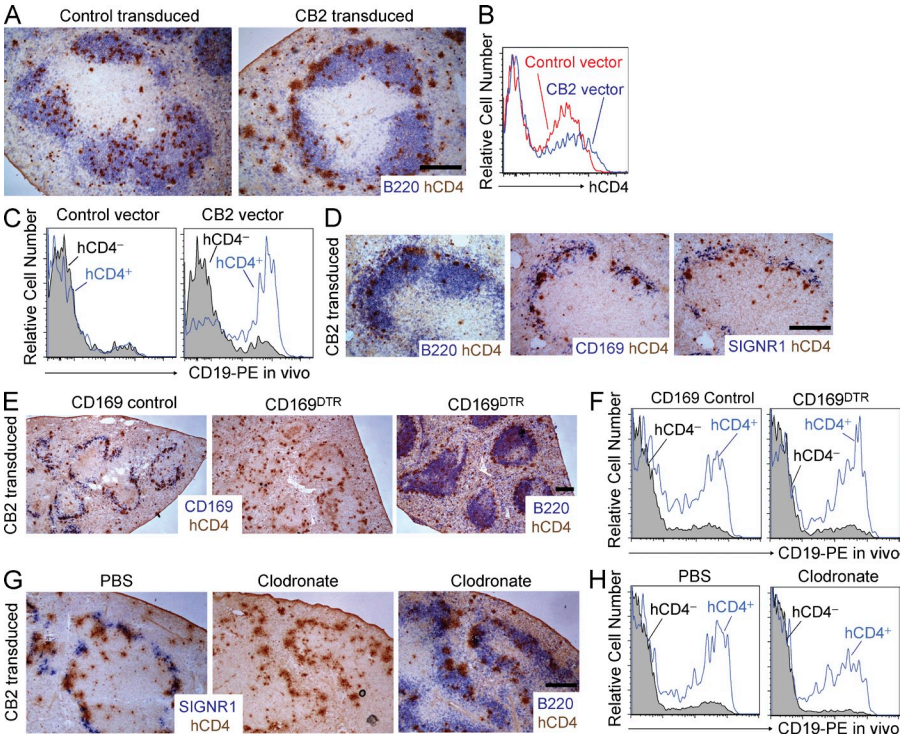

Figure 3. CB2 overexpression is sufficient to position B cells to the MZ. (A–C) CD45.1+ B cells retrovirally transduced with vectors encoding either control surface receptor (truncated nerve growth factor receptor) or CB2, as well as the reporter hCD4, were transferred into CD45.2+ recipient mice. Mice were analyzed 16 h later. (A) Immunohistochemical staining of splenic cryosections shows localization of hCD4+ transduced cells (brown) and endogenous B cells (blue). (B) Flow cytometry of hCD4 expression in transferred retrovirally transduced B cells. (C) Flow cytometry of the binding of CD19-PE antibody 5 min after i.v. injection by donor CD45.1+ B cells in recipient mice. Transduced B cells (hCD4+) are shown in blue, co-transferred nontransduced B cells (hCD4−) in gray. (D) Co-localization of CB2-transduced cells (hCD4; brown) with B cell and macrophage markers (B220, CD169, and SIGNR1; blue) in adjacent sections. Data in A–D are representative of one of three independent experiments. (E and F) CB2-transduced CD45.1+ B cells were transferred into CD169DTR or littermate control mice that had been treated with diphtheria toxin. (E) Immunohistochemical staining of splenic cryosections. (F) Flow cytometry of the binding of anti–CD19-PE 5 min after i.v. injection by transferred CD45.1+ B220+ B cells. (G and H) CB2-transduced CD45.1+ B cells were transferred into mice treated 2.5 wk earlier with liposomal clodronate or PBS. Analysis was as in E and F. Data in (E–H) are representative of two independent experiments of each type. Bars, 100 µm.

(Ohl et al., 2003; Pereira et al., 2009b). Both CCR7 and EBI2 are expressed in MZ B cells at similar levels to follicular B cells, and MZ B cells migrate toward the CCR7 ligand CCL21 and the EBI2 ligand 7α,25-dihydroxycholesterol (unpublished data). These findings demonstrate that CB2 acts as positional cue for MZ B cells and that its positional function is most apparent in the absence of S1P1, S1P3, and CXCR5 signaling.

CB2 retains MZ B cells in the spleen

Although there was no appreciable difference in positioning of MZ B cells after treatment with CB2 antagonist in WT mice, we did notice a small increase in MZ B cells within the blood 3 h after the treatment (Fig. 2, A and B). There was no increase in follicular B cells in the blood nor was there a measurable decrease in MZ B cells in the spleen at this time point (Fig. 2 B). Integrins are involved in retaining MZ B cells within the spleen and MZ B cells exhibit high in vitro adhesive activity to VCAM1 and ICAM1 (Lu and Cyster, 2002). However, MZ B cell adhesion to either VCAM1 or ICAM1 in a static in vitro adhesion assay was not significantly affected by addition of 2-AG or CB2 antagonist (unpublished data). We hypothesized that the release of MZ B cells to the blood after CB2 antagonism was dependent on intact S1P1 signaling. We found that when mice were pretreated with FTY720 to functionally antagonize S1P1, CB2 antagonism was less efficient in releasing MZ B cells into the blood (Fig. 2 C).

Treatment with CB2 antagonist did not alter splenic MZ B cell numbers after 3 h (Fig. 2 B, right). However, it seemed possible that there might be a cumulative effect of continued cell loss over long periods of time. We found that after 16 treatments with SR144528 over an 8-d period, MZ B cells were decreased by approximately one third, whereas there was no statistically significant change in splenic follicular B cell numbers (Fig. 2 D).

It was possible that CB2 deficiency decreased MZ B cell numbers by decreasing the generation of new MZ B cells. CB2-deficient, heterozygous, or WT mice were treated with BrdU containing drinking water for 8 d and then assessed for BrdU incorporation by flow cytometry. There was a similar amount of BrdU incorporation by MZ B cells in CB2 deficient mice compared with either heterozygote or WT controls (Fig. 2 E), suggesting that there are not major differences in MZ B cell turnover in CB2-deficient and control mice but not excluding minor differences.

Collectively, these findings suggest that an important function of CB2 in MZ B cells is to promote their retention in the spleen. We postulate that CB2 antagonism for longer periods of time, or CB2 deficiency, reduces the number of MZ B cells through ongoing loss of normally non-recirculatory MZ B cells into the blood. However, we do not exclude the possibility that CB2 also functions to promote MZ B cell development or survival.

CB2 overexpression is sufficient to position B cells to the MZ

To better understand the role of CB2 in MZ B cell positioning, we tested whether increased CB2 expression was sufficient to alter B cell localization. Activated B cells were

transduced with a retrovirus containing a CB2 insert and a human CD4 (hCD4) reporter, or with a control retrovirus lacking the CB2 insert, and transferred into WT recipient mice, and positioning of transduced B cells was assessed after 16 h. Strikingly, CB2 overexpression was sufficient to position B cells to the MZ (Fig. 3 A). This contrasts with the effect of S1P1 overexpression, where cells distribute through the red pulp without any preference for the MZ (Lo et al., 2005). The stronger and more diffuse staining of the CB2-transduced cells likely reflects the higher reporter expression on these cells compared with vector transduced cells (Fig. 3 B). In vivo labeling with anti–CD19-PE confirmed that many CB2-transduced B cells were positioned in blood-exposed spaces in the spleen, whereas control transduced B cells were not (Fig. 3 C). The distribution of CB2-overexpressing B cells in the MZ suggests that CB2 ligand is concentrated in this area. Macrophage cell lines have been reported to produce the endocannabinoid 2-AG in vitro (Sugiura et al., 2006) and macrophages have been implicated in retaining MZ B cells in the MZ (Karlsson et al., 2003). There are two major macrophage populations in the MZ. Marginal metallophilic macrophages can be identified by their high expression of CD169 and reside at the interface of the MZ and follicle (Mebius and Kraal, 2005). MZ macrophages, identified by expression of the C-type lectin SIGNR1, reside within the MZ (Mebius and Kraal, 2005). CB2-overexpressing B cells colocalized more closely with marginal metallophilic macrophages than MZ macrophages (Fig. 3 D). To assess the contribution of marginal metallophilic macrophages to positioning of CB2-overexpressing B cells, mice expressing diphtheria toxin receptor (DTR) from the CD169 locus ($CD169^{DTR}$ mice; Miyake et al., 2007) or littermate controls were treated with diphtheria toxin to deplete $CD169^{+}$ cells and then given CB2-transduced B cells. The CB2-overexpressing cells could position to the MZ and access vascular spaces as assessed by in vivo labeling despite an absence of $CD169^{+}$ cells (Fig. 3, E and F). To test the possibility that MZ macrophages contributed to ligand production, mice were treated with liposomes containing clodronate. MZ macrophages remain absent for several weeks after treatment with liposomal clodronate (van Rooijen et al., 1989). Such treatments were found to cause an acute disruption in white pulp organization that lasted for several days (unpublished data). To allow time for repopulation of B cells in the follicles, we transferred CB2-transduced B cells into mice between 2 and 3 wk after liposomal clodronate administration. Despite an absence of SIGNR1 staining, CB2-transduced B cells were able to position in the MZ of clodronate-treated animals (Fig. 3, G and H). These results suggest that CB2 ligand is concentrated within the splenic MZ and that neither marginal metallophilic macrophages nor MZ macrophages contribute significantly to the distribution of CB2 ligand in vivo. Given this and the evidence of CB2 expression by some myeloid cell types (Munro et al., 1993; Sugiura et al., 2004), we asked whether CB2 deficiency affected splenic macrophage populations. Combined CD169 and SIGNR1 staining of spleen sections showed that both macrophage populations

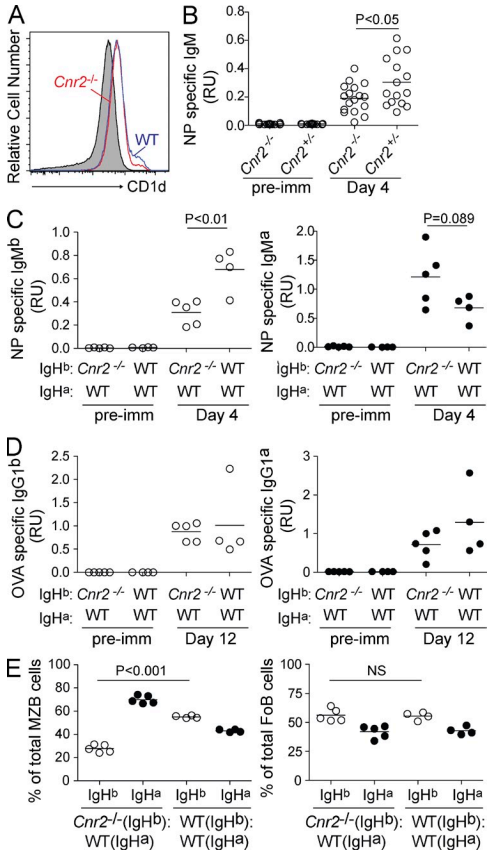

Figure 4. Diminished early antibody production to CD1d-restricted antigen in CB2-deficient mice. (A) Flow cytometric analysis of CD1d expression on CB2-deficient and WT splenic B cells from a mixed chimera. Histogram is gated on $B220^{+}$ cells. Background CD1d staining of non–B cells is shown in gray. A representative of five mice is shown. (B) NP-specific IgM assessed by ELISA in serum from CB2-deficient or heterozygous littermates that had been immunized i.v. with NP-αGalCer. Serum was collected before (preimm) and 4 d after immunization. Data are shown in relative units (RU) and are pooled from three independent experiments with four to seven mice per group. (C–E) ELISA analysis of serum from 1:1 mixed CB2-deficient or WT IgH^{b} and WT IgH^{a} BM chimeric mice. (C) Mixed chimeras were immunized i.v. with NP-αGalCer. NP-specific IgM^{b} (left) and IgM^{a} (right) in serum from the indicated time points. (D) Mixed chimeras were immunized i.p. with OVA in alum. OVA-specific $IgG1^{b}$ (left) and $IgG1^{a}$ (right) in serum from the indicated time points. (E) Contribution of CB2-deficient or WT (IgH^{b}) BM compared with WT BM (IgH^{a}) in MZ B cells (left) or follicular B (FoB) cells (right) of mixed chimeras. Data in C–E are representative of at least two independent experiments with four or more mice per group. In all dot plots, dots indicate individual mice and lines indicate means.

were similarly abundant in CB2-deficient mice compared with WT (unpublished data).

CB2 is required for optimal CD1d-restricted humoral responses

It has been difficult to establish the contribution of MZ B cells to T cell–independent humoral responses to model antigens. A previous study showed that mice lacking MZ B cells as a result of impaired Notch signaling had essentially normal

humoral responses to haptenated T cell–independent antigens such as lipopolysaccharide or Ficoll (Tanigaki et al., 2002). MZ B cells express high levels of the MHC class I–like molecule CD1d that can present lipid antigens to iNKT cells (Pillai and Cariappa, 2009). CB2-deficient mice have a lower frequency of CD1d^{hi} B cells in the spleen than WT mice, consistent with the reduced number of MZ B cells (Fig. 4 A). MZ B cells activate T cells more rapidly and efficiently than follicular B cells (Attanavanich and Kearney, 2004) and recent in vitro experiments have indicated that lipid-loaded MZ B cells promote greater iNKT cell activation than follicular B cells (Barral et al., 2008; Leadbetter et al., 2008). To determine if CB2 deficiency impaired the ability of mice to mount CD1d-restricted humoral responses, we i.v. immunized CB2-deficient or littermate control mice with the haptenated lipid antigen 4-hydroxy-3-nitrophenyl (NP)-αGalactosyl-Ceramide (NP-αGalCer; Leadbetter et al., 2008). Because the MZ B cell response to T cell–independent antigens peaks at day 3–4, earlier than the follicular response (Martin and Kearney, 2001), we examined serum antibody levels at this early phase of the response. CB2-deficient mice showed an impaired antigen-specific IgM antibody production to NP-αGalCer 4 d after immunization (Fig. 4 B). To determine whether this diminished response reflected a B cell–intrinsic defect, we generated mixed chimeras using CB2-deficient or CB2-WT IgH^b donors and WT IgH^a donors. Serum collected from CB2-deficient mixed chimeras 4 d after immunization showed impaired IgM^b responses compared with CB2-WT mixed chimeras (Fig. 4 C, left). In contrast, IgM^a, produced by WT B cells in CB2-deficient mixed chimeras, trended toward increased responses compared with WT mixed chimeras (Fig. 4 C, right). We next assessed humoral responses of mixed chimeras immunized with the conventional T cell–dependent antigen ovalbumin in the adjuvant alum. We found no difference in IgG1^b or IgG1^a production between CB2-deficient and CB2-WT mixed BM chimeras (Fig. 4 D). Analysis of the B cell contribution to the MZ compartment revealed a decreased contribution of CB2-deficient compared with WT B cells, whereas follicular B cells of the two genotypes were equally represented (Fig. 4 E). The decrease in MZ B cells derived from CB2-deficient BM was proportional to the decrease in NP-specific IgM^b production in these animals. Conversely, the trend toward increased NP-specific IgM^a production in CB2-deficient mixed chimeras was proportional to the increase in MZ B cells derived from WT IgH^a BM. These results suggest that decreases in the size of the MZ B cell compartment as a result of CB2 deficiency lead to decreased CD1d-restricted humoral responses.

In summary, we show that CB2 provides a Gαi-coupled input that promotes B cell tropism for the splenic MZ. Previous work has led to a model where MZ B cells shuttle between the MZ and follicle in cycles promoted by alternating responses to S1P and CXCL13 (Cinamon et al., 2008). However, S1P is unlikely to be more abundant in the MZ than in the blood or red pulp, raising the question of what factors prevent MZ B cell egress from the spleen. One factor is likely to be the high integrin adhesive function of MZ B cells and the abundant integrin ligand expression in the MZ (Lu and Cyster, 2002). We suggest that an additional factor is CB2-mediated attraction to locally produced ligand. MZ B cells respond more strongly to the endocannabinoid 2-AG than follicular B cells, and previous work has shown that small in vitro differences in chemotactic responsiveness can be associated with large in vivo effects on cell position (Reif et al., 2002; Bromley et al., 2005; Hardtke et al., 2005). In CB2-deficient animals, ongoing loss to the blood may account, at least partially, for the decreased number of MZ B cells. We do not exclude the possibility that CB2 also contributes to promoting the development or survival of MZ B cells. We propose that stromal cells within the MZ, such as the marginal sinus lining cells, are responsible for CB2 ligand generation. The CD1d-restricted humoral response to αGalCer-containing antigens depends on interactions with iNKT cells (Barral et al., 2008; Leadbetter et al., 2008). Although it has so far been problematic to selectively detect iNKT cell distribution in situ within nontransgenic mouse tissues, by in vivo CD45 antibody labeling and flow cytometry we found that a higher fraction of CD1d-tetramer binding iNKT cells than conventional CD3 T cells were in blood-exposed regions of the spleen (54 ± 1 vs. 20 ± 2%; $n = 10$; $P < 0.001$), consistent with the presence of some iNKT cells in the MZ. Although we cannot exclude the possibility that CB2 deficiency affects CD1d-restricted B cell responses in additional ways, we feel that the present data support the conclusion that MZ B cells make a prominent contribution to the early antibody response against CD1d-restricted systemic antigens.

MATERIALS AND METHODS

Mice and BM chimeras. Adult C57BL/6 (CD45.2$^+$) and Ly5.2 (CD45.1$^+$) mice 7–10 wk of age were from the National Cancer Institute. *Cnr2*$^{-/-}$ mice (005786; The Jackson Laboratory; MGI 3604531) were backcrossed to C57BL/6 mice for 7–10 generations. ROSA26^{PTX} mice were on a mixed B6-129 background (Regard et al., 2007). CD169^{DTR} mice (Miyake et al., 2007; MGI 3720841) were backcrossed to C57BL/6 for at least seven generations. *CXCL13*$^{-/-}$ mice (MGI 1888499) and *S1pr3*$^{-/-}$ (MGI 1339365; provided by R. Proia, National Institutes of Health, Bethesda, MD) were on a B6 background. BM chimeras were made as previously described (Pereira et al., 2009a) and analyzed at least 7 wk after reconstitution. Mice were housed in a specific pathogen-free environment in the Laboratory Animal Research Center at the University of California, San Francisco (UCSF), and all animal procedures were approved by the Institutional Animal Care and Use Committee.

Treatments. For short-term CB2 blocking experiments, mice were injected once in the tail vein with 200 µl of 0.1% ethanol in PBS (carrier) or with 10 µg CB2 antagonist SR144528 (obtained from the National Institute of Mental Health's Chemical Synthesis and Drug Supply Program), dissolved in 0.1% ethanol in PBS. For long-term blocking of CB2 function, mice were injected i.p. twice a day with either 10 µg SR144528 or carrier for 8 d. For FTY720 pretreatment, mice were injected intraperitoneally at 1 mg FTY720 per kg body weight or an equivalent volume of saline 1.5 h before administration of CB2 antagonist. Liposomal clodronate or PBS was obtained from N. van Rooijen (clodronateliposomes.org; Free University Medical Center, Amsterdam, Netherlands) and was administered at a dose of 10 µl/g by i.v. injection by tail vein. Diphtheria toxin (EMD Biosciences) was administered at a dose of 30 ng/g intraperitoneally 3 and 1 d before transfer of cells. For BrdU

labeling, mice were given drinking water containing 0.5 mg/ml BrdU and 10 mg/ml glucose (Sigma-Aldrich) to avoid taste aversion for 8 d. Detection of BrdU-labeled cells was with FITC-labeled anti-BrdU (3D4; BD) according to the manufacturer's protocol.

In vivo labeling of B cells. For in vivo labeling of B cells, mice were i.v. injected with 1 µg PE–anti-CD19 (BD) 5 min before sacrifice. Tissues were processed on ice and samples were analyzed by flow cytometry.

Flow cytometry, chemotaxis, and immunohistochemical analysis. A FACSCalibur or LSRII (BD) was used for flow cytometry. All antibodies were obtained from BD. MZ and follicular B cells were gated on $B220^+$ cells. MZ B cells were gated as $CD23^{lo}CD21^{hi}$ and follicular B cells were gated as $CD23^{hi}CD21^{int}$. iNKT cells were detected using PBS57-loaded CD1d tetramers (National Institutes of Health Tetramer Core Facility). Chemotaxis assays were performed using total splenocytes as previously described (Cinamon et al., 2004). Immunohistochemistry of spleen sections was done as previously described (Cinamon et al., 2004). Anti-CD169 (Moma-1) was obtained from AbD Serotec. Anti-SIGNR1 (22D1) was a gift from C.G. Park (Rockefeller University, New York, NY).

Retroviral constructs and transductions. The CB2 retroviral construct was made by inserting the mouse *Cnr2* open reading frame into the MSCV2.2 retroviral vector in frame with a preprolactin leader and FLAG epitope–encoding sequence and followed by an internal ribosome entry site and cytoplasmic domain truncated human CD4 as an expression marker (Reif et al., 2002). The control vector contained cytoplasmic domain truncated human nerve growth factor receptor as an irrelevant insert. To generate activated B cells that can be efficiently retrovirally transduced, MD4 Ig-transgenic mice (MGI 2384500) containing lysozyme-specific B cells were injected with 5 mg hen egg lysozyme, splenocytes were harvested 6 h later, and the B cells further activated by culturing with 20 µg/ml anti-CD40 (FGK4.5; UCSF Hybridoma Core) for 24 h as in past studies (Reif et al., 2002). The activated B cells were spin infected for 2 h with retroviral supernatant and cultured overnight before transfer into host mice. Transferred cells were analyzed after 16 h by flow cytometry and immunohistochemistry.

Immunizations and ELISAs. Mice were immunized i.v. with 1 µg NP-αGalCer (Leadbetter et al., 2008) in 200 µl PBS containing 0.05% BSA that had been briefly sonicated or i.p. with 50 µg OVA (Sigma-Aldrich) in alum (Sigma-Aldrich). Serum was prepared from blood collected from the retroorbital space 4 or 12 d later. For NP or OVA-specific antibody detection, microtiter plates were coated overnight at 4°C with 10 µg/ml NP(30)-BSA (Biosearch Technologies Inc.) or 20 µg/ml OVA diluted in carbonate buffer, pH 9.6. Washing was with PBST (0.01% Tween-20), blocking was with PBST/5% BSA, and serum and antibodies were diluted in PBST/5% BSA. Serum dilutions were incubated in the coated wells for 1–2 h, and bound antibodies were detected using biotinylated rat anti–mouse IgM (BioLegend), IgM^a, IgM^b, $IgG1^a$, or $IgG1^b$ (BD), followed by HRP-conjugated streptavidin and developed with ABTS (SouthernBiotech). Absorbance was measured at 405 nm in a VersaMax microplate reader using SoftMax pro 5.2 (Molecular Devices). Standard curves and relative serum antibody concentrations were calculated using SoftMax pro 5.2 software.

Statistical analysis. Data were analyzed with a two-sample Student's *t* test.

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