The sequential role of Mst1/mTORC1/STAT1 activity in chemokine receptor 2-regulated B cell receptor signaling

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Abstract

Background: Chemokine (C-C motif) receptor 2 (CCR2) contributes to autoimmune pathogenesis. However, the effect of CCR2 on B cell signaling and its role in autoimmunity remains unclear. Herein, we investigated the role of CCR2 in the B cell receptor (BCR) signaling pathway and aimed to illustrate its potential molecular mechanisms of action. Methods: To investigate the alterations in B cell signaling and the immune response, we used flow cytometry, western blotting, microscopic techniques, Seahorse assay, and immunofluorescence assay on samples from C57BL/6 mice and germinal CCR2-deletion mice. Results: The absence of CCR2 disturbed follicular B cell development. Furthermore, CCR2 absence was correlated with increased mTORC1-mediated energy metabolism and enhanced early B cell activation, which were induced by the up-regulation of BCR proximal signaling and F-actin accumulation. Mst1 and STAT1 were key factors in up-regulating the B cell activation in CCR2 deficient mice. The disrupted peripheral B cell differentiation and enhanced B cell signaling were associated with the inhibition mTORC1, Mst1, and STAT1. Moreover, loss of CCR2 caused a weakened T cell dependent antigen response, resulting in decreased antibody secreting cells and diminished antigen specific IgM levels. Conclusion: CCR2 is involved in the regulation of BCR signaling pathway by sequentially activating signaling pathways dominated by Mst1, mTORC1, and STAT1. Our study suggests that CCR2 might represent a novel therapeutic targeted for autoimmune diseases.

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Author contributions

Yingzi Zhu is responsible for the execution of the experiments, analysis of data and manuscript drafting. Heng Gu is responsible for the execution of the experiments and analysis of data. Lu Yang, Na Li, Qiuyue Chen, Danqing Kang, Yukai Jing, Panpan Jiang, Qianglin Chen, Li Luo, Ju Liu, Jiang Chang, Zhenzhen Li, Yi Wang, Xin Dai are involved in assistance in the experimental process and performed a part of the experiments. Heather Miller, Lisa S Westerberg, Chan-Sik Park and Masato Kubo revised the manuscript critically and proposed constructive comments. Lingli Dong and Chaohong Liu have made substantial contributions to conception, study design and manuscript revision.11Yingzi Zhu & Heng Gu should be considered joint first author; Lingli Dong & Chaohong Liu should be considered joint corresponding author.

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Conflict of Interest

The authors have no conflict of interest to declare.

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investigated the role of CCR2 in the B cell receptor (BCR) signaling pathway and aimed to illustrate its potential molecular mechanisms of action.

Methods : To investigate the alterations in B cell signaling and the immune response, we used flow cytometry, western blotting, microscopic techniques, Seahorse assay, and immunofluorescence assay on samples from C57BL/6 mice and germinal CCR2-deletion mice.

Results : The absence of CCR2 disturbed follicular B cell development. Furthermore, CCR2 absence was correlated with increased mTORC1-mediated energy metabolism and enhanced early B cell activation, which were induced by the up-regulation of BCR proximal signaling and F-actin accumulation. Mst1 and STAT1 were key factors in up-regulating the B cell activation in CCR2 deficient mice. The disrupted peripheral B cell differentiation and enhanced B cell signaling were associated with the inhibition mTORC1, Mst1, and STAT1. Moreover, loss of CCR2 caused a weakened T cell dependent antigen response, resulting in decreased antibody secreting cells and diminished antigen specific IgM levels.

Conclusion : CCR2 is involved in the regulation of BCR signaling pathway by sequentially activating signaling pathways dominated by Mst1, mTORC1, and STAT1. Our study suggests that CCR2 might represent a novel therapeutic targeted for autoimmune diseases.

Key words : Autoimmune diseases; B cell receptor; CCR2; Mst1; mTOR

Introduction

B cells are derived from bone marrow (BM) and travel to peripheral lymphoid organs to fight pathogens.[1] Following B cell receptor (BCR) activation, sequential signaling pathways involving adaptors, kinases, second messengers, and phosphatases produce a variety of B cell responses.[2-4]Actin cytoskeleton is a major target of BCR signaling that can regulate B cell morphological features, immune synapse formation, and BCR internalization.[5, 6]

As a member of the chemokine family, the contribution of chemokine (C-C motif) receptor 2 (CCR2) in autoimmunity has been previously demonstrated. A previous study suggested that CCR2 expressed on regulatory T cells (Tregs) can restrain the proliferation of T cell*in vitro.* [7]Furthermore, CCR2 was shown to mediate arthritis progression by enhancing Tregs migration.[8] Although not as well studied as T cells, a growing body of evidence indicate a potential connection between CCR2 and B cell regulation. While CXCR4 as well as functional and constitutive CCR2 are expressed in B cells, the transcription of CCR2 occurs in immature B cells and dampens in mature B cells.[9] Furthermore, mice lacking CCR2 are a specifically well suited animal model to investigate B cell expansion under infection and non-infection condition.[10] Following infections, the lymphatic follicles are notably enlarged, which represents an increased B cell proliferation and outgrowth.[11]Additionally, in CCR2 deficient immature B cells, actin polymerization is increased, as well as the migration and homing to the lymph nodes, all of which being mediated by the interaction between CCR2 and its ligand CCL2.[12]. Taken together, these studies suggest that CCR2 might play an important role in the B cell downstream signaling pathways.

Several downstream pathways dominated by BCR activation are particularly prominent in autoimmunity. First, the mammalian target of rapamycin (mTOR) was shown to be involved in the autoimmune pathogenesis, while rapamycin has been demonstrated to represent an effective treatment of rheumatism.[13, 14] MTOR aids the expanded T follicular helper (TFH) cells to facilitate B cell activation and autoantibody production.[15]Furthermore, CCR2 can mediate HIF-1α expression via the PI3K-Akt-mTOR pathway; thus, controlling the cell metabolic process.[16] Another pathway mainly regulated by Mst1 plays an essential role in lymphocyte migration and adhesion during immunosurveillance. During T cell-regulated B cell activation, Mst1 functions as a molecular brake to balance immune tolerance, and its depletion leads to hypergammaglobulinemia in mice.[17] Mst1 deficient mice exhibited peripheral lymphoid tissue hypertrophy, decreased marginal zone (MZ) B cells in the spleen, and the emigration of single-positive thymocytes was also influenced.[18] Moreover, Mst1 depleted B cells show an attenuated response to mitogens and a down-regulated BCR signaling as well as clustering.[19]Additionally, the Janus kinase/signal transduction and activator of transcription

(JAK/STAT) signaling pathway is involved in the development of systemic lupus erythematosus (SLE), which is supported by the effective use of JAK inhibitor as a therapeutic method.[20] CCL2-CCR2 has been shown to associate with the JAK/STAT signaling pathway by activating STAT1, STAT3, and STAT5.[21] Furthermore, CCL2 and CCR2 dimerization induce tyrosine phosphorylation of STAT5.[22] However, the interaction between these three main pathways during the course of autoimmunity and the manner in which they are regulated by CCR2 and B cell signaling activity, remains unknown.

Therefore, we hypothesized that CCR2 interacts with BCR signaling potentially through sequential Mst1-mTOR-STAT1 activation. Herein, we utilized CCR2 deficient mice and revealed that the loss of CCR2 leads to up-regulation of BCR proximal signaling molecules, enhancement of the F-actin remodeling related BCR clustering, and increased expression levels of various transcriptional factors. Changes being underlined by the effect of CCR2 on PI3K-Akt-mTORC1, Mst1-mTORC1-Dock8-WASP, and Mst1-mTORC1-STAT1 axes, respectively.

Materials and Methods

Animals

CCR2 knockout (KO) mice on C57BL/6 (B6) background were purchased from Jackson laboratory (Bar Harbor, ME, USA). Wildtype (WT) C57BL/6J (CD45.2$^+$ and CD45.1$^+$) mice were purchased from Charles River (Beijing, China). CCR2 KO mice displayed a stable phenotype based on sufficient backcrossing with B6 mice. Mice were bred and placed in a specific-pathogen–free animal department. Male and female mice between 2-6 months old were used for the experiments. All studies were conducted in a non-blinded fashion. Animal experimentation was reviewed and approved by the Institutional Animal Care and Ethics Committee of Animal Experimentation of Tongji Medical College.

Cell isolation and purification

Splenic mononuclear cells used for flow cytometry (FCM) were separated from red blood cells using a Ficoll-Hypaque solution (17-1440-02, GE healthcare, Boston, MA, USA). The mononuclear cells were collected from the upper layer following centrifugation at 2000 rpm for 20 min at room temperature (RT). After washing, cells were suspended in ice-cold PBS containing 2% fetal bovine serum (2% PBS-FBS) (10437028, Thermo Fisher, Waltham, MA, USA). Splenic B cells used for other assays were further purified from mononuclear cells by removing T cells with anti-CD90.2 mAb (105310; BioLegend, San Diego, CA, USA) and guinea pig complement (C300-0500; Rockland Immunochemicals, Gilbertsville, PA, USA), followed by incubation for 1 h in a cell culture flask to remove mononuclear cells. For BM isolation cells from the femoral and tibial cavities were flushed with 5 ml ice-cold 2% PBS-FBS. Following centrifugation for 5 min at 2000 rpm (4 °C), cell pellets were lysed with Red Cell Lysis Buffer (RT122-02, Tiagen, Beijing, China), resuspended and subsequently passed through a cell strainer. For peritoneal cells isolation, euthanize mice were injected 5 ml of ice-cold PBS into the peritoneal cavity and then the abdomen was massaged gently for 1-2 min to dislodge attached cells before collection. This was repeated twice to get as many cells as possible. Next, the peritoneum was cut and the liquid in the cavity was collected. The cell suspension was centrifuged at 2000 rpm for 5 min (4 °C), the supernatant was removed, and the cells were resuspended in 2 ml 2% PBS-FBS.

Flow cytometry and phosphorylation flow

Following incubation with anti-CD16/CD32 mAb (101319; BioLegend) to block the Fc receptors, splenocytes or BM cells were seeded into 96-well plates at a concentration of 2×10^6/well. The following antibodies (Abs) were acquired from BioLegend: FITC channel: anti-CD127 (135008), anti-CD19 (101506), anti-B220 (103206), anti-Annexin V (640906), anti-CD5 (100622), anti-CD11b (101226), and anti-CD138 (142521). APC channel: anti-CD43 (143208), anti-CD21 (123412). PE channel: anti–BP-1 (108307), and anti-CD23 (101608). PE/Cy7 channel: anti-CD24 (101822). PerCP/Cy5.5 channel: anti-IgD (405710). Brilliant Violet (BV) 510 channel: anti-B220 (103247) and anti-CD45.2 (109838). BV421 channel: anti-IgM (406518). Alexa Fluor 647 (AF647) channel: anti-GL7 (144606). APC/Cy7 channel: anti-CD45.1 (110716). Lastly, the PE-anti-NP (N-5070-1) was acquired from Biosearch Technologies (Novato, CA, USA).

Phosphorylation flow (phosflow) was performed to determine the level of phosphorylated WASP and F-actin in primary B cells. Cells were incubated with anti-B220 to mark B cells and soluble antigen (sAg), which consist of biotin-conjugated F(ab')2 anti-mouse Ig (M + G) (115-066-068; Jackson ImmunoResearch, West Grove, PA, USA) and streptavidin (16000114, Jackson ImmunoResearch), then activated at 37 °C for 5, 10, and 30 min. Following activation, cells were fixed, permeabilized, and then stained with anti-pWASP (A300-205A; Bethyl Laboratories, Montgomery, TX, USA) and AF488–phalloidin (R37110; Thermo Fisher), followed by goat-anti-rabbit (G/R) IgG AF405 antibody (A-31556, Thermo Fisher).

For the T-bet expression assay, splenic B cells were incubated with 10 μg/ml $F(ab')_2$ goat anti-mouse (G/M) IgM (115-156-020, Jackson ImmunoResearch) for 1 and 2 h, respectively. Subsequently, the splenic B cells were fixed, permeabilized, and stained with anti-B220 as well as anti-T-bet (644809, BioLegend). Samples were collected using a Attune NxT sonic focused flow cytometer (Thermo Fisher) and data were analyzed using the FlowJo 10 software (BD Biosciences, Franklin lakes, NJ, USA).

Confocal microscopy and total internal reflection fluorescence microscopy

To observe the surface distribution of B cell receptors (BCR) and their related physiological activities, confocal microscopy (CFm) and total internal reflection fluorescence microscopy (TIRFm) were utilized. For CFm assay, purified B cells were incubated with AF594-mB-F(ab')2-anti-Ig (M + G) (115-586-068; Jackson ImmunoResearch) and activated at 37 °C for 5, 10, and 30 min. The cells were fix and permeabilize before staining. The Abs used were purchased from Cell Signaling Technology (Danvers, MA, USA): pSHIP (3941S), pSTAT1 (9167S), pSTAT5 (4322S), and pNF-ϰB (3033S). From Abcam (Cambridge, MA, USA): pBtk (ab52192), pCD19 (ab203615). From Thermo Fisher: F-actin (R37110), AF488 G/R IgG (A-11008), AF405 G/R IgG, and AF405 G/M IgG (A-31553). From Bethyl Laboratories: pWASP (A300-205A). From Merck-Millipore (Darmstadt, Germany): pY (05-321).

For TIRFm assay, B cells were stimulated with membrane-tethered antigens (mAg) using lipid bilayers consisting of biotinylated F(ab')2 and streptavidin tethered to lipids attached to the bottom of chambers. As previously described,[23]cells were labeled with AF546-Fab' before being stimulated with mAg for 3, 5, and 7 min. Following stimulation, the cells were fixed, permeabilized, and stained with the same Abs used in the CFm assays.

Representative images were taken and analyzed for mean fluorescence intensity (MFI), area of B cell spreading (IRM), and colocalization using NIS elements AR 5.01 software (Nikon Eclipse Ti-PFS, Tokyo, Japan). Data from three independent experiments using more than 30-50 individual cells were included for each parameter. In order to minimize the variability, a unified evaluation standard to evaluate the morphology and contact area at different time points was set in all our studies.

Immunoblotting

Purified B cells from WT and CCR2 KO mice were activated at 37 °C with sAg. At the 5-, 10-, and 30-min activation time-points, 1 ml of PBS was added to terminate the activation. Cell lysates were obtained using a mixed RIPA buffer (P0013B, Beyotime, Shanghai, China), protease inhibitor cocktail (G2006, Servicebio, Wuhan, China), NaF (1 M, G2007-1, Servicebio), and Na3VO3 (100 Mm, G2007-1, Servicebio). Lysates were run through SDS-PAGE and western blotting. In addition to the Abs mentioned in the CFm assay, some other Abs were used. From Cell Signaling Technology: Btk (8547S), pAkt (4060L), Akt (9272S), SHIP (2728S), pFoxo-1 (9461S), Foxo-1 (2880S), pS6 (4856S), S6 (2217S), pPI3K (4228S), PI3K (4292S), pMst1 (3681S), Mst1 (PA5-22015), pmTOR (5536S), mTOR (2983S), pEzrin (3726S), Stat1 (14994S), P65 (4764S), pIKKB (2697S), and IKKB (8943S). From Abcam: Stat5 (ab194898). From Santa Cruz Biotechnology (Santa Cruz, CA, USA): WASP (sc-13139). Loading controls: anti-mouse β-actin (60008-1-IG-10, Proteintech, Chicago, IL, USA). Western blotting imaging was performed using the ChemiDocXRS + imaging systems (Bio-Rad, Hercules, CA, USA).

In vitro **inhibitory treatment with Rapamycin, XMU-MP-1, and Fludarabine**

Purified CCR2 KO B cells were pretreated with 20 nM rapamycin (HY-10219, MedChem Express, Monmouth

Junction, NJ, USA), 3 μM XMU-MP-1 (T4212, TargetMol, Boston, MA, USA) or 5 μg fludaradine (T1038, TargetMol) and incubated at 37 °C for 2 h before being activated with sAg. The remaining steps were the same as the untreated control.

Seahorse analysis

Assays using the Seahorse XF 24 cell metabolism analyzer (XFe24, Seahorse Bioscience, Billerica, MA, USA) were performed following the manufacturer's instructions. Purified WT and CCR2 KO B cells were pre-stimulated with 10 μg/ml LPS (L2880, Sigma Aldrich, St. Louise, CA, USA) for 1 h and detected in XF medium under basal conditions. Subsequently, 1.5 μM oligomycin (abs42024304, Absin Bioscience, Shanghai, China), 1 μM fluoro-carbonylcyanide phenylhydrazone (FCCP) (C2920, Sigma Aldrich) and 500 nM rotenone (R8875, Sigma Aldrich) plus 1 μM antimycin A (abs42013402, Absin Bioscience) were sequentially added, at the appropriate time of detection. Oxygen consumption rates (OCR) were quantified to reflect the cellular energy metabolism level.

In vitro **B cell proliferation and apoptosis**

To track B cell growth rate, 2×10^6 purified splenic B cells were labeled with 5 μM Cell Trace Violet (CTV; C34557, Thermo Fisher) before being seeded into 96-well plates with 1 ml complete RPMI1640 medium (Gibco, C11875500BT) containing 10% FBS (Gibco, 10091-148), 10 μM HEPES (Gibco, 1804629) and 50 μM β-mercaptoethanol (M2650, Sigma Aldrich). Next, cells were stimulated with 5 μg/ml lipopolysaccharide (LPS) or C_PG for 72 h. After collecting cell, FCM was performed.

Calcium flux assay

Purified B cells were incubated with 0.5 μM calcium-sensitive dye Fluo-4 AM (S1060, Beyotime) at 37 °C for 25 min and then stained with anti-B220 Ab for 30 min. Using a LSRII flow cytometer (BD Biosciences, Franklin lakes, NJ, USA), a baseline fluorescence was recorded for the first 30 s and then cells were immediately stimulated with 10 μg/ml pre-warmed biotin-conjugated $F(ab')_2$ anti-mouse Ig (M + G). The fluorescence intensity was monitored for the next 300 s. Calcium flux kinetics were analyzed using the FlowJo 7.6 software.

Scanning electron microscopy (SEM)

For SEM experiments, antigen coated coverslips were used. Sterile coverslip were coated in 24-well plates with 60 μl poly-D-lysine solution (50 μg/ml) (C0312, Beyotime) overnight at 4 °C. The next day, the coverslips were washed with sterile PBS, dried, and incubated with 100 μl 10 μg/ml F(ab')2 antibody at 37 °C for 3 h. Purified B cells at a concentration of 3×10^6 cells/ml were added gently onto the antigen coated coverslips and stimulated at 37 °C for 10 min. A total of 400 μl of 2.5% glutaraldehyde was used to terminate the stimulation and fix the cells for 20 min (on ice). Scanning electron micrographs were captured using a Ultra-high Resolution Scanning Electron Microscope (SU8010, HITACHI, Tokyo, Japan). Cellular filopodia expansion was imaged and the number and length of filopodia were quantified using ImageJ software (NIH, Bethesda, MD, USA).

Immunization and Enzyme-linked immunosorbent assay (ELISA)

WT and CCR2 KO mice were injected intraperitoneally (i.p.) with T cell dependent antigen 4-hydroxy-3-nitrophenylacetyl conjugated keyhole limpet hemo-cyanin (NP-KLH, 40 μg/mouse) (N-5060-25, Biosearch Technologies), supplemented with Freund's incomplete adjuvant (FIA) (S6322-1VL, Sigma Aldrich). Fourteen days post-prime, 100 μl of blood was taken from the tail vein, then mice were boosted with the same reagents. Five days later, the mice were euthanized and the spleens were harvested. FCM was performed to test the ratio and absolute cell number of B cell subsets. ELISA was performed using NP30-bovine serum albumin (BSA)-coated the ELISA kit (Bethyl Laboratories) was perform as indicated in the manufacturer's information to determine the titer of the NP-specific IgM and IgG1. Serum anti-dsDNA antibody was also quantified by ELISA as previously described.[24]

Bone marrow chimeras

Recipient mice were 2–3-month-old B6 mice (CD45.1). Mice were irradiated with 7 Gy X-ray and then immediately injected intravenously (i.v.) with 5×10^6 BM cells from WT or CCR2 KO (CD45.2) under aseptic condition. Cells from WT or CCR2 KO mice were mixed with that of CD45.1 mice at a 1:1 ratio. One week before the radiation and 2 weeks after the injection, mice were given water with antibiotics (gentamicin and erythromycin). Eight weeks after the transfer and the sacrifice of the mice, FCM was performed.

Tissue immunofluorescence assay

Spleens and kidneys from WT and CCR2 KO mice were frozen in Tissue-Tek® O.C.T. embedding medium (Sakura Finetek, Torrance, CA, USA), and cut into 10 μm-thick cryosections, followed by fixation with ice-cold acetone for 5 min. Sections were incubated in 5% BSA (4240GR100, BioFroxx, Einhausen, Germany) containing 1% anti-CD16/CD32 mAb to block Fc receptors and then incubated with the primary Abs overnight at 4 °C. Next, sections were incubated with Dylight 650 (84547, Thermo Fisher) or AF488 G/R IgG (A11006, Thermo Fisher). Images were captured and analyzed using the NIS elements AR 5.01 software (Nikon).

Statistical analysis

Student's t -test and MannWhitney U test were performed using Prism 8.0.1 software (GraphPad Software, Chicago, IL, USA). Colocalization was displayed using the Pearson's correlation coefficient. Data are pooled from at least 3 individual experiments. Figures were shown as mean value ± SEM. P values lower than 0.05 were considered statistically significant.

Results

CCR2 deficiency disrupts the peripheral differentiation of B cells

To investigate whether the lack of CCR2 influences B cell development and differentiation, we first examined B cell subsets using FCM. The CCR2 KO B cells extracted from BM were incubated with anti-BP-1 and -CD24 Abs to distinguish B cell precursors (**Fig. 1A-B**). Our results suggest that CCR2 deficiency caused little to no alterations to the development of B cell in BM since the proportion and absolute cell number of precursor B cell subsets in both WT and CCR2 KO mice were similar (**Fig. 1C**). However, this was not the case for the peripheral B cell differentiation process. By testing peripheral B cell subsets (**Fig. 1D-F**), we discovered that the proportion and cell number of follicular (FO) B cells ($B220^+IgM^{low}IgD^{high}$) as well as the cell number of transitional type-2 (T2) ($B220^+IgM^{high}IgD^{high}$) B cells from the CCR2 KO mice were significantly diminished compared to those observed in WT mice (**Fig. 1G-H**). Nevertheless, both the ratio and cell number of transitional type-1 (T1) ($B220^+IgM^{low}IgD^{low}$), marginal zone (MZ) ($B220^+CD23^{low}CD21^{high}$), and germinal center (GC) ($B220^+GL\text{-}7^{high}CD95^{high}$) B cells in WT and CCR2 KO mice were comparable (**Fig. 1I-K**). Furthermore, no differences were observed for B1a ($CD19^+IgD\text{-}IgM^+CD5\text{-}CD11b^+$) and B1b ($CD19^+IgD\text{-}IgM^+CD5^+CD11b^+$) B cells (**Fig. 1L**) when comparing WT and KO mice (**Fig. 1M-N**). To rule out the potential effect of other immune cells on the abnormally differentiated B cells observed in CCR2 KO mice, BM chimeras were generated using irradiated C57BL/6J ($CD45.1^+$) recipients through tail vein injection of BM cells extracted from WT and CCR2 KO ($CD45.2^+$) mice. Thus, we examine the inherent reduction of FO B cells in CCR2 deficient mice (**Fig. S1A-H**). Since the kidneys are most susceptible to develop systemic autoimmune disease, we analyzed the glomerulus of CCR2 KO mice and observed a significant increase in IgG immune complex deposition. Additionally, CCR2 KO mice showed increased anti-dsDNA Ab titers (**Fig. 1O-P**). While examining other organs, we observed that CCR2 KO mice exhibited more severe lung lymphocyte infiltrations compared to that of WT mice (**Fig. 1Q, Fig. S2A**). The transcription factor T-bet expressed by B cell has been previously associated with autoimmunity,[25] and as expected, CCR2 KO B cells exhibited higher expression levels of T-bet than those of the WT B cells (**Fig. 1R**). Collectively, these findings suggest that CCR2 plays an important role in the integrity maintenance of peripheral B cell differentiation and the regulation the peripheral autoimmunity.

CCR2 KO mice exhibit up-regulated BCR proximal signaling, including positive and negative signals

Surface BCRs stimulated by antigens initiate B cell signaling transduction, and the intensity of this signaling was enhanced by CD19. CD19 recruits Bruton's tyrosine kinase (Btk) by activating PI3K, which is critical for amplifying downstream signaling. To explore the molecular mechanism through which CCR2 mediates the regulation on BCR signaling, we examined the aggregation of phosphorylated CD19 (pCD19) following stimulation with sAg for 5, 10, and 30 min. Compared to WT B cells, the colocalization between BCR and pCD19 in CCR2 KO B cells was significantly increased at 5 min and gradually decreased to the 30 min time-point when pCD19 was internalized along with BCR. Similar results were seen using western blotting to examine the pCD19 protein levels after 5 min of activation, with CCR2 KO B cells showing significantly higher pCD19 levels than those of WT B cells (**Fig. 2A-D**). Notably, although the colocalization between pCD19 and BCR after 10 min of stimulation decreased in CCR2 KO B cells, the aggregation of pCD19 remained at higher levels than that observed in WT B cells. This indicates that CCR2 deficiency might affect the spatial organization of CD19 also play a potential role in B cell activation. Next, we evaluated the total levels of BCR signaling-protein phosphorylated tyrosine (pY); typical BCR related negative signal molecule-phosphorylated Src homology 2 (SH2) domain-containing inositol polyphosphate (pSHIP); and phosphorylated Btk (pBtk). Compared to WT B cells, the pY and pBtk levels were significantly increased from 5 to 10 min post-stimulation and then decreased after 30 min of stimulation (**Fig. 2E-H**). This was consistent with the immunoblotting results (**Fig. 2I**). Likewise, the colocalization between pY or pBtk and BCR was increased in CCR2 KO B cells (**Fig. 2J-K**). Similarly, the pSHIP level in CCR2 KO B cells also increased after 5 and 10 min of activation (**Fig. 2L-M**), and its colocalization with BCR peaked at 10 min, which was also confirmed by immunoblotting (**Fig. 2N-O**).

Since the phospholipase C gamma 2 (PLCγ) induced-calcium (Ca^{2+}) mobilization is responsible for the BCR signaling pathways,[26]and CCR2, as G protein-coupled receptor, can trigger a flux of intracellular Ca^{2+}, we investigated the Ca^{2+}mobilization using FCM. We discovered that the Ca^{2+}mobilization was enhanced in CCR2 KO B cells upon sAg stimulation (**Fig. 2P**). These findings suggest that the CCR2 deficiency leads to an up-regulation of BCR proximal signaling molecules.

mTORC1-mediated energy metabolism during B cell differentiation was enhanced in mice lacking CCR2

Cell differentiation-associated molecules, mTOR complex 1 (mTORC1) have been reported to function via the PI3K/Akt signaling pathway to mediate hypoxia-inducible factor 1 (HIF-1) expression and consequently influence the cell energy metabolism process.[16] Thus, we examined PI3K-Akt-mTORC1 activity in B cells lacking CCR2. Following sAg stimulation, the B cell lysate was incubated with Abs specific for phosphorylated PI3K (pPI3K), mTORC1 (pmTORC1), Akt (pAkt), S6 (pS6), and Akt distal glucose metabolism-related transcriptional factor forkhead box protein O1 (pFoxo-1). The protein expression levels of pPI3K, pAkt, pS6, pmTORC1, and pFoxo-1 were increased in CCR2 KO B cells following stimulation (**Fig. 3A**). To clarify whether the effects of CCR2 on mTORC1 and its downstream pathways affected the B cell metabolic molecular signaling, we used mTORC1 inhibitors in CCR2 KO B cells to obstruct the upstream effectors of the mTOR pathway. Additionally, CCR2 KO B cells were pretreated with rapamycin to examine the activation of B cell signaling molecules under the same conditions as for the uninhibited groups. As expected, following rapamycin treatment, the activation of pSHIP, pBtk, pCD19, pAkt, pFoxo-1, and pS6 in CCR2 KO B cells was brought to similar levels as the ones observed in WT B cells (**Fig. 3B**). This further indicates that mTORC1 pathway activity is necessary for CCR2-regulated B cell metabolism. In addition, Seahorse assay was performed to analyze the real-time oxidative phosphorylation based on the oxygen consumption rate (OCR). Following stimulation with $F(ab')_2$ anti-mouse Ig (M + G), CCR2 KO B cells exhibited higher ATP production levels, maximal respiratory capacity, and maximal respiratory potential than those of WT B cells (**Fig. 3C**). This change in energy metabolism can facilitate either proliferation or apoptotic processes in CCR2 KO B cells. In support of this, upon*in vitro* lipopolysaccharide (LPS) stimulation, CCR2 KO B cells proliferated faster than LPS stimulated WT B cells (**Fig. 3D-E**). Furthermore, upon *in vitro* C_PG stimulation, CCR2 KO B cells proliferated and proceeded through apoptosis at higher rates than those of C_PG stimulated WT B cells (**Fig. 3F-G**).

Engagement of BCR induces heightened aerobic glycolysis by up-regulating glucose and oxygen utilization; thus, B cell activation drives MYC-dependent up-regulation of glucose transporter 1 (Glut1) and HIF-1α-mediated up-regulation of oxygen transport.[27, 28] HIF-1 and MYC have been reported to coordinately regulate immune cell metabolic reprogramming.[29] Thus following our immunoblotting analysis of these two molecules we discovered that CCR2 KO B cells exhibited increased levels of HIF-1α and c-MYC (**Fig. 3H**).

Taken together, loss of CCR2 disrupts the differentiation of FO B cells through the up-regulation of the PI3K/Akt/mTORC1 metabolic pathway.

CCR2 deficiency boosts F-actin accumulation and BCR internalization

Actin remodeling has been shown to play a crucial role during the process of BCR clustering and formation of B cell morphological features.[6] To examine the involvement of CCR2 in actin remodeling and polymerization, we assessed F-actin accumulation and the activation of actin nucleation promoting factor-WASP in CCR2 KO B cells after sAg stimulation using confocal microscopy (CFm). The F-actin levels observed in activated CCR2 deficient B cells were consistently higher than those of the control B cells (**Fig. 4A-B**). The colocalization between F-actin and BCR was significantly increased after 5 min of stimulation (**Fig. 4C**). Similarly, the pWASP levels in activated CCR2 deficient B cells remained higher than those in WT B cells (**Fig. 4D**). Furthermore, after 5 min of stimulation, the colocalization of pWASP and BCR in CCR2 KO B cells was enhanced (**Fig. 4E**). Additionally, phosflow was performed to measure the pWASP and F-actin levels which coincided with the CFm results. Examining the dynamics of actin polymerization, we found that the mean fluorescence intensity (MFI) of F-actin in CCR2 KO B cells increased slower during the first 5 min of stimulation, after which it accelerated rapidly and peaked at 10 min (**Fig. 4F**). The MFI of pWASP raised rapidly during the first 5 min of stimulation and declined thereafter (**Fig. 4G**). Further, the Mst1-Dock8-WASP axis protein expression levels were examined using western blotting. In the absence of CCR2, the levels of Dock8, pMst1, and pWASP were increased (**Fig. 4H**). To get clearer image of the B cell membrane morphology, we utilized scanning electron microscopy (SEM) to observe B cell morphology and quantify their filopodia (**Fig. 4I**). Compared to the WT B cells, the filopodia number and length were remarkably increased in CCR2 KO B cells (**Fig. 4J-K**). Furthermore, in order to illustrate the latent mechanism through which CCR2 regulates the BCR signaling via the Mst1/Dock8/WASP axis, we pretreated CCR2 KO B cells with the Mst1 inhibitor, XMU-MP-1, and reexamined the BCR signaling molecules upon sAg stimulation. Remarkably, the levels of Dock8, pMst1, pSHIP, pBtk, pPI3K, pAKT, pFoxo-1, pmTOR, and pWASP in CCR2 KO B cells decreased following XMU-MP-1 treatment (**Fig. 4L**). Subsequently, to verify whether the inhibition of mTORC1 in CCR2 KO B cells could also adjust the Dock8/WASP axis, we pretreated CCR2 KO B cells with rapamycin. After the inhibition, the levels of Dock8, pMst1, and pWASP in CCR2 KO B cells were rescued to a comparable degree to WT B cells (**Fig. 4M**). Taken together, CCR2 deletion augments actin accumulation, which is achieved through the modulation of the Dock8-WASP activity, and the latter, at least in part, implemented via the Mst1-mTORC1 pathway.

Loss of CCR2 leads to enhanced early event of BCR activation

BCR clustering and spread are essential for the initiation of the BCR signal. Based on the boosted F-actin accumulation in CCR2 KO B cells, we investigated the influence CCR2 depletion has on the early activation of B cells. Upon stimulation with mAg for 3, 5, and 7 min, the distribution pattern of F-actin in CCR2 KO B cells appeared at the outer edge of the central BCR cluster, which was similar to the pattern observed in WT B cells (**Fig. 5A**). However, the MFI of F-actin in the contact zone of KO B cells increased over time from 3 to 5 min post-stimulation, which was significantly higher than that in WT B cells (**Fig. 5B-C**). Consistent with the augmented F-actin accumulation, the recruitment of pWASP in KO B cells was also increased at 3 min after stimulation (**Fig. 5A, D**). Moreover, these changes were accompanied by the increased accumulation of pCD19 in KO B cells at 3 and 5 min post-stimulation, compared to that of WT B cells (**Fig. 5E-F**). As was the case for pCD19, the recruitment of pY, pBtk, and pSHIP in KO B cells remained higher than that of WT B cells (**Fig. 5G-K**). Convincingly, these findings suggest that CCR2 KO promotes the aggregation of BCR microclusters and accumulation of signalosome at an early phase of

B cell activation, which subsequently influences the formation of the central clusters at the terminal phase; thus, positively regulating B cell signaling.

CCR2 depletion triggers the activation of STAT1 to enhance BCR signaling

CCR2 is associated with the activity of the JAK/STAT pathway and its downstream transcriptional factors.[21] Furthermore, the inhibition on the CCL2-CCR2 axis was shown to affect the activity of NF-ϰB.[30] To ascertain the effect of CCR2 KO on the transcriptional factors downstream of JAK/STAT pathway, we determined the activation of STAT1, NF-ϰB, and STAT5 in B cells using CFm and western blotting. Upon sAg stimulation, the colocalization between pSTAT1 and BCR in CCR2 deficient B cells significantly increased within 10 min after stimulation (**Fig. 6A-C**), which was in contrast to the observations for WT B cells. For pNF-ϰB, its colocalization with BCR was notably higher in CCR2 deficient B cells at 5 and 30 min post-stimulation (**Fig. 6D-F**). Lastly, we observed increased colocalization of pSTAT5 and BCR at 5 min after stimulation in CCR2 depleted B cells (**Fig. 6G-I**). While the pSTAT1, pNF-ϰB, and pIKKB protein expression levels increased in CCR2 KO B cells upon sAg stimulation, there was no difference in pSTAT5 protein expression (**Fig. 6J**).

Previous studies showed that Mst1 plays a role in cell proliferation by influencing the AKT/mTOR signaling pathway,[31] and the JAK/STAT downstream transcriptional factor activation.[32] To identify the possible sequential effect of Mst1-mTORC1-STAT1 on BCR signaling, we pretreated CCR2 KO B cells with the STAT1 inhibitor, fludarabine, prior to sAg stimulation. Following the fludarabine treatment, the levels of pCD19, pSHIP, pAkt, pS6, Dock8, pNF-kB, pIKKB, and pMst1 were corrected towards the expression levels observed in WT B cells (**Fig. 6K**). Similarly, following pretreatment with XMU-MP-1 (**Fig. 6L)** or rapamycin (**Fig. 6M)** , the levels of pSTAT1 and pNF-kB in CCR2 KO B cells were rescued to levels comparable to WT B cells, with the exception of pSTAT5 levels, which displayed little to no changes before and after the rapamycin treatment. This exception may reflect that STAT5 is not particularly affected by Mst1 and mTORC1 suppression. Altogether, by connecting the upstream and downstream signaling pathways, CCR2 might regulate BCR signaling via the Mst1-mTORC1-STAT1 pathway.

CCR2 deficiency compromises antibody response to T cell-dependent antigens

To address the impact of CCR2 deletion on B cell function, we examined the T cell-dependent antigen immune response in WT and CCR2 KO mice. Mice were first injected i.p. with 40 μg NP-KLH and boosted at 14 days post-prime, then euthanized 5 days later. Splenic B cells were harvested to determine the peripheral B cell subsets and antibody-secreting cells (ASCs) (**Fig. S1J**). In contrast to the un-immunized CCR2 KO mice, immunized KO mice exhibited a notable increase in FO, MZ, T1, and T2 B cell number, albeit with no significant difference in their proportion to the whole splenic B cells, except for T2 B cells (**Fig. 7A-F**). Furthermore, there were no changes in GCB cells before and after immunization (**Fig. 7G-H**). Moreover, the percentage or the absolute cell number of ASCs, including plasma cells (PC) and plasmablast cells (PBC), were dramatically reduced in CCR2 KO mice (**Fig. 7I-K**). Additionally, in CCR2 KO mice, there was a trend toward a decrease of the percentage and cell number in memory B cells (MBC) (**Fig. 7L-M**).

NP-specific IgM and IgG levels in serum from primed and boosted mice were measured using ELISA. We observed that the NP-specific IgM levels of CCR2 KO mice were significantly reduced compared to those of WT mice (**Fig. 7N-O**), particularly after the initial immunization. Paradoxically, we noticed an increased number of lymphatic follicles that contained anatomical GCs in the spleen of immunized CCR2 KO mice, as well as enlarged structures (**Fig. 7P**). The enlarged lymphatic follicles but decreased ASCs in CCR2 deficient mice might indicate an impairment in the differentiation from FO B cells to ASCs; however, future research is necessary to confirm this hypothesis. Collectively, CCR2 plays an important role in the immune response of B cells, and its absence was sufficient to lead to a compromised immune response.

Discussion

In this study, we dissected the influence of CCR2 deficiency on BCR signaling pathways by using germinal

CCR2-deletion mice. This is the first study focusing on the correlation between CCR2 and BCR signaling, and the first to systematically illustrate the sequential involvement of three typical signaling pathways regulated by CCR2 which ultimately influence BCR signaling. We discovered that CCR2 participates in a series of biological signal changes following B cell activation and its absence guides the up-regulation of key BCR signaling molecules, which were associated with disturbed FO B cell differentiation, enhanced early event BCR activation, and compromised B cell function. Furthermore, we showed that these effects were mediated by CCR2 through the synergy of the Mst1/mTORC1/STAT1 signaling pathways.

Mst1, mTOR, STAT1, and their associated pathways have been extensively studied due to their general involvement in various physiological processes. Separately, they are likely responsible for the occurrence and development of autoimmunity. Defective Mst1/Foxo-1 signaling results in the collapse of immune tolerance,[17] and activation of mTORC1 represent one of the major molecules responsible for the SLE pathogenesis and other autoimmune diseases caused by oxidative stress.[33]Additionally, in neuro-autoimmunity, STAT1 phosphorylation and CCR2 expression co-clustered with $CD8^+$ T cells.[34] In the present study, during B cells differentiation, PI3K/Akt pathway enhanced the activity of mTORC1 which promoted B cell energy metabolism; which also bring about attenuation of FO B cells in CCR2 KO mice. Alternatively, CCR2 deficiency induced Mst1 up-regulation, which promoted F-actin accumulation and the interaction of BCR with other signaling molecules via the mTORC1/Dock8/WASP axis. Moreover, by taking advantage of multiple downstream transcription factors of the JAK/STAT pathway, Mst1 and mTORC1 modulated STAT1 to control BCR signaling. Mechanistically, the pSTAT1 and pNF-kB expression levels were corrected following treatment with Mst1 and mTOR inhibitors, which indicates a feedback loop in the CCR2-regulated BCR signaling pathway. Taken together, these findings supported the notion that CCR2 regulates B cell signaling via the Mst1-mTORC1-STAT1 axis.

Based on a different study investigating the interaction between CCL2 and B cell signaling (unpublished data), there are some worth noting differences between CCL2 and CCR2 deficient mice. Amongst the similarities between CCL2 and CCR2 deficient mice, are the up-regulated BCR signaling, increased F-actin accumulation, decreased ASCs, and attenuated antibody production. However, there are significant differences in immune system characteristics. First of all, considering the peripheral B cell differentiation, CCL2 deficiency leads to a reduction in MZ B cells and increase in GC B cells as well as formation of spontaneous germinal centers (Spt-GCs), while CCR2 deficiency leads to fewer FO B cells and has no obvious impact on MZ or GC B cells. An explanation for this discrepancy may be that CCR2 is expressed on non-GC B cells while absent on GC B cells.[35] Furthermore, the distinctions between CCL2 and CCR2 deficiencies might also be explained by the different stages during B cell development at which each marker is expressed. Another difference lies within the downstream activities mediated by the Mst1/mTORC1/STAT1 axis, among which the activity of STAT5 is noteworthy. The absence of CCL2 positively regulated the STAT1 and STAT5 expression levels, while CCR2 depletion only enhanced STAT1 expression levels. This might indicate that CCR2 exerts its autoimmune-inducing effects by targeting STAT1 rather than STAT5. Nevertheless, CCL2 and CCR2 might have complementary roles in the BCR downstream Mst1/mTORC1/STAT1 signaling pathway. Last but not least, their impact on B cell function also differs. Upon T cell dependent immunization, splenic lymphatic follicles of immunized CCR2 KO mice were augmented and enlarged, while that of CCL2 KO mice were atrophied and diminished, which further supports the differences in B subsets seen increased during B cell peripheral differentiation. As was the case for CCL2 deficiency, the reduction of ASCs and antibody production might be attributed to the underlying physiological thresholds required for triggering the appropriate immune responses.

Since Mst1, mTOR, and STAT1 have been linked to the pathogenesis of SLE autoimmune disorders, targeting these pathways may allow simultaneous suppression of multiple cytokines. CCR2 can directly participate in the pathogenesis of SLE by means of cooperation with its ligands. In this study, the increased level of anti-dsDNA and T-bet observed in CCR2 KO mice would definitely aggravate the autoimmune progression of SLE. Thus, the existence of a signalling feedback loop in the CCR2-regulated BCR signaling pathway is highly probable and it is worth investigating it since it might prove to be a candidate therapeutic target for autoimmune diseases. Moreover, the correction of signaling expression levels following the targeted

treatment with specific inhibitors in CCR2 deficient mice further supports this hypothesis. In conclusion, our findings highlight the dual role of the CCR2-regulated BCR signaling pathway. On the one hand, as proposed elsewhere, BCR signaling pathways are strongly controlled by the synergistic effects of various signaling axes, in which our study complements the bridging role of CCR2. On the other hand, as previously suggested, biological signals remain within an immune homeostasis maintenance range and either CCR2 or CCL2 abnormalities can trigger to the development of autoimmune disorders, albeit with varied phenotypes or target molecules.

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Figure legends

Fig. 1. Disrupted peripheral differentiation of B cells in CCR2KO mice. Splenic mononuclear cells were isolated from mice and stained with corresponding antibodies. Flow cytometry (FCM) was performed. Organs extracted from mice were used for immunohistochemical and immunofluorescence experiments. (**A-B**) B cell subsets derived from BM cells (n = 8). Pre-pro B cell (A), pro B cell (B), early-pre B cell (C), late-pre B cell (D), immature B cell (E) and recirculating mature B cell (F).**(C)** Quantitative analysis of BM subsets. **(D-F)**Peripheral B cell subsets including follicular B cell (FO), transitional type-1 B cell (T1), transitional type-2 B cell (T2), marginal zone B cell (MZ), and germinal center B cells (GCB) (n = 8). **(G-K)**Quantitative analysis of peripheral B subsets. **(L)** B-1a and B-1b B cells in the peritoneal cavity were counted (WT: n = 7, KO: n = 6). **(M-N)** Quantitative analysis of B1 B cell subsets.**(O)** Representative immunofluorescent analysis (60x, scale bar = 10 μm) of IgG deposits in glomeruli. **(P)** Serum anti-dsDNA Ab titer was quantified using ELISA (n = 7). **(Q)** Hematoxylin and eosin (HE) staining of lung anatomical structure (10×, scale bar = 25 μm). **(R)** Quantitative analysis of T-bet expression level in WT and CCR2 KO $B220^+$ B cells. Quantitative analyses were shown as representative histograms with the specific populations, average percentages (± SEM), and cell number indicated. Each symbol represents a mouse. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: no statistical significance.

Fig. 2. CCR2 KO mice exhibit enhanced BCR proximal signaling. Purified B cells were incubated with AF594-mB-F(ab')2-anti-Ig (M + G) and activated at 37 °C for 5, 10, and 30 min, fixed and permeabilized before staining, confocal microscopy (CFm) was performed. Cells were incubated with soluble antigen (sAg) (biotin-conjugated F(ab')2 anti-mouse Ig (M + G) and streptavidin), then activated at 37 °C for 5, 10, and 30 min, western blotting was performed. **(A)** CFm images of pCD19 and BCR localization. **(B)** Phosphorylated CD19 protein expression levels in $B220^+$ B cells. **(C)**Quantitative analysis of pCD19 mean fluorescence intensity (MFI).**(D)** Colocalization of pCD19 and BCR. **(E)** CFm images of pY and BCR movement. **(F)** CFm images of pBtk and BCR movement.**(G)** Quantitative analysis of pY MFI. **(H)** Quantitative analysis of pBtk MFI. **(I)** The pBtk and pY protein expression levels in $B220^+$ B cells. **(J)** Colocalization of pBtk and BCR. **(K)** Colocalization of pY and BCR. **(L)**CFm images of pSHIP and BCR localization. **(M)** Quantitative analysis of pSHIP MFI. **(N)** Colocalization of pSHIP and BCR.**(O)** Phosphorylated SHIP levels in $B220^+$ B cells. **(P)** Intracellular Ca^{2+} flux in B cells following stimulation with 10 μg/ml biotin-conjugated $F(ab')_2$ anti-mouse Ig (M + G). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: no statistical significance.

Fig. 3. CCR2 attenuates B cell metabolic process through the negative regulation of PI3K-Akt-mTORC1 signaling pathway. (A) pPI3K, PI3K, pmTOR, mTOR, pAkt, Akt, pFoxo-1, Foxo-1, pS6, and S6 protein expression levels in B cells activated with sAg. **(B)** pSHIP, pBtk, pCD19, pAkt, pFoxo-1, and pS6 protein expression levels in CCR2 KO B cells treated with 20 nM rapamycin. **(C)** Seahorse assay was performed to quantify the oxidative respiration of WT and CCR2 KO B cells. **(D)** Celltrace Violet (CTV) dilution of LPS-stimulated (5 μg/ml) B cell proliferation. **(E)** Quantification of *in vitro* proliferation and apoptosis of B cells following a 72-h stimulation with LPS (5 μg/ml). **(F)** Celltrace Violet (CTV) dilution of C_PG-stimulated (5 μg/ml) B cell proliferation. **(G)** Quantification of *in vitro*proliferation and apoptosis of B cells following a 72-h stimulation with C_PG (5 μg/ml). **(H)** HIF-1a and c-Myc protein expression levels**.** *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: no statistical significance.

Fig. 4. CCR2 KO mice exhibit increased accumulation of F-actin through the enhancement of the Mst1-mTORC1-Dock8-WASP pathway. (A) CFm images of pWASP, F-actin, and BCR localization. **(B)**Quantitative analysis of F-actin MFI. **(C)** Quantitative analysis of F-actin and BCR colocalization.

(D) Quantitative analysis of pWASP MFI. **(E)** Quantitative analysis of pWASP and BCR colocalization. **(F-G)** Phosflow detection of pWASP and F-actin levels in B220$^+$ B cells. **(H)** Dock8, pMst1, Mst1, pWASP, WASP, and pEzrin protein expression levels. **(I)**Filopodia dilatation visualized using electron microscopy (scale bars = 5 μm). **(J-K)** Quantitative analysis of number and length of the filopodia. **(L)** Dock8, pMst1, pSHIP, pBtk, pCD19, pMTOR, pAkt, pPi3k, pFoxo-1, and pS6 protein expression levels following XMU-MP-1 treatment on CCR2 KO B cells. **(M)** Dock8, pMst1, and pWASP protein expression levels following rapamycin treatment on CCR2 KO B cells. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: no statistical significance.

Fig. 5. CCR2 depletion promotes spatial aggregation of F-actin which is accompanied by the enhancement of early BCR activation. Purified WT and CCR2 KO B cells were stimulated with membrane antigen (mAg) (AF546–mB-Fab–anti-Ig tethered to lipid bilayers) for 3, 5, and 7 min, total internal reflection fluorescence microscopy (TIRFm) was performed. **(A)** Representative TIRFm images of F-actin and pWASP (scale bars = 2.5 μm). **(B-D)** Quantitative analysis of B cell area in the contact zone and the MFI of F-actin as well as pWASP.**(E)** Representative TIRFm images of pCD19 (scale bars = 2.5 μm).**(F)** Quantitative analysis of pCD19 MFI. **(G)**Representative TIRFm images of pY and pBtk (scale bars = 2.5 μm).**(H-I)** Quantitative analysis of pY and pBtk MFIs.**(J)** Representative TIRFm images of pSHIP (scale bars = 2.5 μm).**(K)** Quantitative analysis of pSHIP MFI. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: no statistical significance.

Fig. 6. CCR2 attenuates the effect of STAT1 activation in BCR signaling pathway. (A) Representative CFm images of pSTAT1 (scale bars = 2.5 μm). **(B-C)** Quantitative analysis of the pSTAT1 MFI and its colocalization with BCR. **(D)** Representative CFm images of pNF-κB (scale bars = 2.5 μm). **(E-F)** Quantitative analysis of the pNF-κB MFI and its colocalization with BCR. **(G)**Representative CFm images of pSTAT5 (scale bars = 2.5 μm).**(H-I)** Quantitative analysis of the pSTAT5 MFI and its colocalization with BCR. **(J)** Protein expression levels of pSTAT1, STAT1, pSTAT5, STAT5, pNF-κB NF-κB, pIKKB, and IKKB in WT and CCR2 KO B cells. **(K-M)** Protein expression levels of the markers mentioned in panel J following fludarabine, XMU-MP-1, or rapamycin treatment of CCR2 KO B cells. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: no statistical significance.

Fig. 7. CCR2 KO mice generate fewer plasma cells and produce less antibodies during T cell dependent immune responses. WT and CCR2 KO mice were injected intraperitoneally (i.p.) with T cell dependent antigen 4-hydroxy-3-nitrophenylacetyl conjugated keyhole limpet hemo-cyanin (NP-KLH, 40 μg/mouse). Boost mice with the same reagents 14 days post-prime. **(A-B)** FCM gating strategy of peripheral B cell subsets after immunization. **(C-F)** Quantitative analysis of the proportion and cell number of FO, MZ, T1, and T2 groups. **(G)**FCM gating strategy of GC B cells. **(H)** Quantitative analysis of the proportion and cell number of GC B cells. **(I-L)** FCM gating strategy of PBC, PC, and MBC. **(J-M)** Quantitative analysis of the proportion and cell number of PBC, PC, and MBC. **(N-O)** ELISA was performed on serum extracted from blood, and NP-specific IgM as well as IgG1 levels were quantified post-prime and post-boost. **(P)**Images of HE stained anatomical spleens (scale bars = 200 μm). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: no statistical significance.

Fig. S1. CCR2 deficiency cause intrinsic peripheral B cell differentiation impairment. B cell subsets in WT (n = 5) and CCR2 KO (n = 3) mice after bone marrow chimeras were determined using FCM.**(A)** Gating strategy. **(B-D)** Representative histogram of peripheral B cell subsets with the specific populations. **(E-I)**Quantitative analysis of the proportion and cell number of peripheral B cell subset from WT and CCR2 KO B mice. **(J)** The procedure of mice immunization**.** Each symbol represents a mouse. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: no statistical significance.

Fig. S2. The anatomical structure of organs stained with hematoxylin eosin (HE).

(A) Kidney, colon, and liver of mice were harvested, sectioned, and stained with HE.