Generation and characterization of CD19-iCre mice as a tool for efficient and specific conditional gene targeting in B cells

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The Cre/loxP system is a powerful tool for generating conditional gene knockout (KO) mice and elucidate gene function in vivo. CD19-Cre and Mb1-iCre transgenic mice are commonly used for generating B cell-specific KO mice and investigate the development, as well as the physiological and pathophysiological roles of B cells. However, the CD19-Cre line low efficiency and the Mb1-iCre line occasional ectopic recombination represent challenges for their use. Thus, we developed a CD19-codon-improved Cre (CD19-iCre) knock-in mouse with the T2A-iCre sequence inserted into the Cd19 locus, just before the stop codon. The CD19-iCre mice were compared with existing models, crossed with the Rosa26-EYFP reporter mice, and their recombination activity in B cells carrying different Cre alleles was assessed. CD19-iCre mice showed more effective Cre recombination in the early B cell developmental stages compared with the CD19-Cre mice. The efficiencies of the CD19-iCre and Mb1-iCre lines were similar; however, the B lineage-specific recombination was more stringent in the CD19-iCre line. Furthermore, the utility value of the CD19-iCre model was superior than that of the CD19-Cre mice regarding deletion efficiency in IL10-floxed mice. Thus, the CD19-iCre line is a valuable tool for highly efficient gene targeting specific to the B cell compartment.

B cells play a vital role in humoral immunity against pathogens1,2. In the context of diseases such as autoimmun-ity, inflammation, and allergy, B cells can act as effector cells and initiate an immune response through antibody production, antigen presentation, and secretion of inflammatory cytokines3. In addition, recent evidence shows that B cells can function as negative regulators of immunity via anti-inflammatory cytokines such as interleukin (IL)-10 and IL-355–7. The peripheral B cell pool is derived from hematopoietic stem/progenitor cells in the bone marrow. The development of B-lineage cells proceeds through several stages, which are orchestrated by successive steps involved in the expression and assembly of immunoglobulin genes and a complex network of transcription factors1,9.

B cell-specific conditional knockout (BKO) mouse models have opened invaluable avenues for determining the molecular mechanisms regulating the development of B cells and the pathophysiological functions in which they are involved. The current options for generating pan-BKO mice are by employing CD19-Cre10 or Mb1-codon-optimized improved Cre (iCre)11 mice, in which Cre recombinase is able to delete the regions of the genome flanked by loxP sites (termed as floxed). These Cre driver lines (CD19-Cre and Mb1-iCre) have been well characterized and used to successfully assess the function of genes through their deletion or genetic manipulation in a B cell-specific manner10,11. However, Cre-mediated deletion in the CD19-Cre and Mb1-iCre lines exhibits flaws with regard to its efficiency and specificity, respectively. When B cell-specific Cre mice were crossed with mice harboring the Rosa26-enhanced yellow fluorescent protein (EYFP) reporter (R26EYFP+/fl; hereafter referred to as R26EYFP mice)12 which express EYFP directly from the ubiquitous Rosa26 promoter following Cre-dependent deletion of a loxP-flanked stop element, it was possible to evaluate the specificity and efficiency of Cre expression in B cells using flow cytometry. A very small population of EYFP+ cells was identified among pro-B cells of CD19-Cre/Rosa26-EYFP double transgenic mice (Cd19Cre/T2A-EYFP)13, and less than half of total CD19+ B cells in the bone marrow of these mice expressed EYFP, whereas CD19 expression began at the pro-B

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a

wild type Cd19 locus

Targeted allele

KI allele (CD19-iCre)

b

c

CD19-Cre

CD19-iCre

Homo

Hetero

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cell stage in the bone marrow during B cell development. In contrast, the other B cell-specific Cre driver line Mbi-iCre, which was created by replacing the coding exons of the Cd19 coding sequence (immediately upstream of the stop codon) (Fig. 1a). Following the induction of double-strand DNA breaks by the Cas9 enzyme mediated by a guide RNA (gRNA), the homology arms guided the T2A-iCre to be inserted in-frame with the Cd19 open reading frame by homologous directed repair. This targeting strategy using self-cleaving 2A sequences was designed to mediate bicistronic translation, which is potentially more efficient than the well-described internal ribosome entry site sequence. Upon translation of the chimeric Cd19-T2A-iCre mRNA, the T2A sequence led to ribosome skipping, resulting in the co-expression of Cd19 and iCre as discrete proteins in Cd19-expressing cells. Consequently, iCre expression was regulated by the Cd19 promoter in tandem with the endogenous Cd19 expression. In the present study, the targeting construct was correctly introduced in embryonic stem (ES) cells, which was confirmed by polymerase chain reaction (PCR) analysis. This was followed by deletion of a neomycin resistance gene (Neo) cassette (Neo cassette) by Flp cDNA transfection into targeted ES cells to avoid unpredictable Cre activity. After confirming the Neo cassette deletion by PCR, the targeted ES cells were transferred into blastocysts and the mouse germline. Heterozygous and homozygous Cd19-iCre mice (referred to as Cd19<i>Cre</i> and Cd19<i>C<sub>i</sub>Cre</sub>, respectively) were found to be viable, fertile, and born at the expected Mendelian frequencies (data not shown).
To understand the consequences of the CD19-iCre targeted insertion strategy on CD19 expression, we analyzed peripheral B cells in mice heterozygous or homozygous for the knock-in allele by flow cytometry and compared the collected data with that of a previously published CD19-Cre line\(^1\), in which the Cre was inserted in exon 2 and the CD19 coding sequence was disrupted, leading to a CD19 deficiency in the homozygous situation. CD19-Cre homozygous mice showed complete loss of CD19 expression, whereas the CD19-iCre homozygous mice retained CD19 expression, albeit there was a significant reduction in its levels compared with that in B cells from wild-type (WT) mice. The mean fluorescence intensity (MFI) of CD19-stained B cells in CD19-iCre mice decreased by about fourfold compared with WT mice (Fig. 1c). Moreover, Cd19 mRNA and protein levels were decreased in splenic B cells from CD19-iCre mice (Fig. 1d, e), as assessed by quantitative RT-PCR and western blot analysis, respectively. It should be noted that high levels of released iCre and CD19 along with subtle levels of the uncleaved CD19-T2A-iCre fusion protein were detected, indicating efficient T2A self-processing in B cells (Fig. 1e). These results suggest that the targeted insertion of T2A-iCre might interfere with CD19 expression or mRNA stability. Heterozygous mice for the Cre allele are commonly used as Cre deleter lines. Mice heterozygous for the Cre insertion in CD19-Cre line, which retained one functional CD19 allele, showed reduced surface CD19 levels (the MFI was approximately half of that of WT mice) (Fig. 1c). Cd19\(^{Cre/}\) mice also showed reduced CD19 expression, but significantly higher compared with CD19-Cre mice (Fig. 1c). However, B cells from the Cd19\(^{Cre/}\) mice did not show any further apparent alterations and exhibited normal development in the bone marrow, spleen, lymph nodes, and peritoneal cavity (Fig. 1f).

**Efficient recombination at different B cell developmental stages with the CD19-iCre transgene.** To determine the efficiency of Cre expression, CD19-iCre mice were crossed with the R26\(^{EYFP}\) line to generate Cd19\(^{Cre/\text{iCre}/}\) mice. Almost all CD19\(^{+}\) B cells expressed EYFP in the spleen and mesenteric lymph nodes (mLN), but EYFP was not detected in CD19\(^{-}\) non-B cells (Fig. 2a), suggesting high B cell-specific Cre expression in the CD19-iCre lines. Next, we examined the efficiency of Cre-mediated deletion in B cell subsets of CD19-iCre mice and compared them side-by-side with previously published B cell specific Cre-driver lines: the CD19-Cre and the Mb1-iCre mice that were also crossed with the R26\(^{EYFP}\) line. A previous study demonstrated that Mb1-iCre mice have an earlier and/or more efficient loxP site recombination in developing B cells than CD19-Cre mice\(^1\). Our results also confirmed these findings, since almost all of the pro-B cells expressed EYFP in Mb1\(^{Cre/\text{iCre}/}\) mice, whereas only ~50% of the same subset of cells were EYFP-positive in Cd19\(^{Cre/\text{iCre}/}\) mice (Fig. 2b).

A comparison of our newly developed CD19-iCre line showed highly efficient Cre-mediated activation of EYFP expression in pro-B cells, similar to that in the Mb1-iCre line. In the case of the very early B lineage stage...
Highly specific recombination in B cells with the CD19-iCre transgene. A previous study indicated aberrant loxP recombination in T cells from $Mb^{1}\text{Cre}^+\text{R26}^{\text{EYFP}}$ mice, albeit with a relatively low frequency. To detect ectopic Cre-mediated activation of EYFP expression in $Cd19\text{Cre}^+\text{R26}^{\text{EYFP}}$ and $Mb^{1}\text{Cre}^+\text{R26}^{\text{EYFP}}$ mice, flow cytometry analysis was conducted. In agreement with a previous report, a small number of splenic CD4+ and CD8+ TCRβ+ T cells from $Mb^{1}\text{Cre}^+\text{R26}^{\text{EYFP}}$ mice were found to be EYFP+. In contrast, EYFP expression was virtually absent in T cells from $Cd19\text{Cre}^+\text{R26}^{\text{EYFP}}$ mice (Fig. 3). Together, these data indicate the highly targeted expression of iCre in CD19-iCre mice, thereby demonstrating that this transgenic line is a very valuable resource to study B cell-specific gene functions.
CD19-iCre mice are a valuable line for studying B cell function. We have previously shown that lipopolysaccharide (LPS)-activated B cells produce IL-10 after stimulation with B cell antigen receptor (BCR)25. To test the utility of the CD19-iCre driver line for general knockout studies, this line was crossed with IL10 floxed (Il10^floxed) mice26 to generate Cd19^Cre/+Il10^floxed animals. We compared these Cd19^Cre/+Il10^floxed mice with Cd19-Cre/Il10^floxed (Cd19^-Cre/Il10^floxed) mice regarding the recombination efficiency of floxed alleles in splenic B cells. The deleted Il10 floxed alleles in B cells, but not T cells, sorted from spleens with high purity (>98%) were confirmed by genomic PCR in Cd19^Cre/+Il10^floxed and Cd19^Cre/+Il10^f/f mice (Fig. 4a). However, it is noteworthy that the recombination efficiency in B cells from Cd19^Cre/+Il10^floxed mice was superior to that in Cd19^Cre/+Il10^f/f mice. Enzyme-linked immunosorbent assay (ELISA) showed that Cd19^Cre/+Il10^f/f B cells had compromised IL-10 secretion compared with control Cd19^Cre/+ B cells (Fig. 4b), in agreement with a previous study27. Notably, IL-10 production was significantly reduced in Cd19^Cre/+Il10^floxed B cells than in Cd19^Cre/+Il10^f/f B cells (Fig. 4b). Collectively, these data suggest that CD19-iCre mice are valuable Cre drivers to manipulate genes in B cells.

Discussion

In this study, we describe a new B cell-specific Cre line, named CD19-iCre, which is the third-generation line after CD19-Cre and Mb1-iCre. Our findings indicate that CD19-iCre can recombine loxp sites with high efficiency in pro-B cells of Cd19^Cre/+R26^EYFP mice and show efficient Cre-mediated deletion of loxp sites during B cell development in several tissues, which is comparable to that of the Mb1-iCre line but greater than that of the CD19-Cre mice. Indeed, the Il10 floxed allele was more efficiently deleted in splenic B cells from Cd19^Cre/+Il10^floxed mice compared with those from Cd19^Cre/+Il10^f/f mice, and, consequently, Cd19^Cre/+Il10^f/f B cells expressed less IL-10 than Cd19^Cre/+Il10^floxed B cells.

Despite the previously reported studies on Mb1-iCre mice attributing the efficiency of the Cre deletion to promoter-specific differences between the Mb1-iCre and the CD19-Cre mouse lines, our present study shows that this may not be the case as the results exhibited highly efficient Cre-mediated recombination in pro-B cells of Cd19^Cre/+R26^EYFP mice, which was comparable to that of Mb1^Cre/+R26^EYFP mice. Given the endogenous expression of Cd19 in the pro-B cell population, these results suggest that CD19-iCre mice can excise the floxed sequence more effectively than CD19-Cre mice. The enhanced efficiency of the CD19-iCre mice may be due to the use of the improved iCre, which has been reported to be more efficiently expressed in mouse cells28. Another possible underlying reason can be the difference in the design strategy of the Cre-transgenic lines. In the CD19-Cre line, in addition to the regulatory elements of the endogenous Cd19 locus, a β-globin polyadenylation site was introduced to permit polyadenylation of the Cre mRNA. In the CD19-iCre line, we introduced the T2A-iCre sequence before the 3′ promoter-specific differences between the Mb1-iCre and the CD19-Cre mouse lines, our present study shows that the recombination efficiency in B cells from Cd19^Cre/+Il10^floxed mice was superior to that in Cd19^Cre/+Il10^f/f mice regarding the recombination efficiency of floxed alleles in splenic B cells. The deleted Il10 floxed alleles in B cells, but not T cells, sorted from spleens with high purity (>98%) were confirmed by genomic PCR in Cd19^Cre/+Il10^floxed and Cd19^Cre/+Il10^f/f mice (Fig. 4a). However, it is noteworthy that the recombination efficiency in B cells from Cd19^Cre/+Il10^floxed mice was superior to that in Cd19^Cre/+Il10^f/f mice. Enzyme-linked immunosorbent assay (ELISA) showed that Cd19^Cre/+Il10^f/f B cells had compromised IL-10 secretion compared with control Cd19^Cre/+ B cells (Fig. 4b), in agreement with a previous study27. Notably, IL-10 production was significantly reduced in Cd19^Cre/+Il10^floxed B cells than in Cd19^Cre/+Il10^f/f B cells (Fig. 4b). Collectively, these data suggest that CD19-iCre mice are valuable Cre drivers to manipulate genes in B cells.

Conditional gene targeting based on the Cre/loxP system is a powerful technology for the analysis of gene function, but it is key that the expression of Cre transgenes occurs in a targeted cell-specific manner. Despite the differential sensitivity of individual loxp-flanked target gene alleles to Cre-mediated recombination, at least in the R26^EYFP reporter system, Mb1-iCre mice can exhibit undesired recombination in T cells. By contrast, our CD19-iCre mouse line did not show such ectopic recombination. Nonetheless, the proportion of detectable EYFP+ T cells was minor in Mb1^Cre/+R26^EYFP mice, in cases in which the target genes that are to be deleted and analyzed in B cells are critical for T cell development and function. Thus, it is important to carefully assess the recombination in T cells and interpret the phenotype. Until now we did not observe Cre-mediated recombination in the germline of Cd19^Cre/+R26^EYFP mice; however, we cannot exclude the possibility that the CD19-iCre line induce germline deletions.

The T2A-based approach in CD19-iCre mice may produce the equimolar amounts of iCre relative to CD19 protein from a single multicistronic transcript. Generally, a 2A peptide, when placed between genes can induce ribosomal skipping during protein translation, thereby producing discrete proteins, whereas unsuccessful skipping and continued translation may result in a fusion protein. We could detect very little CD19-T2A-iCre fusion protein in Cd19^Cre/+ mice, which might explain the observed reduction in surface CD19 levels in CD19-iCre mice. However, given the reduced Cd19 expression in Cd19^Cre/+ mice, it seems most likely that T2A insertion may affect the expression or mRNA stability of Cd19. Although minor reduction in Cd19 expression did not induced apparent alterations of B cell development in Cd19^Cre/+ mice, we advocate using Cd19^Cre/+ mice as controls for conditional BKO mice generated with this strain, as is the case with CD19-Cre or Mb1-iCre lines.

In summary, our newly developed CD19-iCre mice showed more effective Cre recombination at the early B cell stages, as well as throughout B cell development, when compared with that of the commonly used CD19-Cre line. Although the efficiency of the CD19-iCre line was comparable to that of the Mb1-iCre line, more stringent B lineage-specific recombination was observed in the novel model. Thus, its high efficiency and specificity make the CD19-iCre line the most reliable and robust model available for gene targeting and manipulation of B cells in vivo.

Methods

Mice. C57BL/6 mice were purchased from CLEA Japan. CD19-Cre30 and Rosa26-EYFP12 were obtained from Jackson Laboratories. The Mb1^Cre/+ (kindly provided by Dr. Michael Reth)31 and Il10^floxed mice (kindly provided by Dr. Werner Muller)32 were housed and bred under specific pathogen-free conditions in the animal facility of Kyushu University and used at the age of 7 to 15 weeks. All studies and procedures were approved by the Kyushu University Animal Experiment Committee. All animal experiments were conducted in accordance with the ARRIVE guidelines and the ethical guidelines of Kyushu University.
Generation of the CD19-iCre knock-in mice. For the targeting vector construction, the 5′ homology arm containing the intron and exon 14 of Cd19 (1.0 kb) and 3′ homology arm containing the 3′ UTR of Cd19 (1.0 kb) were amplified by PCR from C57BL/6 mouse genome. Gene encoding Cre recombinase optimized for mammalian codon usage and nuclear localization signal (iCre) was obtained from Mb1-iCre mouse genome [11,20]. T2A self-cleaving peptide sequence was synthesized as oligo nucleotides adding to PCR primer to amplify iCre gene. The neomycin resistance gene (Neo<sup>+</sup>) cassette (Neo cassette) under the tk promoter flanked by FRT site sequences for Flippase (Flp) site-directed recombination was obtained from a Flp-ER<sup>+</sup> targeting vector (Yasuda and Rajewsky, unpublished). Four PCR fragments were assembled in pBlueScript II plasmid vector using NEBuilder HiFi DNA assembly master mix (NEB) to generate targeting vector (pCD19_T2A_iCre Neo). The targeting vector was designed for integration of the T2A-iCre sequence just before the stop codon of Cd19 to produce CD19 and iCre from same transcript. The Neo cassette was inserted downstream of iCre, right after the stop codon. All PCR amplifications were performed using high fidelity KOD DNA polymerase (TOYOBO). 1 × 10<sup>6</sup> C57BL/6 ES cells (EGR-101) kindly provided by Dr. Masahito Ikawa [39] were electroporated with 100 ng/µl (0.61 µM) recombinant Cas9 Nuclease 3NLS (IDT #1074181), 36 ng/µl (1 µM) gCD19-1 crRNA (5′-CTAGTCACTTGG GAGTCA/CTTG-3′, stop codon is underlined):tracrRNA duplex, and 100 ng/µl pCD19_T2A_iCre Neo in 100 µl of iXOpti-MEM medium (Thermo Fisher) using NEPA 21 super electroporator (NEPA GENE) and 2 mm gap cuvette. The following parameters were used for electroporation; Poring pulse (voltage 125 V; pulse length 2.5 ms; pulse interval 50 ms; number of pulses 2; decay rate 10%; polarity +) and Transfer pulse (voltage 20 V; pulse length 50 ms; pulse interval 50 ms; number of pulses 5; decay rate 40%; polarity -). The electrical resistivity was typically 0.03 to 0.05 kΩ. Electroporated cells were selected for 6–7 days under ES medium (StemSure D-MEM, 15% FCS, L-glutamine, non-essential amino acids, β-mercaptoethanol, and LIF) containing 200 µg/ml G418 (Nacalai Tesque). 60 ES cell colonies were subjected to PCR screening, and 30 clones (50%) showed correct recombination in both 5′ and 3′ side. A clone, A4, was subjected to pCAG-Fippe electroporation (Addgene) to delete Neo cassette. Neo<sup>−</sup> deleted 5–10 ES cells were injected into Balb/c blastocysts. A chimeric male (80–90% chimerism) was crossed to C57BL/6 females to obtain germine transmitted offspring.

**Antibodies.** For flow cytometry, single-cell suspensions prepared from bone marrow, spleen, LNs and peripheral blood were stained with the following biotin- or fluorochrome-conjugated antibodies purchased from BioLegend, BD Biosciences or eBioscience: Biotin-conjugated anti-Cd19 (1D3), and anti-Cd93 (AA4.1); allophycocyanin (APC)-conjugated anti-Cd5 (53-7.3), anti-Cd19 (1D3), anti-Cd138 (281-2), anti-TCRβ (H57-597) and anti-IgM (RMM-1); allophycocyanin (APC)-Cy7-conjugated anti-B220 (RA3-6B2), anti-CD19 (6D5); peridinin chlorophyll protein complex-cyanine 5.5 (PerCP-Cy5.5)-conjugated anti-IgD (11-26c.2a).

**Flow cytometry and cell sorting.** Tissues were disrupted by passing through a 40-µm cell strainer (BD Biosciences). After red blood cell lysis with ammonium chloride potassium buffer, cells were incubated with an anti-CD16/CD32 (2.4G2; BD Pharmingen) to reduce nonspecific labeling of the cells before staining. Single cells were stained with fluorochrome- or biotin-labeled antibodies. Data were acquired on a Cytoflex (Beckman Coulter) and analyzed with FlowJo software (Tree Star) to detect the following populations: pre-pro-B (IgM<sup>−</sup>IgD<sup>−</sup>B220<sup>+</sup>CD19<sup>−</sup>CD43<sup>+</sup>), pro-B (IgM<sup>−</sup>IgD<sup>−</sup>B220<sup>−</sup>CD19<sup>−</sup>CD43<sup>+</sup>), pre-B (IgM<sup>−</sup>IgD<sup>−</sup>B220<sup>−</sup>CD19<sup>−</sup>CD43<sup>−</sup>) immature B (IgM<sup>−</sup>IgD<sup>−</sup>B220<sup>−</sup>CD19<sup>−</sup>CD43<sup>−</sup>), recirculating B (Rec; IgM<sup>−</sup>IgD<sup>−</sup>B220<sup>−</sup>CD19<sup>−</sup>CD43<sup>−</sup>) and plasma cells (CD138<sup>−</sup>TAC1<sup>−</sup>) in the bone marrow; follicular (FO; CD19<sup>−</sup>Cd93<sup>−</sup>CD21<sup>−</sup>CD23<sup>−</sup>), and marginal zone (MZ; CD19<sup>−</sup>Cd93<sup>−</sup>CD21<sup>−</sup>CD23<sup>−</sup>) in spleen; B1a (IgM<sup>−</sup>IgD<sup>−</sup>B220<sup>−</sup>CD19<sup>−</sup>CD43<sup>−</sup>), B1b (IgM<sup>−</sup>IgD<sup>−</sup>B220<sup>−</sup>CD19<sup>−</sup>CD43<sup>−</sup>), and B1c (IgM<sup>−</sup>IgD<sup>−</sup>B220<sup>−</sup>CD19<sup>−</sup>CD43<sup>−</sup>) in peritoneal cavity (PEC); germinal center cells (B1D′fas<sup>−</sup>CD38<sup>−</sup>) in mesenteric lymph node (mLN). Cell sorting was performed on a FACSMedley (BD Biosciences) to isolate the following populations: B cells (CD19<sup>−</sup>TCRβ<sup>−</sup> and T cells (TCRβ<sup>−</sup>CD19<sup>−</sup>) in the spleen.

**Genomic PCR analysis.** Genomic DNA isolated from sorted cells or tail biopsies was used for PCR analysis. For CD19-iCre detection, the following primer pair were used: sense primer 5′-TGAAGGTTGCTGTTTCTCGTG-3′ and antisense primer 5′-TAGAGACTCTGCTCCCTGTTCC-3′ and antisense primer 5′-TGGAGAGGGTCAGTGGTTCCCG-3′ and antisense primer 5′-ATCAAGGCTACATCTGACTGACCCATCATCTGGGATC-3′ (wild-type). For Il10<sup>lox</sup>-flxed gene detection, the following primer pair were used: sense primer 5′-CCAGCATAGAGGCTTGATGAC-3′ and antisense primer 5′-GAGAGGTAAGGATATGTCTTGGCAGTCCCA-3′ (Il10<sup>lox</sup>-flxed); sense primer 5′-GTGAGCCAAAACACTTCTCAGATCCCAGC-3′ and antisense primer 5′-GAGTCCGTTGACTGATGATGTTCCAGC-3′ (Il10<sup>−</sup>-A).

**Quantitative RT-PCR analysis.** RNA was isolated and purified using the RNeasy kit (Qiagen) from purified B cells. CDNA was generated using the ReverTra Ace qPCR RT Master Mix (TOYOBO). Real-time PCR was performed on a LightCycler 96 (Roche) using Thunderbird SYBR qPCR mix (TOYOBO). The following primer pairs were used: Cd19, sense 5′-GGAGTGTTCCCTGGGCTCTAT-3′ and antisense 5′-CATCCCTGGGAAACTGTTCC-3′; β-actin, sense 5′-GCCCTTTGGCCTCCT-3′ and antisense 5′-CGGAGTTGGGTCACAGCACA-3′.

**B cell isolation, stimulation and enzyme-linked immunosorbent assay.** For B cell isolation, splenic B cells were purified by the negative selection of CD43<sup>+</sup> cells with anti-CD43 magnetic beads (Miltenyi Biotec). The purified B cell population was >95% positive for B220 staining. For B cell stimulation assays,
purified B cells (5 × 10^5 cells/ml) were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) FCS, β-mercaptoethanol and antibiotics for 48 h with 10 µg/ml of lipopolysaccharide (LPS) (Sigma-Aldrich), and then stimulated with 10 µg/ml of anti-IgM F(αb) (Jackson Immunoresearch). IL-10 in the culture medium was detected by enzyme-linked immunosorbent (ELISA) assay according to the manufacturer’s protocol (Biolegend).

**Western blotting.** Purified B cells were lysed in lysis buffer (10 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% [vol/vol] Triton X-100, 0.5 mM EDTA) plus protease inhibitor cocktails (Nacalai Tesque). Samples were transferred to polyvinylidifluoride membranes by electrophoresis and antibodies against the following proteins were detected by enzyme-linked immunosorbent (ELISA) assay according to the manufacturer’s protocol (Biolegend).

**Statistical analysis.** GraphPad Prism 6 (GraphPad Software) was used for all statistical analyses. Statistical significance was determined by two-tailed paired or unpaired Student’s t test for two groups or one-way ANOVA with Tukey’s post hoc test for multiple groups. Comparisons of two nonparametric data sets were done by the Mann–Whitney U test. A p value of less than 0.05 was considered statistically significant.

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Author contributions
T.Y., Y.S., C.O., K.K, and A.B. conducted the experiments. Y.B., Y.S., and C.O. analyzed the data. Y.B. supervised the study and wrote the manuscript with input from all authors.

Competing interests
The authors declare no competing interests.

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