NFATc1 affects mouse splenic B cell function by controlling the calcineurin–NFAT signaling network

Sankar Bhattacharyya,1 Jolly Deb,1 Amiya K. Patra,1 Duong Anh Thuy Pham,1 Wen Chen,1 Martin Vaeth,1 Friederike Berberich-Siebelt,1 Stefan Klein-Hessling,1 Edward D. Lamperti,3 Kurt Reifenberg,4 Julia Jellusova,5 Astrid Schweizer,5 Lars Nitschke,3 Ellen Leich,2 Andreas Rosenwald,2 Cornelia Brunner,6 Swen Engelmann,7 Ursula Bommhardt,7 Andris Avots,1 Martin R. Müller,3 Eisaku Kondo,8 and Edgar Serfling1

1Department of Molecular Pathology, Institute of Pathology, University of Würzburg, D-97080 Würzburg, Germany
2Institute of Pathology, University of Würzburg, D-97080 Würzburg, Germany
3Central Animal Facility, University of Erlangen-Nürnberg, D-91058 Erlangen, Germany
4Department of Physiology, University of Erlangen-Nürnberg, D-91058 Erlangen, Germany
5Department of Pathology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama 700-8558, Japan
6Department of Pathology, Aichi Cancer Center Research Institute, Nagoya 464-8681, Japan
7Institute of Medical and Clinical Immunology, University Magdeburg, D-39120 Magdeburg, Germany
8Department of Oncological Pathology, Aichi Cancer Center Research Institute, Nagoya 464-8681, Japan

Abbreviations used: AICD, activation-induced cell death; BAC, bacterial artificial chromosome; Btk, Bruton’s tyrosine kinase; ChIP, chromatin immunoprecipitation; CsA, cyclosporin A; EAE, experimental autoimmune encephalomyelitis; GC, germinal center; IP3, inositol 1,4,5-trisphosphate; MZB, marginal zone B cell; PI, propidium iodide; PLCγ2, phospholipase Cγ2; TPA (12-O-tetradecanoylphorbol-13-acetate) + ionomycin.

By studying mice in which the Nfatc1 gene was inactivated in bone marrow, spleen, or germinal center B cells, we show that NFATc1 supports the proliferation and suppresses the activation-induced cell death of splenic B cells upon B cell receptor (BCR) stimulation. BCR triggering leads to expression of NFATc1/Xα, a short isoform of NFATc1, in splenic B cells. NFATc1 ablation impaired Ig class switch to IgG3 induced by T cell–independent type II antigens, as well as IgG3+ plasmablast formation. Mice bearing NFATc1−/− B cells harbor twofold more interleukin 10–producing cells. NFATc1−/− B cells suppress the synthesis of interferon-γ by T cells in vitro, and these mice exhibit a mild clinical course of experimental autoimmune encephalomyelitis. In large part, the defective functions of NFATc1−/− B cells are caused by decreased BCR-induced Ca2+ flux and calcineurin (Cn) activation. By affecting CD22, Rcan1, CnA, and NFATc1/Xα expression, NFATc1 controls the Ca2+-dependent Cn–NFAT signaling network and, thereby, the fate of splenic B cells upon BCR stimulation.

© 2011 Bhattacharyya et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.rupress.org/terms). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/).
cells by tonic BCR signals in the periphery (Srinivasan et al., 2009). Although these and other studies elucidated important signaling molecules for the survival of resting peripheral B cells, they did not address which signaling pathways control the survival and function of peripheral B cells upon BCR stimulation.

In a typical immune response, triggering of immune receptors by cognate antigens culminates in the massive clonal expansion of peripheral lymphocytes, followed by elimination of most of the amplified effector cells by apoptosis toward the end of the immune response (Strasser and Pellegrini, 2004; Krammer et al., 2007). Although immune reactions depend on numerous parameters, immune receptor signals are key determinants that control the strength of an immune response and the onset of apoptosis, and thus the termination of the immune response. Triggering of BCR results in the rapid phosphorylation of several proximal signaling molecules, such as Bruton\'s tyrosine kinase (Btk), phospholipase Cγ2 (PLC-γ2), and others, by the tyrosine protein kinases Lyn and Syk. Along with tyrosine phosphorylated B cell adaptor proteins, such as SLP-65/BLNK, these molecules assemble in supramolecular complexes that transmit BCR signals to downstream serine/threonine protein kinases and, finally, to transcription factors. By hydrolyzing phosphatidylinositols to diacylglycerol and inositol 1,4,5-triphosphate (IP3) that, in turn, binds to and stimulates IP3 receptors, PLC-γ2 affects the release of Ca2+ from intracellular stores and the subsequent influx of extracellular Ca2+ (King and Freedman, 2009). An increase in intracellular Ca2+ levels leads to the activation of the Ca2+-calmodulin-dependent Ser/Thr-specific phosphatase calcineurin (Cn; also designated as PP2B), which dephosphorylates and activates members of NFAT transcription factors by facilitating their nuclear translocation.

Together with NFATc2 (also designated as NFAT1), NFATc1 (or designated as NFAT2) belongs to the most prominent NFAT factors in activated lymphocytes. However, contrary to NFATc2, which is constitutively expressed in most peripheral lymphocytes, the expression of NFATc1 in peripheral lymphocytes is strongly induced at the transcriptional level. In CD4+ T cells, the transcription of Nfat1 gene is induced by TCR triggering and co-stimulatory signals (Chuvpilo et al., 1999, 2002; Nurieva et al., 2007), whereas, as we show here, BCR triggering induces NFATc1 expression in splenic B cells. The induction of NFATc1 expression upon immune receptor stimulation is an important control level of NFAT activity. The observation that NFAT factors have to reach a certain threshold level for the induction of the IL-2 (Il2) promoter in T cells was made nearly 20 yr ago (Fiering et al., 1990). The induction of Nfat1 gene is controlled by a switch from the constitutively active promoter P2 to the inducible P1 promoter, whose activity directs the predominant synthesis of short isoform NFATc1/αA (Chuvpilo et al., 2002). This isoform differs from other NFATc proteins in its short C terminus, and thus lacks a second transactivation domain present in longer NFATc1 isoforms (Avots et al., 1999) that harbor two highly conserved sumoylation motifs (Nayak et al., 2009). NFATc1/αA contains an individual N-terminal peptide, designated as α-peptide, which differs markedly from that in NFATc2 and other NFATc proteins, including the NFATc1/β isoforms (Serfling et al., 2006). Although the functional relevance of these protein domains remains to be shown in detail, it is very likely that along with its high expression level, NFATc1/αA exerts specific functions in T cell and B cell physiology.

NFAT factors were originally described as regulators of cytokine genes in T cells, such as of IfI2, Il3, Il4, Il5, Ifng, and Tifa genes (Serfling et al., 2000; Hogan et al., 2003; Macian, 2005), but they also orchestrate the expression of numerous other genes in other cells, including B cells. However, apart from a study on the inactivation of Cn B1 subunit (Winslow et al., 2006), which affects all NFATc members and probably more downstream effectors, there is only a restricted number of comprehensive analyses on NFAT function in B cells. Investigations of B cell differentiation in mice bearing NFATc1−/− lymphocytes upon reconstitution of RAG-deficient mice with Nfat1−/− fetal liver cells indicated a crucial role for NFATc1 in the development of peritoneal B1a cells (Berland and Worts, 2003). In mice created by complementation of RAG−/− blastocysts with Nfat1−/− embryonic stem cells, a strong decrease in α-IgM-mediated proliferation of NFATc1−/− B cells was observed (Yoshida et al., 1998).

By using mice in which the Nfat1 gene was inactivated in BM, spleen, or germinial center (GC) B cells, we show that NFATc1 controls the fate of splenic B cells upon BCR stimulation. NFATc1 inactivation impaired BCR-mediated proliferation and facilitated activation–induced cell death (AICD). Although, apart from the loss of peritoneal B1a cells, NFATc1 ablation did not markedly affect B cell differentiation in BM and periphery, it resulted in a strong decrease of Ig class switch to IgG3 upon immunization with T cell–independent type II antigens and IgG3+ plasmablast formation. NFATc1 ablation also resulted in a decreased capacity of NFATc1−/− B cells to stimulate T cells to proliferate and to secrete IL-2 and IFN-γ in vitro. This is caused in part by an increase in IL-10–producing CD14hiCD5+ B cells, which might also contribute to the mild clinical course of experimental autoimmune encephalomyelitis (EAE) in mice bearing NFATc1−/− B cells in vivo. Several of these defects seem to depend on intracellular Ca2+ levels, which were found to be decreased in NFATc1−/− splenic B cells. In this study, we show that, in addition to NFATc1/αA induction, NFATc1 regulates the expression and activity of further members of the Ca2+-dependent Cn network, such as CD22, Rcan1, CnA, and NFATc2, and thus affects the survival and function of peripheral B cells.

RESULTS

BCR triggering of naïve splenic B cells leads to strong induction of NFATc1/αA

Stimulation of naïve splenic B cells from 6–8-wk-old mice with 10 µg/ml anti-IgM antibody (α-IgM; the F(ab)2 fragment of anti–mouse IgM) in vitro leads to strong induction of NFATc1 after ~24 h. A similar strong induction of NFATc1 was also detected by TPA+ionomycin (T+I) treatment,
whereas a much weaker induction was observed after stimulation of B cells with 10 µg/ml LPS or 2 µg/ml α-CD40 (Fig. 1 A and Fig. S1 A). The short NFATc1 protein induced by α-IgM reacted with an antibody raised against the NFATc1 α-peptide and corresponds to NFATc1/αA, whereas the NFATc1 version induced by LPS did not react (Fig. 1 B). The Western blot data on the important role of BCR signals in NFATc1 induction are supported by the strong induction of an Egfp reporter gene controlled by the Nfatc1 locus upon α-IgM stimulation. When splenic B cells from mice bearing a BAC-Nfatc1/Egfp transgene were stimulated by α-IgM, LPS, or α-CD40 for 24 h, a much stronger GFP induction was detected after α-IgM than after LPS or α-CD40 treatment (Fig. 1 C).

To show that BCR-mediated induction of NFATc1 is affected by prototypical signaling molecules of the BCR-signaling pathways, we investigated the effect of α-IgM stimulation on NFATc1 induction in splenic B cells from mice deficient for the protein tyrosine kinase Btk (Kerner et al., 1995; Khan et al., 1995) or the adaptor molecule SLP-65 (Jumaa et al., 1999). As shown in Fig. 1 (D and E), inactivation of either Btk or SLP-65 led to complete loss of NFATc1 induction, indicating that the strong induction of NFATc1/αA is mediated through these signaling molecules, which are known to be key components of BCR signaling.

NFATc1 ablation suppresses the formation of peritoneal B1a cells, but does not interfere with B cell development in BM and spleen

To investigate the role of NFATc1 in B cell physiology we used mice bearing “floxed” exon 3 alleles of Nfatc1 (Nfatc1flx/flx). To generate mice in which the murine Nfatc1 gene was inactivated in B cells, Nfatc1flx/flx mice were crossed with mice that specifically express the cre enzyme in B cells of BM (mb1-cre; Hobeika et al., 2006), spleen (Cd23-cre; Kwon et al., 2008), or GC (Aicda-cre; Kwon et al., 2008). Crossing of Nfatc1flx/flx mice with the mb1-cre and Cd23-cre lines resulted in the ablation of NFATc1 expression in splenic B cells (Fig. S1, B–D).

Flow cytometric analyses of B cell development in BM from WT and mb1-cre x Nfatc1flx/flx mice did not show significant differences in the expression of B220, CD43, BP-1, CD24, IgM, and IgD on pro-B cells (Hardy fractions B and C; Hardy and Hayakawa, 2001; CD24+BP-1− and CD24+BP-1+ fractions in B220+CD43hi cells), pre-B cells, and immature and mature B cells (Hardy fractions D–F; Hardy and Hayakawa, 2001; Fig S2 A). Likewise, the differentiation of LN and splenic NFATc1−/− B cells appeared to be relatively unaffected (Fig. S2 B), although only 75–80% B cells were counted in preparations of whole splenic B cells from 7 mb1-cre x Nfatc1flx/flx mice compared with WT mice. Stains of splenic B cells for CD21+CD23 revealed similar ratios of follicular B cells and marginal zone B cells (MZBs) from WT and mb1-cre x Nfatc1flx/flx mice (Fig. S2 C). Also, the maturation of splenic B cells did not markedly differ between WT and NFATc1−/− cells (Fig. S2 D). However, in agreement with published data (Berland and Wortis, 2003), in peritoneal washes from mb1-cre x Nfatc1flx/flx mice a 5–10-fold decrease in the population of B1a cells was detected. In the peritoneum of Cd23-cre x Nfatc1flx/flx mice, B1a cells that do not express CD23 were found in normal numbers (Fig. S2, E and F).
Impaired BCR-induced proliferation and increased AICD of NFATc1<sup>−/−</sup> splenic B cells

Investigations of the α-IgM–mediated proliferative capacity of splenic NFATc1<sup>−/−</sup> B cells in CFSE dilution assays and [3H]thymidine incorporation experiments showed a marked decrease in their proliferation in vitro, compared with WT B cells. In contrast, no differences in proliferation were observed between NFATc1<sup>−/−</sup> and WT B cells upon LPS or α-CD40 stimulation (Fig. 2 A and Fig. S3 A). In addition, NFATc1<sup>−/−</sup> splenic B cells showed a distinct increase in α-IgM–mediated apoptosis. This is reflected in the relative drop of living cells (as shown by their SSC–FSC features; Fig. S3 B) and the increase of Annexin V–positive apoptotic NFATc1<sup>−/−</sup> cells compared with WT B cells (Fig. 2 B). Similar differences in survival between WT and NFATc1<sup>−/−</sup> B cells were detected in cell cycle analysis by PI staining upon α-IgM stimulation for 48 h, showing a pronounced increase in hypoploid sub-G1 cells from ~20–40% (Fig. S3 C). The increase in α-IgM–mediated AICD is a specific feature of NFATc1<sup>−/−</sup> B cells because contrary to those, NFATc2<sup>−/−</sup> B cells were resistant to α-IgM–mediated apoptosis (Fig. 2 B), which agrees with the pro-apoptotic activity of NFATc2 in activated T cells, as previously described (Chuvpilo et al., 2002).

NFATc1 controls numerous genes that affect the AICD of B cells

For the identification of NFATc1 target genes that control the proliferation and/or AICD of B cells, we investigated gene expression of splenic B cells by using mouse whole-genome expression arrays. Freshly isolated B cells from WT and mb1<sup>cre</sup> x Nfatc1<sup>flx/flx</sup> mice were either left uninduced or induced by α-IgM for 3, 8, or 16 h. We identified 53 genes whose expressions were enhanced by more than twofold and 37 genes whose expression was suppressed more than twofold by NFATc1 upon α-IgM stimulation for 16 h (Tables S1 and S2). Among the enhanced “top ten” genes are the Spp1, Rcan1, Pdcd1, Tnfsf14, and Fasl genes that encode apoptosis regulators (Fig. 2 C). The Spp1 gene encodes the multifunctional protein osteopontin (Hur et al., 2007); the Rcan1 gene encodes the Cn modulator calcipressin (Vega et al., 2003); the Pdcd1 gene encodes PD-1 (CD279), a member of the Ig supergene family which is expressed on T and B cells (Keir et al., 2008); and the Tnfsf14 gene encodes LIGHT (CD258), a member of the TNF family that is a ligand of the lymphotxin β receptor (LTβR) and herpes virus entry mediator (HVEM; Scheu et al., 2002). Although the RNA levels of all five genes were almost undetectable in nonstimulated B cells, the expression of Spp1 and Rcan1 genes was rapidly induced by α-IgM treatment for 3 h and decreased (Spp1) or remained quite constant (Rcan1) afterward in NFATc1<sup>−/−</sup>, but not WT B cells. In contrast, the expression of Pdcd1, Tnfsf14, and Fasl genes was undetectable upon 3 h induction and increased strongly after 16 h of α-IgM stimulation in WT but not NFATc1<sup>−/−</sup> cells (Fig. 2 D).

To show that the Spp1, Rcan1, Pdcd1, Tnfsf14, and Fasl genes are genuine NFATc1 targets in splenic B cells, we performed chromatin immunoprecipitation (ChIP) assays using splenocytes that were either left noninduced or induced with T+I or α-IgM for 16 h. Upon immunoprecipitation with an NFATc1–specific antibody, we detected a marked increase in NFATc1 binding to the promoters of Pdcd1, Tnfsf14, and Fasl genes after both stimulations (Fig. 2 E). In contrast, the Spp1 and Rcan1 promoters already showed NFATc1 binding in nonstimulated cells, but stimulation increased their NFATc1 binding. As expected, for the I2 promoter an increase in NFATc1 binding was observed upon T+I stimulation but not upon α-IgM stimulation (Fig. 2 E).

To show whether in our assays PD-1 and LIGHT might affect the survival of B cells, we stimulated splenic B cells from PD-1<sup>−/−</sup> and LIGHT deficient mice by α-IgM. Compared with WT B cells, both types of B cells showed a strong reduction in AICD (Fig. S3 D). This indicates that three of the most prominent NFATc1 targets in primary B cells, the Pdcd1, Tnfsf14 and Fasl genes, code for proapoptotic proteins.

Apart from the Ccnb1/Cyclin B1 gene, among the 53 genes that showed a twofold and stronger increase in expression upon BCR stimulation in WT (compared with NFATc1<sup>−/−</sup> B cells) we did not detect genes that directly control cell cycle and proliferation (Table S1). This suggests that NFATc1 enhances the expression of (numerous) genes controlling proliferation only to moderate levels. Such view finds support in gene set enrichment analyses (GSEA) of microarray data in which numerous gene signatures enhancing proliferation were significantly enriched in WT B cells compared with NFATc1<sup>−/−</sup> B cells. Among those are eleven signatures that are significantly associated with cell cycle and proliferation (Table I), and numerous cell cycle–associated genes. In addition to the Ccnb1 gene, these include several cyclin genes, which are listed as signature genes in leading edge analyses (Table S3).

NFATc1 supports the proliferation of B cells by controlling their Ca<sup>2+</sup> flux and Cn activity

Among the 10 genes whose expression was most strongly suppressed by NFATc1 in B cells are the Cd22, Fcer2a/CDC23, and S100a4 genes, whose activity modulates (or is modulated by) intracellular Ca<sup>2+</sup> levels in B cells (Fig 3 A; Kolb et al., 1990; Nitschke, 2009; Matsuura et al., 2010). These findings suggest that NFATc1, as one of the most prominent NFATc1 targets (along with Rcan1; Figs. 2, C and E), controls BCR-mediated, store-operated Ca<sup>2+</sup> entry, and thus the Ca<sup>2+</sup>–dependent Cn network, for its own induction in B cells.

Sustained Ca<sup>2+</sup> entry is necessary for many physiological processes controlling lymphocyte activity, including their proliferation and apoptosis (Hogan et al., 2010). This is illustrated for splenic B cells in Fig. 3 B. Almost all cells proliferated when freshly prepared splenic B cells were cultured in X-vivo medium, a medium rich in Ca<sup>2+</sup>, whereas in a 1:1 mixture of X-vivo and RPMI media (the latter contains about one-third the amount of Ca<sup>2+</sup> as the X-vivo medium) <50% of B cells were found to proliferate. Adding 10 mM Ca<sup>2+</sup> to this medium restored the proliferative capacity, whereas inhibiting Cn by cyclosporin A (CsA), either at the start of
the experiment or 1 d later, blocked any proliferation of B cells (Fig. 3 B). A similar strong effect has been observed with 10 µM NC13, a novel Cn inhibitor (Sieber et al., 2007), and 2.5-µM Vivit-11R (Fig. S4, A and C), a specific NFAT inhibitor (Aramburu et al., 1999). The mild proliferative defect of NFATc1<sup>−/−</sup> B cells upon α-IgM stimulation (Fig. 2 A and Fig. 3 B) is in line with the observation that NFATc1<sup>−/−</sup> B cells exhibit a marked reduction in Ca<sup>2+</sup> flux (Fig. S4 E),

**Figure 2.  NFATc1 is required for optimal proliferation and survival of splenic B cells.** (A) CFSE-labeled splenic B cells from a WT and mb1-cre x Nfatc<sup>f/f</sup> mouse were incubated for 72 h in the absence (Unstim.) or presence of 10 µg/ml LPS, 10 µg/ml α-IgM, or 2 µg/ml α-CD40. CFSE dilution was measured by flow cytometry. (B) Splenic B cells from WT, mb1-cre x Nfatc<sup>f/f</sup>, and Nfatc2<sup>−/−</sup> mice were incubated for 72 h; cells were stained with PI and Annexin V and analyzed by flow cytometry. (C) WT and mb1-cre x Nfatc<sup>f/f</sup> (KO) splenic B cells were stimulated with α-IgM, as indicated. Gene expression was analyzed by DNA microarray. Key at the bottom indicates the fold changes in gene expression (log2 values). (D) Induction of 10 NFATc1 target genes by α-IgM stimulation in WT and NFATc1<sup>−/−</sup> splenic B cells. Error bars show ± the SEM. (E) ChIP assay showing the in vivo binding of NFATc1 in splenocytes to the promoters of Il2, Rcan1, Spp1, Fasl, Pdcd1, and Tnfsf14 genes upon stimulation for 16 h with α-IgM or T+I. Lanes 1–3, ChIP assays using splenocytes that were left unstimulated (-) or stimulated by T+I or α-IgM for 16 h. ChIP assays were also performed using an antibody directed against GST protein. As input control, 1% of chromatin of all assays was used. In A and B, one typical experiment is shown from more than three assays; in C and D, mean values from three individual DNA microarray analyses are presented, and in E one typical ChIP assay from three assays is shown.
in both the release of Ca$^{2+}$ from intracellular stores and in influx of extracellular Ca$^{2+}$ to $\sim$60% of level to that in WT cells (Fig. 3 C).

CD22 is an inhibitory co-receptor of BCR that is transiently expressed on splenic B2 cells (Nitschke, 2009). Its surface expression is impaired by BCR stimulation (Lajaunias et al., 2002) and, as flow cytometric assays of WT and NFATc1$^{-/-}$ B cells imply, NFATc1 contributes to CD22 suppression (Fig. 3 D). This conclusion is supported by the DNA microarray data (Fig. 3 A and Table S2) and semi-quantitative RT-PCR assays that showed higher CD22 RNA levels in NFATc1$^{-/-}$ than WT B cells treated by $\alpha$-IgM for 16 h (Fig. 3 D). Splenic B cells from CD22$^{-/-}$ mice show, on the other hand, a strong NFATc1/\(\alpha\)A expression in noninduced and LPS-treated B cells, whereas WT B cells express isofoms other than NFATc1/\(\alpha\)A under these conditions (Fig. 1 A and Fig. S4 F).

In addition to the R<em>am1</em> gene, the <em>P<sub>pp3cb</sub></em> gene encoding the large CnA \(\beta\) subunit that is predominantly expressed in lymphocytes is a further direct NFAT target. This has been shown previously for cardiomyocytes (Oka et al., 2005), and in ChIP assays we detected the in vivo binding of NFATc1 to the 5' region of <em>P<sub>pp3cb</sub></em> gene (not depicted). In Western blots using an antibody recognizing CnA \(\alpha/\beta\) proteins, a strong decrease in the \(\alpha\)-IgM-mediated induction of one CnA-specific protein was detected in protein extracts from NFATc1$^{-/-}$ B cells (Fig. 3 E, middle), suggesting an important role of NFATc1 in CnA expression and function. This conclusion is supported by the marked decrease of Cn activity in extracts from freshly prepared NFATc1$^{-/-}$ B cells, compared with WT B cells (Fig. 3 E, bottom).

The diminished Cn activity suggests that the activation of other NFATc factors might also be impaired in NFATc1$^{-/-}$ B cells. In immunohistochemical stainings of B cells from WT and <em>mb1-cre x NFATc<sup>flox/flo</sup></em> mice stimulated for 1 or 2 d with \(\alpha\)-IgM and \(\alpha\)-CD40 and restimulated for 30 min with T+1, we detected markedly less NFATc2 in nuclei of NFATc1$^{-/-}$ B cells than WT cells (Fig. 3 F). This suggests an impaired NFATc2 activation in the absence of NFATc1. In RNA levels, no differences were detected for NFATc2 RNA between WT and NFATc1$^{-/-}$ B cells, whereas a slight decrease in NFATc3 RNA levels was observed for NFATc1$^{-/-}$ B cells, compared with WT cells (Fig. 3 F). However, because no distinct increase in NFATc3 RNA levels was detected upon $\alpha$-IgM stimulation in WT B cells (Fig. 3 F), NFATc1 appears to indirectly affect <em>Nfatc3</em> gene expression.

Collectively, these data indicate that NFATc1 controls R<em>can1</em> and CnA expression, i.e., its immediate upstream control molecules, which, in a feedback loop, affect the transcriptional induction of NFATc1. In addition, NFATc1 seems to also affect upstream BCR signals, probably through CD22 and other targets. A scheme of this network connecting NFATc1 with its upstream activators in B cells is shown in Fig. S5A.

### NFATc1 affects Ig class switch by T cell–independent type II antigens

Interuption of BCR signaling by inactivating Btk, SLP-65, PLC-$\gamma_2$, and further components of the BCR signaling cascade not only leads to a drastic decrease in Ca$^{2+}$ flux, but also to defects in Ig class switching upon immunization with T cell–independent type II (TI-II) antigens (Kerner et al., 1995; Khan et al., 1995; Jumaa et al., 1999; Wang et al., 2000). When we measured Ig levels in sera from nonimmunized WT mice and mice bearing NFATc1$^{-/-}$Cd23$^{-/-}$ B cells, we did not detect statistically significant differences in the concentration of Ig classes (except IgM in <em>Cd23<sup>−/−</sup> x Nfatc<sup>flox/flo</sup></em> mice; Fig. S5 B). However, upon immunization with the TI-II antigen NP-Ficoll, we detected 10-fold lower IgG3 serum levels in <em>Cd23<sup>−/−</sup> x Nfatc<sup>flox/flo</sup></em> mice, compared with those from WT mice. Slight, but statistically significant, differences were also detected in IgM levels 2 wk after immunization (Fig. 4 A). Very similar results were obtained upon immunization of <em>Aicda-cre x Nfatc<sup>flox/flo</sup></em> mice that resulted in tenfold lower IgG3 serum levels compared with nonimmunized mice.
Figure 3. NFATc1 controls Ca2+/Cn signaling in splenic B cells. (A) DNA microarray assay showing 10 genes whose expression was enhanced in B cells from mb1-cre x Nfatc1fl/fl mice compared with WT B cells. Mean values of 3 individual assays are shown. (B) Manipulation of Ca2+/Cn signaling by adding 10 mM CaCl2 or 100 ng/ml CsA to proliferation assays. Splenic B cells from WT or mb1-cre x Nfatc1fl/fl mice were stained with CFSE and treated with 10 µg/ml α-IgM or CsA in X-vivo medium or 1:1 X-vivo+RPMI medium that contains less Ca2+. Numbers indicate the percentage of cycling cells. One typical set of more than three experiments is shown. (C) Ca2+ release and influx in B cells from WT and mb1-cre x Nfatc1fl/fl (KO) mice. One typical experiment from more than three assays is shown. (D) Effect of NFATc1 ablation on CD22 expression. (left) Semiquantitative RT-PCR assay showing an increase in CD22 RNA in NFATc1−/− splenic B cells. One typical assay from three individual assays is shown. (right) Flow cytometric detection of CD22 expression on WT and Cd23-cre x Nfatc1fl/fl splenic B cells left unstimulated or stimulated with LPS or α-IgM for 2 h. Two more assays with B cells from two mice each were performed and gave similar results. (E) CnA assays. (top) ChIP assay for the binding of NFATc1 to the Ppp3cb and Il2 promoter in vivo. Splenocytes were left unstimulated (−) or stimulated with α-IgM for 16 h. As indicated, either an NFATc1-specific or an unrelated antibody (α-GST) was used. As input control, 1% of chromatin of all assays was used. (middle) Immunoblot using whole cellular protein from B cells and a CnAα/β-specific antibody. Below the blot, Ponceau red staining is shown as loading control. (bottom) Mean values of Cn activity from three assays are shown (P ≥ 0.05 between WT and mb1-cre x Nfatc1fl/fl cells). (F) Effect of NFATc1 ablation on NFATc2 expression. (top) Decrease in nuclear NFATc2 in NFATc1−/− B cells. Splenic B cells were treated with α-IgM and α-CD40 for 24 h, and for the last 30 min, the cells were also treated with T+I. Splenic B cells stained with an NFATc2-specific antibody. (right) Nuclear staining of 100 cells measured by confocal microscopy is compiled. (bottom) Semiquantitative RT-PCR assay. Error bars show ± the SEM.
Figure 4. NFATc1 deficiency results in a defect in IgG3 class switch by the TI-type II antigen NP-Ficoll. (A) IgM and IgG3 levels in sera of WT, Cd23-cre, and Aicda-cre x Nfatc1flx/flx mice after immunization with the TI-type II antigen NP-Ficoll. Each symbol represents one mouse. (B) Appearance of circular IgG3 and IgG1 transcripts in WT and NFATc1−/− splenic B cells after in vitro culture for 2 d in the presence of LPS. (C) ELISA showing a decrease in IgG3 production of B cells from Cd23-cre x Nfatc1flx/flx mice upon culture for 6 d. (D) Decreased IgG+ plasmablast formation of B cells from Cd23-cre x Nfatc1flx/flx mice upon 3 d in culture. Flow cytometry of CD19+ gated B cells. In the columns data from three assays are compiled. (E and F) Diminished...
NFATc1/GFPhigh cells also expressed high levels of the GC
expression was detected for IgM+IgG3+ B cells, whereas B
with WT mice 1, 2, or 3 wk after primary injection. Again,
These in vivo data are supported by the Ig class switch to
Although in cultures of splenic B cells circular RNA transcripts of IgG3 switch region could be detected in WT B cells after 2 d, no circular transcripts were observed in NFATc1−/− B cells, whereas circular IgG1 transcripts could be detected in both WT and NFATc1−/− B cells (Fig. 4 B). Measuring of secreted IgM and IgG3 antibodies revealed a decrease in IgG3 secretion of ∼50% compared with WT cells (Fig. 4 C). After culturing B cells for 5 d, the percentage of CD138+ IgG3+ plasmablasts generated from NFATc1−/− cells decreased to ∼30% of IgG3+ WT plasmablasts (Fig. 4 D). This might be caused, in part, by a decrease in proliferation and increase in AICD. Indeed, this was found for NFATc1−/− CD138+ plasma cells in vitro (Fig. 4, E and F).

The Ig switch to IgG3 by Ti-type II antigen takes place in B cells expressing high levels of NFATc1. This was shown by measuring NFATc1, IgM, and IgG3 levels in preparations of splenocytes from Nfatc1-Egfp reporter mice upon immunization with NP-Ficoll by flow cytometry. A high level of NFATc1 expression was detected for IgM+IgG3+ B cells, whereas B cells expressing only IgM expressed less NFATc1 (Fig. 4 G). Injections of four WT and four Cd23-cre x Nfatc1flx/flx mice with NP-KLH (in alum) revealed only subtle, albeit statistically significant differences in serum levels of IgG2a one week after injection (Fig. S5 C). On the same hand, no differences were detected in the affinity of IgG1 serum antibodies generated in WT and Cd23-cre x Nfatc1flx/flx mice (Fig. S5 D). This shows that in contrast to the strong effect on Ig class

When we injected TNP-Ficoll into mice and measured its binding to B cells, we observed a decrease in binding of TNP-Ficoll to NFATc1−/− MZB cells, compared with MZB cells in WT mice. This is shown in Fig. 5 A for 8–9 mo old mice, whereas in younger 7–8-wk-old mice a 40–60% reduction in TNP-Ficoll binding was observed (unpublished data). 1 h after i.v. injection, TNP-Ficoll formed a ring like structure in the marginal zone of B cell follicles, distal of IgDhigh B2 cells and associated with metallophilic macrophages (Fig. 5, B and C). Whereas in spleen from WT mice TNP-Ficoll was found to be evenly distributed and closely associated with IgMhigh MZBs, large aggregates of antigen were detected around, but not associated with CD169+ macrophages and IgMhigh B2 cells and associated with metallophilic macrophages (Fig. 5, B and C). Similar deposits of antigen aggregates were described upon TNP-Ficoll immunization of mice deficient in Pyk-2, complement C3, or complement receptor CR1/2. All of these mice exhibit marked defects in MZB cells (Guinamard et al., 2000).

Contrary to the immunizations with Ti-type II antigen, we did not detect remarkable differences in Ig class switch upon immunization with NP-KLH, a T cell–dependent (TD) antigen. Injections of four WT and four Cd23-cre x Nfatc1flx/flx mice with NP-KLH (in alum) revealed only subtle, albeit statistically significant differences in serum levels of IgG2a one and two wks of immunization, but no differences in IgM, IgG1 and IgG2b levels (Fig. S5 C). On the same hand, no differences were detected in the affinity of IgG1 serum antibodies generated in WT and Cd23-cre x Nfatc1flx/flx mice (Fig. S5 D). This shows that in contrast to the strong effect on Ig class
switch by TI-type II antigens, inactivation of NFATc1 in B cells exerts a mild, if any effect on Ig class switch by TD antigens in the primary response.

NFATc1 affects the regulatory capacity of B cells in vitro and in vivo

B cells control immune reactions by presenting antigens to T cells by releasing cytokines and secreting humoral antibodies. When we tested the effect of NFATc1−/− C57BL/6 B cells on allogenic T cells from BALB/c mice in MLCs we observed a stronger stimulatory activity of WT B cells than NFATc1−/− B cells on T cell proliferation and IL-2 synthesis. This indicates NFATc1−/− B cells as poor activators of T cells (Fig. 6, A and B). This conclusion found support in the weak stimulatory capacity of NFATc1−/− B cells presenting an OVA peptide (OVA323-339) to syngenic CD4+ T cells from OTII mice that are transgenic (tg) for an OVA-specific TCR (Fig. 6, C and D; Barnden et al., 1998), and NFATc1−/− c2−/− double KO B cells displayed even less stimulatory activity (Fig. S6).

Several lines of evidence indicated that B cells can suppress immune reactions by secreting IL-10, and thereby affect the course of autoimmune diseases (Bouaziz et al., 2008; Lampropoulou et al., 2010). Compared with splenic B cells from WT mice, splenic B cells from Cd23-cre x Nfatc1flx/flx mice harbor twofold more CD19+CD5+CD1dhigh B cells that were described to produce IL-10 (Fig. 7 A; Matsushita et al., 2008). Upon treatment by LPS for 24 h (and, in addition, by T+I for the last 8 h) in vitro, the percentage of IL-10−producing CD5+CD1dhigh B cells increased twofold (Fig. 7 B; note the lack of any IL-10 signal for splenic B cells from IL-10−/− mice). A similar increase in the percentage of IL-10−producing CD138+ plasmablasts was detected upon differentiation of NFATc1−/− splenocytes for 3 d in vitro (Fig. 7 C). Incubation with LPS, but not α-IgM, led to a strong increase in IL-10 secretion of both WT and NFATc1−/− B cells, again with higher IL-10 levels for NFATc1−/− than for WT B cells (Fig. 7 D). By adding B cells from WT or Cd23-cre x NFATc1flx/flx mice, which were stimulated overnight with α-IgM or LPS or left unstimulated, to allogenic T cells, NFATc1−/−

![Figure 6. Ablation of NFATc1 in B cells impairs their T cell help.](https://jem.rupress.org/)

(A and B) MLC. In A, 5 × 10^4 B cells from C57/BL6 WT or Cd23-cre x Nfatc1flx/flx mice were incubated with 5 × 10^4 CFSE-labeled CD4+ T cells from a BALB/c WT mouse. After 3 d, proliferation of T cells was measured. In B, B and T cells were cultured as in A and IL-2 secretion was measured by ELISA. In A, one typical assay out of three is shown; in B mean values of two assays are compiled. (C and D) Splenic B cells from WT and Cd23-cre x Nfatc1flx/flx mice were treated overnight with 10 µg/ml α-IgM and 1 µM (C) or 10 µM OVA323-339 peptide (D) followed by incubation with CFSE-stained 5 × 10^4 T cells from an OTII tg mouse (Barnden et al., 1998) for 3 d. In C, one typical assay out of three is shown. In D, mean values of three experiments are presented.
B cells suppressed the IFN-γ production of T cells, whereas WT B cells did not or only slightly affected T cell IFN-γ expression. This suppressive effect of NFATc1−/− B cells was markedly reduced by adding the IL-10–specific antibody JES5-2A5, which relieved IL-10–mediated inhibition of cytokine production of T cells by B cells (Lampropoulou et al., 2008). A very strong suppressive effect on IFN-γ production was exerted by recombinant IL-10 that could be relieved by adding α-IL-10. By adding α-IL-10 to T cell co-cultures, in part the suppressive effect of NFATc1−/− B cells could be relieved (Fig. 7 E), suggesting that an increase in IL-10 production by NFATc1−/− splenic B cells contributes to suppression of T cell proliferation in vitro.

These in vitro findings prompted us to investigate whether NFATc1−/− B cells affect the clinical course of mouse EAE (Hur et al., 2007), a mouse model for human multiple sclerosis.

Figure 7. NFATc1 ablation leads to an increase in IL-10 production and amelioration of the clinical course of EAE. (A) Increased CD19+CD5+CD1dhi B cell number in spleen freshly prepared from Cd23-cre x Nfatc1flx/flx mice, compared with WT mice. Six individual mice were investigated, and mean values are shown. (B) IL-10 production of unstimulated CD5+CD1dhi NFATc1−/− B cells or cells treated with α-IgM or LPS (10 µg/ml each) for 24 h and with T+I for the last 8 h. Cells were stained with antibodies directed against CD1d and CD5, and against IL-10 for intracellular flow cytometry. Shown are the results of one (out of five) typical experiment of IL-10 stains of CD5+CD1dhi B cells. (C) Increase in IL-10–producing CD138+CD19+ plasmablasts after 3 d in culture with LPS (and T+I for the last 6 h) in preparations of splenocytes from mice bearing Cd23-cre x Nfatc1flx/flx B cells. One typical assay out of three is shown. (D) IL-10 production by unstimulated WT and NFATc1−/− splenic B cells and cells stimulated by α-IgM or LPS for 72 h in culture. IL-10 was measured in ELISA, and mean values of three assays are shown. (E) MLC. Decreased IFN-γ production by CD4+ T cells incubated for 48 h in the presence of 0.5 µg/ml α-CD3 and, during the last 6 h, with T+I, without B cells or with unstimulated B cells or B cells prestimulated by α-IgM or LPS for 24 h from WT or mb1-cre x Nfatc1flx/flx mice. As indicated, 5 ng/ml recombinant IL-10 and/or 1 µg/ml of IL-10–specific antibody JES5-2A5 (Lampropoulou et al., 2008) were added to the cultures. One typical set out of three assays is shown. (F) EAE. By injection of MOG35-55 peptide in complete Freund’s adjuvant, and pertussis toxin at day 2 (Pierau et al., 2009), EAE was induced in mb1-cre x Nfatc1flx/flx and WT mice as indicated. The data show the mean clinical score + SEM from 3 independent experiments, with n = 9 (WT) and n = 14 (mb1-cre x Nfatc1flx/flx) mice.
which is strongly affected by IL-10 (Fillatreau et al., 2002; Bouaziz et al., 2008). By inducing adjuvant-driven EAE (Pierau et al., 2009) in mb1-cre x Nfatc1flx/flx and WT mice, we observed that NFATc1 ablation in BM B cells resulted in a much milder EAE than in control mice. Whereas in control mice the onset of EAE was detected 7–8 d after MOG35-55 peptide injection reaching EAE scores of 2–2.5 at day 20, for mb1-cre x Nfatc1flx/flx mice first EAE signs were obvious at day 11 upon MOG35-55 injection, reaching scores of 0.5–1.0 (Fig. 7 F). This shows that the expression of NFATc1 in B cells enhances the severity of EAE.

**DISCUSSION**
The results of this study show that ablation of NFATc1 in committed BM pro-B cells or in splenic B cells exerts a relative mild effect on the overall differentiation of peripheral B cells. Peritoneal CD5+ B1a cells are the notable exception because their generation is strictly dependent on NFATc1 expression (Fig. S2, E and F; Berland and Wortis, 2002, 2003). However, although NFATc1 activity seems to play a minor role in the development of B cells in BM and periphery, it is of crucial importance for the fate and function of B cells upon BCR triggering. Our data show that NFATc1 controls (a) the proliferation and AICD of splenic B cells (and plasmablasts) upon BCR stimulation, (b) the Ig class switch by IgG3 by the T1-type II antigen NP-Ficoll and the formation of IgG3+ plasmablasts in vitro, (c) the IL-10 production of CD1dhighCD5+ B cells that suppressed the IFN-γ production by and proliferation of co-cultivated CD4+ T cells, and (d) the clinical course of MOG-peptide/adjuvant-driven EAE, a model mouse of human multiple sclerosis. Because NFATc1 ablation led to a markedly decreased in the release of Ca2+ from endoplasmic stores, Ca2+ influx and Cn activity as well as in distinct changes in expression of genes that affect (or are affected by) Ca2+ levels, one may conclude that NFATc1 controls Ca2+ influx and Cn activity in peripheral B cells.

NFATc1−/− B cells share several characteristics with B cells from mouse models in that individual components of BCR signaling cascade have been inactivated. Such as with mice deficient for Btk (Kerner et al., 1995; Khan et al., 1995), PLC-γ2 (Hashimoto et al., 2000; Wang et al., 2000), and SLP-65/BLNK (Jumaa et al., 1999; Hadri et al., 2006), but studies on IP3R1 expression in T cells (Nagaleekar et al., 2008) did not reveal a positive effect of NFATs on IP3R1 expression in these cells. Likewise, the expression of PLCγ2, Btk, and SLP-65 appeared to be quite normal in NFATc1−/− B cells (unpublished data). However, large proteins such as IP3R1 and PLCγ2 are expressed from very complex genes that, like the Ran1 and Nfatc1 genes, might be under the control of multiple promoters and/or poly A sites and alternately spliced. It remains to be shown whether this is true for other molecules of BCR signaling.

Prominent upstream NFAT targets of Ca2+/Cn cascade are Ran1, the gene encoding the Cn inhibitor calcipression/DSCR1, and the Ppp3cb gene expressing CnA B, the B form of catalytic subunit A of Cn in lymphocytes. For both the Ran1-4 and Ppp3cb promoters, arrays of NFAT motifs have been described, and the binding of NFAT factors to these promoters has been reported for nonlymphoid cells (Rothermel et al., 2003; Oka et al., 2005). Our data indicate that in B cells Rcan1, CnA B, and NFATc1 form a regulatory network (Fig. S5 A) that helps to fine tune the proper expression of NFATc1, in particular of NFATc1-αA, and the activation of further NFATc factors during immune responses.

NFATc1 expression supports BCR-mediated proliferation and suppresses AICD of primary B cells (Fig. 2), e.g., in the early clonal expansion phase of an immune response. Although there are several studies on a direct NFAT-mediated
regulation of cell cycle mediators in T cells (Baksh et al., 2002; Caetano et al., 2002; Carvalho et al., 2007), in our microarray studies among the numerous cyclin, cdk, or cell cycle inhibitor genes we detected only the Cdk1 gene, encoding Cdc2 in B1, as a putative NFATc1 target gene in B cells (Table S1). This finding led us to conclude that in B cells, NFATc1 enhances numerous genes controlling cell cycle at moderate levels, and gene set enrichment analyses (Table I) support this view. One exception might be the Bcl2 gene (Table S2) that, in addition to its role in apoptosis, was described to retard cell cycle entry (Vairo et al., 2000). However, the detection of proapoptotic factors FasL, PD-1, and Light molecules as direct NFATc1 target and own experimental data (unpublished data) suggest that at very high concentrations NFATc1/αA could also act in a proapoptotic manner, killing the majority of amplified lymphocytes at the end of an immune response. But further experiments using more appropriate mouse models are necessary to substantiate this hypothesis.

The increase in CD1d<sup>high</sup>/CD5<sup>+</sup> B cells producing IL-10 suggests that NFATc1 affects the regulatory capacity of B cells. The Il10<sup>−/−</sup> gene belongs to the 37 genes whose expression was markedly increased in NFATc1<sup>−/−</sup> B cells (Table S2), and in 2 out of 3 microarray assays a similar increase was detected for the Il10 gene (unpublished data). This implies that the induction of NFATc1 in B cells suppresses, either directly or indirectly, IL-10 production. This conclusion is supported by the α-IgM−mediated decrease in IL-10 synthesis of CD1d<sup>high</sup>/CD5<sup>+</sup> B cells upon BCR triggering and NFATc1 induction, and suggests that the increase in IL-10−producing B cells contributes to the clinical course of EAE in mb1<sup>−/−</sup>× NFATc<sup>θh/θh</sup> mice. It has previously been shown that B cells play a key role in recovery from EAE by the secretion of IL-10 (Fillatreau et al., 2002). Along with studies on a regulatory role of B cells in the generation of EAE (Yanaba et al., 2008; Pöllinger et al., 2009), our findings lend support to this observation, but also suggest that an impaired antigen−presentation by NFATc1<sup>−/−</sup> B cells might ameliorate EAE symptoms (Fig. 6, C and D). In addition to IL-10, other candidates that affect EAE are IFN-γ and osteopontin/Spp1 (Hurst et al., 2007), which are both encoded by top ten NFATc1 target genes (Fig. 2 and Table S1).

Collectively, the data of our study show that the induction of NFATc1 plays an important role in the activation and function of splenic B cells upon BCR stimulation. Missing or incomplete NFATc1/αA induction appears to be one reason for the generation of B cell unresponsiveness whereas uncontrolled NFATc1/αA expression could lead to unbalanced immune reactions and autoimmune diseases. Experiments are in progress to elaborate more details of this view.

MATERIALS AND METHODS

Mice, cell isolation and cell cultures. The B cell stage-specific cre lines mb1 (Cd79a<sup>−/−</sup>-cre) (Hobeika et al., 2006), Cd23-cre, and Aida-cre (Kwon et al., 2008) have been described previously. Mice bearing a conditional allele of Nfatc1 (Nfatc1<sup>fl</sup>; also designated as Nfatc1<sup>2K</sup>) were generated by K. Mark Ansel, M.R. Müller, and A. Rao (Harvard Medical School, Boston, MA) by flanking exon 3 of the mouse Nfatc1 gene with loxP sites (to be described in detail elsewhere). All of the following gene-deficient mice were published previously: Btk (Khan et al., 1995); SLP-65 (Jamaa et al., 1999); IL-10 (Kühn et al., 1993); CD22 (Nitschke et al., 1997); Light (Scheu et al., 2002); and PD-1 (Nishimura et al., 1999). Transgenic Nfatc1-Egfp reporter mice were created upon insertion of a Egfp cDNA into exon 3 of mouse Nfatc1 locus cloned in a BAC vector spanning ~210 kb Nfatc1 DNA. Details of those BAC-tg mice will be published elsewhere. Animal experiments were performed according to project licenses (55.2-2531.01-53/10B), which are approved and controlled by the Regierung von Unterfranken, Würzburg. In most of experiments, 6-8 wk-old mice were used. Naïve splenic B cells were isolated using Miltenyi Biotec cell isolation kit and own experimental data (unpublished data) suggest that at very high concentrations NFATc1/αA could also act in a proapoptotic manner, killing the majority of amplified lymphocytes at the end of an immune response. But further experiments using more appropriate mouse models are necessary to substantiate this hypothesis.

EAE induction. Mice were immunized with 200 µg of myelin oligodendrocyte (MOG<sub>35-55</sub>) peptide in CFA (Sigma-Aldrich) containing 800 mg of heat-killed Mycobacterium tuberculosis (Difco) as described previously in detail (Perian et al., 2009). Animal experiments were approved by the state authorities of the Land Sachsen-Anhalt. Mice with a clinical score >2.5 were killed according to the animal statues. The MOG peptide was provided by D. Rheinhold (Institute of Medical and Clinical Immunology, Magdeburg, Germany).

Gene expression analysis and statistical evaluation. B cells from WT and mb1<sup>−/−</sup>× Nfatc1<sup>θh/θh</sup> mice were left unstimulated or stimulated with 10 µg/ml α-lgM. IL-2 secretion of T cells was measured with a Ready-Set-Go ELISA kit (eBioscience).
Western blotting. Western blots were performed by separation of whole protein extracts on PAGE-SDS gels followed by immunodetection of NFATc1 using the 7A6 mAb (obtained from BD or Santa Cruz Biotechnology) or polyclonal antibodies (pAb) specific for NFATc1 or NFATc2 (ImmunoGlobe). For detecting NFATc1/αA (Fig 1 B), a pAb raised against the NFATc1 α-peptide (ImmunoGlobe) was used. As loading control, filters were stained by Poncet red. Signals were developed by chemiluminescence detection using Super Signal (Thermo Fisher Scientific).

Apoptosis assays and flow cytometric analysis. Apoptosis assays were performed by staining with propidium iodide (PI, 125 µg/ml) and Annexin V-FITC (BD), incubated for 15 min at 37°C and analyzed on FACS®Calibur using CellQuest software (BD) and FlowJo (Tree Star, Inc.). Electronic compensation of the instrument was done to exclude overlapping emission spectra. In total, 10,000 events were acquired, the cells were gated, and dual parameter dot plots of FL1-H (x-axis; FITC-fluorescence) versus FL2-H (y-axis; PI-fluorescence) were gated in logarithmic fluorescence intensity. For IL-10 detection in specific B cell subset, nonsaturated cells or cells stimulated with α-IgM or LPS were treated by T+I and GolgiPlug for the last 8 h, blocked with mouse Fc receptor specific mAb (BD) and stained with CD5 PE and CD1d FITC mouse-specific antibodies (BD). Cells were fixed and permeabilized using the eBioscience fixing and permeabilizing kit according to manufacturer’s instructions. Next, cells were stained with anti-mouse IL-10 APC antibody (eBioscience). Unstimulated cells and isotype controls were also used in flow cytometric analysis.

For the determination of cell cycle phase distribution of nuclear DNA, cells were first fixed in 100% methanol, permeabilized, and nuclear DNA was labeled with PI (125 µg/ml) after RNase (10 µl from 2 mg/ml stock) treatment. Cell-cycle phase distribution of nuclear DNA was determined on a FACS®Calibur using CellQuest software, fluorescence (FL2-A) detector equipped with a 488-nm Argon laser light source and a 623-nm band pass filter (linear scale). In total, 10,000 events were acquired for analysis. CellQuest and FlowJo statistics was used to quantify the data at different phases of the cell cycle. Histogram display of FL2-A (x-axis, PI-fluorescence) versus counts (y axis) has been displayed in Fig S3 C.

Immunizations and ELISAs. NP-Ficoll (10 µg/mouse in PBS) or NP-KLH (100 µg/mouse, Alum precipitated) was i.p. injected into age-matched mice. Levels of NP-specific antibodies were determined by ELISA with 10 µg/ml NP-BSA coated MaxiSorp plates (Nunc). A sample of pooled sera from days 7 and 21 was used as standard and included on every ELISA plate. Total Ig titers from naive mice were determined by ELISA on PolySorp plates (Nunc) coated with isotype-specific antibodies and monoclonal antibody, and isotype antibodies served as standards (SouthernBiotech). Serum samples were applied as serial dilutions, and their concentration was quantified against the standard curve. Isotype-specific antibodies coupled to alkaline phosphatase were used for detection.

In vitro class-switching assays. 105 splenocytes were cultured in 1 ml X-vivo medium without or with LPS (10 µg/ml) or with LPS + IL-4 (10 ng/ml) for 2 d, followed by RNA extraction with TRIzol and cDNA synthesis using the Script cDNA Synthesis kit (Bio-Rad Laboratories). PCR reactions were performed with the primers for the detection of IgG3 and IgG1 circular transcript as described (Kinoshita et al., 2001), 2.5 × 106 splenocytes were cultured in 1 ml X-vivo medium and stimulated without or with LPS for 6 d. Supernatants were collected and subjected to ELISA. Unlabeled goat anti-mouse IgM and IgG3 were used to coat ELISA plates, mouse IgM and IgG3 (0.1 mg/ml each) were used as standards, and alkaline phosphatase coupled isotype-specific antibodies were used for detection. Signals were displayed in Fig S3 C.

In vitro IL-10 secretion assay. 4 × 106 cells were incubated in 200 µl X-vivo medium with or without α-IgM or LPS for 72 h, and supernatants were collected. ELISA was performed using a mouse IL-10 ELISA kit (eBioscience) according to the manufacturer’s instructions.

Immunohistochemistry. For antigen localization (Fig 5), 150 µg TNP-Ficoll was injected into the tail vein of mice. 60 min later, B cells were prepared for flow cytometry from one part of the spleen, and the other part was used for histological examination. Cryosections were stained with biotin-conjugated hamster α-TNP-Ficoll IgG (BD), α-CD169/MOMA-1 (Serotec), α-lgD or α-lgM (SouthernBiotech).

For nuclear localization of NFATc2 (Fig 3 F), splenic B cells stimulated for 24 h by 10 µg/ml α-IgM and 5 µg/ml α-CD40 and stimulated by T+I for 30 min were harvested by cytopsin, dried overnight, fixed in acetone, and stained with antibodies directed against NFATc2 (BD or Santa Cruz Biotechnology). Nuclear localization of NFATc2 in 100 cells from three individual experiments was determined by confocal microscopy.

Calcium measurements. 5 × 105 of freshly prepared murine splenic B cells were incubated in 700 µl X-vivo medium or RPMI 1640 (Invitrogen) containing 5% FCS (PAN), 1 µM Indo-1-AM (Invitrogen), and 0.015% Phorlec F127 (Invitrogen) at 30°C for 25 min. The cell suspension was then diluted with 700 µl medium containing 10% FCS and incubated at 37°C for another 10 min. The cells were washed twice with Krebs-Ringer solution containing 10 mM Hepes, pH 7.0, 140 mM NaCl, 4 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM glucose, and 0.5 mM EGTA and diluted to a concentration of 106 cells per ml. For measurement, the cells were diluted 1:10 in Krebs-Ringer solution and a baseline was recorded for 60 s. Ca2+ movement was assessed after stimulation of the cells with α-IgM (Jackson ImmunoResearch Laboratories), followed by the addition of Drag to denoted concentrations after 2 min of recording. Increases in free intracellular Ca2+ were measured in real-time on a LSR II (BD). Raw data files were transferred to FlowJo software and are presented as a median and in comparative overlay analyses.

Determination of Cn activity and cellular CnA levels. Cn activity of freshly prepared naive splenic B cells was determined using BIOMOL’s non-radioactive Cn kit according to the manufacturer’s instructions. In brief, 3 × 105 splenic B cells were lysed in 50 µl of lysis buffer (+ protease inhibitors) by pipetting and vortexing, followed by high speed centrifugation for 45 min at 4°C in an Eppendorf centrifuge. For removing free phosphate, supernatants were passed through a desalting spin column, stored on ice, or quick-frozen in liquid nitrogen before storage at −70°C. Aliquots of supernatant corresponding to ~3 × 108 of B cells were tested in 50 µl assays, containing the R11 phosphopeptide Cn substrate. The release of free phosphate was determined in a Malachite green assay. Incubations of parallel assays containing either no substrate, or 10 mM EGTA and EGTA+okaic acid indicated the specificity of Cn reaction. In immunoblots, a CnA α/β-specific antibody (BD) was used to determine CnA levels (Fig 3 E).

ChIP assays. ChIP assays were performed according to a previously published protocol (Soutoglou and Talalabnis, 2002) and slightly modified. In brief, the chromatin of 0.5–1 × 108 splenocytes was cross-linked using 1% formaldehyde, and the reaction was quenched with 125 mM glycine. Nuclei were isolated and treated with DNase I, sonicated, and, after preclaring with protein A–Sepharose that was blocked with salmon sperm DNA, protein–DNA complexes were immunoprecipitated using an NFATc1 mAb (BD), or an unrelated GST antibody as control. After washing, elution, and reversal of cross-links, DNA was isolated by phenol/chloroform and used in PCR reactions that were performed with the following primers amplifying the promoters of the following genes: B2, forward 5’-CCTGTGTCGCAATTT AGCTCA-3’ and reverse 5’-CCTTTTCTGTAGACTCCTGGA-3’; Fasl, forward 5’-ACTTGTGTAGTGTGACCTC-3’ and reverse 5’-GATTCTCAGTGTTAGCTCA-3’; Pdld1, forward 5’-CATGGTAGTGAAGGATCACA-3’ and reverse 5’-GCGGAATACTTCCACTGGA-3’; Rcan1, forward 5’-GGGGAATTGATGCTTGGA-3’ and reverse 5’-GAGTCGTCGGCTTCTCATTCCA-3’; Spp1, forward 5’-GACACGACAGGCTGTTGATT-3’ and reverse 5’-AAACGGAGTTGAGGACCC-3’; Rcan1, forward 5’-GAGTCGTCGGCTTCTCATTCCA-3’; Tnfsf14, forward 5’-GTGCGCGTCTGTTGTCACTGCA-3’ and reverse 5’-GGTGAAGATGTTGCACTGGA-3’; Hpy, forward 5’-TTTCTTCCA GGTTGCCAGTC-3’ and reverse 5’-CAGTCTGTTCCACTCACC-3’;
**REFERENCES**


