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Canonical NF-κB signaling is uniquely required for the long-term persistence of functional mature B cells

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ABSTRACT

While canonical NF-κB signaling is crucial to generate a normal mature B cell compartment, its role in the persistence of resting mature B cells is controversial. To resolve this conflict, we ablated NEMO and IKK2, two essential mediators of the canonical pathway, either early on in B cell development or specifically in mature B cells. Early ablation severely inhibited the generation of all mature B cell subsets, but follicular B cell numbers could be largely rescued by ectopic expression of Bcl2, despite a persisting block at the transitional stage. Marginal zone B and B1 cells were not rescued, indicating a possible role of canonical NF-κB signals beyond the control of cell survival in these subsets. When canonical NF-κB signaling was ablated specifically in mature B cells, the differentiation and/or persistence of marginal zone B cells was still abrogated, but follicular B cell numbers were only mildly affected. However, the mutant cells exhibited increased turnover as well as functional deficiencies upon activation, suggesting that canonical NF-κB signals contribute to their long-term persistence and functional fitness.

SIGNIFICANCE

Mature B cells are long-lived cells responsible for the antibody production in the immune system. Canonical NF-κB signaling, one of the two discrete pathways activating transcription factors of the NF-κB family, participates in the generation of a normal mature B cell compartment. However, the role of these signals specifically in mature B cells is still imperfectly defined. Notably, their role in the persistence of follicular B cells, the main mature B cell subset, is controversial. Here, we show that canonical NF-κB signaling does not contribute to immediate (short-term) survival of follicular B cells, contrasting with the crucial tonic B cell antigen receptor survival signals, but is required for their long-term persistence as well as functional fitness.

INTRODUCTION

Mature B cells comprise three major subsets: follicular and marginal zone (MZ) B and B1 cells (1). Two receptors, the B cell antigen receptor (BCR) and B cell-activation factor of the tumor necrosis factor family (BAFF) receptor (BAFFR) have been shown to critically control the generation and/or persistence of mature B cells (2-5).

Numerous stimuli activate the NF-κB signaling pathways in mature B cells. In mammals, the NF-κB family of transcription factors comprises 5 members (RelA, RelB, c-Rel, NF-κB1 and NF-κB2) whose activation is initiated by two major signaling pathways (5). The canonical pathway depends on the IKK complex, consisting of the structural protein NEMO and the IκB kinases IKK1 and IKK2. This complex triggers the degradation of a specific set of inhibitors of NF-κB and, the induction of dimers containing RelA and/or c-Rel. The canonical pathway regulates NF-κB activation downstream of the BCR (5). The stimulation of the alternative pathway, mediated by NF-κB inducing kinase and IKK1, leads to the partial proteolysis of the inhibitory precursor NF-κB2 and the activation of dimers containing RelB and/or the processed form of NF-κB2 through another set of receptors including BAFFR (4).

There is abundant evidence for a critical role of the canonical pathway in the generation and/or maintenance of mature B cells. The ablation of NEMO or IKK2 in the B cell lineage, as well as conditional replacement of the latter by a kinase-dead IKK2, impaired the generation of the three mature B subsets and transitional 2 (T2) cells, a developmental stage preceding the mature stage (6-9). In the case of the kinase-dead IKK2 some mutant cells made it into the mature compartment, but were completely outcompeted by wild type cells over a period of four weeks, upon blockade of B cell generation in the bone marrow (6). Mechanistically, tonic BCR signaling could increase the production of NF-κB2 via canonical NF-κB activation in T2 and follicular B cells, sensitizing B cells to the pro-survival effect of BAFFR (10).

However, other work suggested that canonical signals downstream of the BCR may not be critical for the maintenance of mature follicular B cells. Ectopic expression of a constitutively active form of IKK2 only slightly rescued the acute loss of B cells upon

ablation of the BCR in these cells, whereas a full rescue was obtained through signals along the PI3K – FOXO1 axis (11). In addition, a study of the role of B cells in a prostate cancer model suggested that the absence of canonical signaling may not result in the rapid disappearance of mature B cells (12).

In the present work, using conditional ablation of NEMO and IKK2 either early during B cell development or specifically in mature B cells as well as ectopic expression of Bcl2, we show that canonical NF-κB signaling contributes to the functional integrity of follicular B cells, and supports their long-term persistence through its pro-survival activity. However, absence of canonical NF-κB signaling in these cells only mildly affects their numbers in steady state, in contrast to its severe impact on transitional B cell numbers.

RESULTS

Ectopic expression of Bcl2 allows accumulation of follicular B cells upon NEMO deletion early in development

Consistent with previous work (6, 8, 9), the generation of mature B cells was impaired upon ablation of NEMO in the B cell lineage *using Mb1-cre* (Fig. 1 *A* and *B*; Figs. S1*A* and S2*A*) (13). Within the mature compartment, MZ B and B1 cells were more affected than follicular B cells. As over-expression of Bcl2 in *c-Rel*$^{-/-}$*RelA*$^{-/-}$ hematopoietic cells has been shown to rescue the generation of mature B cells and promote their survival *in vitro* (14), we assessed whether ectopic expression of Bcl2 leads to the accumulation of mature B cells in the absence of NEMO. Indeed, the generation of follicular B was largely rescued in *Nemo*^{fl} *Mb1-cre*$^{+}$ *Bcl2Tg* mice (Fig. 1*A*; Fig. S1*B*). In contrast, mutant MZ B cellularity was lower (2 – 5.5 fold) compared to controls and was similar to the MZ B cell numbers observed in *Nemo*^{fl} *Mb1-cre*$^{+}$ mice (Fig. 1*A*; Fig. S1*B*). However, the difference between *Mb1-cre*$^{+}$ *Bcl2Tg* and *Nemo*^{fl} *Mb1-cre*$^{+}$ *Bcl2Tg* mice did not reach statistical significance. Surprisingly, MZ B cellularity was also reduced in *Mb1-cre*$^{+}$ *Bcl2Tg* compared to *Mb1-cre*$^{-}$ *Bcl2Tg* control mice. In addition, the ectopic expression of Bcl2 in B cells did not rescue NEMO-deficient B1 cells in the peritoneal cavity (Fig. 1*B*; Fig. S2*B*).

The absence of canonical NF-κB signaling in B cells has previously been shown to affect splenic B cell development also at the T1 to T2 transition (8, 9). We thus investigated whether the accumulation of mutant follicular B cells could be due to the rescue of T2 cell generation in *Nemo*^{fl} *Mb1-cre*$^{+}$ *Bcl2Tg* mice. T2 cell numbers demonstrated a positive correlation with T1 cellularity (Fig. 1*C*; Fig. S3), in agreement with T2 cells arising from the T1 subset (15). Notably, the production of NEMO-deficient T2 cells was clearly reduced compared to controls, independently of the over-expression of Bcl2 (Fig. 1*C*). CD23, used to discriminate T1 and T2 cells (15), has been reported to be an NF-κB target gene (16). We therefore confirmed the identity of the mutant transitional populations using an independent marker, CD93, which is expressed at lower levels in T2 cells (Fig. 1*D*; Fig. S4) (3, 9). Comparable distributions of CD93^{lo}

cells were seen in the transitional subsets of *Nemo*^{fl} *Mb1-cre*$^{+}$ *Bcl2Tg* and control mice, supporting that genuine T1 and T2 cells were detected in the mutant mice.

Thus, ectopic expression of Bcl2 permitted the accumulation of NEMO-deficient follicular B cells close to normal cellularity despite a persisting developmental block at the transitional stage. In contrast, the generation of MZ B and B1 cells was not rescued, possibly due to a role for canonical NF-κB signaling beyond cell survival (17), consistent with the inability of a Bcl2 transgene regulated by *vav* gene regulatory elements to promote the development of MZ B cells in NF-κB1-deficient mice (18).

Peripheral B cells from *Ikk2*^{fl} *CD19-cre*$^{+}$ mice showed altered *in vitro* and *in vivo* responses to stimulation (7), but these results could have been partly due to the reduced numbers of mature B cells in these mice. The large numbers of follicular B cells in *Nemo*^{fl} *Mb1-cre*$^{+}$ *Bcl2Tg* allowed us to examine their responses to various kinds of stimulation. The NEMO-deficient Bcl2Tg B cells exhibited an impaired proliferative response to various mitogenic stimuli *in vitro* compared to control *Bcl2Tg* B cells (Fig. 2*A*). Consistent with defective B cell functions, IgM and IgG serum antibody levels were strongly reduced or undetectable (Fig. 2*B*). In the T cell dependent response to immunization with (4-hydroxy-3-nitrophenyl)acetyl (NP)–chicken γ-globulin, the frequency of germinal center cells was strongly reduced and the production of anti-NP IgG_1 antibodies abolished (Fig. 2 *C* and *D*). These data show that canonical NF-κB signals are essential for the functional activity of follicular B cells, and complement a large body of evidence for the multi-faceted role of this pathway in the control of humoral immunity (5, 17, 19).

Long-term persistence of follicular B cells requires canonical NF-κB signaling

To directly evaluate the contribution of canonical signaling to the maintenance of mature B cells, we ablated NEMO using *CD21-cre* (3). We excluded B1 cells from the analysis since, in our hands, *CD21-cre* proved to be poorly expressed in the prototypical $CD5^{+}$ B1a subset (Fig. S5). A large follicular B cell population was detected in the spleens of *Nemo*^{fl} *CD21-cre*$^{+}$ mice, while MZ B cells were essentially lost (Fig. 3 *A* and *B*). Similar results were obtained upon IKK2 ablation through *CD21-cre* (Fig. 3 *C* and *D*). These data

contrast with the small mature B cell compartment detected in the absence of NEMO or IKK2 using the *CD19-cre* or *Mb1-cre* alleles (6-9). *Nemo*^{fl} and *Ikk2*^{fl} loxP-flanked exons are efficiently eliminated upon Cre-mediated recombination in B cells (6, 8), which we verified in the case of follicular B cells from *Ikk2*^{fl} *CD21-cre*$^{+}$ mice (Fig. 4). The mild reduction of follicular B cell numbers in *Ikk2*^{fl} *CD21-cre*$^{+}$ mice was not due to the accumulation of cells that had escaped IKK2 deletion. Indeed, the majority of these cells expressed a Cre-inducible truncated human CD2 reporter gene (Fig. 4 *A* and *B*), and the reporter positive cells demonstrated dramatically reduced IKK2 protein levels compared to controls (Fig. 4*C*).

Residual splenic mature B cells expressing a kinase-dead IKK2 have been shown to display an increased turnover compared to controls (6). Thus, we assessed whether follicular B cell persistence was altered in *Ikk2*^{fl} *CD21-cre*$^{+}$ mice by measuring 5-bromo-2'-deoxyuridine (BrdU) incorporation into their DNA after two weeks of labeling. Labeling efficiency was similar between the various mice as indicated by comparable proportions of BrdU^{+} transitional B cells (Fig. 4*D*). In agreement with previous work (3, 20), follicular B cells from wild-type (*CD21-cre*$^{-}$) and *Ikk2*$^{fl/+}$ *CD21-cre*$^{+}$ controls showed low proportions (12 – 13%) of BrdU^{+} cells. In contrast, 40% of IKK2-deficient follicular B cells had incorporated BrdU (Fig. 4*D*), suggesting increased turnover.

Overall, the present results suggest that the main function of canonical NF-κB signaling in follicular B cell homeostasis is indeed the control of cell survival, as demonstrated by the ability of a Bcl2 transgene to rescue a substantial compartment of these cells even under conditions where the ablation of canonical signaling in the B cell lineage leads to a severe developmental block at the transitional B cell stage (8, 9). This is in line with earlier work showing the accumulation of mature B cells in mice reconstituted with RelA and c-Rel double deficient Bcl2 transgenic fetal liver cells (14).

DISCUSSION

While ablation of components of the BCR in mature B cells led to a steady state in which BCR-deficient cells were a minority of the mature B cell population because of their rapid elimination (2, 3, 11), NEMO or IKK2 ablation by *CD21-cre* resulted in an only moderate reduction of follicular B cell numbers. This indicates that follicular B cells do not require continuous canonical NF-κB signaling for their persistence, and contrasts with the rapid loss of B cells upon BCR deletion (2, 3). Quite fittingly the latter process can be rescued by constitutive PI3K activation, but not canonical NF-κB activity (11). However, the homeostasis of follicular B cells unable to signal through the canonical NF-κB pathway is clearly different from that of their wild type counterparts. While the latter are long-lived cells with average life spans of months (21), the mutant follicular B cells appear to have a limited life span, probably in the order of a few weeks as indicated by the BrdU labeling data. These results complement our previous demonstration of the failure of such cells to compete with wild type cells *in vivo* (6). Together with the accumulation of NEMO-deficient follicular B cells upon ectopic expression of Bcl2, these data raise the possibility that canonical NF-κB signals may determine the fitness of mature follicular B cells in their competition for survival niches in the peripheral immune system (22).

The ablation of NEMO or IKK2 early during B cell development results in strongly reduced numbers of follicular B cells (6-9), in stark contrast to the effects of the deletion of these two molecules specifically in mature B cells. This difference likely reflects the critical role of canonical NF-κB signaling in transitional B cells (6, 8, 9). In combination with the limited life span of follicular B cells, the shortage of newly generated mature B cells is expected to lead to the suboptimal filling of the follicular B compartment, in the absence of canonical NF-κB signaling.

Compared to follicular B cells, MZ B and B1 cells demonstrate a stronger dependency on canonical NF-κB signals for their development and/or persistence. Thus, MZ B cell numbers were strongly reduced in $Nemo^{fl}$ and $Ikk2^{fl}$ $CD21\text{-}cre^{+}$ mice, and the ectopic expression of Bcl2 failed to rescue the generation and/or maintenance of NEMO-deficient MZ B and B1 cells. These results are suggestive of a role beyond the control of

survival for canonical NF-κB signaling in MZ B and B1 cells. It is noteworthy in this context that Cyclin D2, a regulator of the cell cycle and a target of NF-κB, contributes to the development of B1 cells (23).

While an important signaling pathway activated in B cells upon engagement of BAFFR is the alternative NF-κB signaling pathway (4), there is also evidence for a cross-talk between the alternative and canonical pathways, both in the sense of BAFFR signaling resulting in canonical NF-κB activity (8, 24), and of canonical signals upregulating the expression of components of the alternative pathway (8, 10, 17, 19). Thus, BAFFR might be involved in the activation of survival signals in mature B cells through both the canonical and the alternative NF-κB signaling pathway, and/or canonical signals could contribute to an enhanced sensitivity of the corresponding cells to survival signals downstream of BAFFR. In this scenario, the occasional engagement of the BCR on resting mature B cells by antigens in the environment, with a resulting activation of the canonical NF-κB pathway, might enhance the competitive fitness of the cells in their ability to access survival niches (22). The activation of canonical NF-κB signaling downstream of the BCR and/or BAFFR could be mediated by the kinase Btk (5, 25). Indeed, similar to the behavior of follicular B cells in the absence of canonical NF-κB signals, ectopic expression of Bcl2 rescues the generation of follicular B cells in *xid* mice which bear a mutation in *Btk*, and $CD23^+$ transitional and follicular *xid* B cells are outcompeted by wild-type B cells (26, 27).

MATERIALS AND METHODS

Mice. *Nemo*^{fl}, *Ikk2*^{fl}, *Mb1-cre*, *CD21-cre* and *R26-Stop*^{fl}*-hCD2* mice have been described (3, 6, 13, 28, 29). Bcl2 transgenic Eμ-Bcl-2-22 (*Bcl2Tg*) mice were obtained from The Jackson laboratory (30). Mice were generated on or backcrossed to the C57BL/6 genetic background. Animal care and mouse work were conducted according to the guidelines of the Institutional Animal Care and Use Committee of Harvard University, the Immune Disease Institute (USA), the Max Delbrück Center for Molecular Medicine, the Landesamt für Gesundheit und Soziales (Germany) and the Bundesministerium für Wissenschaft und Forschung (Austria).

For simplicity and clarity of the manuscript, the given denominations include genotypes as follows (+, *f*, Δ and *y* indicate wild type, loxP-flanked, deleted alleles and the y sex chromosome, respectively): *Mb1-cre*$^{-}$: *Nemo*$^{f/f}$, *Nemo*$^{f/y}$ and *Nemo*$^{+/y}$; *Nemo*^{fl} *Mb1-cre*$^{+}$: *Nemo*$^{f/f}$ *Mb1-cre*$^{+}$ and *Nemo*$^{f/y}$ *Mb1-cre*$^{+}$; *CD21-cre*$^{-}$: *Nemo*$^{f/y}$ or, *Ikk2*$^{f/\Delta}$ and *Ikk2*$^{+/\Delta}$; *Nemo*^{fl} *CD21-cre*$^{+}$: *Nemo*$^{f/y}$ *CD21-cre*$^{+}$; *Ikk2*$^{fl/+}$ *CD21-cre*$^{+}$: *Ikk2*$^{f/+}$ *CD21-cre*$^{+}$ and *Ikk2*$^{+/\Delta}$ *CD21-cre*$^{+}$; *Ikk2*^{fl} *CD21-cre*$^{+}$: *Ikk2*$^{f/\Delta}$ *CD21-cre*$^{+}$. Previous work (6) suggested that B cell development is unaffected in mice bearing either a heterozygous deletion of *Ikk2* or loxP-flanked *Ikk2* or *Nemo* alleles. The presence of single *Ikk2*-deleted alleles in some of our mice resulted from the occasional deletion of loxP-flanked alleles by *CD21-cre* in the germ line (11).

Flow cytometry. Cell suspensions from spleen and peritoneal cavity were stained with the following antibodies coupled to FITC, PE, PerCP, PerCP-Cy5.5, APC, PE-Cy7, Pacific Blue, Bv421, Bv605 or Bv785: anti-CD1d (1B1), anti-human CD2 (TS1/8), anti-CD19 (6D5 and 1D3), anti-CD21 (7G6 and 7E9), anti-CD23 (B3B4), anti-CD38 (90), anti-CD93 (AA4.1), anti-CD95 (Jo2), anti-B220 (RA3-6B2) and anti-IgM (II/41 and goat anti-mouse Fab) purchased from affymetrix eBioscience, BD Biosciences, Biolegend and Jackson ImmunoResearch Laboratories. Data were recorded on FACSCalibur, FACSCanto II or LSRFortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star).

***In vitro* B cell proliferation and isotype class switching.** Splenic B cells were purified by magnetic depletion using anti-CD43 beads (Miltenyi Biotec), labeled with 10 μM Cell Proliferation Dye eFluor® 450, 10 μM Cell Proliferation Dye eFluor® 670 (affymetrix eBioscience) or 2.5 nM Carboxyfluorescein Diacetate Succinimidyl Ester (Molecular probes), and cultured in 6-well plates at 10^6 cells / 4 ml DMEM (Gibco) supplemented with 10% fetal calf serum, 2 mM L-Glutamine, 10 mM Hepes, 1 mM sodium pyruvate, 1x non essential amino acids, 1x penicillin / streptomycin and 50 μM β-mercaptoethanol. Cells were left either untreated or stimulated with 10 μg/ml $F(ab')_2$ fragment anti-IgM (Jackson ImmunoResearch Laboratories), 20 μg/ml lipopolysaccharides (Sigma) and 1 or 2 μg/ml anti-CD40 (Biolegend) plus 25 or 100 ng/ml Il-4 (R&D Systems) for 4 days. Dead cells were stained using 1 μg/ml propidium iodide (Sigma), 10 nM TO-PRO-3 (Molecular Probes) or AnnexinV-FITC (Biolegend). Cell division of live B cells was subsequently determined by flow cytometry.

Immunization and serum antibody titers. Mice were immunized and antibody titers determined as previously described (31). Briefly, animals were injected intraperitoneally with 100 μg of (4-hydroxy-3-nitrophenyl)acetyl (NP) – chicken γ-globulin (NP-CGG) (Biosearch Technologies) in Alum (Sigma). Presence of germinal center B cells in the spleen was determined by flow cytometry at day 14 post-immunization. Blood was collected from unimmunized mice as well as 7 and 14 days after the injection of NP-CGG to measure serum Ig titers or NP-specific IgG_1 by enzyme-linked immunosorbent assay.

Western blotting. Flow cytometry-purified $B220^+CD93^-IgM^+CD1d^+hCD2^+$ or $B220^+CD93^-IgM^+CD1d^+hCD2^-$ follicular B cells were lysed using a whole cell extract buffer (25 mM Hepes pH 7.9, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% Triton X100, 10 mM NaF, 10 mM Na-Pyrophosphate, 100 μM Na-o-Vanadate, 2 mM DTT) completed with protease inhibitors (Aprotinin and PMSF). Proteins were separated by SDS-PAGE, and transferred on PVDF membranes (Millipore). The expression of IKK2 and PLCγ2 was visualized using 10AG2 (Millipore) and Q20 (Santa Cruz Biotechnology)

primary antibodies, anti-mouse and anti-rabbit IgG secondary antibodies coupled to HRP (Jackson ImmunoResearch Laboratories), and ECL detection reagent (Amersham).

5-bromo-2'-deoxyuridine labeling. Mice were given 1mg/ml of 5-bromo-2'-deoxyuridine (Sigma) in the drinking water for 14 days. BrdU incorporation into the DNA of transitional and follicular B cells was determined by flow cytometry using the FITC BrdU Flow Kit (BD Biosciences).

Statistics and graphs. The Prism software (GraphPad Software) was used to perform statistical analysis of the data, compute best-fitting linear function and generate graphs. Statistical significance of data was determined using a one-way analysis of variance (ANOVA) followed by a post hoc Tukey's multiple comparisons test or a two-tailed unpaired Student's *t* test.

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FIGURE LEGENDS

Fig. 1: Ectopic expression of Bcl2 rescues follicular B cell generation in the absence of NEMO. (*A*) Absolute cell numbers of follicular (Fo) and marginal zone (MZ) B cells in the spleen of *Mb1-cre*$^{-}$, *Mb1-cre*$^{+}$ and *Nemo*^{fl} *Mb1-cre*$^{+}$ mice in (left) the absence (- *Bcl2Tg*) or (right) upon ectopic expression of Bcl2 (+ *Bcl2Tg*). Data are pooled from 4-15 experiments (n = 4-19 per genotype). (*B*) Percent B1 cells within B cells in the peritoneal cavity. Data are cumulative from 5-14 experiments (n = 5-15 per group). (*C*) Transitional 2 (T2) cellularity as a function of transitional 1 (T1) cell numbers. Data are pooled from 6-15 experiments (n = 5-19 per genotype). Solid (controls) and dotted (*Nemo*^{fl} *Mb1-cre*$^{+}$) lines represent best-fitting linear function, forced to go through the origin. (*D*) Percent CD93^{lo} T1 and T2 cells in the spleen of *Mb1-cre*$^{-}$, *Mb1-cre*$^{+}$ and *Nemo*^{fl} *Mb1-cre*$^{+}$ mice upon ectopic expression of Bcl2 (+ *Bcl2Tg*). Data are pooled from 9 experiments (n = 7-9 per group). One *Mb1-cre*$^{+}$ and 1 *Nemo*^{fl} *Mb1-cre*$^{+}$ mouse were excluded from the analysis in *A* (left panel) and in *B* (left panel), respectively, due to aberrantly high cell numbers compared to the other mice of their group. Each symbol indicates one mouse and horizontal lines signify mean in *A*, *B*, *D*. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. One-way ANOVA in *A*, *B*, *D*.

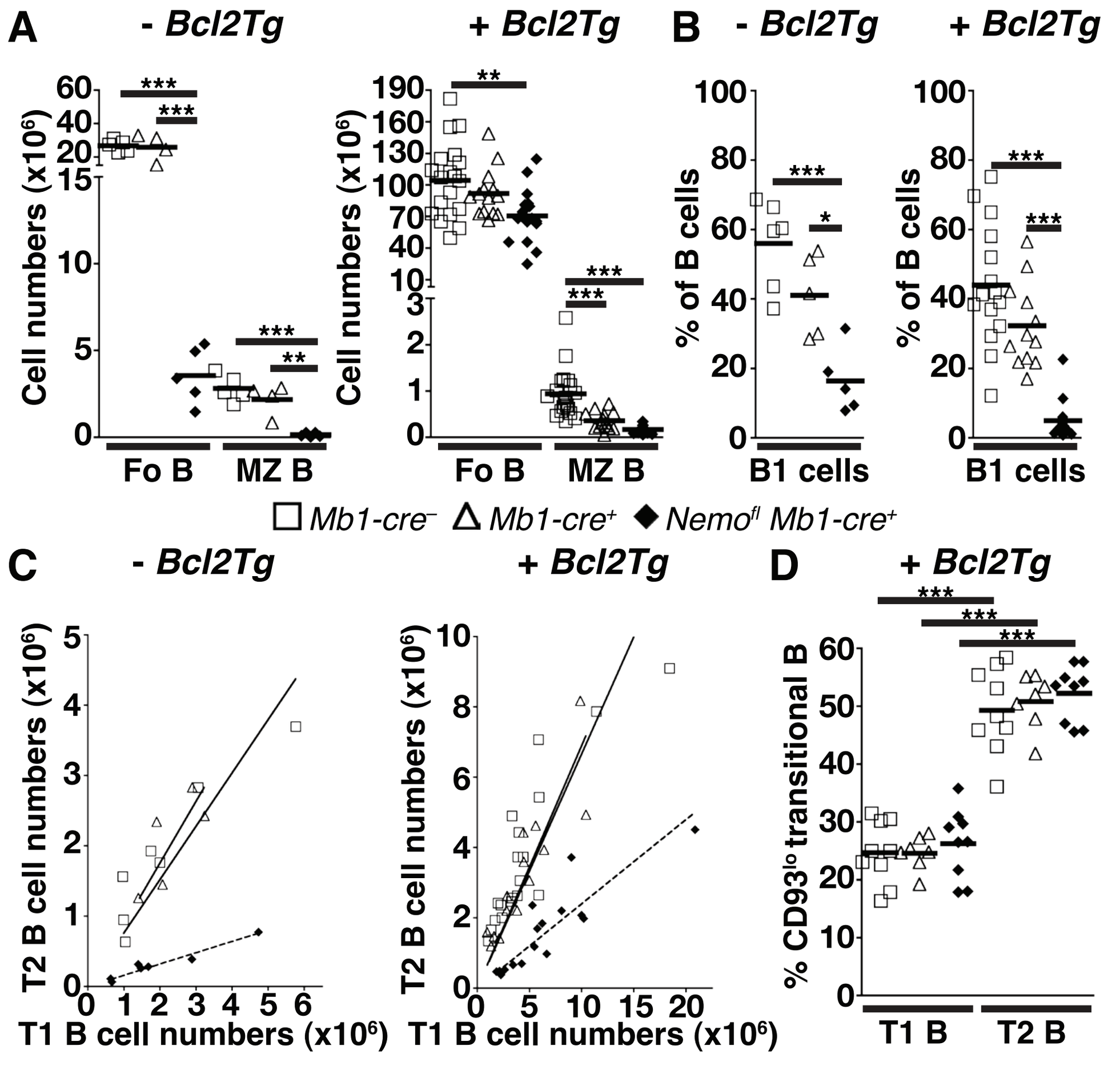
Fig. 2: Mature B cells from *Nemo*^{fl} *Mb1-cre*$^{+}$ *Bcl2Tg* mice are functionally defective. (*A*) Proliferation of live mature B cells from *Mb1-cre*$^{-}$ *Bcl2Tg* (light grey filled histogram), *Mb1-cre*$^{+}$ *Bcl2Tg* (black histogram) and *Nemo*^{fl} *Mb1-cre*$^{+}$ *Bcl2Tg* (black histogram) mice, MACS-purified, labeled with Cell Proliferation Dye eFluor® 450 and stimulated with 10 μg/ml anti-IgM (α-IgM), 20 μg/ml lipopolysaccharides (LPS) or 1 μg/ml anti-CD40 + 25 ng/ml IL-4 (α-CD40+IL-4) for 4 days. Dark grey filled histogram, resting *Mb1-cre*$^{-}$ *Bcl2Tg* B cells. At least 3 mice per genotype were analyzed in independent experiments. (*B*) Serum antibody isotype titers in sera of naïve mice (n = 4-7 per genotype). (*C*) Flow cytometry of CD38^{lo}CD95^{+} germinal center B cells within splenic B220^{+} cells of mutant and control mice 14 days after immunization with (4-hydroxy-3-nitrophenyl)acetyl (NP) – chicken γ-globulin. Numbers adjacent to outlined areas specify percent cells in each gate. Five mice were analyzed per genotype in two experiments. (*D*)

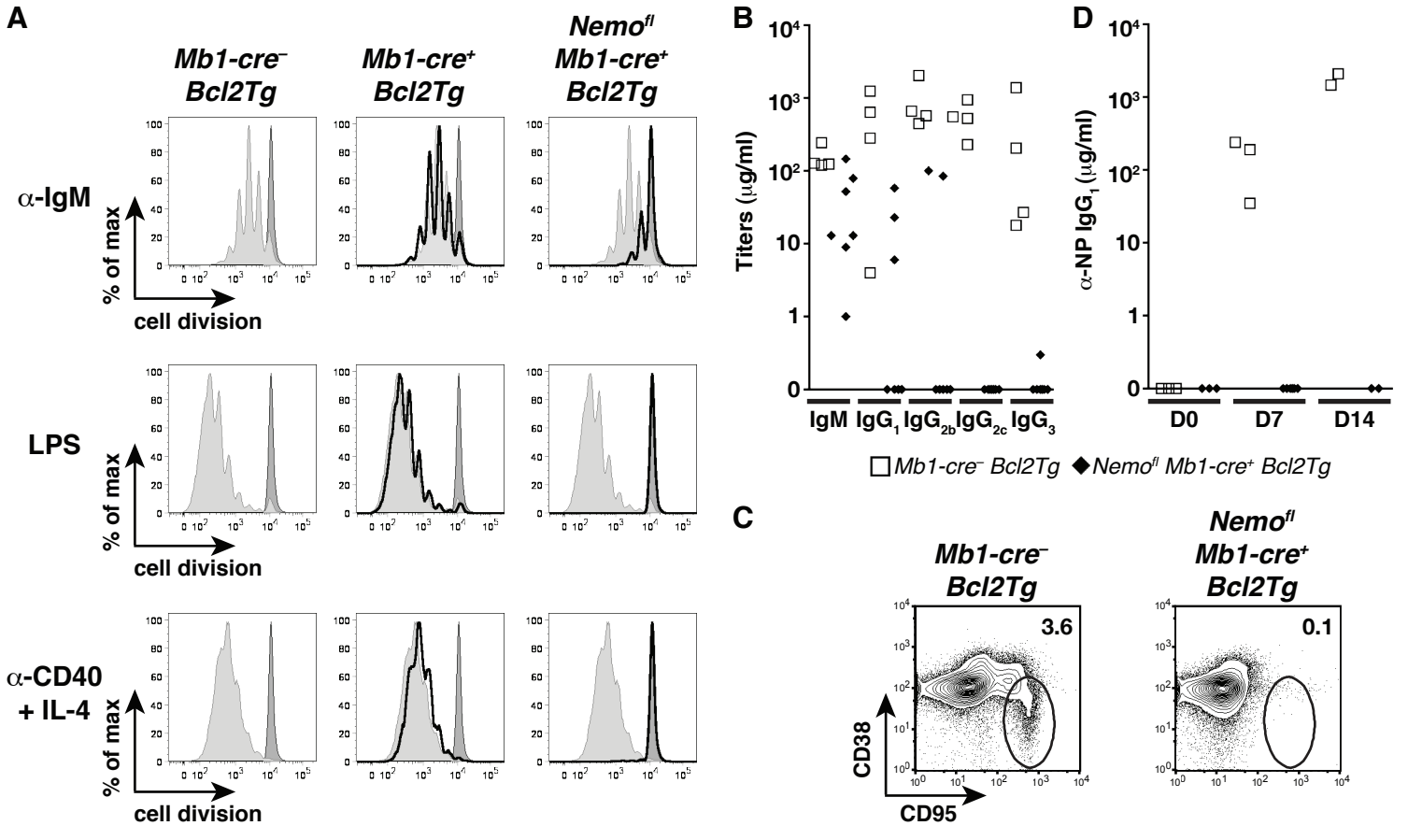
Serum titers of NP-specific IgG_1 (n = 2-6 per group). D0, unimmunized mice; D7 and D14, day 7 and 14 post immunization. One control mouse at day 7 did not respond to the immunization and was excluded from the analysis. Data are representative of two experiments. In *B* and *D*, symbols indicate individual mice.

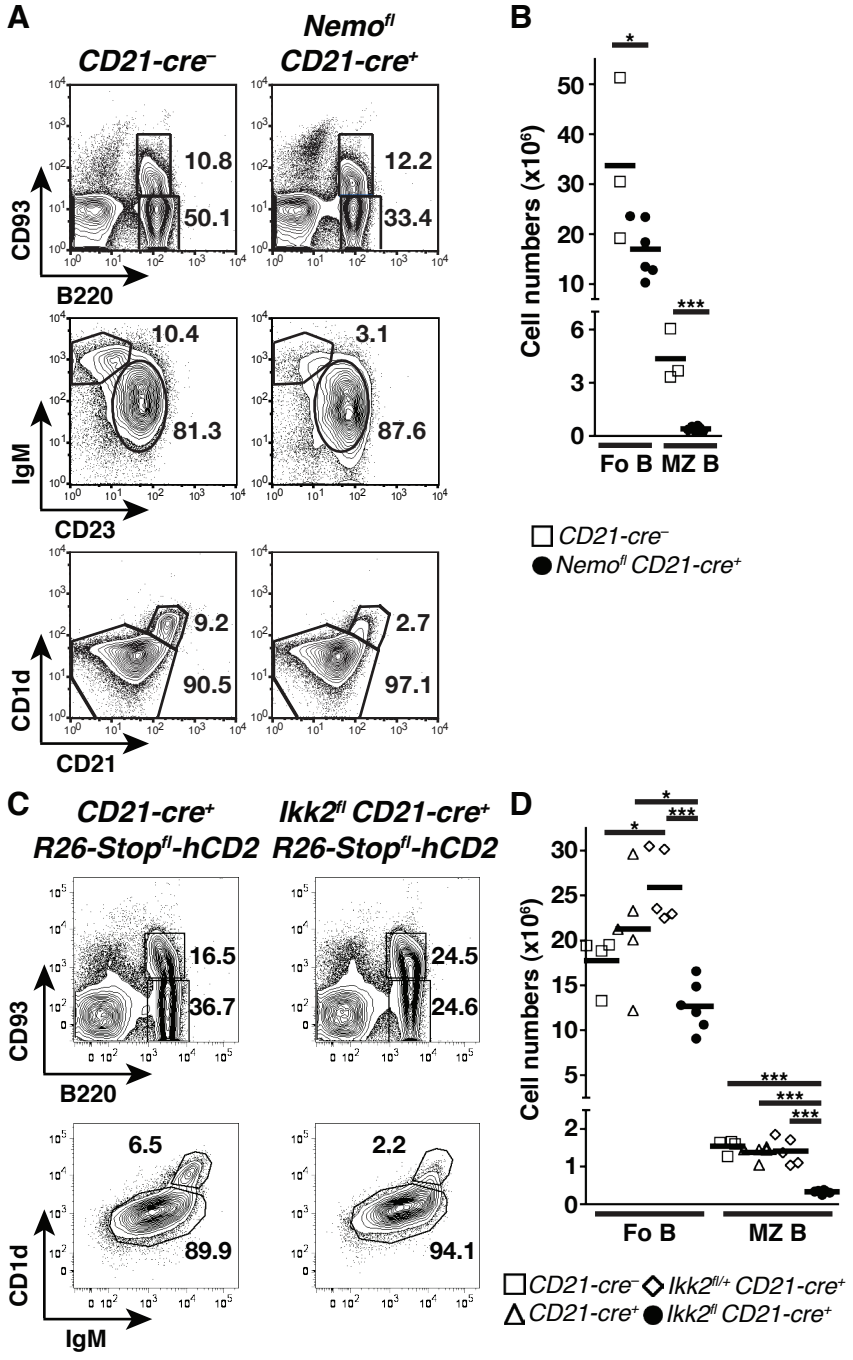
Fig. 3: Mild reduction of follicular B cell numbers upon deletion of NEMO or IKK2 in mature B cells. (*A*) Flow cytometry of $B220^+CD93^+$ transitional and $B220^+CD93^-$ mature B cells within lymphocytes (top), IgM^+CD23^+ follicular B cells within mature B cells (middle), and $CD1d^{hi}CD21^{hi}$ MZ B cells within $CD19^+$ B cells (bottom) in the spleen of *CD21-cre*$^-$ and *Nemo*^{fl} *CD21-cre*$^+$ mice. (*B*) Absolute cell numbers of follicular (Fo) and MZ B cells in *CD21-cre*$^-$ and *Nemo*^{fl} *CD21-cre*$^+$ mice. Data are cumulative from 2 experiments (n = 3-6 per group). (*C*) Flow cytometry of (top) transitional and mature B cells among lymphocytes, and (bottom) $CD1d^+IgM^+$ follicular and $CD1d^{hi}IgM^{hi}$ MZ B cells within mature B cells in the spleen of *CD21-cre*$^+$ *R26-Stop*^{fl}*-hCD2* and *Ikk2*^{fl} *CD21-cre*$^+$ *R26-Stop*^{fl}*-hCD2* mice. (*D*) Absolute cell numbers of follicular (Fo) and MZ B cells in *CD21-cre*$^-$, *CD21-cre*$^+$, *Ikk2*$^{fl/+}$ *CD21-cre*$^+$ and *Ikk2*^{fl} *CD21-cre*$^+$ mice. Data are pooled from 6 experiments (n = 4-6 per genotype). In *A* and *C*, numbers adjacent to outlined areas specify percent cells in the gate. Symbols represent individual mice and horizontal bars signify mean in *B*, *D*. *, $P \leq 0.05$; ***, $P \leq 0.001$. Student's *t* test in *B*, one-way ANOVA in *D*.

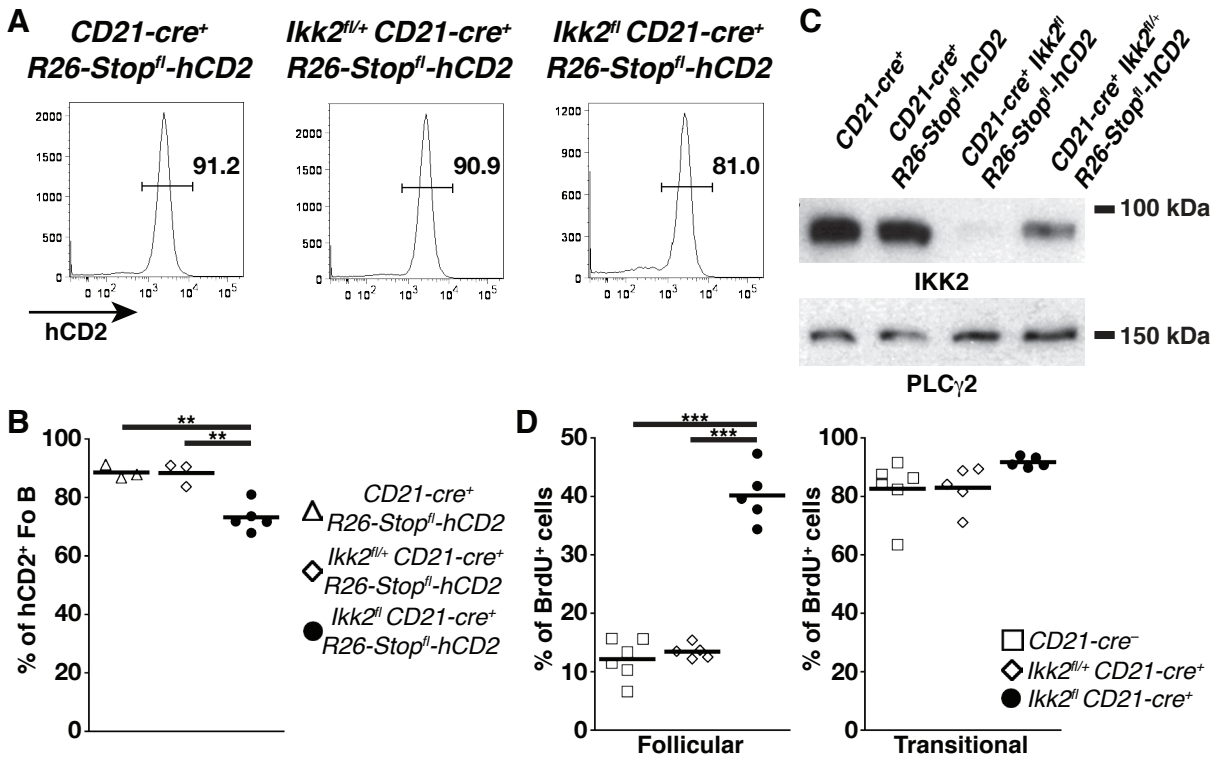
Fig. 4: IKK2-deficient follicular B cells persist but exhibit increased turnover. (*A*) Flow cytometry of human CD2 (hCD2) expression on follicular B cells in the spleen of *CD21-cre*$^+$ *R26-Stop*^{fl}*-hCD2*, *Ikk2*$^{fl/+}$ *CD21-cre*$^+$ *R26-Stop*^{fl}*-hCD2* and *Ikk2*^{fl} *CD21-cre*$^+$ *R26-Stop*^{fl}*-hCD2* mice. Numbers adjacent to outlined areas specify percent cells in each gate. (*B*) Proportions of $hCD2^+$ follicular (Fo) B cells. Data are cumulative from 5 experiments (n = 3-5 per group). (*C*) Western blot analysis of IKK2 and PLCγ2 protein levels in flow cytometry-purified $B220^+CD93^-CD1d^+IgM^+hCD2^+$ follicular B cells from control and mutant mice; follicular B cells from *CD21-cre* mice were $hCD2^-$. Western blot performed twice. (*D*) Proportions of $BrdU^+$ (left) $B220^+CD19^+CD93^-IgM^+CD23^+$ follicular and (right) $B220^+CD19^+CD93^+$ transitional B cells in mice given BrdU in

drinking water for 14 days, as determined by flow cytometry. Data are cumulative from 2 experiments (n = 5-6 per group). One *Ikk2*^{fl} *CD21-cre*$^{+}$ mouse was excluded from the analysis due to sickness in *D*. In *B* and *D*, symbols represent individual mice and horizontal bars signify mean. **, $P \leq 0.01$; ***, $P \leq 0.001$. One-way ANOVA in *B* and *D*.


A
- Bcl2Tg
+ Bcl2Tg
Cell numbers (x10⁶)
Fo B
MZ B
B
% of B cells
B1 cells
Mb1-cre⁻
Mb1-cre⁺
Nemo^fl Mb1-cre⁺
C
T2 B cell numbers (x10⁶)
T1 B cell numbers (x10⁶)
D
% CD93^lo transitional B
T1 B
T2 B


A
Mb1-cre⁻ Bcl2Tg
Mb1-cre⁺ Bcl2Tg
Nemo^fl Mb1-cre⁺ Bcl2Tg
α-IgM
LPS
α-CD40 + IL-4
% of max
cell division
B
Titers (μg/ml)
IgM
IgG1
IgG2b
IgG2c
IgG3
D
α-NP IgG1 (μg/ml)
D0
D7
D14
Mb1-cre⁻ Bcl2Tg
Nemo^fl Mb1-cre⁺ Bcl2Tg
C
Mb1-cre⁻ Bcl2Tg
Nemo^fl Mb1-cre⁺ Bcl2Tg
3.6
0.1
CD38
CD95


A
CD21-cre⁻
Nemo^fl CD21-cre⁺
CD93
B220
10.8
50.1
12.2
33.4
IgM
CD23
10.4
81.3
3.1
87.6
CD1d
CD21
9.2
90.5
2.7
97.1
B
Cell numbers (x10⁶)
Fo B
MZ B
CD21-cre⁻
Nemo^fl CD21-cre⁺
C
CD21-cre⁺ R26-Stop^fl-hCD2
Ikk2^fl CD21-cre⁺ R26-Stop^fl-hCD2
16.5
36.7
24.5
24.6
6.5
89.9
2.2
94.1
D
Fo B
MZ B
CD21-cre⁻
Ikk2^fl/+ CD21-cre⁺
CD21-cre⁺
Ikk2^fl CD21-cre⁺


A
CD21-cre+ R26-Stopfl-hCD2
Ikk2fl/+ CD21-cre+ R26-Stopfl-hCD2
Ikk2fl CD21-cre+ R26-Stopfl-hCD2
91.2
90.9
81.0
hCD2
C
CD21-cre+
CD21-cre+ R26-Stopfl-hCD2
CD21-cre+ Ikk2fl R26-Stopfl-hCD2
CD21-cre+ Ikk2fl/+ R26-Stopfl-hCD2
100 kDa
IKK2
150 kDa
PLCγ2
B
% of hCD2+ Fo B
CD21-cre+ R26-Stopfl-hCD2
Ikk2fl/+ CD21-cre+ R26-Stopfl-hCD2
Ikk2fl CD21-cre+ R26-Stopfl-hCD2
D
% of BrdU+ cells
Follicular
Transitional
CD21-cre−
Ikk2fl/+ CD21-cre+
Ikk2fl CD21-cre+

SUPPLEMENTARY FIGURE LEGENDS

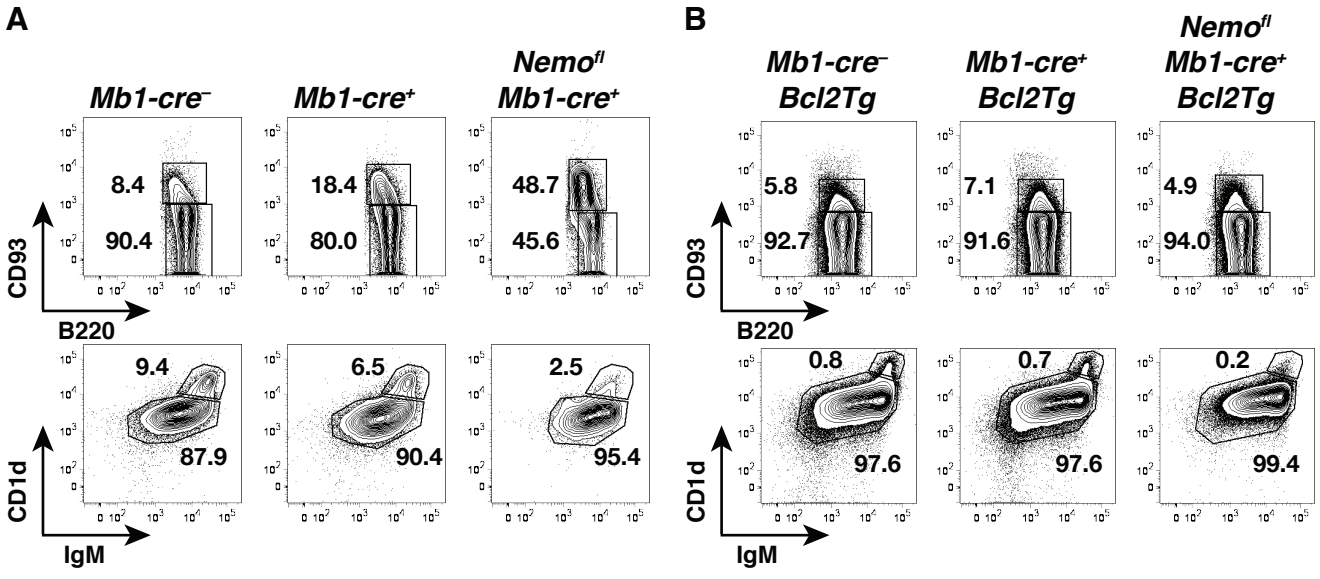
Fig. S1: Identification of mature follicular and marginal zone B cells. Flow cytometry of (*Top*) $B220^{+}CD93^{+}$ transitional and $B220^{+}CD93^{—}$ mature B cells within splenic $B220^{+}CD19^{+}$ B cells, as well as (*Bottom*) $IgM^{+}CD1d^{+}$ follicular and $IgM^{hi}CD1d^{hi}$ MZ B within splenic mature B cells of *Mb1-cre*$^{—}$, *Mb1-cre*$^{+}$ and *Nemo*^{fl} *Mb1-cre*$^{+}$ mice in the absence (n = 4-5 per genotype in 4 experiments) (*A*) or upon ectopic expression of Bcl2 (n = 14-19 per genotype in 15 experiments) (*B*; *Bcl2Tg*). Numbers adjacent to outlined areas specify percent cells in each gate.

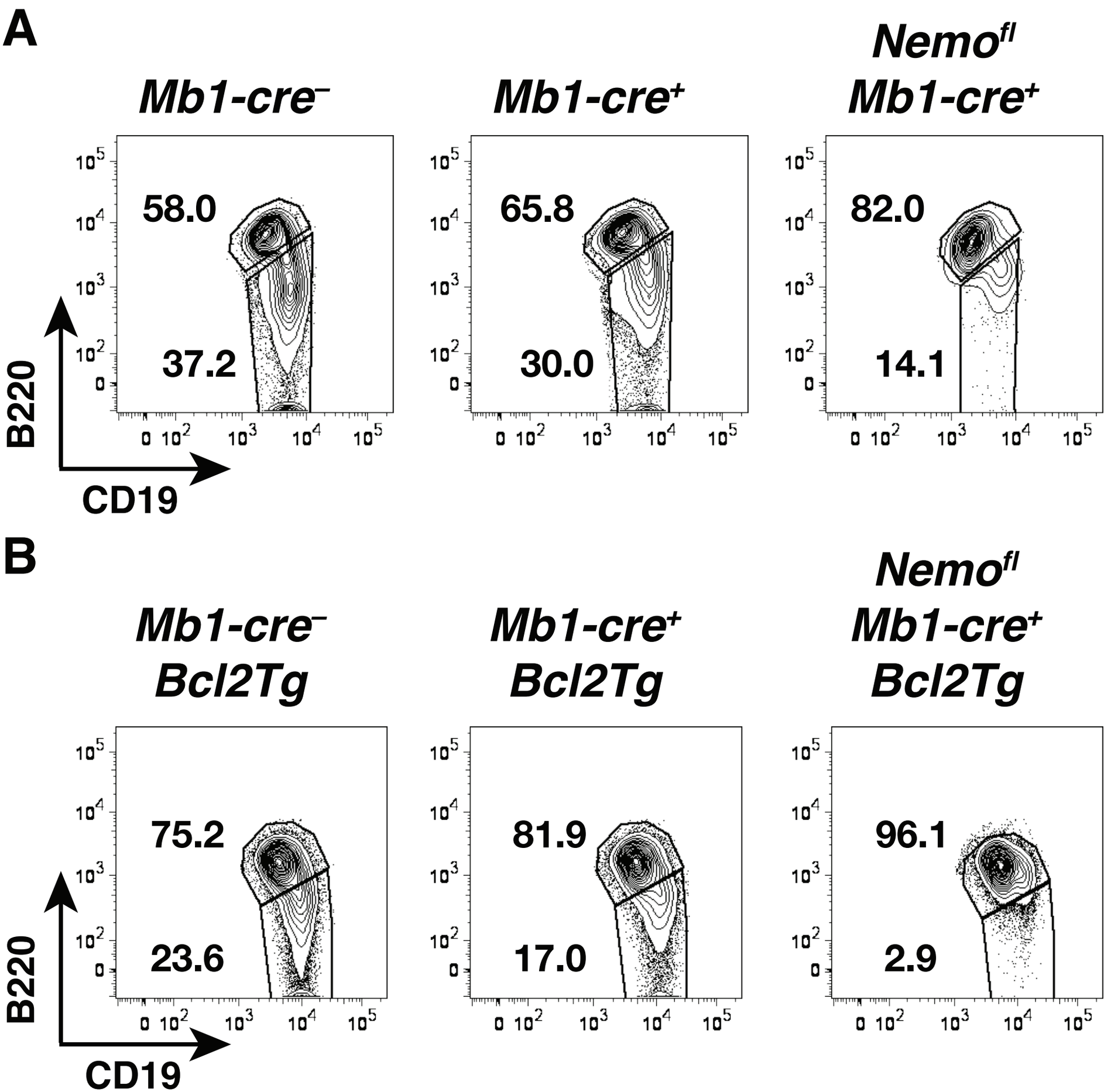
Fig. S2: Evaluation of B1 cell proportions. Flow cytometry of $B220^{lo/—}CD19^{+}$ B1 and $B220^{+}CD19^{+}$ B2 cells gated on $CD19^{+}$ B cells in the peritoneal cavity of *Mb1-cre*$^{—}$, *Mb1-cre*$^{+}$ and *Nemo*^{fl} *Mb1-cre*$^{+}$ mice in the absence (n = 5-6 per genotype in 5 experiments) (*A*) or upon ectopic expression of Bcl2 (n = 12-15 per genotype in 14 experiments) (*B*; *Bcl2Tg*). Numbers adjacent to outlined areas indicate percent cells in the gate.

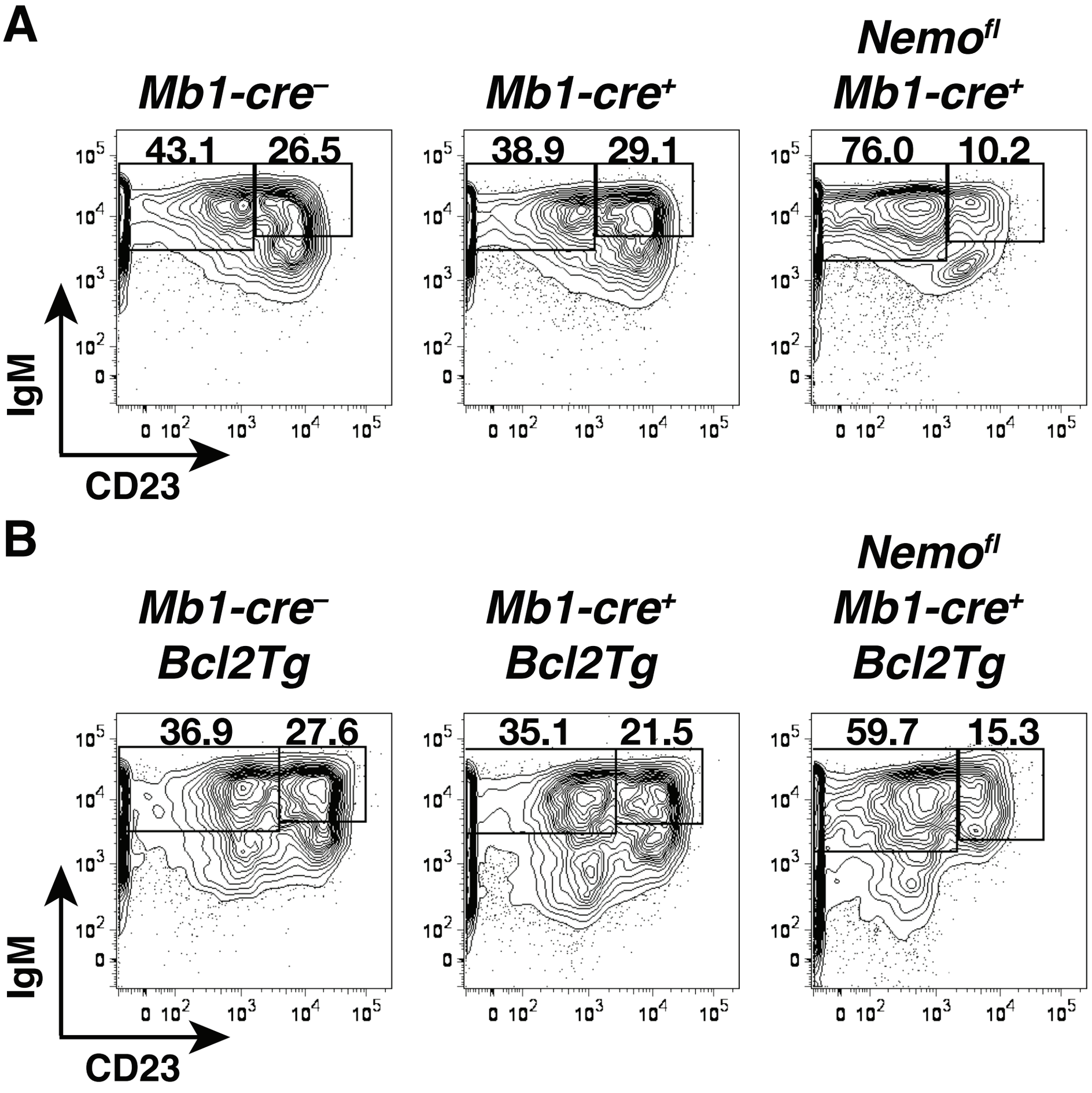
Fig. S3: Detection of Transitional 1 and 2 B cells. Flow cytometry of $IgM^{hi}CD23^{—}$ transitional 1 and $IgM^{hi}CD23^{+}$ transitional 2 subsets within $B220^{+}CD19^{+}CD93^{+}$ transitional B cells in the spleen of *Mb1-cre*$^{—}$, *Mb1-cre*$^{+}$ and *Nemo*^{fl} *Mb1-cre*$^{+}$ mice in the absence (n = 5-7 per genotype in 6 experiments) (*A*) or upon ectopic expression of Bcl2 (n = 14-19 per genotype in 15 experiments) (*B*; *Bcl2Tg*). Numbers adjacent to outlined areas specify percent cells in the gate.

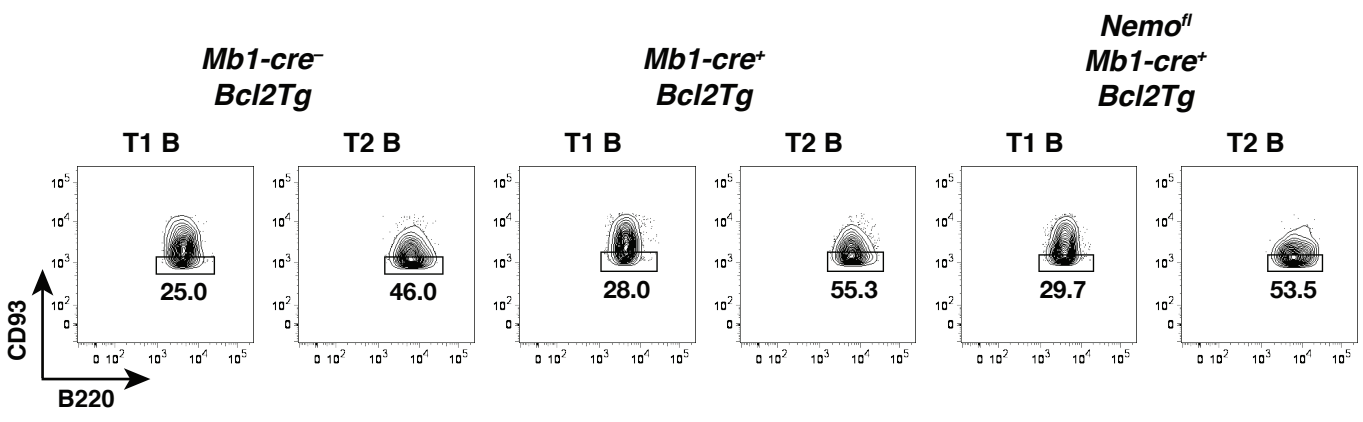
Fig. S4: Determination of the percentage of $CD93^{lo}$ cells within transitional 1 and 2 populations. Proportions of $CD93^{lo}B220^{+}$ cells within splenic $B220^{+}CD19^{+}CD93^{+}IgM^{hi}CD23^{—}$ transitional 1 and $B220^{+}CD19^{+}CD93^{+}IgM^{hi}CD23^{+}$ transitional 2 B cells measured by flow cytometry in *Mb1-cre*$^{—}$ *Bcl2Tg*, *Mb1-cre*$^{+}$ *Bcl2Tg* and *Nemo*^{fl} *Mb1-cre*$^{+}$ *Bcl2Tg* mice (n = 7-9 per genotype in 9 experiments). Numbers adjacent to outlined areas specify percent cells in the gate. Transitional 2 cells from control mice were used as the reference to set the $CD93^{lo}$ gate.

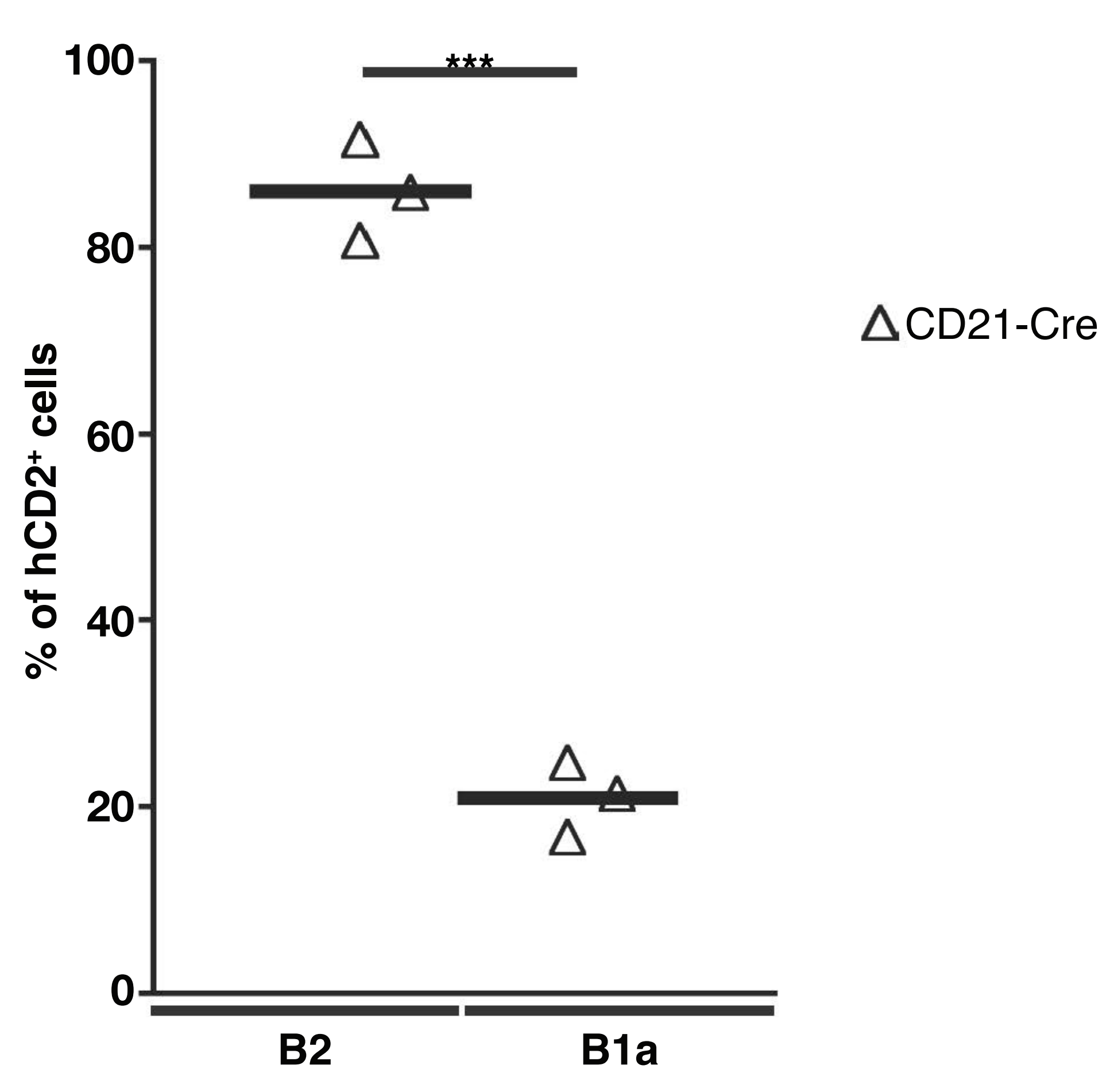
Fig. S5: Proportions of hCD2^{+} cells within the B1a and B2 cell subsets. Percent hCD2^{+} B220^{+}CD19^{+} B2 and B220$^{lo/—}$CD19^{+}CD5^{+}CD43^{+} B1a cells in the peritoneal cavity of *CD21-cre*$^{+}$ *R26-Stop*^{fl}*-hCD2* mice determined by flow cytometry. Data are pooled from 3 experiments (n = 3). Each symbol indicates one mouse and horizontal lines signify mean. ***, P ≤ 0.001. Student's t test.


A
Mb1-cre–
Mb1-cre+
Nemofl
Mb1-cre+
CD93
B220
8.4
90.4
18.4
80.0
48.7
45.6
CD1d
IgM
9.4
87.9
6.5
90.4
2.5
95.4
B
Mb1-cre–
Bcl2Tg
Mb1-cre+
Bcl2Tg
Nemofl
Mb1-cre+
Bcl2Tg
5.8
92.7
7.1
91.6
4.9
94.0
0.8
97.6
0.7
97.6
0.2
99.4


A
Mb1-cre–
Mb1-cre+
Nemofl
Mb1-cre+
58.0
37.2
65.8
30.0
82.0
14.1
B220
CD19
B
Mb1-cre–
Bcl2Tg
Mb1-cre+
Bcl2Tg
Nemofl
Mb1-cre+
Bcl2Tg
75.2
23.6
81.9
17.0
96.1
2.9
B220
CD19


A
Mb1-cre–
Mb1-cre+
Nemofl
Mb1-cre+
43.1
26.5
38.9
29.1
76.0
10.2
IgM
CD23
B
Mb1-cre–
Bcl2Tg
Mb1-cre+
Bcl2Tg
Nemofl
Mb1-cre+
Bcl2Tg
36.9
27.6
35.1
21.5
59.7
15.3
IgM
CD23


Mb1-cre⁻
Bcl2Tg
Mb1-cre⁺
Bcl2Tg
Nemo^fl
Mb1-cre⁺
Bcl2Tg
T1 B
T2 B
T1 B
T2 B
T1 B
T2 B
25.0
46.0
28.0
55.3
29.7
53.5
CD93
B220


100
80
60
40
20
0
% of hCD2+ cells
***
B2
B1a
CD21-Cre