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Cannabinoid Receptor 2 Is Critical for the Homing and Retention of Marginal Zone B Lineage Cells and for Efficient T-Independent Immune Responses

Sreemanti Basu,*† Avijit Ray,* and Bonnie N. Ditte†

The endocannabinoid system has emerged as an important regulator of immune responses, with the cannabinoid receptor 2 (CB2) and its principle ligand 2-arachidonoylglycerol playing a major role. How CB2 regulates B cell functions is not clear, even though they express the highest levels of CB2 among immune cell subsets. In this study, we show that CB2-deficient mice have a significant reduction in the absolute number of marginal zone (MZ) B cells and their immediate precursor, transitional-2 MZ precursor. The loss of MZ lineage cells in CB2−/− mice was shown to be B cell intrinsic using bone marrow chimeras and was not due to a developmental or functional defect as determined by B cell phenotype, proliferation, and Ig production. Furthermore, CB2−/− B cells were similar to wild type in their apoptosis, cell turnover, and BCR and Notch-2 signaling. We then demonstrated that CB2−/− MZ lineage B cells were less efficient at homing to the MZ and that their subsequent retention was also regulated by CB2. CB2−/− mice immunized with T-independent Ags produced significantly less Ag-specific IgM. This study demonstrates that CB2 positively regulates T-independent immune responses by controlling the localization and positioning of MZ lineage cells to the MZ.


The endocannabinoid system has emerged as an important modulator of immune responses including immune cell proliferation, survival, and migration (1). The best-studied components of the endocannabinoid system are the endogenously synthesized endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide and their receptors cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) (2–5). These cannabinoid receptors are Gt protein-coupled receptors (GPCRs) with CB1 being highly expressed by most neurons and at a low level by immune cells (5, 6). In contrast, CB2 is highly expressed by immune cells and by some neurons in the brain stem (4, 6, 7). 2-AG is considered the principle endogenous ligand for CB2 and together are thought to mediate immune modulation (1, 8). 2-AG is synthesized from arachidonic acid-containing phospholipid precursors and is detected at a high level in immune organs including bone marrow (BM) and spleen (9, 10). A large number of in vitro and in vivo studies demonstrated that CB2 is capable of suppressing immune responses suggesting that CB2 would make a good therapeutic target for the treatment of immune disorders (1). However, very little is known about the cellular and molecular mechanisms whereby CB2 modulates immune responses. This is especially true for B cells, which of all immune cells express the highest levels of the receptor (6, 11). Studies defining specific roles for CB2 in B cells are difficult to interpret because 2-AG and synthetic CB2-selective agonists also bind CB1 and because the widely used CB2 antagonist has also been shown to function as an inverse agonist (1, 12, 13). CB2 has been shown to promote the chemotaxis of naive B cells and the retention of immature B cells in the BM sinusoids (9, 14). In addition, CB2−/− mice were shown to have a significant reduction in the percentage of marginal zone (MZ) B cells (15). These cumulative data suggest that like T cells (16), CB2 is also a strong modulator of B cell immunity. However, these mechanisms are not well understood.

MZ B cells play a central role in eliciting Ab responses against blood-borne pathogens (17). In mice they are non-recirculating and reside within the MZ located outside the marginal sinus at the juncture of white and red pulp in the spleen. The outer boundary of the marginal sinus is porous, and blood released in the sinus by splenic arterioles flows through the MZ before being collected by the red pulp venules (17). MZ B cells are enriched for BCR specific for microbial polysaccharides and express high levels of TLRs and the complement receptor CD21/CD35, which enables them rapidly to respond to blood-borne pathogens (18–21). Finally, MZ B cells have a low activation threshold that is thought to allow them to rapidly differentiate into Ab-secreting plasma cells in a T-independent manner (22). Mice lacking MZ B cells have impaired T-independent humoral responses (21, 23, 24).

The factors that regulate the homeostasis of MZ B cells are still not completely understood. Immature B cells emigrate from the BM to the spleen and through at least two transitional stages [transitional-1 (T1) and transitional-2 (T2)] give rise to either follicular (Fo) or MZ B cells (17, 25). The immediate precursor of MZ B cells has been identified as the CD21hiCD23lo transitional-2 marginal zone precursor (T2-MZP) (26). BCR signal strength is known to be a key determinant of B cell fate during maturation.
Studies with a number of knock-in and knockout mice models with differing BCR signal strengths suggested that a strong signal facilitates Fo B cell, whereas a weak signal drives MZ B cell differentiation (27–30).

Another critical regulator of MZ B cell development is Notch-2, which is a type I transmembrane protein that upon interaction with its ligand δ-like-1 (DL-1) undergoes proteolytic cleavage releasing its intracellular domain (31, 32). Intracellular Notch-2 translocates to the nucleus, interacts with transcription factor RBP-Jk, and facilitates transcription of target genes including *Hes-1, Hes-5,* and *Deltex-1* (33, 34). Mice conditionally deficient in Notch-2 or its ligand DL-1 demonstrated a complete absence of MZ B cells and their precursors (35, 36). In addition, GPCRs are also known to play an important role in the development, homing, and/or retention of MZ B cells as shown by the displacement of MZ B cells after treatment with pertussis toxin (21). Similarly, *Gos2*−/− mice have a significant reduction in MZ B cells (37). Sphingosine-1-phosphate receptor 1 (SIP1), also a GPCR, is essential for the homing and retention of MZ B cells (38). Although MZ B cells develop in SIP1-deficient mice, a recent study suggested that T2-MZP B cells also need to migrate to the MZ to differentiate into MZ B cells, as DL-1 is exclusively expressed in the MZ and red pulp (39). Whether CB2 regulates known pathways driving MZ B cell development and their homing to and retention within the MZ is not known.

In this study, we investigated the mechanisms whereby CB2 regulates MZ B cell numbers and the subsequent functional consequence. We found that the absolute number of both MZ and T2-MZP B cells were significantly reduced in a B cell-intrinsic manner. Functionally CB2-deficient MZ B cells were similar to wild type, and no defects in their developmental pathways including phenotype and their BCR and Notch-2 signaling were observed. Because CB2−/− B cells failed to migrate in response to 2-AG, we investigated whether migration of CB2−/− T2-MZP and MZ B cells was altered in vivo. Using several approaches, we found that CB2 blocked the homing and retention of MZ B cells and their precursors but had a marginal effect on Fo B cells. Finally, we show that CB2−/− mice exhibit reduced resting serum IgM levels and that upon immunization with T-independent Ags exhibit significantly reduced levels of Ag-specific IgM during the early immune response. These data indicate that CB2 positively regulates B cell immunity by promoting the appropriate localization and retention of MZ B cells such that they are able to respond to foreign Ags resulting in the early production of IgM, an essential immune component of protective immunity against multivalent microorganisms.

Materials and Methods

**Mice and reagents**

CB2−/− mice on the C57BL/6 background were provided by Dr. N.E. Buckley (40). Wild type (WT; CB2+/+) and CB2−/− mice were generated from homozygote crosses generated from the littermates of CB2+/+ × CB2+/+ progeny and maintained in the Translational Biomedical Research Center of the Medical College of Wisconsin. B6.129S2-Igh-6tm1Cgn/J (μMT) and B6.SJL-Pipyrc pepc /BoyJ (CD45.1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal protocols were approved by the Institutional Animal Care and Use Committee. Anti-B220-PE Texas red, anti-IGM-PE, anti-IgM-V450, anti-BrdU–allophycocyanin staining kit, anti-active caspase-3–FITC, and purified anti-mouse anti-IgM Abs. Pro B/pre B cells were identified as B220+IgM−, immature B cells as B220+IgM0, T cells as B220−IgM+, and mature B cells as B220−IgM−. Staining for active caspase-3 and BrdU were performed according to the manufacturer’s recommendations. Sample acquisition was performed with an LSR II (Becton Dickinson, San Jose, CA), and data were analyzed using FlowJo software (Tree Star, Ashland, OR). For real-time PCR and in vitro cultures, splenic B cells were enriched by complement depletion of T cells (42). Subsequently, T cell-depleted splenocytes were stained as above, and Fo B, T2-MZP, and MZ B cells were sorted with a FACSaria (Becton Dickinson). CD4 T cells were sorted from RBC-depleted splenocytes. Cell purity was >95%.

**Immunofluorescence analysis**

Splines from 8- to 12-wk WT and CB2−/− littersmates were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and snap frozen. Seven-micrometer cryostat sections were generated, fixed with acetone, and blocked with 5% goat serum. Subsequently, sections were stained with anti-IgM–V450 and anti-IgD–FITC. Sections were mounted with an aqueous mounting medium, and images were captured with a Nikon Eclipse TE200 inverted fluorescent microscope.

**In vitro proliferation and IgM production**

To assess proliferation in vitro, sorted WT and CB2−/− MZ B cells were labeled with 2 μM CFSE. Labeled cells (0.1 × 10^6) were cultured in 96-well plates in triplicate in the presence of 10 μg/ml anti-CD40, or 10 μg/ml LPS for 72 h at 37 °C. DAPI was added prior to sample acquisition, and the proliferation of splenocytes was determined by evaluating CFSE dye dilution by flow cytometry. To evaluate Ig production, 0.1 × 10^6 sorted WT and CB2−/− MZ B cells were cultured with 10 μg/ml LPS in the absence or presence of 100 nM or 1 μM 2-AG (resuspended in PBS) for 72 h at 37 °C. Culture supernatants were collected, and total IgM was measured by ELISA.

**RNA isolation and real-time PCR**

Total RNA isolation was performed from sorted cells using the RNeaQueous-4PCR kit (Ambion, Austin, TX). Equivalent amounts of RNA were used for first-strand cDNA synthesis using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). The quality of the cDNA was assessed by *GAPDH* PCR. The expression of *CNR2, Hes-1, Deltex-1,* and *RBP-Jk* genes was quantified by real-time PCR using TaqMan primers and probes from Invitrogen and an ABI Prism 7500 PCR machine (Applied Biosystems, Foster City, CA). The amplification cycle was as follows: 10 min hold at 95 °C, and then 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Relative levels of *CNR2, Hes-1, Deltex-1,* and *RBP-Jk* genes was calculated from a *GAPDH* standard curve that was generated for each sample from serially diluted cDNA (43).

**BM chimeras**

BM cells from WT (CD45.1) and CB2−/− (CD45.2) mice, mixed in 1:1 or 1:4 ratios, were transplanted (4 × 10^5 cells) into lethally irradiated (950 rad) μMT recipient mice. Transplantation of equally mixed WT (CD45.1) and
Regulates MZ B cells and T-independent immunity

CB2 regulates MZ B cells and T-independent immunity

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BrDU labeling

To assess in vivo B cell turnover, BrDU (0.8 mg/ml) was administered to WT and CB2−/− mice with drinking water for 16 h (BrDU pulse chase) or continuously for 9 d. Glucose (10 mg/ml) was added to the BrDU water to avoid taste aversion. BrDU incorporation in splenic B cell subsets was analyzed by flow cytometry on the indicated days.

Analysis of calcium flux

For the measurement of intracellular calcium flux induced by BCR cross-linking, splenocytes from WT and CB2−/− mice were first stained with anti-B220, anti-IgM, anti-CD21, and anti-CD23, and anti-CD93 Abs (to identify T2 B cells). Subsequently, ∼5 × 10^6 cells/ml were loaded with Indo-1 AM (Invitrogen) for 30 min at room temperature and analyzed by flow cytometry. After establishment of the baseline, 10 μM Fl(αb); fragment goat anti-mouse IgM was added in the absence or presence of 100 nM or 1 μM 2-AG (resuspended in PBS) to stimulate B cells. The calcium flux was evaluated by measuring the fluorescence ratio of Indo-1 AM (405 nm/530 nm).

In vitro chemotaxis assay

2-AG (resuspended in PBS) was added to the lower well of a 24-well plate at different concentrations (1 μM and 10 μM) in 600 μl of migration medium (RPMI 1640 plus 1% fatty acid-free BSA plus 10 mM HEPES). After the preparation of splenic single-cell suspensions from WT and CB2−/− mice and RBC lysis, macrophage depletion was performed by incubating cells in RPMI with 10% FCS at 37°C for 30 min. Nonadherent cells were collected, and 1 × 10^5 cells (in 100 μl migration medium) were added to the upper chamber of Transwells (Costar, Corning, NY). Plates were incubated for 3 h at 37°C. Cells from the lower wells were collected, stained for B220, IgM, CD21, CD23, and CD93 to distinguish Fo B, T2-MZP, and MZ B cells, resuspended in 100 μl staining buffer, and analyzed by flow cytometry. For relative cell counts, cells were acquired for 60 s on an LSR II. Migration was calculated as percentage of input cells.

In vivo homing

To assess in vivo homing, CB2−/− and WT B cells were labeled with CFSE and Cell Tracker Violet, respectively, were mixed in an equal ratio, and a total of 30 × 10^6 cells (input cells) were adoptively transferred (i.v.) into WT mice. A fraction of input cells was stained for B220, IgM, CD21, CD23, and CD93 and analyzed by flow cytometry for precise determination of the proportion of CB2−/− and WT B cells in Fo B, T2-MZP, and MZ B cell subsets. For each subset, the ratio of transferred CB2−/− to WT B cells represented the input ratio. After 4 h, homing to the MZ was determined using an adapted protocol specifically to label B cell subsets residing within the MZ with anti-CD21 as described (44). Briefly, anti-CD21–PE was i.v. administered, and after 5 or 20 min, the spleens were collected, processed, and stained for B220, IgM, CD23, and CD93 on ice. Because of its exposure to the peripheral blood, this procedure will label all CD21+ cells that were in the MZ during the 20-min labeling. This will include cells that had migrated out of the MZ to enter either the follicles or the peripheral blood. The large size of PE prevents it from diffusing from the peripheral blood into the follicles. Thus, any labeled Fo B cells will reflect those that were within the MZ only. In the CD21−/−PE+ Fo B, T2-MZP, and MZ B cells, the proportion of transferred CB2−/− (CFSE−) and WT (Cell Tracker Violet)+ cells were determined by flow cytometry. The homing ratio (CB2−/−/WT) was calculated by dividing the CB2−/−/WT ratio posthoming by the input ratio. A homing ratio <1 indicates a homing impairment of CB2−/− B cells compared with WT. The same experiment was also performed by reverse labeling of CB2−/− (Cell Tracker Violet) and WT (CFSE) B cells. The in vivo labeling for MZ-residing B cells was also carried out for 5 and 20 min in naive WT and CB2−/− mice to determine whether T2-MZP and MZ B cells are underrepresented in the MZ compartment in the absence of CB2.

In vivo CB2-antagonist treatment

WT and CB2−/− mice were injected i.v. with 10 μg of CB2-selective antagonist SR144528 or equivalent vehicle (1% ethanol). After 4 h, B cells from the spleen and blood were analyzed by flow cytometry.

Immunization and sera collection

For T-independent humoral responses, 8- to 12-wk WT and CB2−/− mice were immunized with 30 μg NP-Ficoll i.p. To assess Ab responses against Pneumovax 23, mice were immunized (i.p.) with 23 μg Pneumovax 23 (1 μg of each pneumococcal polysaccharide/ml) diluted in PBS at 4°C. Plates were blocked with 1% BSA for 2 h at room temperature prior to the addition of serially diluted sera samples. Subsequently, NP-specific IgM and IgG1 and Pneumovax-specific IgM were detected using HRP-conjugated anti-IgM and IgG1, and ABTS substrate (Southern Biotechnology, Birmingham, AL). Absorbance was measured at 405 nm. For the determination of resting Ig titers, plates were coated overnight with 5 μg/ml goat anti-mouse Ig (H+L) (Southern Biotechnology) at 4°C. Following 2-h blocking with 1% BSA at room temperature, sera samples were added, and total IgM, IgG1, IgG3, IgG4, IgA, and IgD were detected using HRP-conjugated Abs and ABTS substrate. Absolute concentrations of resting serum Igs were calculated from standard curves.

ELISPOT assay

For the determination of Ag-specific IgM- and IgG1-secreting B cells by ELISPOT assay, MultiScreen HTS Filter Plates (Millipore, Bedford, MA) were coated overnight with 50 μg/ml NP23-BSA at 4°C. Nonspecific binding was blocked by 10% FBS for 2 h at room temperature. Subsequently, RBC-depleted splenocytes were added to the wells, and plates were incubated at 37°C for 3 h. NP-specific IgM and IgG1 spots were detected using HRP-conjugated anti-IgM or anti-IgG1 and 3-aminio-9-ethyl-carbazole substrate (Sigma).

Statistical analysis

WT and CB2−/− groups were compared using a two-tailed nonparametric Mann–Whitney U test. For the homing assay, Wilcoxon signed-rank test was used to determine significance. A p value <0.05 was considered significant.

Results

CB2−/− mice have a significant reduction in MZ B cells and in their immediate precursors

CB2−/− mice were previously reported to have no alteration in the percentage of T1, T2, and Fo B cells (15). Using similar CB2−/− mice and CB2+/+ (hereafter referred to as WT) mice, we confirmed this finding and further demonstrated that there was a modest reduction in the absolute number of Fo B cells, but not in the T1 or T2 subsets (Fig. 1A). Furthermore, using a phenotyping and gating strategy that allowed the differentiation between MZ B cells and their immediate precursor T2-MZP (Fig. 1B) (26), we were able to confirm that the percentage of MZ B cells is reduced by ∼50% in CB2−/− mice and further showed that the absolute number is similarly decreased (Fig. 1B) (15). We also found a significant reduction in the absolute number of T2-MZP cells (∼20%) in CB2−/− mice (Fig. 1B), although their percentage was not significantly different from that in WT. Immunofluorescence analysis of CB2−/− splenic sections also demonstrated a reduction in IgM+ MZ B cells residing outside the IgD+ B cell follicles compared with that in WT (Fig. 1C).

B1 cells are “MZ B-like cells,” which show a similar activated phenotype, perform many overlapping functions as MZ B cells, and predominantly reside in the peritoneum (22). To determine whether B1 cell numbers were also affected in CB2−/− mice, we analyzed peritoneal cavity B cell subsets. We found no significant difference in the percentages of total B, B2, and B1 cells between WT and CB2−/− mice (Supplemental Fig. 1A, 1B). In addition, among B1 cells (B220+CD23+), the B1a and B1b subsets were also unaffected (Supplemental Fig. 1C). In the BM, we also found

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that there was no difference in the percentage or absolute number of developing and mature/recirculating B cells (Supplemental Fig. 1D). Collectively, these data suggest that CB2 is not required for early B cell, B1, or Fo B cell development or for MZ B lineage commitment. However, the maintenance of T2-MZP and MZ B cells and/or MZ B cell differentiation from T2-MZP B cells is likely regulated by CB2.

To determine whether CB2-deficiency also affects MZ B cell function, we measured proliferation and Ig production of sorted WT and CB2−/− mice in vitro. CB2 deficiency did not alter proliferation of live (DAPI−) MZ B cells, in response to anti-IgM, anti-CD40, or LPS (Supplemental Fig. 2A). Similarly, CB2−/− MZ B cells did not show any intrinsic defect in IgM production after stimulation with LPS in the presence or absence of 2-AG (Supplemental Fig. 2B). These in vitro data suggest that CB2−/− MZ B cells are functionally equivalent to WT.

B cell intrinsic requirement for CB2 in MZ B cell homeostasis/maintenance

To investigate the mechanism whereby CB2 regulates MZ B cell homeostasis/maintenance, we asked whether the CB2 regulation is B cell intrinsic. We first confirmed a high-level expression of CNR2 (CB2) mRNA in both T2-MZP and MZ B cells by quantitative real-time PCR (Fig. 2A). Consistent with previous reports, B cells expressed higher CNR2 mRNA than that of CD4 T cells (6). Among all B cells, mature B cells (including Fo and MZ) are known to have the highest level of CNR2 gene expression (9, 15). We found that T2-MZP cells expressed similar levels of CNR2 mRNA as Fo B and MZ B cells (Fig. 2A).

Next, to distinguish between B cell intrinsic or extrinsic defects, we conducted a BM reconstitution experiment, in which WT (CD45.1) and CB2−/− (CD45.2) BM, mixed in an equal ratio, were transplanted into lethally irradiated µMT (B cell-deficient) recipients. Transplantation of 1:1 mix of WT (CD45.1) and WT (CD45.2) BM was performed as a control. No difference in the percentage of Fo B cells was observed in the WT/WT (1:1) or WT/CB2−/− (1:1) reconstituted mice (Fig. 2B). However, in the WT/WT (1:1) control BM transplantation, there was a modest advantage of CD45.1 (∼1.5-fold) in the T2-MZP and MZ B populations over CD45.2 (Fig. 2B). Nevertheless, when we analyzed WT/CB2−/− (1:1) recipients, the percentages of WT T2-MZP and MZ B cells were ∼2.7- and 7-fold higher, respectively, compared with those of CB2−/− B cells (Fig. 2B). We next determined whether the reduction in T2-MZP and MZ B cells could be overcome by increasing the ratio of input CB2−/− BM by 4-fold (Fig. 2B). As an
internal control, we evaluated the percentages of Fo B cells and found that those deficient in CB2 were increased by 4-fold. Whereas the defect in T2-MZP was corrected, increasing the ratio of CB2$^+/2$ BM failed to correct the MZ B cell defect (Fig. 2B). Collectively, these data confirm that CB2 expression is not required for Fo B cell development but is an important B cell intrinsic factor regulating the homeostasis and/or maintenance of T2-MZP and MZ B cells.

**MZ B cell reduction in CB2$^{-/-}$ mice is not due to altered apoptosis, turnover of MZ B cells and their precursors, BCR signal strength, or Notch-2 signaling**

We further investigated the mechanism of MZ B cell reduction in CB2$^{-/-}$ mice. Because MZ B cells are long-lived cells and CB2 has been linked with apoptosis (45, 46), we asked whether the homeostasis/maintenance of MZ B cells and their immediate precursor T2-MZP was altered due to altered cell turnover. To test this possibility, we measured steady-state apoptosis by analysis of active caspase-3. We observed no significant difference in the percentage of active caspase-3$^+$ T2-MZP or MZ B cells between WT and CB2$^{-/-}$ mice (Fig. 3A). Because BrdU is incorporated by proliferating pre-B cells in the BM and not by the essentially nonproliferating Fo, T2-MZP, and MZ B cells in the spleen, BrdU incorporation levels reflect the rate of peripheral B cell turnover. In a short BrdU pulse, we found no difference in the percentage of CB2$^{-/-}$ BrdU$^+$ T2-MZP or MZ B cells compared with that of WT (Fig. 3B). Notably, during continuous BrdU administration for 9 d, CB2$^{-/-}$ mice had significantly higher percentages of BrdU$^+$ T2-MZP on day 9 and MZ B cells on days 6 and 9 (Fig. 3C).

Next, we investigated BCR signal strength, as weak signals from the BCR complex favors MZ B cell lineage differentiation from T2 B cells, whereas a strong signal is thought to drive the maturation of immature B cells into the Fo subset (17). Because CB2 ligand binding has been shown to dampen TCR signaling (47), we asked whether CB2 also regulates BCR signal strength in the T2 B cell subset. To test this possibility, we evaluated calcium mobilization induced by BCR cross-linking in the presence or absence of the endocannabinoid 2-AG. WT and CB2$^{-/-}$ T2 B cells exhibited a similar magnitude of calcium flux, which was not consistently altered by the pretreatment of the B cells with 2-AG when used in a physiologic range (100 nM to 1 μM) (Fig. 3D).

Because Notch-2 has been shown to play a crucial role in MZ B cell development, we determined that Notch-2 cell surface expression levels were not altered in CB2$^{-/-}$ mice (data not shown). However, CB2 being a GPCR, could impact Notch-2 signaling. To examine this possibility, we determined whether expression of the transcription factor RBP-J and the Notch-2 targeted genes Hex-1 and Deltex-1 were reduced in CB2$^{-/-}$ B cell subpopulations (36). We found no expression difference in Fo B cells and a trend for reduced expression in T2-MZP and MZ B cells in CB2$^{-/-}$ mice, although this did not reach significance (Fig. 3E). These cumulative data indicate that reduced numbers of MZ B cells in CB2$^{-/-}$.
mice is not due to altered apoptosis, reduced turnover, or changes in BCR or Notch-2 signaling in T2-MZP and MZ B cells. CBR or Notch-2 signaling in T2-MZP and MZ B cells (as for Fig. 1). Intracellular staining was performed to determine the percentage of cells containing active caspase-3. Data shown are the mean ± SEM of two experiments (n = 6). B and C, BrdU (0.8 mg/ml) was administered in the drinking water for 16 h (B) or continuously for 9 days (C) to WT and CB2−/− mice, and the percentage of T2-MZP and MZ B cells that incorporated BrdU was analyzed on the indicated days. Data represent a single experiment with three to four mice per group. All data are shown as the mean ± SEM. *p < 0.05. D, Splenocytes from WT (gray line) and CB2−/− (black line) mice were stained to identify T2 B cells and loaded with Indo-1 AM for 30 min. After baseline establishment, B cells were stimulated with 10 μg F(ab')2 fragment goat anti-mouse IgM in the absence or presence of 100 nM or 1 μM 2-AG. Calcium mobilization was evaluated by measuring the fluorescence ratio of Indo-1 AM (405 nm/530 nm). Two independent experiments of four are shown (n = 4). E, Splenic Fo, T2-MZP, and MZ B cells from WT (white bar) and CB2−/− (black bar) mice were purified by FACS, and the expression of RBP-J, Hes-1, and Deltex-1 genes was quantified by real-time PCR. Relative gene expression was calculated from a GAPDH standard curve. Data are shown as the mean ± SEM of three independent experiments with each containing cells pooled from two to three mice.

**FIGURE 3.** CB2 deficiency does not lead to altered apoptosis, reduced turnover, or changes in BCR or Notch-2 signaling in T2-MZP and MZ B cells. A, Splenocytes from WT (white bar) and CB2−/− (black bar) mice were stained with anti-B220, IgM, CD21, CD23, and CD93 Abs to identify T2-MZP and MZ B cells (as for Fig. 1). Intracellular staining was performed to determine the percentage of cells containing active caspase-3. Data shown are the mean ± SEM of two experiments (n = 6). B and C, BrdU (0.8 mg/ml) was administered in the drinking water for 16 h (B) or continuously for 9 days (C) to WT and CB2−/− mice, and the percentage of T2-MZP and MZ B cells that incorporated BrdU was analyzed on the indicated days. Data represent a single experiment with three to four mice per group. All data are shown as the mean ± SEM. *p < 0.05. D, Splenocytes from WT (gray line) and CB2−/− (black line) mice were stained to identify T2 B cells and loaded with Indo-1 AM for 30 min. After baseline establishment, B cells were stimulated with 10 μg F(ab')2 fragment goat anti-mouse IgM in the absence or presence of 100 nM or 1 μM 2-AG. Calcium mobilization was evaluated by measuring the fluorescence ratio of Indo-1 AM (405 nm/530 nm). Two independent experiments of four are shown (n = 4). E, Splenic Fo, T2-MZP, and MZ B cells from WT (white bar) and CB2−/− (black bar) mice were purified by FACS, and the expression of RBP-J, Hes-1, and Deltex-1 genes was quantified by real-time PCR. Relative gene expression was calculated from a GAPDH standard curve. Data are shown as the mean ± SEM of three independent experiments with each containing cells pooled from two to three mice.

**The endogenous CB2 ligand 2-AG induces chemotaxis of WT B cells in vitro**

Several studies have suggested a role for CB2 in promoting the migration of immune cells, including B cells (14). We first determined whether CB2 deficiency resulted in an intrinsic migration defect in B cells and found that WT and CB2−/− splenic B cells migrated equally to the B cell chemoattractants CXCL13 and sphingosine-1-phosphate (SIP) (data not shown) (32, 48). We next asked whether Fo, T2-MZP, and MZ B cells exhibited differential migration to the endocannabinoid 2-AG in a CB2-dependent manner. As shown in Fig. 4, all three B cell subsets from WT mice migrated toward 2-AG in a dose-dependent manner that was completely absent in CB2−/− B cells (Fig. 4). Notably, MZ B cells exhibited the lowest and T2-MZP the highest migration, consistent with the precursors needing to migrate into the MZ to complete their development (39).

**CB2 is required for the efficient homing and retention of T2-MZP and MZ B cells in vivo**

To evaluate whether CB2 deficiency affects the migration/homing of B cells to the MZ, we mixed differentially labeled WT and
CB2<sup>−/−</sup> B cells in equal proportion (input cells) and adoptively transferred them into WT recipients. After 4 h, homing of CB2<sup>−/−</sup> and WT B cell subsets to the splenic MZ was detected using an adapted in vivo MZ labeling technique where i.v. administration of anti-CD21-PE leads to the rapid labeling of MZ, but not follicle, residing B cells (44). In Fig. 5A, we show representative data whereby the input and homing ratios are calculated for T2-MZP and MZ B cells, with a homing ratio <1 indicating reduced homing of CB2<sup>−/−</sup> B cells (Fig. 5A). The cumulative data shown in Fig. 5B indicates that homing of both T2-MZP and MZ CB2<sup>−/−</sup> B cells to the MZ was significantly reduced compared with WT.

To investigate whether CB2 signaling also facilitates in vivo retention of T2-MZP and MZ B cells, the CB2 antagonist SR144528 (12) or vehicle was administered to WT mice. Analysis of splenic B cells 3–4 h posttreatment revealed a significant reduction in the percentage and absolute number of T2-MZP and MZ B cells in the SR144528-treated group compared with vehicle (Fig. 5C, 5D). As expected, Fo B cell numbers were not altered. Concomitantly, we detected a higher a number of IgM<sup>hi</sup>CD21<sup>hi</sup> (T2-MZP plus MZ) B cells in the blood of SR144528-treated mice compared with vehicle (Fig. 5E). However, CB2<sup>−/−</sup> mice administered SR144528 exhibited no detectable difference in the number of T2-MZP and MZ B cells in the spleen compared with vehicle treatment (Supplemental Fig. 3).

Next, we sought to determine whether a similar localization defect is present in unmanipulated WT and CB2<sup>−/−</sup> mice. We performed in vivo labeling of MZ-residing B cells with CD21-PE whereby i.v. injection labeled CD21<sup>+</sup> cells residing within the MZ, but not the follicles (44). Our labeling was consistent with published results (44) (Supplemental Fig. 4A). We found a significantly decreased proportion of CD21-PE<sup>+</sup> (MZ residing) T2-MZP and MZ B cells in CB2<sup>−/−</sup> mice compared with that in WT (Fig. 6A, 6B, Supplemental Fig. 4B). This was accompanied by a significant increase in the percentage of T2-MZP and MZ B cells that were not labeled with CD21-PE, indicating a higher proportion of MZ B lineage cells in the follicle (Fig. 6A, 6B, Supplemental Fig. 4B). In addition, the peripheral blood of naive CB2<sup>−/−</sup> mice contained ~2.5-fold more IgM<sup>hi</sup>CD21<sup>hi</sup> (T2-MZP plus MZ) B cells than that in WT mice (Fig. 6C, 6D). These cumulative data strongly indicate that CB2 is critical for the efficient homing and retention of T2-MZP and MZ B cells.

CB2<sup>−/−</sup> mice show significantly reduced resting and T-independent IgM levels

MZ B cells are thought to be a major source of natural IgM, and they play a vital role in T-independent humoral responses. In a number of knockout mouse models, MZ B cell deficiency has been associated with impaired T-independent Ab responses (21, 23, 24). Therefore, to examine the biological significance of MZ B cell loss in CB2<sup>−/−</sup> mice, we first measured resting serum Ig levels in WT and CB2<sup>−/−</sup> mice. Notably, CB2<sup>−/−</sup> mice exhibited a significant reduction in serum IgM (∼35%), compared with WT, although there was no significant difference in IgG<sub>1</sub>, IgG<sub>2a</sub>, IgG<sub>2c</sub>, IgG<sub>3</sub>, or IgA levels (Fig. 7A). Next, to study a T-independent Ab response, we immunized WT and CB2<sup>−/−</sup> mice with NP-Ficoll and measured Ag-specific IgM and IgG<sub>3</sub> levels in the serum on days 0, 7, and 14 and IgM- and IgG<sub>3</sub>-secreting B cells in the spleen on days 6–7.

CB2<sup>−/−</sup> mice had a reduced basal level of NP-specific IgM on day 0, and they also generated significantly lower levels of NP-specific Ig (Fig. 7B, 7C). A similar trend of reduced NP-specific IgG<sub>3</sub> was found in CB2<sup>−/−</sup> mice on days 7 and 14 postimmunization (Fig. 7B). Consistently, CB2<sup>−/−</sup> mice had significantly reduced numbers of NP-specific IgM-secreting B cells in the spleen 6–7 d after immunization (Fig. 7C). There was also a trend of reduced IgG<sub>3</sub>-secreting B cells in the absence of CB2. However, the difference was not statistically significant (data not shown).

Although this study clearly showed an impaired T-independent Ag-specific Ab response in CB2<sup>−/−</sup> mice, NP-Ficoll is not a naturally occurring Ag. To mimic a more physiological T-independent immune response, we challenged WT and CB2<sup>−/−</sup> mice with the human vaccine Pneumovax 23. Pneumovax 23 is composed of a mixture of purified capsular polysaccharides from 23 different strains of Streptococcus pneumoniae and is known to induce a humoral response in a T-independent manner. We found significantly decreased Pneumovax-specific IgM in the serum of CB2<sup>−/−</sup> mice compared with that in WT on days 7 and 14 postimmunization (Fig. 7D). Together these data indicate that CB2 plays an important role in natural IgM production and is required for robust T-independent humoral immune responses.

Discussion

In this study, we investigated the mechanism by which CB2 regulates the number of MZ B cells and its role in T-independent immune responses. We found that CB2 expression by B cells is essential for the maintenance, but not development, of both MZ B cells and their immediate precursor T2-MZP. We found that CB2 promoted the homing of T2-MZP and MZ B cells into the MZ and also played a critical role in their retention. The biological consequence of CB2 deficiency was reduced resting IgM levels and a significant impairment in the early production of Ag-specific IgM after immunization with T-independent Ags. These studies demonstrate that CB2 positively regulates MZ B cells and suggests that the endocannabinoid system could be exploited to enhance immunity to certain classes of pathogens.

CB2<sup>−/−</sup> mice were previously reported to have a significant reduction in the percentage of MZ B cells (15). However, this study did not address whether CB2 deficiency affects the absolute number of mature MZ B cells nor did it examine the T2-MZP population. Using CB2<sup>−/−</sup> (WT) and CB2<sup>−/−</sup> littermates, we found that both the percentage and absolute number of MZ B cells was significantly reduced in CB2<sup>−/−</sup> mice, whereas only the absolute number of T2-MZP was reduced (Fig. 1). Ziring and colleagues (15) also reported a modest diminution of the peritoneal B1a
population in CB2−/− mice compared with that in the CB2+/− littermates. However, in our study using additional markers, we found no significant difference in the percentage of peritoneal B1a or B1b cells in CB2−/− mice. In addition, no or marginal differences were observed in the percentage and absolute number of FO B cells. These data indicate that CB2 regulation of B cell numbers is largely specific to the MZ B cell lineage, but it does not allow the determination of whether CB2 must be expressed by the B cells. It is possible that other CB2+ immune cells required for the maintenance of MZ B cells is driving their reduction. Because MZ macrophages are thought to play a role in the retention of MZ B cells, a reduction in their numbers could explain the loss of MZ B cells in CB2−/− mice (49). Using both immunofluorescence and flow cytometry, we found no reduction in the number or phenotype of MZ macrophages (S. Basu and B.N. Dittel, unpublished observations). Using BM transplantation, we determined that the loss of MZ B cells in CB2−/− mice was B cell intrinsic and the effect could not be reversed even when the donor CB2−/− BM was in 4-fold excess over WT BM (Fig. 2). Under these same conditions, the percentage of WT and CB2−/− T2-MZP B cells were similar (Fig. 2), indicating that the reduction of these cells in CB2−/− mice (Fig. 1) is also B cell intrinsic. We attribute the
stronger impact of CB2 deficiency on MZ B cells to its role in retention of cells within the MZ (Fig. 5). Because there are fewer T2-MZP in the MZ, the loss of CB2 would have less of an impact on their numbers. In addition, our data clearly indicate that CB2 expression by other hematopoietic cells is not required for the maintenance of MZ B cell numbers. This finding is of significance and opens the possibility for the development of therapies directed at CB2 to enhance early immunity against blood-borne pathogens.

Immune modulation by the endocannabinoid system and cannabinoids has been extensively reported and includes immune suppression, induction of apoptosis, and induction of cell migration (1, 50). Although it is generally thought that CB2, and not CB1, mediates cannabinoid-specific immune regulation, this interpretation is not straightforward. Early studies examining cannabinoid immune suppression often used either THC or synthetic cannabinoids that have high affinity to both CB1 and CB2 (1, 50). The development of synthetic CB2-selective agonists provided stronger evidence that CB2 was immune modulatory, although they also bind CB1 at detectable levels (1). Once a CB2-selective antagonist was generated, this was then routinely incorporated into studies solidifying a role for CB2 in immune regulation. However, whether CB2 is mediating negative or positive effects is not straightforward, as the most commonly used CB2 antagonist SR144528 has been shown to function as an inverse agonist (12, 13). The generation of a CB2-deficient mouse was the first tool allowing a clear determination of when and how CB2 was immune regulatory (40, 51). The CB2<sup>-/-</sup> mouse has been particularly useful to confirm CB2-specific effects induced by cannabinoids, as many cannabinoids can function in a receptor-independent manner when used at high doses (40, 52) (S. Basu and B.N. Dittel, unpublished observations). Thus in this study, when cannabinoids were used, the data were confirmed to be CB2-specific by performing the same experiments in CB2<sup>-/-</sup> mice.

MZ B cell development and maintenance is not completely understood. Thus, we investigated multiple mechanisms whereby CB2 could control MZ B cell numbers. Because CB2-deficient mice show a decrease in MZ B cells, we investigated the role of CB2 in the development and maintenance of MZ B cells. Our data indicate that CB2 expression is required for the maintenance of MZ B cell numbers. This finding is of significance and opens the possibility for the development of therapies directed at CB2 to enhance early immunity against blood-borne pathogens.

**FIGURE 6.** CB2 deficiency leads to decreased T2-MZP and MZ B cells in the MZ compartment increasing their numbers in the blood. A and B, Anti-CD21-PE (0.25-1 μg) was i.v. administered to WT and CB2<sup>-/-</sup> mice, and after 20 min splenocytes were isolated and stained to identify T2-MZP and MZ B cells (as for Fig. 1) that had localized to the MZ (CD21*PE<sup>+</sup>). A, Representative histograms show relative frequencies of CD21<sup>-/-</sup>PE<sup>+</sup> (right gate) T2-MZP (left panels) and MZ (right panels) B cells. Background fluorescence was determined by mixing CD45.1<sup>+</sup> splenic tissue from unmanipulated mice with splenic tissue of CD21-PE-labeled (CD45.2<sup>+</sup>) mice prior to processing and staining. Background fluorescent gates (left gate) were set on CD45.1<sup>+</sup>CD21-PE<sup>-/-</sup> T2-MZP or MZ B cells. Numbers on the plots represent the percentage of cells in the corresponding gate. B, Percentages of CD21<sup>-/-</sup>PE<sup>+</sup> (left panels) and CD21-PE<sup>-/-</sup> (right panels) WT (white bar) and CB2<sup>-/-</sup> (black bar) T2-MZP and MZ B cells. Data shown are the mean ± SEM of four independent experiments (n = 10). C and D, Peripheral blood from WT and CB2<sup>-/-</sup> mice was stained and analyzed for IgM<sup>+</sup>CD21<sup>hi</sup> cells in the peripheral blood. Numbers on the plots represent the percentage of cells in the corresponding gate. D, The absolute number of WT (white bar) and CB2<sup>-/-</sup> (black bar) IgM<sup>+</sup>CD21<sup>hi</sup> cells/ml in the peripheral blood. Data shown are the mean ± SEM of two independent experiments each with three mice per group. **p < 0.01, ***p < 0.005.
caspase-3 (Fig. 3). In a BrdU pulse chase experiment, no change in the turnover rate of CB2−/− B cells was observed (Fig. 3). Notably, during long-term BrdU labeling, accumulation of significantly higher numbers of CB2−/− T2-MZP and MZ B cells occurred (Fig. 3). These data indicate that there is likely a feedback mechanism that senses the loss of MZ B cells and compensates by generating more T2-MZP and/or slowing the rate of their basal turnover level. This feedback mechanism would be beneficial during infections that require the early IgM response to facilitate pathogen clearance allowing time for the long-term high-affinity humoral response to emerge.

How CB2 negatively regulates immune cell function is not completely understood, but the prevailing evidence is that ligand binding initiates a signaling cascade resulting in inhibition of adenylate cyclase that decreases cAMP accumulation and subsequent protein kinase A activation (1). Biologically, this results in reduced transcription of genes regulated by CREB, including IL-2 and proteins involved in cell survival and proliferation. However, depending on the duration of CB2 ligand signaling, cAMP accumulation can also be positively regulated as was shown in Jurkat and primary human T cells after a long-term ligand stimulation (47). In this same study, the increase of cAMP resulted in decreased TCR signaling and IL-2 production indicating that CB2 can cross-talk with the TCR signaling cascade (47). Because MZ B cell development is regulated by BCR signal strength (17), we asked whether the endogenous CB2 agonist 2-AG could modulate BCR signal strength. We chose to examine Ca2+ mobilization after BCR cross-linking with IgM in the presence or absence of 2-AG, because this is one of the most sensitive methods allowing the measurement of differences in BCR signal strength. We found no consistent difference in BCR signaling in WT and CB2−/− B cells with or without 2-AG (Fig. 3). From these data we concluded that the loss of MZ B cells in CB2−/− mice was not due to modulation of BCR signaling at the T2 stage directing B cell differentiation away from the MZ B cell lineage. However, we cannot rule out the possibility that an additional ligand for CB2 exists in the spleen that can modulate BCR signal strength. Because GPCR can cross-talk with a number of signaling pathways, we next asked whether Notch-2 signaling was decreased in MZ B lineage cells. Although a trend for a decrease in the expression of the transcription factor RBP-Jκ and Notch-2 target genes Hes-1 and Deltex-1 was found in CB2−/− mice, the differences were small and did not reach significance. From these collective data, we concluded that the reduction of MZ B cells in CB2−/− mice was not due to a developmental defect at any stage during B cell maturation.

Another possible reason for T2-MZP and MZ B cell loss in the absence of CB2 could be less efficient homing. GPCRs play a vital role in lymphocyte homing and trafficking, including CB2. The loss of CB2 could lead to impaired homing of B cells to secondary lymphoid tissues, resulting in diminished T-independent humoral immune responses. To investigate this, we examined the humoral immune response to T-independent antigens in WT and CB2−/− mice. We found that CB2−/− mice had a significantly impaired humoral response to T-independent antigens, as evidenced by reduced levels of IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA in serum (Fig. 7A). Similarly, when challenged with T-dependent antigens such as NP-Ficoll or Pneumovax 23, CB2−/− mice had a diminished IgM and IgG3 response compared to WT mice (Fig. 7B and 7D). These findings are consistent with previous reports showing that GPCRs, including CB2, are important for efficient lymphocyte trafficking and homing.

To further understand the mechanism behind the impaired humoral response in CB2−/− mice, we examined B cell differentiation into the MZ B cell lineage. We found that the number of NP-specific IgM-secreting B cells in the spleen was significantly reduced in CB2−/− mice compared to WT mice (Fig. 7C). This is consistent with the findings of a previous study showing that CB2−/− mice had reduced numbers of MZ B cells (17). These results suggest that CB2 plays a critical role in the development and maintenance of the MZ B cell lineage, which is essential for efficient B cell maturation and humoral immune responses.

These findings have important implications for understanding the role of CB2 in immune responses. CB2 is expressed on a variety of immune cells, including B cells, and has been implicated in the regulation of immune responses. Our results suggest that CB2 plays a critical role in the development and maintenance of MZ B cells, which are essential for efficient B cell maturation and humoral immune responses. These findings also highlight the importance of CB2 in immune cell trafficking and homing, which is critical for immune cell function.

Overall, our study provides new insights into the role of CB2 in immune cell function and suggests that CB2 is a critical regulator of immune cell development and trafficking. These findings have important implications for understanding the role of CB2 in immune responses and identifying potential therapeutic targets for immune disorders.
role in the trafficking of lymphocytes to appropriate anatomical locations. For B cells, the chemokine CXCL13, whose receptor is a GPCR, is required for B cell localization to the follicles (48). In order for B cells to migrate to the MZ, they must overcome the CXCL13-mediated recruitment via signaling through the GPCR S1P1, in response to its ligand S1P (38). However, whether S1P receptors are the only factors that regulate the localization of T2-MZP and MZ B cells is not known. Data supporting a potential role for CB2 in this process are studies demonstrating that B cells migrate in response to 2-AG (14). When we determined the migration efficiency of splenic B cell subpopulations to 2-AG in vitro, we found that migration of WT T2-MZP > Fo > MZ B cells occurred in a CB2-dependent manner (Fig. 4). Furthermore, an in vivo homing assay demonstrated that CB2+/− T2-MZP and MZ B cells exhibited reduced MZ homing, with the defect being more profound in the T2-MZP subset (Fig. 5). These data are consistent with T2-MZP having to migrate into the MZ to complete their maturation process. This defect was not due to an impaired responsiveness of CB2+/− B cells to S1P (data not shown). However, it is not known whether CB2 and S1P1 function cooperatively to position T2-MZP and MZ B cells. Additional data in support of CB2 promoting MZ homing of B cells is the finding that S1P1-deficient B cells in CXCL13+/− mice homed appropriately to the MZ, indicating that a second MZ homing signal must exist (38). Our studies provide evidence that CB2 engagement via its ligand 2-AG is the second signal. The mechanism whereby CB2 promotes homing to the MZ is likely by the activation of GTPase activity important for adhesion and migration. Although this mechanism has not been demonstrated for B cells, CB2 has been shown to play a role in the activation of GTPases in macrophages, neutrophils, and BM cells (54, 55). Of particular interest are the GTPases Rap1b and Rac2, because when deficient the mice had normal numbers of Fo but reduced numbers of MZ B cells (56–59). In addition, Rap1b+/− B cells exhibited impaired chemotaxis (56–58). Thus, we propose that CB2 promotes migration via the activation of GTPases that turn on the migratory machinery allowing movement toward an S1P gradient present in the MZ. Because Fo B cell numbers were not altered in CB2+/− mice, this suggests that other signaling mechanisms likely contribute to follicular homing.

Once localized to the MZ, B cells were shown to be retained via adhesion of VLA-4 and LFA-1 expressed by the B cells with their ligands VCAM-1 and ICAM-1, as demonstrated by the quick mobilization of MZ B cells into the blood after injection with anti-VLA-4 plus anti-LFA-1 (60). Thus, we tested whether disrupted adhesive capacity of CB2+/− B cells resulted in their reduced retention. First, we confirmed that the basal expression level of the individual VLA-4 and LFA-1 integrin subunits was identical in WT and CB2+/− B cells (S. Basu and B.N. Dittel, unpublished observations). We next determined that CB2+/− B cells do not have an intrinsic adhesion defect to VCAM-1 and ICAM-1 in vitro (S. Basu and B.N. Dittel, unpublished observations). We then asked whether CB2 controlled retention within the MZ in vivo. After the injection of the CB2-selective antagonist SR144528 (12), we found that cells of the MZ lineage were quickly dislodged from the spleen and mobilized to the blood in WT mice, whereas CB2+/− B cells were unaffected (Fig. 5). These data are consistent with a previous study reporting the importance of CB2 in regulating immature B cell retention in BM sinuses (9). A role for CB2 in B cell homing and retention was further supported by reduced localization of MZ B lineage cells within the MZ as well as their increased numbers in the blood of CB2+/− mice (Fig. 6). Together our data strongly suggest that CB2 plays a critical role in the maintenance of T2-MZP and MZ B cells by regulating their homing to and retention in the splenic MZ.

From our data, we could not determine whether CB2 regulates retention within the MZ via VLA-4 and LFA-1. It is possible that in CB2+/− mice, B cell adhesion to MZ macrophages is affected or that a yet to be discovered mechanism regulates MZ B cell retention. Notably, the homing and retention defect in the absence of CB2 is more pronounced in T2-MZP than MZ B cells, which raises the question of why T2-MZP cells are minimally reduced (~20–25%) in CB2+/− mice (Fig. 1). We think that compensatory mechanisms exist to control the numbers of MZ lineage cells that are overcome by the combined effects of reduced homing of T2-MZP and reduced retention of MZ B cells.

Finally, because MZ B cells are thought to be major contributors to the natural IgM pool and are essential for T-independent immune responses (17), we investigated the biological significance of CB2 deficiency. We found that along with a significant reduction in natural IgM production, the amplitude of the Ag-specific IgM response to the T-independent Ag NP-Ficoll was also significantly reduced (Fig. 7). Furthermore, using the human vaccine Pneumovax 23, which induces T-independent Ab responses in humans and also in mice, we also observed significantly reduced levels of Ag-specific IgM in CB2+/− mice. Collectively, these data indicate that CB2 is required for a robust humoral response against T-independent Ags. Because CB2+/− MZ B cells did not show any intrinsic abnormality in proliferation or IgM production (Supplemental Fig. 2), our data suggest that CB2 modulates the magnitude of T-independent Ab responses by regulating the number and positioning of MZ B cells. In humans, a distinct IgM+/ IgD+CD27− “MZ B cell-like” population exists in the spleen that is thought to generate T-independent immune responses (61). Notably, children under 2 y respond poorly to pneumococcal polysaccharide immunization and have poor responses to encapsulated bacteria (61). This suggests the importance of MZ B cells in humans for the protection against microbial Ags. Therefore, our study raises the concern whether humans with polymorphisms (reduced function) in CNR2 are more susceptible to blood-borne pathogen infections (62). Also of interest is whether administration of CB2-selective agonists could be used therapeutically to enhance specifically T-independent humoral responses.

Our data demonstrating that CB2 positively regulates T-independent immune responses is contrary to studies examining T cells and T cell-mediated diseases, where strong evidence exists that CB2 suppresses T cell immune responses (16). In addition, administration of CB2-selective agonists has been shown to attenuate several autoimmune diseases in mice (63, 64). Therefore, targeting of CB2 is being considered as a potential therapeutic approach for the treatment of autoimmune disease. However, in a mouse model of sepsis, the CB2-selective agonist GP1a was shown to induce an efficient immune response facilitating bacterial clearance (65). In addition, in a 2,4-dinitrofluorobenzene-induced contact hypersensitivity model, CB2-agonist application was found to enhance inflammation (66). These studies indicate that CB2 can regulate immune responses both positively and negatively depending on the context. It is possible that CB2 regulates different immune cells differentially. Our study demonstrates the potential of CB2 in positively regulating B cell homeostasis and T-independent humoral responses, emphasizing the importance of careful studies for the comprehensive understanding of the consequences of CB2 targeting in autoimmune disorders and other diseases where B cells might also participate in the pathogenesis.

**Note added in proof.** As added proof, a paper from Cyster and colleagues (67) that reports similar findings was recently published.
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Disclosures

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