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The Calcium Sensors STIM1 and STIM2 Control B Cell Regulatory Function through Interleukin-10 Production

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SUMMARY

A chief Ca^{2+} entry pathway in immune cells is store-operated Ca^{2+} (SOC) influx, which is triggered by depletion of Ca^{2+} from the endoplasmic reticulum (ER). However, its physiological role in B cells remains elusive. Here, we show that ER calcium sensors STIM1- and STIM2-induced SOC influx is critical for B cell regulatory function. B cell-specific deletion of STIM1 and STIM2 in mice caused a profound defect in B cell receptor (BCR)-induced SOC influx and proliferation. However, B cell development and antibody responses were unaffected. Remarkably, B cells lacking both STIM proteins failed to produce the anti-inflammatory cytokine IL-10 because of defective activation of nuclear factor of activated T cells (NFAT) after BCR stimulation. This resulted in exacerbation of experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis. Our data establish STIM-dependent SOC influx as a key signal for B cell regulatory function required to limit autoimmunity.

INTRODUCTION

B cells play an essential role in the regulation of immune responses. Upon encountering their cognate antigens through their B cell receptors (BCRs), B cells take on multiple functions including antibody production, antigen-presentation, and effector T cell differentiation. In addition to protective roles against pathogenesis, B cells also serve as negative regulators of autoimmunity by secreting anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) (Lund and Randall, 2010), which have been shown to contribute to the suppression of experimental autoimmune encephalomyelitis (EAE) (Fillatreau et al., 2002; Matsushita et al., 2008), inflammatory bowel disease (Mizoguchi et al., 2002), and collagen-induced arthritis (Evans et al., 2007; Mauri et al., 2003).

A central response to BCR stimulation is intracellular Ca^{2+} elevation, which leads to activation of many transcription factors to regulate the gene expression that mediates diverse genetic programs (Feske, 2007; Macian, 2005). Increasing cytosolic Ca^{2+} concentrations can occur via two main mechanisms: Ca^{2+} release from endoplasmic reticulum (ER) stores and Ca^{2+} influx from outside the cell across the plasma membrane. BCR stimulation activates several protein tyrosine kinases and subsequently phospholipase C (PLC)-γ2. Activated PLC-γ2 generates the second messenger inositol-1,4,5-trisphosphate, which binds to its receptor in the ER membrane, leading to the rapid and transient release of Ca^{2+} into the cytosol from ER stores. In turn, the decrease of ER luminal Ca^{2+} concentration triggers the opening of Ca^{2+} channels in the plasma membrane, which can induce a sustained influx of extracellular Ca^{2+}. These processes are known as store-operated Ca^{2+} (SOC) influx, which is an essential pathway for maintaining sustained Ca^{2+} signaling in immune cells (Baba and Kurosaki, 2009; Parekh and Putney, 2005). The induction of SOC influx critically requires the ER Ca^{2+} sensor, stromal interaction molecule 1 (STIM1) (Liou et al., 2005; Roos et al., 2005), and the SOC channel Orai1 (also called CRACM1) (Feske, 2007). STIM1 functions as the chief ER-resident protein to activate SOC influx by detecting ER Ca^{2+} depletion in many cells including mast cells (Baba et al., 2008), T cells (Oh-Hora et al., 2008), platelets (Varga-Szabo et al., 2008), and macrophages (Braun et al., 2009). STIM2, a related homolog of STIM1, also activates SOC influx, but is less effective than STIM1 in T cells (Oh-Hora et al., 2008). It has an additional distinct function in regulating basal cytosolic and ER Ca^{2+} concentrations (Brandman et al., 2007).

The importance of Ca^{2+} influx is underscored by reports that patients with mutations in the *Orai1* or *Stim1* genes presented with severe combined immunodeficiency and defects in SOC influx in lymphocytes (Feske et al., 2006; Picard et al., 2009). *Stim1* mutation has also been associated with autoimmune symptoms. However, abnormal T cell functions in these diseases make it difficult to understand B cell-intrinsic functions. Although Ca^{2+} signaling in B cells is widely assumed to be essential, the importance of SOC influx for physiological B cell functions was unclear.

To directly assess the involvement of SOC influx in B cells, we generated mice with B cell-specific deletions of *Stim1* and *Stim2*. We found that STIM1 and STIM2 critically regulated BCR-induced SOC influx and proliferation in vitro, and yet they were dispensable for B cell development and antibody responses in vivo. The ablation of STIM1 and STIM2 in B cells

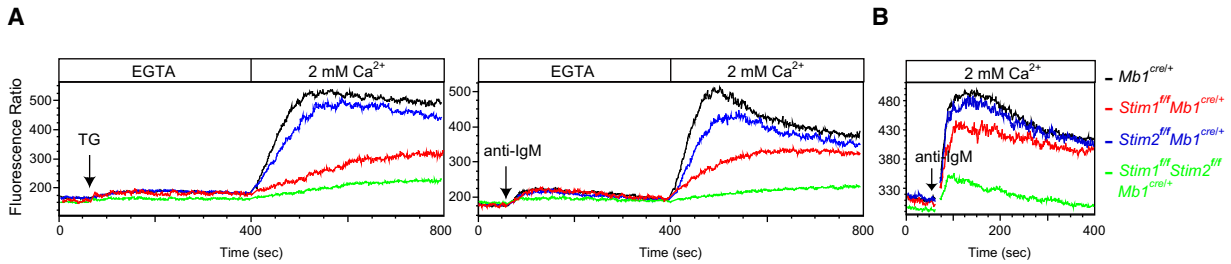

Figure 1. STIM1 and STIM2 are Required for SOC Influx in B Cells

(A) Ca^{2+}-mobilization profiles, monitored by Indo-1 AM imaging. Ca^{2+} release was elicited in $Mb1^{cre/+}$, $Stim1^{f/f}Mb1^{cre/+}$, $Stim2^{f/f}Mb1^{cre/+}$, and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells by stimulation with thapsigargin (TG) or anti-IgM F(ab)'$_2$ (anti-IgM) in Ca^{2+}-free conditions (0.5 mM EGTA), and Ca^{2+} influx was induced by restoration of the extracellular Ca^{2+} concentration to 2 mM.

(B) Ca^{2+}-mobilization profiles in the presence of 2 mM extracellular Ca^{2+} in $Mb1^{cre/+}$, $Stim1^{f/f}Mb1^{cre/+}$, $Stim2^{f/f}Mb1^{cre/+}$, and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells after stimulation with anti-IgM. (A and B) $B220^+$ cells were gated and analyzed. All values are plotted as the FL5/FL4 fluorescence ratio (FL4 = 500–520 nm; FL5 = 400–420 nm). Data are representative of at least three independent experiments.

caused defects in activation of nuclear factor of activated T cells (NFAT), B cell IL-10 production, and suppression of an EAE model of autoimmune disease. These results provide genetic evidence for the significance of STIM-dependent Ca^{2+} signaling in B cell regulatory function.

RESULTS

B Cell-Specific Deletions of STIM1 and STIM2 in Mice

We generated mice with specific deletions of the genes encoding STIM1 and STIM2 in B cells by using the cre recombinase–*loxP* system. *LoxP*-flanked (floxed [f]) *Stim1* (Baba et al., 2008) and *Stim2* (Figure S1A available online) were deleted from B cells by crossing of $Stim1^{f/f}$ and $Stim2^{f/f}$ mice with mb-1 cre mice carrying a *cre* transgene in one of the *Mb1* alleles ($Mb1^{cre/+}$) (Hobeika et al., 2006). We confirmed that $Stim1^{f/f}Mb1^{cre/+}$, $Stim2^{f/f}Mb1^{cre/+}$, and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice had recombination of these genes in B cells, resulting in the complete ablation of STIM1 and STIM2 proteins in splenic B cells by immunoblot analysis (Figure S1B). Of note, B cell development in spleen, bone marrow, and peritoneal cavity of $Stim1^{f/f}Mb1^{cre/+}$, $Stim2^{f/f}Mb1^{cre/+}$, and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice was equivalent to that of $Mb1^{cre/+}$ mice (Figure S1C). Therefore, STIM1 and STIM2 are dispensable for normal B cell development.

Deficiency of STIM1 and STIM2 in B Cells Impairs BCR-Mediated SOC Influx

To determine the effects of STIM1 and STIM2 on SOC influx in B cells, we monitored cytosolic Ca^{2+} in $Stim1^{f/f}Mb1^{cre/+}$, $Stim2^{f/f}Mb1^{cre/+}$, $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$, and control $Mb1^{cre/+}$ B cells after Ca^{2+} depletion from ER stores in response to anti-IgM or thapsigargin (TG), an ER Ca^{2+} pump inhibitor. $Stim1^{f/f}Mb1^{cre/+}$ B cells showed considerably less Ca^{2+} influx after treatment with TG or anti-IgM, whereas $Stim2^{f/f}Mb1^{cre/+}$ B cells had a mild decrease (Figure 1A). The loss of both STIM1 and STIM2 led to almost complete suppression of SOC influx induced by stimulation with TG or anti-IgM (Figure 1A). To further assess the effect of STIM proteins on Ca^{2+} mobilization, we stimulated B cells with anti-IgM in the presence of extracellular Ca^{2+}. STIM1- or STIM2-deficient B cells exhibited a modest reduction in Ca^{2+} rises, whereas cytosolic Ca^{2+} concentrations were much lower when both STIM1 and STIM2 were absent (Figure 1B). These results collectively indicate that both STIM1 and STIM2 positively and cooperatively regulate SOC influx after BCR stimulation and that the effect of STIM2 is smaller than STIM1.

BCR-Mediated Proliferation and Survival Require SOC Influx

We next examined proliferative responses to mitogenic stimuli. Purified B cells were labeled with cabroxyfluorescein diacetate succinimidyl ester (CFSE), and cell division was then monitored by CFSE dilution. BCR stimulation induced several rounds of cell division in control B cells, whereas $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells failed to divide in response to anti-IgM stimulation (Figure 2A). The absence of STIM1, but not STIM2, reduced the frequency of BCR-evoked proliferation. On the other hand, anti-CD40 or lipopolysaccharide (LPS)-mediated proliferation was normal in B cells lacking STIM proteins (Figure 2A). Importantly, stimulation with anti-CD40 or LPS did not induce Ca^{2+} influx even in control B cells (data not shown), indicating that anti-CD40 and LPS activate B cells in a STIM-independent manner. We also characterized the proliferative response to costimulation of anti-IgM with IL-4 or anti-CD40. The defective proliferation in BCR-stimulated $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells was considerably restored by the addition of anti-CD40, while IL-4 had a smaller effect (Figure 2B). In accordance with the proliferation results, B cells lacking both STIM1 and STIM2 did not survive after BCR stimulation, but normally did in response to anti-CD40 or LPS (Figure 2C). Again, costimulation with anti-IgM and anti-CD40 or IL-4 significantly restored the impaired BCR-induced survival of $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells, although IL-4 did considerably less for their survival than did anti-CD40 (Figure 2D). These results demonstrate an essential STIM-mediated Ca^{2+} signaling requirement for B cell proliferation and survival when induced by stimulation with BCR, but not anti-CD40 or LPS. Signals evoked by anti-CD40 or IL-4 can compensate for STIM protein deficiencies after BCR stimulation.

Normal Antibody Responses in Mice with B Cells Lacking STIM1 and STIM2

To assess the consequences of altered in vitro B cell functions on the immune system, we investigated the abilities of gene

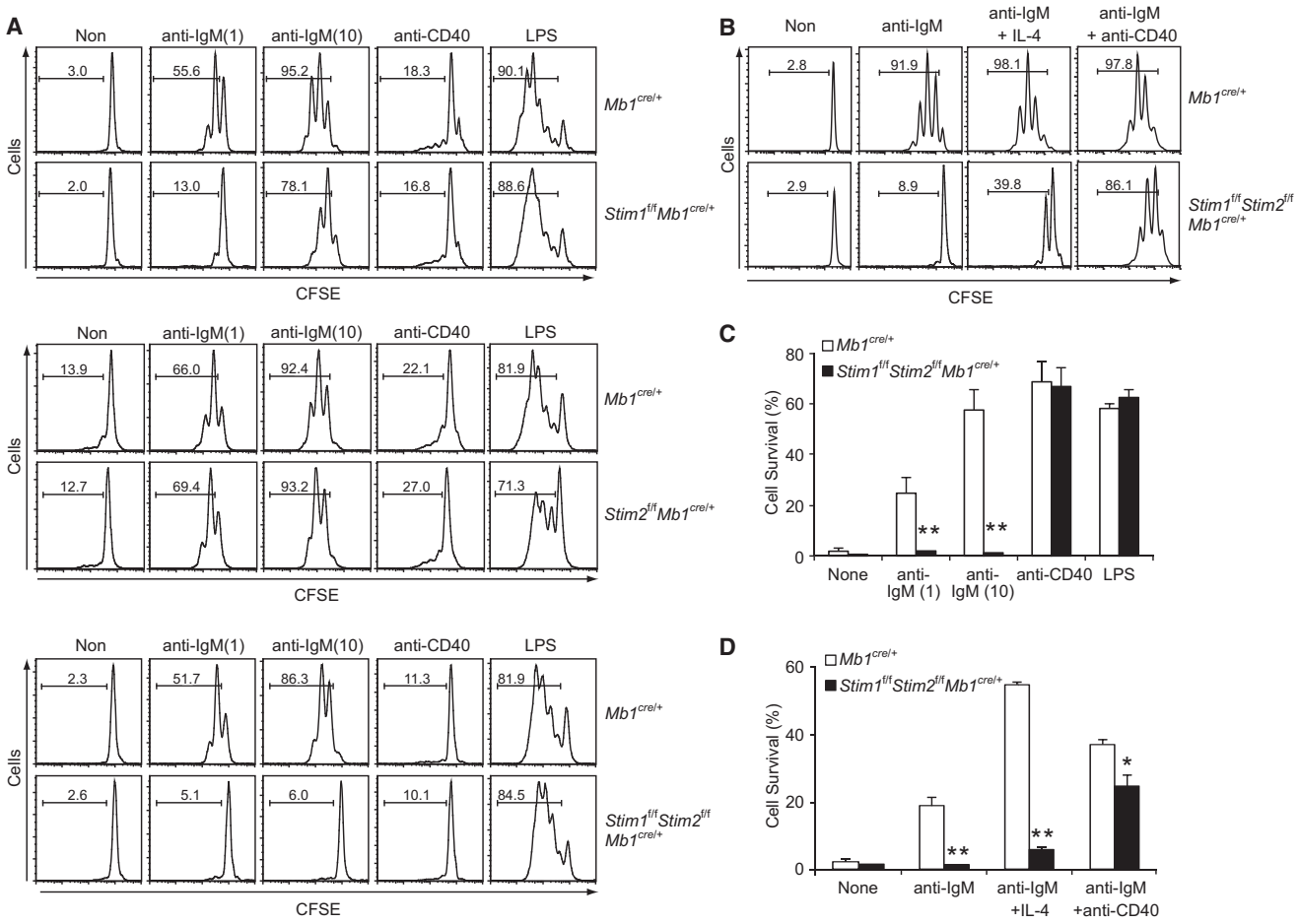

Figure 2. BCR-Mediated Proliferation and Survival Require SOC Influx

(A and B) Proliferation of $Mb1^{cre/+}$, $Stim1^{f/f}Mb1^{cre/+}$, $Stim2^{f/f}Mb1^{cre/+}$, and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells labeled with CFSE and stimulated for 72 hr with anti-IgM (1 or 10 μg/ml), anti-CD40 (10 μg/ml) or LPS (10 μg/ml) (A) or with a combination of anti-IgM (10 μg/ml) and either IL-4 (40 ng/ml) or anti-CD40 (10 μg/ml) (B). Percentages of proliferating B cells were assessed by CFSE dilution.

(C and D) Survival of $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells cultured as described in (A) (for C) and (B) (for D), stained with TO-PRO-3, and presented as a percentage of viable TO-PRO-3^{-} cells.

(A–D) Results represent one of three similar experiments. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.001$ versus $Mb1^{cre/+}$ B cells.

targeted mice to produce antibodies in vivo. Serum titers of IgM, IgG1, IgG2b, IgG3, and IgA were comparable among unimmunized $Stim1^{f/f}Mb1^{cre/+}$, $Stim2^{f/f}Mb1^{cre/+}$, $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$, and control $Mb1^{cre/+}$ mice (Figure S2A). To evaluate T cell-independent (TI) antibody responses, we elicited immunization of mice of all four genotypes with the 4-hydroxy-3-nitrophenyl (NP)-LPS or NP-Ficoll. The mutant mice showed normal antigen-specific IgM and IgG3 responses to both TI antigens (Figures 3A and 3B and Figure S2B). We also examined T cell-dependent (TD) antibody responses by immunization with NP-chicken γ globulin (CGG) and monitored the production of antigen-specific IgM and IgG1 antibodies. Mutant mice showed comparable primary immune responses to those of control mice (Figure 3C and Figure S2C). After rechallenge with NP-CGG, antigen-specific antibody production was also within the normal range, indicating that the ability to mount primary and memory responses is not affected by the complete absence of STIM proteins. During TD responses, proliferating B cells in lymphoid organs form germinal centers essential for the normal generation of memory B cells and plasma cells that produce high-affinity antibodies (Klein and Dalla-Favera, 2008). As expected, $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice had similar percentages of germinal center B cells (defined as $B220^{+}Fas^{hi}GL7^{+}$; Figure S2D), and the affinity maturation of the antibody responses was normal (Figure S2E). In vitro plasma cell formation of $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells in response to LPS or IL-4 plus anti-CD40 was also intact (Figure S2F). Thus, we conclude that STIM-mediated Ca^{2+} influx is not essential either for TI or TD antibody responses.

STIM-Dependent Ca^{2+} Influx Is Required for B Cell IL-10 Production

Published studies have shown that in addition to protection against pathogens, B cells negatively regulate hyperinflammation during autoimmune diseases by production of anti-inflammatory cytokines such as IL-10 and TGF-β (Mauri and Ehrenstein, 2008). To determine whether B cell regulatory

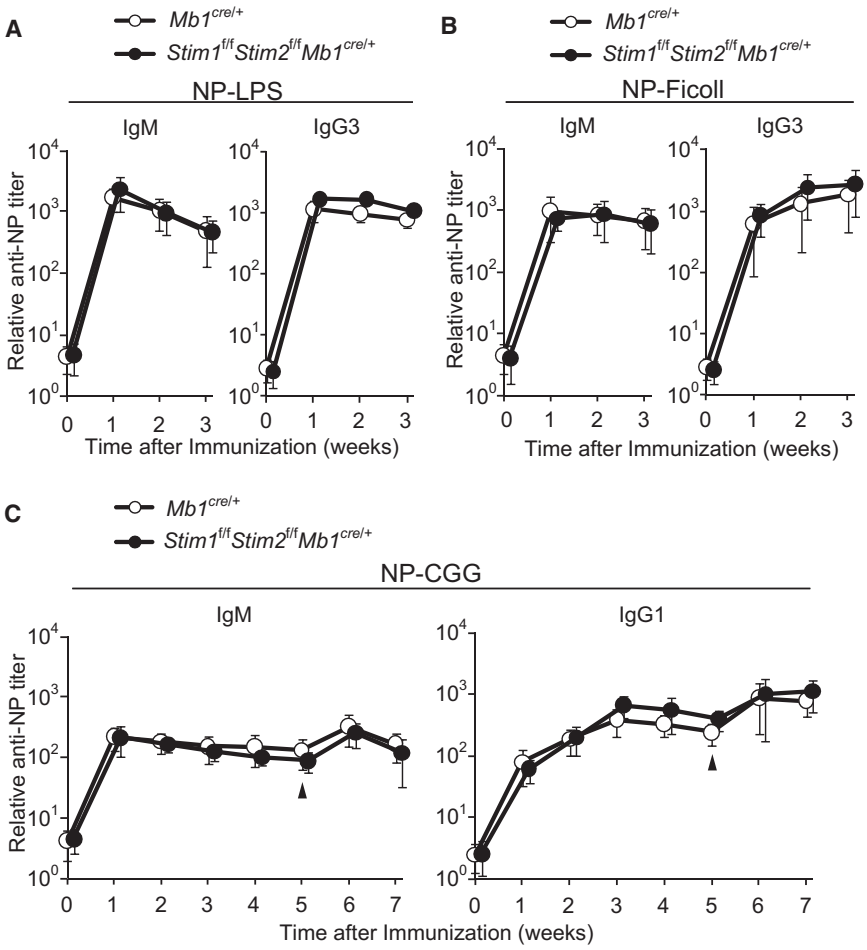

Figure 3. STIM1 and STIM2 are Dispensable for Antigen-Specific Antibody Production

(A and B) IgM and IgG3 NP-specific antibody responses of $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice immunized with NP-LPS (A) or NP-Ficoll (B), as assessed by NP-specific ELISA.

(C) IgM and IgG1 NP-specific antibody responses of $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice immunized with NP-CGG in alum and re-challenged with NP-CGG 5 weeks after primary immunization, as assessed by NP-specific ELISA. Arrowheads indicate the day of secondary immunization. Data are presented as mean ± SD of five (A and B) or ten (C) mice for each genotype at each time point.

function was affected by loss of STIM1 and STIM2, we first measured synthesis of mRNA encoding IL-10 and TGF-β in splenic B cells after mitogenic stimulation with phorbol myristate acetate (PMA) and ionomycin. We found that transcripts of IL-10, but not TGF-β, were considerably impaired in the absence of STIM proteins (Figure 4A and data not shown). In addition, intracellular cytokine staining and ELISA assay showed that $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells had compromised IL-10 production compared with control or single-deficient B cells (Figures 4B and 4C).

We further evaluated the requirement of STIM proteins for BCR-induced IL-10 secretion. BCR ligation alone did not induce IL-10 secretion by naive $Mb1^{cre/+}$ B cells (Figure 4D) given that this requires signals from CD40 and/or Toll-like receptors (TLRs) (Lampropoulou et al., 2008; Yanaba et al., 2009). Given that BCR stimulation is assumed to potentiate IL-10 secretion when B cells are first preactivated with anti-CD40 or TLRs agonists (Lampropoulou et al., 2010), this point was investigated with B cells by a two-step stimulation that first applied anti-CD40, and CpG (a TLR9 agonist) or LPS (a TLR4 agonist), and then anti-IgM. Small amounts of IL-10 were secreted equivalently by $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells after stimulation with CpG or LPS, but not anti-CD40 (Figure 4D). The following stimulation of LPS-activated $Mb1^{cre/+}$ B cells with anti-IgM markedly augmented IL-10 production compared to LPS alone, whereas CpG preactivation resulted in no increase of IL-10 secretion (Figure 4D). Although preactivation of $Mb1^{cre/+}$ B cells with anti-CD40 enhanced BCR-induced IL-10 production, the effect was much less than with LPS. These results indicate that BCR signaling reinforced IL-10 production efficiently in LPS-activated B cells. In striking contrast, $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells secreted much less IL-10 after restimulation with anti-IgM (Figure 4D). $Stim1^{f/f}Mb1^{cre/+}$ or $Stim2^{f/f}Mb1^{cre/+}$ B cells exhibited a modest reduction of IL-10 secretion, although less pronounced than $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells (Figure S3). Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis confirmed that synthesis of IL-10 mRNA was also considerably inhibited in the absence of STIM proteins (Figure 4E).

To address the capacity of the putative regulatory B cell subset with a $CD1d^{hi}CD5^{+}$ phenotype (Yanaba et al., 2008) to produce IL-10 in the absence of STIM proteins, we measured the secretion of IL-10 by purified $CD1d^{hi}CD5^{+}$ B cells from $Mb1^{cre/+}$ or $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice after stimulation with LPS and anti-IgM. $CD1d^{hi}CD5^{+}$ B cells, but not $CD1d^{low}CD5^{-}$ B cells, from $Mb1^{cre/+}$ mice showed marked IL-10 secretion whereas $CD1d^{hi}CD5^{+}$ B cells from $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice secreted much less IL-10 (Figure 4F). It is noteworthy that the absolute numbers of $CD1d^{hi}CD5^{+}$ B cells was not affected by loss of STIM proteins (Figure 4G), suggesting that impaired IL-10 secretion in $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells was not due to the aberrant development of these regulatory B cells. Thus, we conclude that BCR-induced IL-10 production is dependent on STIM proteins.

STIM-Dependent IL-10 Production Requires Calcineurin-NFAT Activation

The results described above suggested that the impaired Ca^{2+} influx in $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells leads to defects in the intracellular signaling events required for IL-10 production. In

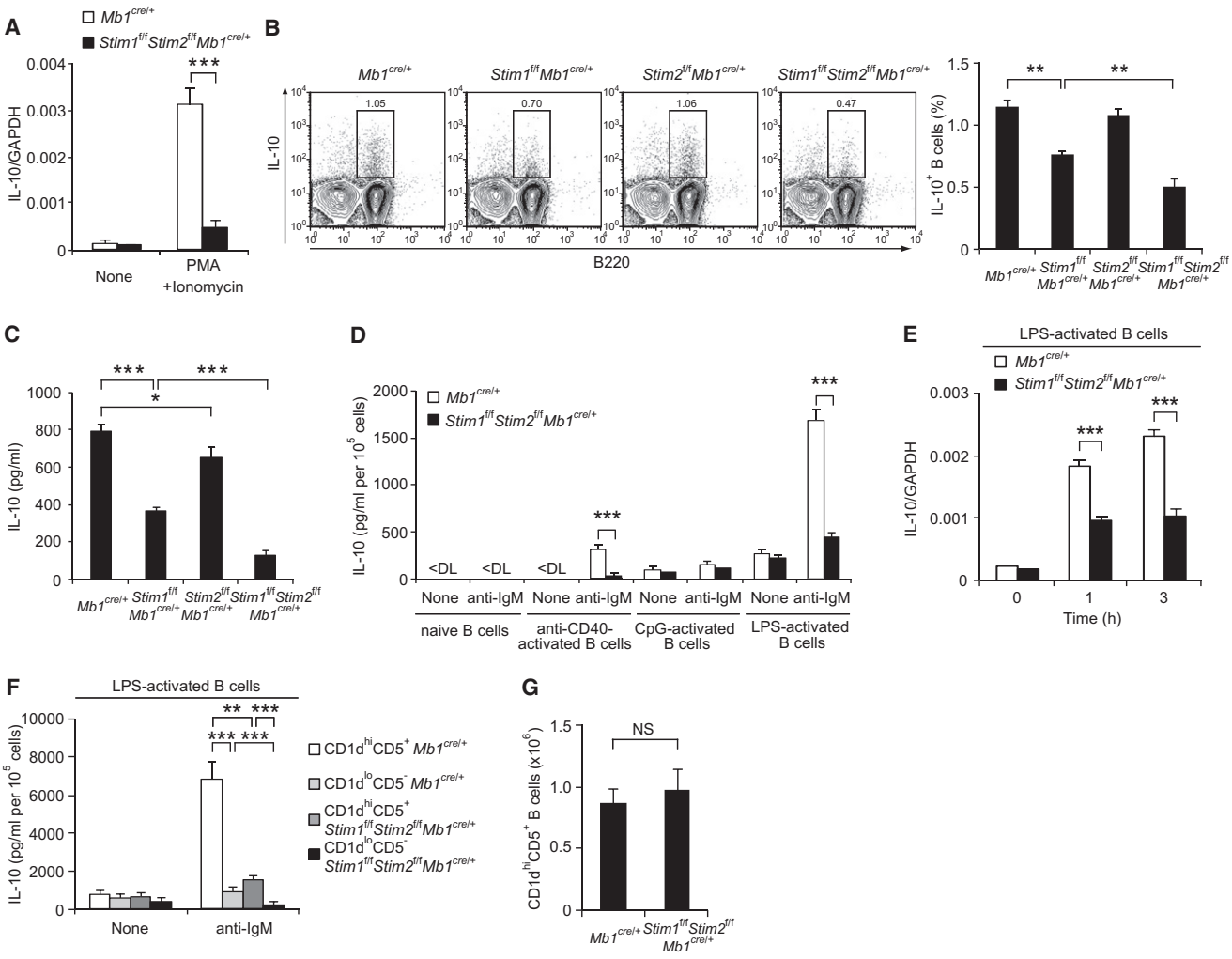

Figure 4. Loss of STIM1 and STIM2 Impairs IL-10 Production by B Cells

(A) Quantitative RT-PCR of mRNA encoding IL-10 in splenic B cells from $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice after stimulation with PMA (100 ng/ml) and ionomycin (1 μM) for 4 hr, normalized to the expression of GAPDH.

(B) Production of IL-10 by splenic B cells from $Mb1^{cre/+}$, $Stim1^{f/f}Mb1^{cre/+}$, $Stim2^{f/f}Mb1^{cre/+}$, and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice after stimulation with PMA and ionomycin for 5 hr in the presence of monensin, as assessed by intracellular cytokine staining. Numbers above the outlined area indicate the percentage of cells in the gate. Data are representative of three independent experiments. On the right, the frequency of $IL\text{-}10^+$ B cells is shown. Data shown are pooled from three independent experiments.

(C) ELISA of IL-10 by splenic B cells after stimulation with PMA and ionomycin for 24 hr.

(D) ELISA of IL-10 by $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells (naive B cells) and their B cells cultured with anti-CD40, CpG, or LPS for 48 hr (anti-CD40-, CpG-, and LPS-activated B cells) followed by stimulation with anti-IgM for 24 hr. < DL, below detection limit.

(E) Quantitative RT-PCR of mRNA encoding IL-10 in LPS-activated $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells after stimulation with anti-IgM for 1 hr or 3 hr, normalized to the expression of GAPDH.

(F) ELISA of IL-10 by $CD1d^{hi}CD5^+B220^+$ and $CD1d^{lo}CD5^-B220^+$ B cells isolated from $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice and cultured with LPS for 48 hr followed by stimulation with anti-IgM for 24 hr.

(G) Absolute number of $CD1d^{hi}CD5^+B220^+$ B cells in spleen from $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice. NS, no significance.

(A, D, E, and F) Data are representative of at least three independent experiments.

(C and G) Data shown are pooled from three independent experiments.

All data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

a variety of cells, IL-10 expression is regulated by mitogen-activated protein kinases (MAPKs) and various Ca^{2+}-activated transcription factors such as CREB, NF-κB, and NFAT (Saraiva and O'Garra, 2010). Thus, we examined the effects of the deficiency in STIM proteins on the activation of these molecules. To directly compare the intracellular signaling events and IL-10 secretion, we first activated $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells with LPS and then stimulated them with anti-IgM for various time periods. The activation status of CREB, MAPKs (Jnk, p38, Erk), NF-κB, and NFAT were assessed by immunoblotting. $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells had similar phosphorylation of CREB, Jnk, p38, Erk, and the NF-κB inhibitor

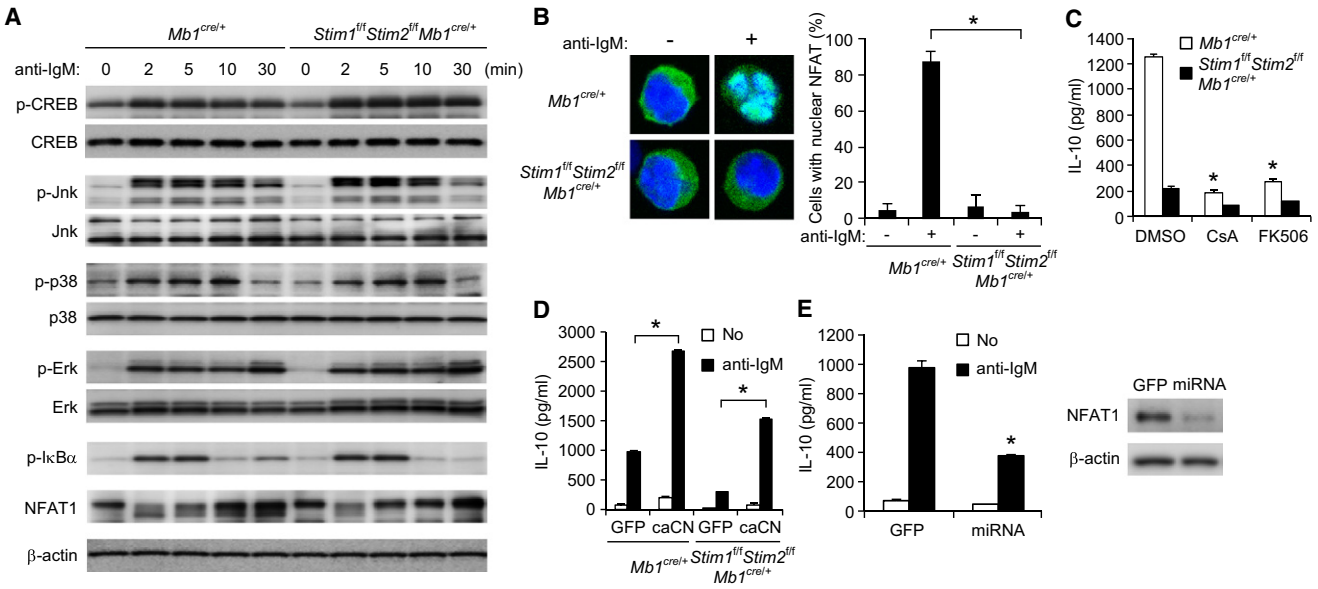

Figure 5. STIM-Mediated IL-10 Production Requires NFAT Activation

(A) Immunoblot analysis of whole-cell lysates of $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells cultured with LPS for 48 hr (LPS-activated B cells) followed by stimulation with anti-IgM. p-, phosphorylated; β-actin, loading control.

(B) Confocal microscopy of NFAT nuclear translocation in $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells retrovirally transduced with GFP-NFAT4 (green) and stimulated with anti-IgM for 30 min followed by DAPI staining of the nucleus (blue). A proportion of cells with NFAT nuclear translocation is shown on the right. Data shown are pooled from three independent experiments. *$p < 0.001$ versus $Mb1^{cre/+}$ B cells.

(C) ELISA of IL-10 by LPS-activated $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells treated with dimethyl sulfoxide (DMSO), cyclosporine A (CsA; 1 μM), and FK506 (1.25 μM) for 1 hr followed by stimulation with anti-IgM for 24 hr. *$p < 0.001$ versus $Mb1^{cre/+}$ B cells treated with DMSO.

(D) ELISA of IL-10 by LPS-activated $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells retrovirally transduced with GFP alone or constitutively active calcineurin (caCN) followed by stimulation with anti-IgM for 24 hr.

(E) ELISA of IL-10 production by LPS-activated $Mb1^{cre/+}$ B cells retrovirally transduced with GFP alone or microRNA for NFAT1 (miRNA) followed by stimulation with anti-IgM for 24 hr. Immunoblot analysis of NFAT1 in whole-cell lysates is shown on the right.

(A, C, D and E) Results represent one of three similar experiments. Data are presented as mean ± SEM. *$p < 0.001$ versus GFP^+ B cells (D and E).

IκBα (Figure 5A), indicating that these signaling events are not globally affected by STIM proteins. In contrast, BCR-induced dephosphorylation of NFAT was markedly impaired in $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells, although transiently observed at 2 min after BCR stimulation (Figure 5A). The loss of STIM1 alone, but not STIM2, also reduced NFAT1 activation (Figure S4A). Very similar results were obtained with naive B cells stimulated with anti-IgM alone (Figure S4B), suggesting the significance of BCR signals themselves. Furthermore, microscopic analysis revealed a drastic defect in the nuclear translocation of green fluorescent protein (GFP)-NFAT4 expressed in $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells after BCR stimulation (Figure 5B). These results suggest the importance of STIM-mediated Ca^{2+} influx for NFAT activation induced by BCR stimulation.

A key question is whether NFAT is vital for BCR-induced IL-10 production. NFAT is activated by Ca^{2+} and calmodulin-dependent phosphatase calcineurin. The calcineurin inhibitors cyclosporine A (CsA) and FK506, which inhibited the activation of NFAT (Figures S4C and S4D), completely suppressed IL-10 secretion (Figure 5C). Reciprocally, expression of a constitutively active form of calcineurin A (caCN) substantially increased BCR-induced IL-10 production in both $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells (Figure 5D). Furthermore, silencing of NFAT1 in $Mb1^{cre/+}$ B cells greatly lowered IL-10 production after BCR stimulation (Figure 5E). These data collectively provide evidence that STIM-dependent NFAT activation promotes IL-10 production during BCR signaling.

Severe EAE Development in $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ Mice

IL-10-producing B cells have a protective role in the T cell-mediated autoimmune disease EAE (Fillatreau et al., 2002). To determine whether STIM-mediated IL-10 production contributes to B cell regulatory function in vivo, we elicited EAE in $Mb1^{cre/+}$ and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice. After immunization with myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}), $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice exhibited intense exacerbation of development and progression of EAE (Figure 6A and Table 1). The times of disease onset were equivalent, but incidences and mean maximum scores were significantly higher in $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice (Table 1). Histopathological analyses revealed enhanced inflammatory cell infiltration and demyelination in the spinal cords of $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice (Figure 6B). We also observed increased numbers of $CD8^+$ and $CD4^+$ T cells, but not $B220^+$ B cells and $Gr\text{-}1^+$ neutrophils, in the spinal cords of $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice (Figure 6C). In addition to effecter T cells such as $CD4^+$ T cells producing IFN-γ (Th1 cells) and IL-17 (Th17 cells), the infiltration of $Foxp3^+$ regulatory T (Treg) cells and IL-10-producing $CD4^+$ T cells was augmented (Figure 6C), which might represent

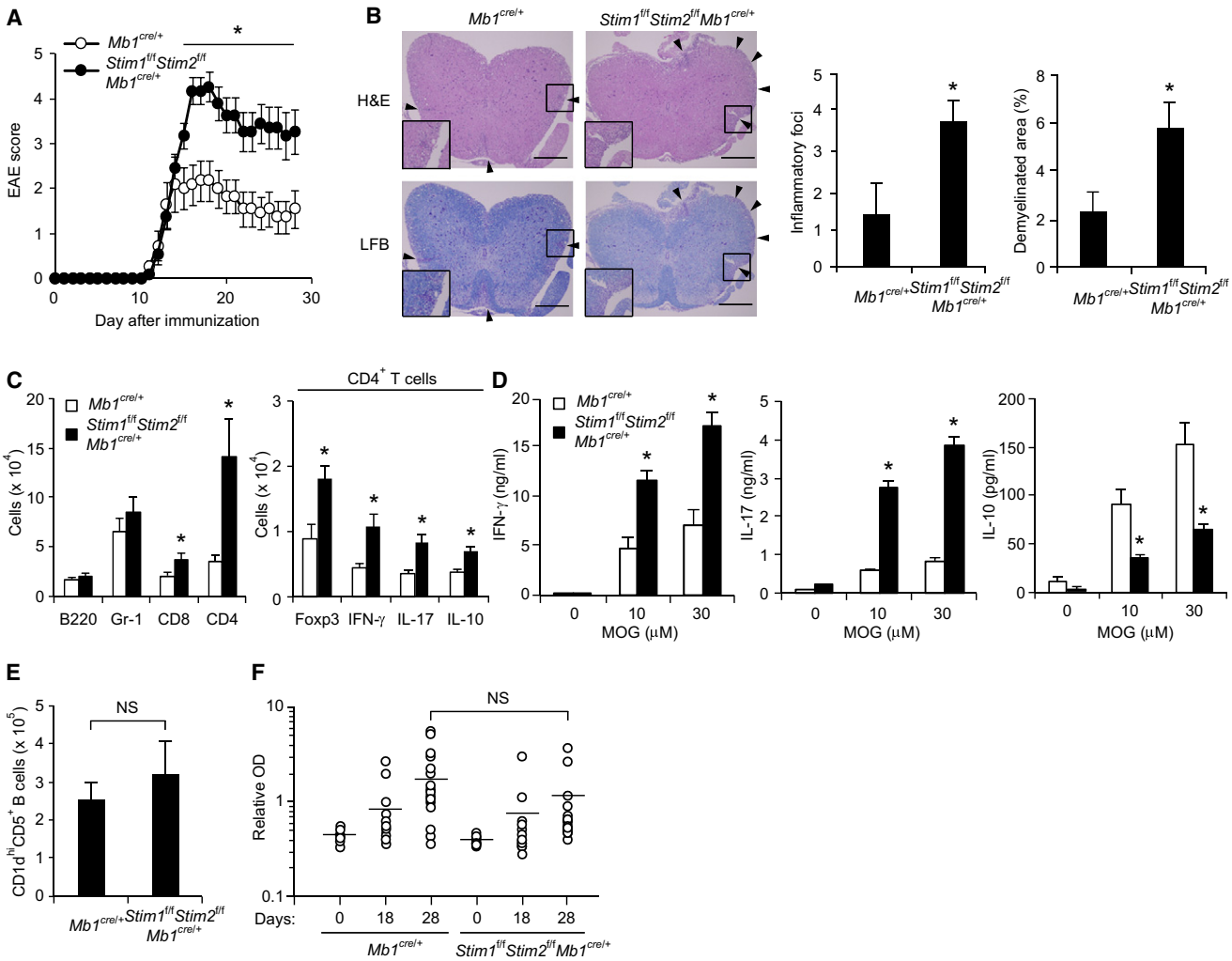

Figure 6. Double Deficiency in STIM1 and STIM2 in B Cells Exacerbates the Severity of EAE In Vivo

(A) Clinical EAE scores for *Mb1*$^{cre/+}$ and *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ mice immunized with MOG_{35-55}. The EAE score is shown as mean ± SEM for 12 mice.

(B) Histological analysis of spinal cords harvested from *Mb1*$^{cre/+}$ and *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ mice 28 days after MOG_{35-55} immunization. Sections were stained with hematoxylin and eosin (H&E) and Luxol Fast Blue (LFB). Arrowheads indicate inflammatory foci and demyelinated area. Insets (bottom-left corners) show inflammatory foci and demyelinated area in boxes in main images. Original magnification, x 60 (main images) or × 100 (insets); scale bars represent 100 μm. The number of inflammatory foci and the demyelinated area is shown on the right.

(C) Absolute number of cells from spinal cords harvested from *Mb1*$^{cre/+}$ and *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ mice 28 days after MOG_{35-55} immunization.

(D) ELISA of IFN-γ, IL-17 and IL-10 by splenocytes isolated from *Mb1*$^{cre/+}$ and *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ mice 28 days after EAE induction followed by stimulation with MOG_{35-55} for 48 hr. *$p < 0.05$ versus *Mb1*$^{cre/+}$ mice (A-D).

(E) Absolute number of $CD1d^{hi}CD5^{+}B220^{+}$ B cells in spleen harvested from *Mb1*$^{cre/+}$ and *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ mice 28 days after MOG_{35-55} immunization. Data are presented as mean ± SEM for five mice.

(F) ELISA of IgG anti-MOG in sera collected from *Mb1*$^{cre/+}$ and *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ mice 18 and 28 days after MOG_{35-55} immunization. Each circle represents an individual mouse. Horizontal bars indicate mean values. NS, no significance (E and F).

(A–F) Data shown are pooled from at least three independent experiments.

a process to compensate for hyperinflammation in *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ mice. It was also noted that the deletion of STIM proteins in B cells did not affect the suppressive capacity of splenic Treg cells (Figure S5A) and IL-10 expression of CNS-infiltrating $CD4^{+}$ T cells, Gr-1^{+} neutrophils and $F4/80^{+}$ macrophages, in EAE-induced mice (Figure S5B). EAE is caused by autoantigen-specific Th1 and Th17 cells that produce IFN-γ and IL-17, respectively (Kuchroo et al., 1993; Park et al., 2005), whereas the resolution of EAE is associated with increased production of IL-10 (Bettelli et al., 1998). Upon in vitro stimulation with MOG_{35-55}, splenocytes from *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ mice 28 days after EAE induction produced more IFN-γ and IL-17, but less IL-10 compared with control cells (Figure 6D). Of note, numbers of $CD1d^{hi}CD5^{+}$ B cells in spleen, which could produce IL-10 to suppress EAE (Matsushita et al., 2008), were not different between *Mb1*$^{cre/+}$ and *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ mice after MOG_{35-55} immunization (Figure 6E). Although it has been suggested that MOG_{35-55}-specific antibodies enhance EAE

Table 1. EAE Clinical Scores

Group	Incidence	Mean Day of Onset (mean ± SEM)	Mean Maximum Score (mean ± SEM)
Mb1$^{cre/+}$	8/10 (80%)	12.4 ± 0.5	2.8 ± 0.6
Stim1$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$	11/11 (100%)	12.8 ± 0.5	4.5 ± 0.3*

Assessment of clinical EAE includes the number of mice that developed disease, the day of disease onset, and the maximum clinical score of each mouse obtained over the entire observation period. Data shown are pooled from two independent experiments; *$p < 0.05$ versus *Mb1*$^{cre/+}$ mice.

severity (Linington et al., 1988; Lyons et al., 1999), serum titers of anti-MOG IgG 18 and 28 days after EAE induction were not significantly influenced by loss of STIM proteins (Figure 6F). Collectively, these results indicate that STIM proteins in B cells are essential to limit the development and progression of EAE. Moreover, this phenomenon was not associated with differentiation of regulatory B cells or antibody responses against autoantigens.

STIM-Dependent IL-10 Production by B Cells Suppresses EAE Development

To determine whether dysregulation of EAE in *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ mice arises from the defective production of antigen-specific IL-10 by B cells, we isolated splenic B cells from mice 28 days after EAE induction (MOG-primed B cells) and measured their IL-10 secretion in recall response to MOG_{35-55}. Stimulation of control B cells with anti-CD40 alone induced modest IL-10 production, but *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ B cells secreted less IL-10 (Figure 7A). Concomitant treatment with anti-CD40 and MOG_{35-55}, but not an irrelevant antigen, proteolipid protein peptide ($PLP_{178-191}$), largely augmented IL-10 production by control B cells, whereas *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ B cells failed to respond to any antigens (Figure 7A). The impaired IL-10 production in mutant B cells was also confirmed by intracellular cytokine staining (Figure 7B). Thus, STIM proteins are required for antigen-specific IL-10 secretion by B cells from EAE-induced mice.

We next examined whether STIM proteins in B cells were responsible for the suppression of EAE severity in vivo. MOG-primed B cells taken from *Mb1*$^{cre/+}$ and *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ mice were adoptively transferred to *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ mice before EAE induction. The mice that received MOG-primed *Mb1*$^{cre/+}$ B cells, but not *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ B cells, resolved EAE symptoms (Figure 7C). Furthermore, to evaluate whether the defective IL-10 production by *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ B cells leads to the exacerbation of EAE, we retrovirally expressed IL-10-internal ribosome entry site (IRES)-GFP constructs in *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ B cells and adoptively transferred them to B cell-deficient μMT mice, which themselves develop severe EAE as a result of their lacking B cells (Wolf et al., 1996). Adaptive transfer of *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ B cells transduced with IL-10-IRES-GFP, but not those transduced with GFP alone, ameliorated EAE clinical signs of μMT mice (Figure 7D). Therefore, we conclude that STIM1 and STIM2 are essential for B cell regulatory function through IL-10 production, which controls the development of EAE disease.

DISCUSSION

In this study, we identified a physiological function for SOC influx in B cells by analyzing mice with B cell-specific deletions of STIM1 and STIM2. Our findings indicate that STIM1 and STIM2 are critical for BCR-induced SOC influx, proliferation, NFAT activation, and subsequent IL-10 production in vitro. Although STIM proteins are not essential for B cell development and antibody responses, these molecules suppressed EAE via an IL-10-dependent mechanism.

B cells from *Stim1*$^{f/f}$*Mb1*$^{cre/+}$ mice had severely impaired SOC influx, in accordance with our previous study investigating chicken STIM1-deficient DT40 B cells (Baba et al., 2006). Moreover, the loss of STIM2 in B cells had less effect on SOC influx, similar to a previous report investigating STIM2-deficient T cells (Oh-Hora et al., 2008). Importantly, B cells lacking both STIM1 and STIM2 showed far more suppressed SOC influx than B cells lacking STIM1 alone. Previous reports have shown that STIM2 is more sensitive for the reduction in the ER luminal Ca^{2+} than STIM1 and elicits persistent SOC influx along with a small amplitude of Ca^{2+} mobilization (Brandman et al., 2007), which suggests that influences of STIM1 and STIM2 on Ca^{2+} influx have different kinetics and duration. Although further investigation is needed to discern the precise functional differences between the two in B cells, our findings clearly point out the positive and cooperative function of STIM1 and STIM2 for efficient Ca^{2+} influx activation in B cells.

The defects in BCR-induced B cell proliferation and survival in the absence of STIM proteins indicate the importance of SOC influx, and CD40 signaling, which is STIM-independent, restores the impaired BCR signals. Because BCR and CD40 signals induce B cell proliferation and survival through several common molecules including Cyclin D2 and the antiapoptotic protein Bcl-x_L (Grillot et al., 1996; Lam et al., 2000), the compensational effect of CD40 may result from their activation. Alternatively, given the synergistic effect between signals through CD40 and BCR (Bishop, 2004), their simultaneous stimulation may allow activation of a Ca^{2+}-independent pathway not used by either receptor alone.

During TD immune responses, B cells undergo clonal expansion by receiving cognate T cell help through cytokine stimulation and CD40 engagement in germinal centers, leading to formation of high-affinity plasma cells and memory cells. In our study, in vitro B cell proliferation induced by BCR stimulation was entirely dependent on STIM proteins, whereas in vivo antibody responses to TD antigens seemed to be STIM independent. These findings raise at least two possibilities. First, requirements for Ca^{2+} signaling may be different between in vitro and in vivo responses. Transient Ca^{2+} release from the ER may be sufficient for activating molecules responsible for in vivo clonal expansion. Second, the signals delivered by cytokines or CD40 ligation through the interaction of B cells with T cells in germinal centers may be sufficient to counteract defects in STIM-dependent responses. This is supported by our data that in vitro costimulation of anti-IgM with anti-CD40 or IL-4, albeit to a lesser degree, can greatly restore impaired BCR-induced proliferative and survival responses of *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ B cells. In addition to TD responses, TI antibody production was not affected by loss of STIM proteins. Because TI responses are

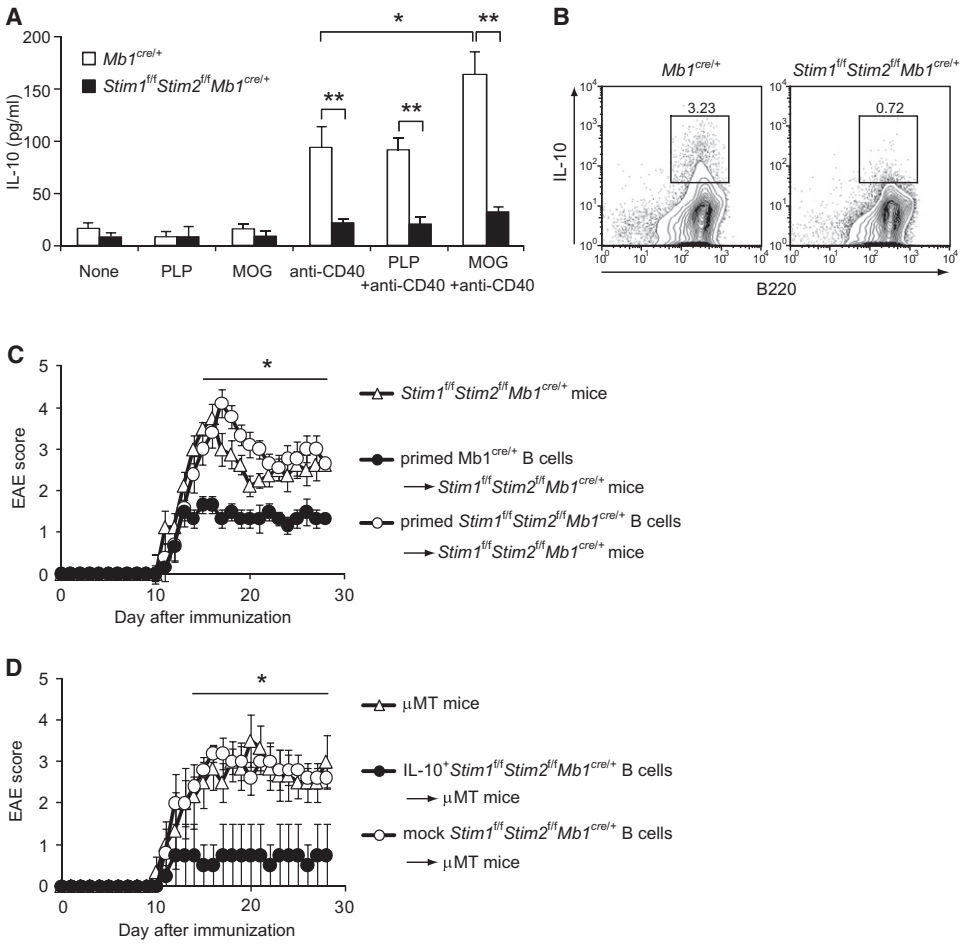

Figure 7. STIM-Dependent IL-10 Production by B Cells Suppresses EAE Development

(A) ELISA of IL-10 by splenic B cells isolated from *Mb1*$^{cre/+}$ and *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ mice 28 days after EAE induction followed by stimulation with MOG_{35-55}, $PLP_{178-191}$ and/or anti-CD40 for 5 days. Data are presented as mean ± SEM. *p < 0.05, **p < 0.001.

(B) Production of IL-10 by splenic B cells isolated from *Mb1*$^{cre/+}$ and *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ mice 28 days after EAE induction, stimulated with MOG_{35-55} and anti-CD40 for 72 hr and then restimulated with PMA and ionomycin for 5 hr in the presence of monensin, as assessed by intracellular cytokine staining. Results represent one of three similar experiments.

(C) Clinical EAE scores for *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ mice immunized with MOG_{35-55} after injecting splenic B cells harvested from *Mb1*$^{cre/+}$ and *Stim1*$^{f/f}$*Stim2*$^{f/f}$ *Mb1*$^{cre/+}$ mice 28 days after EAE induction (primed *Mb1*$^{cre/+}$ and *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ B cells). The EAE score is shown as mean ± SEM for six to ten mice. *p < 0.05 versus primed *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ B cells.

(D) Clinical EAE scores for μMT mice immunized with MOG_{35-55} after injecting *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ B cells retrovirally transduced with GFP alone (mock *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ B cells) or IL-10-IRES-GFP (IL-10^{+} *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ B cells). The EAE scores are shown as mean ± SEM for four to six mice. *p < 0.05 versus Mock *Stim1*$^{f/f}$*Stim2*$^{f/f}$*Mb1*$^{cre/+}$ B cells.

(A, C, and D) Data shown are pooled from two independent experiments.

considered to be BCR-mediated immune responses, our data indicate that in vitro BCR-mediated B cell proliferation does not directly correspond to TI responses in vivo. Given that in vivo immune responses are complicated and influenced by diverse cytokines, complement receptor signals, and interactions with other cells, our results indicate that multiple signals during immunization permit in vivo antibody responses independent of STIM1 and STIM2.

It is known that B cells can secrete IL-10, which is essential for suppression of EAE autoimmune disease (Fillatreau et al., 2002). EAE is induced by immunization of mice with MOG_{35-55} together with *Mycobacterium tuberculosis* that serves as an agonist for TLR2 and TLR4. Ablation of TLR2, TLR4, or TLR signaling adaptor molecule MyD88 in B cells impair IL-10 production and thereby exacerbated EAE development (Lampropoulou et al., 2008). However, TLR stimulation is not enough, given that activation via BCR and CD40 is also necessary for B cells to suppress autoimmunity. This follows from the fact that bone marrow chimeric mice with B cells bearing a fixed surface IgM antigen receptor or lacking CD40 show no recovery from EAE (Fillatreau et al., 2002). Nevertheless, in vitro, TLR stimulation, but not anti-IgM stimulation alone, allows B cells to secrete IL-10. These data suggest that stepwise stimulation or its combination will determine the ability to produce IL-10. Indeed, our results showing that B cells first activated with LPS robustly promote IL-10 secretion after restimulation with BCR support

the idea of a stepwise stimulation mechanism for IL-10 production. Furthermore, our finding that such BCR-dependent IL-10 production is diminished in B cells lacking STIM proteins strongly suggests that SOC influx induced by BCR stimulation drives IL-10 expression.

Although the mechanism for IL-10 production by B cells is unclear, we here have identified NFAT as a crucial transcription factor responsible for IL-10 production during BCR ligation. This implies that the failure of STIM-deficient B cells to produce IL-10 upon BCR stimulation may have resulted from a lack of continuous NFAT activation. This interpretation is supported by many studies showing that optimal cytokine production by T cells requires sustained Ca^{2+} influx and prolonged nuclear localization of NFAT (Feske, 2007). IL-10 expression is controlled by various molecular mechanisms under different stimuli in many types of cells (Saraiva and O'Garra, 2010). In agreement with our study, others have shown that the calcineruin-NFAT pathway is involved in IL-10 production probably by Ca^{2+}-related stimuli in neutrophils, dendritic cells, and Th2 and Th17 cells (Ghosh et al., 2010; Goodridge et al., 2007; Greenblatt et al., 2010). Thus, the calcineurin-NFAT pathway may represent a common mechanism for Ca^{2+}-drived IL-10 expression through STIM proteins. It should be noted that BCR stimulation alone can activate NFAT, but not the induction of IL-10 secretion in naive B cells. Although our results do not directly address the issue of why preactivation with LPS is necessary for BCR-evoked IL-10 production, we have three speculations. First, TLR signaling may cause B cell differentiation, thereby allowing them to produce BCR-dependent IL-10. It has been reported that IL-10 is produced exclusively by $CD138^+CD19^+$ plasmablast and/or plasma cells during *Salmonella* infection (Neves et al., 2010). When cultured with LPS in vitro, naive B cells differentiate into plasma cells via plasmablasts, which suggests that some LPS-activated B cells may become plasmablasts that predominantly secrete BCR-dependent IL-10. Second, TLR-signaling may be required for activation of transcription factors that collaborate with NFAT for IL-10 expression. Indeed, NFAT proteins interact with many transcription factors including AP-1, IRF4, GATA3, C/EBP, cMAF, and Mef2 to produce various cytokines in T cells (Macian, 2005), which suggests that additional components downstream of TLR signals serve as NFAT transcriptional partners to express IL-10 in B cells. Finally, TLR signals may cause changes in the chromatin structure at the *Il10* locus for efficient IL-10 production induced by following BCR signals. Several studies have suggested the importance of chromatin remodeling as initial events leading to IL-10 transcription (Saraiva and O'Garra, 2010). Thus, the mechanism of IL-10 expression merits further investigation.

Our in vitro observations of impaired IL-10 production by B cells in the absence of STIM proteins are consistent with the defective suppression of autoimmune disease in $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice. The functional importance of STIM proteins in B cells was shown by the impaired amelioration of EAE development due to a failure of antigen-specific IL-10 production by B cells. That MOG-primed B cells from $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice secreted less IL-10 in response to in vitro recall MOG and anti-CD40 stimulation compared to control mice emphasizes the importance of SOC influx for IL-10 secretion by autoantigen-specific B cells. In contrast, CD40 ligation alone showed less IL-10 secretion in MOG-primed $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ B cells, which may explain the significance of in vivo BCR stimulation for generating IL-10-producing B cells given that in vitro CD40-mediated signals are independent of STIM proteins. Although the nature of splenic regulatory B cells remains unclear, previous studies have shown that adoptive transfer of a splenic $CD1d^{hi}CD5^+$ B cell subset selectively normalizes EAE in B cell-depleted mice (Matsushita et al., 2008) or CD19-deficient mice (Matsushita et al., 2010), which suggests that it is a central subset of regulatory B cells in EAE. In our study, the numbers of splenic $CD1d^{hi}CD5^+$ B cell subset were not affected during EAE development by loss of STIM proteins, which suggests that the inability to suppress EAE in $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice may be caused by a functional defect in IL-10 production by regulatory B cells, but not its developmental defect. However, the adaptive transfer of $CD1d^{hi}CD5^+$ B cells from naive $Mb1^{cre/+}$ mice into $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice 24 hr before EAE induction did not reduce disease severity (data not shown). It may be possible that $CD1d^{hi}CD5^+$ B cells may not be the main or the only regulatory B cell subset in this model. Another likely explanation is that $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ recipient mice have normal number of $CD1d^{hi}CD5^+$ B cells, which might compete with adoptively transferred $CD1d^{hi}CD5^+$ B cells for antigen response and/or colonization to suppress T cell-mediated inflammation. In line with this possibility, CD19-deficient mice or mice injected with anti-CD20 mAb used in previous studies lack endogenous $CD1d^{hi}CD5^+$ B cells. In addition, when wild-type recipient mice are given $CD1d^{hi}CD5^+$ B cells from naive mice 24 hr before EAE induction, the reduction of EAE severity is not detectable (Matsushita et al., 2010). Therefore, further studies are needed to elucidate the importance of STIM proteins for in vivo suppressive capacity of $CD1d^{hi}CD5^+$ B cells, and the involvement of unidentified autoantigen-reactive B cells that potentiate or produce IL-10 remains possible. In summary, our results define a physiological function of SOC influx in B cells and formally demonstrate that STIM1 and STIM2 are critical mediators of B cell regulatory function. They limit autoimmune disease by supporting IL-10 production.

EXPERIMENTAL PROCEDURES

Mice and Immunization

Mice with the *loxP*-flanked *Stim1* allele have been described previously (Baba et al., 2008). The generation of $Stim2^{f/f}$ mice is described in detail in the Supplemental Experimental Procedures. The mb-1 cre (Hobeika et al., 2006) and μMT mice (Kitamura et al., 1991) have been described previously. We generated $Stim1^{f/f}Mb1^{cre/+}$, $Stim2^{f/f}Mb1^{cre/+}$, and $Stim1^{f/f}Stim2^{f/f}Mb1^{cre/+}$ mice by crossing of $Stim1^{f/f}$ and/or $Stim2^{f/f}$ mice with mb-1 cre mice. Mice were given intraperitoneally (i.p.) with 100 μg of NP-CGG in alum, 50 μg NP-Ficoll, or 50 μg NP-LPS (Biosearch Technologies). For secondary immunization, mice were given i.p. with 50 μg NP-CGG without adjuvant. Mice were maintained under specific pathogen-free conditions, and all protocols were approved by the RIKEN Animal Committee.

Ca^{2+} Measurement

Cytosolic Ca^{2+} concentrations were measured as described previously (Baba et al., 2006). Splenocytes were loaded with indo-1 acetoxylmethylester (Indo-1 AM) and Pluronic F-127 (Invitrogen) and stained with antibody to B220. Cells were stimulated with 10 μg/ml anti-mouse IgM $F(ab)'_2$ (Jackson Immunoresearch) or 2 μM thapsigargin (Calbiochem). Changes in fluorescence intensity were monitored on a LSR flow cytometer (BD Biosciences).

EAE Induction

EAE was induced by subcutaneous immunization with 200 μg of MOG_{35-55} (Toray Research Center) emulsified in complete Freund's adjuvant (CFA) containing 500 μg of heat-killed mycobacterium tuberculosis H37RA (Sigma-Aldrich). Mice also received 200 ng of pertussis toxin (List Biological laboratories) i.p. in 0.2 ml of PBS on the same day and 2 days later. Clinical signs of EAE were assessed daily with a 0-6 scoring system (0, no signs; 1, tail limpness; 2, impaired righting reflex; 3, hind limb weakness; 4, hind limb paralysis; 5, hind limb paralysis with fore limb weakness; and 6, death).

Adoptive Transfer

Purified B cells (1×10^7 cells) from spleens of mice 28 days after MOG_{35-55} immunization were transferred intravenously into *Stim1*$^{f/f}$*Stim2*$^{f/f}$ *Mb1*$^{cre/+}$ mice. B cells retrovirally transduced with GFP alone or IL-10-IRES-GFP were sorted by a FACSAria cell sorter (BD Biosciences). A total of 1×10^6 GFP^+ B cells were transferred intravenously into μMT mice 24 hr before EAE induction.

Statistical Analysis

Statistical analyses were performed with the two-tailed unpaired Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.immuni.2011.03.016.

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