A Role for IRF8 in B Cell Anergy

Simanta Pathak, Shibin Ma, Vipul Shukla and Runqing Lu

*J Immunol* 2013; 191:6222-6230; Prepublished online 11 November 2013;
doi: 10.4049/jimmunol.1301169
http://www.jimmunol.org/content/191/12/6222

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2013/11/12/jimmunol.1301169.DC1

**References**

This article cites 44 articles, 20 of which you can access for free at:
http://www.jimmunol.org/content/191/12/6222.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
A Role for IRF8 in B Cell Anergy

Simanta Pathak,1 Shibin Ma,1 Vipul Shukla, and Runqing Lu

B cell central tolerance is a process through which self-reactive B cells are removed from the B cell repertoire. Self-reactive B cells are generally removed by receptor editing in the bone marrow and by anergy induction in the periphery. IRF8 is a critical transcriptional regulator of immune system development and function. A recent study showed that marginal zone B cell and B1 B cell populations are dramatically increased in IRF8-deficient mice, indicating that there are B cell–developmental defects in the absence of IRF8. In this article, we report that mice deficient for IRF8 produced anti-dsDNA Abs. Using a hen egg lysozyme double-transgenic model, we further demonstrate that B cell anergy was breached in IRF8-deficient mice. Although anergic B cells in the IRF8-proficient background were blocked at the transitional stage of development, anergic B cells in the IRF8-deficient background were able to mature further, which allowed them to regain responses to Ag stimulation. Interestingly, our results show that IRF8-deficient B cells were more sensitive to Ag stimulation and were resistant to Ag-induced cell death. Moreover, our results show that IRF8 was expressed at a high level in the anergic B cells, and an elevated level of IRF8 promoted apoptosis in the transitional B cells. Thus, our findings reveal a previously unrecognized function of IRF8 in B cell anergy induction. The Journal of Immunology, 2013, 191: 6222–6230.

It has been estimated that almost 70% of newly generated immature B cells are self-reactive (1). B cell central tolerance is a process through which self-reactive B cells are rendered nonself-reactive (2). Central tolerance consists of receptor editing, anergy induction, and clonal deletion. In the bone marrow, self-reactive B cells are initially removed from the repertoires through receptor editing and deletion (3). The self-reactive B cells that somehow escape deletion in the bone marrow are functionally inactivated by anergy in the periphery. Anergy refers to a persistent state of unresponsiveness to Ag stimulation, and anergic B cells are arrested in their development at the transitional stage (2). The majority of self-reactive B cells are inactivated by anergy, and breach in B cell anergy has been linked to development of autoimmune diseases (2).

Three groups of proteins are critical for B cell anergy induction. The first group is the molecules involved in BCR signaling. It has been shown that chronic Ag stimulation leads to monophosphorylation of Igα/β, resulting in biased activation of phosphatase SHIP-1 and adaptor Dok-1 (4). In anergic B cells, SHIP-1 and Dok-1 are constitutively phosphorylated, leading to activation of an inhibitory circuit that is critical for B cell anergy induction. SHIP-1 deficiency in B cells is alone sufficient to cause lupus-like autoimmune disease in mice (4). In addition to SHIP-1, signaling molecules, such as Cbl, PKCδ, and PTEN, are also critical for establishment of B cell anergy (5–7).

The second group is the molecules that promote the survival and differentiation of B cells. BAFF is a cytokine that is critical for survival and differentiation of B2 cells (8). Anergic B cells have a shorter lifespan, primarily because of their high dependence on BAFF for survival compared with their normal counterparts (9). The reliance on BAFF for survival and differentiation helps to reinforce B cell anergy, because anergic B cells fail to compete with normal B cells for limited amounts of BAFF. However, when BAFF is overexpressed anergic B cells survive and mature, eventually leading to breakdown of anergy (10). Bim is a proapoptotic Bcl-2–related protein, and Bim deficiency by itself is sufficient to breach B cell anergy (11). It has been shown that Bim deficient anergic B cells are able to survive and mature because they are no longer dependent on BAFF for survival (11).

The third group is transcription factors that mediate B cell responses to Ag stimulation. Transcription factors integrate the signals from BCR and its costimulatory receptors, and in doing so they dictate B cell responses to Ag via regulation of genes that control B cell survival and activation. NFAT1 is one such transcription factor that functions in a calcium-dependent manner, and lack of NFAT1 is sufficient to break B cell anergy (12). NFAT1-deficient B cells are hyperresponsive to Ag stimulation, indicating that NFAT1 is a negative regulator of Ag responses in B cells (13, 14). Similarly, c-Myc is rapidly induced upon Ag engagement and is critical for Ag-induced B cell expansion (15). However, anergic B cells produce low levels of c-Myc and fail to enter the cell cycle upon Ag stimulation (16). Interestingly, overexpression of c-Myc in anergic B cells can restore B cell responses to Ag stimulation and, by itself, is sufficient to breach B cell anergy (17). Although defective distal BCR signaling in anergic B cells renders them unresponsive to Ag stimulation, elevated expression of key transcription factors in the anergic B cells, such as c-Myc, could lower their activation threshold, thereby restoring the response of anergic B cells to Ag stimulation.

IRF8, also known as ICSBP, is a critical transcriptional regulator that controls the development and differentiation of multiple lineages of immune cells, including macrophages, dendritic cells, B cells, and T cells (18). It was demonstrated that IRF8 is required

1S.P. and S.M. contributed equally to this work.
Received for publication April 30, 2013. Accepted for publication October 9, 2013.
This work was supported by the National Institutes of Health (Grant AI67891 to R.L.). S.P. was supported by a predoctoral fellowship from the University of Nebraska Medical Center.
Address correspondence and reprint requests to Prof. Runqing Lu, Department of Genetics, Cell Biology, and Anatomy, University of Nebraska Medical Center, 985805 Nebraska Medical Center, Omaha, NE 68198. E-mail address: Rlu@UNMC.edu
The online version of this article contains supplemental material.
Abbreviations used in this article: Dox, doxycycline; HEL, hen egg lysozyme; HEL-Tg mice, BCR-transgenic mice recognizing HEL; IRF8−/−, IRF8 germline mutant; IRF8(CD19cre), IRF8 conditional mutant mice that are hemizygous for CD19cre; MZ, marginal zone; PI, propidium iodide; rtTA, reverse tetracycline transactivator; shHEL-Tg, HEL-Tg mice expressing secreted HEL Ag; SLE, systemic lupus erythematosus.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/$16.00
www.jimmunol.org/cgi/doi/10.4049/jimmunol.1301169
for macrophage development and, in its absence, the granulocyte population is dramatically increased (19). IRF8 was also shown to induce the expression of PU.1 and EBF1 in hematopoietic progenitor cells to directly promote B cell commitment and specification (20). Our previous studies showed that IRF8, together with its closely related family member IRF4, orchestrates the transition from large pre-B to small pre-B cells (21–23). Additionally, IRF8 was shown to induce Bc1-6 in germlinal center reactions (24). A recent study showed that marginal zone (MZ) B cells and B1 B cells are significantly increased in IRF8-deficient mice. The defects were observed in IRF8 germline mutant (IRF8−/−) mice, as well as conditional-mutant mice, indicating that the defects are B cell intrinsic. Moreover, it was shown that spleens of IRF8 conditional-mutant mice contain twice as many mature B cells as do their counterparts in wild-type mice (25). MZ and B1 B cells are known to harbor B cells with self-reactive BCRs, and expansion of MZ and B1 B cells is often observed in mice with defects in B cell tolerance induction (26). In this report, we show that IRF8-deficient mice, both germline and conditional knockout, produce anti-dsDNA Abs. Using a well-established mouse model of B cell anergy, we further demonstrate that B cell anergy was breached in IRF8-deficient mice. Thus, our findings identify IRF8 as a novel regulator of B cell anergy.

Materials and Methods

Mice

IRF8−/− mice and conditional-mutant mice were described previously (25, 27). The BCR-transgenic mice recognizing hen egg lysozyme (HEL-Tg mice; MD4), as well as the transgenic mice expressing secreted hen egg lysozyme (HEL) were obtained from The Jackson Laboratory. IRF8 conditional-mutant mice were bred with CD19Cre mice to generate IRF8 conditional-mutant mice that are hemizygous for CD19Cre (IRF8fl/fl). All mice were on the C57BL/6 genetic background and were maintained under specific pathogen-free conditions. Experiments were performed according to guidelines from the National Institutes of Health and with an approved protocol from the Institutional Animal Care and Use Committee of University of Nebraska Medical Center. Mice aged 6–30 wk were used for this study.

ELISA

Anti-HEL ELISA was performed using a previously described method (28). Sera (1:200 dilutions) were added to a 96-well plate precoated with HEL Ag (10 μg/ml). After washing, the plate was incubated with biotinylated anti-IgMa Ab. The bound Ig was visualized by addition of alkaline phosphatase and para-nitrophenyl phosphate. The raw ODs were converted to U/ml using a control serum from HEL-Tg mice to allow interplate comparison. Anti-dsDNA IgMa and IgG ELISA were conducted with kits from Alpha Diagnostic (San Antonio, TX), according to the manufacturer’s recommendation.

Cell-proliferation assay with CFSE tracking dye

B cells were isolated from mice spleen through negative selection. Briefly, splenocytes were incubated with a mixture of biotinylated Abs against CD3, CD4, CD8, Gr-1, Ter119, and Dx5. After incubation with streptavidin-conjugated MicroBeads (Miltenyi Biotec), B cells were isolated with a negative-selection column. The isolated cells were incubated with 1 μM CFSE tracking dye (Molecular Probes) in PBS at 37°C for 10 min. The stained cells were plated in a 96-well plate in triplicate at 5 × 10^5 cells/well. The cells were stimulated with different mitogens, including HEL, LPS, and IgM, and analyzed by FACS after 4 d.

Survival assay

The isolated B cells were plated into each well of a 96-well plate in triplicate; incubated with different concentrations of BAFF, HEL, or both; and analyzed at different time points. To distinguish dead and live cells, the treated cells were incubated with propidium iodide (PI) prior to FACS analysis.

FACS analysis

Cells were isolated from mice, preincubated with either 2% rat serum or Fc-Block (2.4G2), and stained with optimal amounts of specific Abs, either biotinylated or directly fluorochrome conjugated. The Abs against B220, IgM, CD93 (AA4.1), CD21, and IgMb were purchased from BD Pharmingen. The stained cells were analyzed with a FACS Calibur flow cytometer.

Measurement of calcium influx

Splenocytes were isolated from mice and stained with Abs against B220. After washing, the stained cells (5 × 10^5 cells/ml) were incubated with 1 μM Indo-1 AM (Molecular Probes) for 30 min at 37°C in RPMI 1640 medium containing 3% FBS. The calcium influx of loaded cells was analyzed with an LSR II flow cytometer. The baseline emission of fluorescence ratio (405/525 nm) was collected for 1 min, followed by stimulation by HEL at 100 ng/ml. The fluorescence ratio of 405/525 nm was recorded for another 4 min. The increase in the fluorescence ratio was used to reflect the intensity of calcium mobilization upon BCR activation. The data were analyzed with FlowJo software.

Western blot analysis

Splenic B cells were isolated via negative selection. The negatively selected B cells were stimulated with different concentrations of HEL at 37°C for the indicated times. The cells were lysed, and proteins were separated by SDS-PAGE. The membranes were probed with indicated Abs, and the signals were revealed with an ECL detection system (Pierce). The Abs against IRF4, IRF8, c-Myc, and cyclin D2 were obtained from Santa Cruz Biotechnology.

Generation of inducible IRF8 transgenic mice in B cells

Previously, we generated an inducible IRF4 transgenic mouse in which the expression of IRF4 can be induced in B cell by doxycycline (Dox) (29). In this study, we used the same approach and generated an inducible IRF8 transgenic mouse. Briefly, a full-length IRF8 cDNA was inserted into the pTet-Splice vector to generate the transgenic construct (IRF8Tg). Expression of the IRF8 transgene is driven by a minimal CMV promoter linked with a tetracycline response element. Three founder lines were established. To induce transgene expression in B cells, we bred IRF8Tg mice with transgenic mice expressing a reverse tetracycline transactivator (rtTA) (30). The rtTA-transgenic construct, which coexpresses GFP, was inserted into the ROSA26 locus (ROSA-rtTA). ROSA-rtTA was inactive because of an internal stop codon that can be cleaved by Cre recombinase. We bred ROSA-rtTA mice with CD19-cre mice to generate B cell-specific expression of rtTA (CD19 rtTA). CD19rtTA mice were subsequently mated with IRF8Tg mice to generate mice in which the expression of IRF8 transgene can be induced exclusively in B cells (CD19rtTAIRF8Tg). In this system, the expression of IRF8 transgene is induced only in the presence of Dox. The founder line expressing the highest level of IRF8 transgene was chosen for subsequent studies. Eight- to twelve-week-old mice were used for this study.

TUNEL assay

The apoptosis status of splenic B cells in mice was examined with a TUNEL assay. The TUNEL assay was conducted as we described previously (31, 32). CD19creIRF8Tg and CD19cre mice were administered Dox water for 4 wk. Splenic B cells were isolated from the mice and stained with surface Abs against CD93, B220, and IgM. TUNEL− cells were revealed with an APO direct kit (BD Pharmingen).

Results

IRF8-deficient mice, both germline and B cell specific, produce anti-dsDNA Abs

To determine whether deficiency in IRF8 leads to breakdown of B cell tolerance, we measured titers of serum anti-dsDNA Abs. As shown in Fig 1A, IRF8−/− mice produced higher titers of anti-dsDNA IgM and IgG Abs than did IRF8+/− mice. Importantly, the titers of anti-dsDNA IgM and IgG were also found to be higher in IRF8−/− mice than in IRF8 heterozygous mutant mice (IRF8+/−) (Fig. 1B). Anti-dsDNA Abs can be detected in IRF8−/− mice at 3 mo of age. In contrast, the anti-dsDNA Abs can only be detected in IRF8+/− mice ≥5 mo of age. Together, our results show that IRF8-deficient B cells produce anti-dsDNA Abs, indicating that IRF8 plays a role in B cell tolerance induction.

B cell anergy is breached in IRF8-deficient mice

The majority of self-reactive B cells are removed via anergy induction in the periphery. However, the role of IRF8 in B cell anergy...
induction is unclear. The most widely used mouse model of B cell anergy is a double-transgenic mouse that consists of a transgenic BCR recognizing HEL and a second transgene expressing secreted HEL (33). B cells in HEL-Tg mice expressing secreted HEL Ag (sHEL-Tg) are anergic and are unable to produce anti-HEL Abs. To determine the role of IRF8 in B cell anergy induction, we bred IRF8\(^{-/-}\) mice with sHEL-Tg mice to generate sHEL-Tg mice deficient for IRF8 (IRF8\(^{-/-}\)sHEL-Tg). Blood was collected from mice, and ELISA was performed to measure titers of anti-HEL Ab. As shown in Fig. 2A, anti-HEL Ab titers were very low in IRF8\(^{+/+}\) and IRF8\(^{-/-}\) mice, indicating that these mice do not make anti-HEL Ab. As expected, both IRF8\(^{+/+}\)HEL-Tg and IRF8\(^{-/-}\)HEL-Tg mice produced high levels of anti-HEL Abs. Interestingly, the titers of anti-HEL Abs were significantly higher in IRF8\(^{-/-}\)HEL-Tg mice than in IRF8\(^{+/+}\)HEL-Tg mice. In contrast, the titers of anti-HEL Abs in IRF8\(^{+/+}\)sHEL-Tg mice were as low as those found in IRF8\(^{+/+}\) mice. This finding is consistent with the fact that B cells in IRF8\(^{+/+}\)sHEL-Tg mice were anergic. However, anti-HEL Abs were detected in IRF8\(^{-/-}\)sHEL-Tg mice at levels comparable to those produced by IRF8\(^{+/+}\)HEL-Tg mice, indicating that B cells in IRF8\(^{-/-}\)sHEL-Tg mice are not anergic. We further examined and compared the numbers of splenic B cells in IRF8\(^{+/+}\)HEL-Tg, IRF8\(^{-/-}\)HEL-Tg, IRF8\(^{+/+}\)sHEL-Tg, and IRF8\(^{-/-}\)sHEL-Tg mice. The numbers of splenic B cells were significantly higher in IRF8\(^{-/-}\)HEL-Tg mice than in IRF8\(^{+/+}\)sHEL-Tg mice. Anergic B cells have a shortened lifespan that leads to their reduced number in mice. Indeed, there was only ~6 million anergic B cells in spleens of IRF8\(^{+/+}\)sHEL-Tg mice; however, the number increased to 22 million in IRF8\(^{-/-}\)sHEL-Tg mice (Fig. 2B). Together, our results show that there is a defect in B cell anergy induction in IRF8-deficient mice.

**Figure 1.** IRF8-deficient mice, both germline and B cell specific, produce anti-dsDNA Abs. (A) Sera were collected from IRF8\(^{+/+}\) and IRF8\(^{-/-}\) mice at 3–4 mo of age. Ten mice from each group were used. The titers of anti-dsDNA IgM and IgG Abs were measured by ELISA. (B) Sera were collected from IRF8\(^{+/+}\) and IRF8\(^{-/-}\) mice at 5–8 mo of age. Eight mice from each group were used. The titers of anti-dsDNA IgM and IgG Abs were measured by ELISA. Horizontal lines indicate average values.

**Figure 2.** Breach in B cell anergy in IRF8-deficient mice. (A) Sera were collected from mice of following genotypes: IRF8\(^{+/+}\), IRF8\(^{-/-}\), IRF8\(^{+/+}\)HEL-Tg, IRF8\(^{-/-}\)HEL-Tg, IRF8\(^{+/+}\)sHEL-Tg, and IRF8\(^{-/-}\)sHEL-Tg. Mice aged from 3 to 5 mo were used for this study. Eight mice from each group were used. The titers of anti-IgMa Ab were measured by ELISA. Horizontal lines indicate average values. (B) Splenic B cell populations were enumerated in mice of the following genotypes: IRF8\(^{+/+}\)HEL-Tg, IRF8\(^{-/-}\)HEL-Tg, IRF8\(^{+/+}\)sHEL-Tg, and IRF8\(^{-/-}\)sHEL-Tg. Six mice from each group were used. Data are mean and SD.

B cells in IRF8\(^{-/-}\)sHEL-Tg mice are more mature than B cells in IRF8\(^{+/+}\)sHEL-Tg mice

Anergic B cells fail to mature in the periphery and are arrested in their development at the transitional stage (34). To determine whether B cells in IRF8\(^{-/-}\)sHEL-Tg mice can overcome this developmental block, we analyzed and compared immunophenotypes of splenic B cells in IRF8\(^{+/+}\)sHEL-Tg and IRF8\(^{-/-}\)sHEL-Tg mice. IRF8\(^{+/+}\)HEL-Tg and IRF8\(^{-/-}\)HEL-Tg mice also were analyzed. Transitional B cells stain positive for CD93, but CD93 staining is lost once they become mature B2 cells. The majority of splenic B cells in IRF8\(^{+/+}\)HEL-Tg and IRF8\(^{-/-}\)HEL-Tg mice are mature B2 cells and, thus, stained negative for CD93 (Fig. 3A, left panel). Because anergic B cells are blocked at the transitional stage, they should be CD93\(^{+}\). Indeed, anergic B cells in IRF8\(^{+/+}\)sHEL-Tg mice stained positive for CD93 (Fig. 3A, right panel). However, the intensity of CD93 staining was decreased dramatically in the splenic B cells of IRF8\(^{-/-}\)sHEL-Tg mice (Fig. 3A, right panel), indicating that B cells in IRF8\(^{-/-}\)sHEL-Tg mice...
matured beyond the transitional stage. One of the key features of anergic B cells is the reduced level of surface IgM. Indeed, the expression level of IgM is much lower in IRF8+/+sHEL-Tg mice than in IRF8+/+HEL-Tg mice (Fig. 3B). However, B cells in IRF8−/−sHEL-Tg mice expressed a higher level of surface IgM than did the anergic B cells in IRF8+/+sHEL-Tg mice. It was shown that anergic B cells in sHEL-Tg mice express low levels of CD21, and upregulation of CD21 expression has been linked to a breach in B cell anergy (11). Consistent with this previous report, CD21 was expressed at a low level in the anergic B cells of IRF8+/+sHEL-Tg mice (Fig. 3B). However, the expression level of CD21 on B cells was much higher in IRF8+/+sHEL-Tg mice than in IRF8+/+HEL-Tg mice. Self-reactive B cells can evade tolerance induction and mature in the periphery by co-opting an additional BCR (35). To determine whether B cells in IRF8−/−sHEL-Tg mice use dual BCRs to subvert anergy induction, we stained splenic B cells with anti-IgMb allotype Ab. The transgenic BCR is of the IgMa allotype, whereas the endogenous Ig locus is of the IgMb allotype. As shown in Fig. 3B, 98% of splenic B cells in IRF8+/+sHEL-Tg mice were IgMb+, indicating that they only express transgenic BCR on their surface. Together, our results show that B cells in IRF8+/+sHEL-Tg mice are more mature than are their counterparts in IRF8+/+sHEL-Tg mice.

**FIGURE 3.** B cells in IRF8−/−sHEL-Tg mice are more mature than B cells in IRF8+/+sHEL-Tg mice. Splenocytes were isolated from 3-4-mo-old IRF8+/+HEL-Tg, IRF8−/−HEL-Tg, IRF8+/+sHEL-Tg, and IRF8−/−sHEL-Tg mice. The cells were stained with biotinylated HEL or Abs against B220, CD93, IgM, CD21, or IgMa. (A) The intensity of CD93 staining on isolated splenocytes was examined under a B220 gate. (B) Expression of IgM, IgMb was examined in isolated splenocytes. The binding to biotinylated HEL was also examined in isolated splenocytes. The analysis was done under a lymphocyte gate. The numbers represent the percentages of the cells that fall into each quadrant. The result is representative of at least three independent experiments.

B cells in IRF8−/−sHEL-Tg mice are responsive to Ag stimulation

Anergic B cells exhibit defects in BCR signaling and are unresponsive to Ag stimulation. To further characterize splenic B cells in IRF8−/−sHEL-Tg mice, we examined Ag-induced calcium influx and proliferation in IRF8−/−sHEL-Tg mice. Splenocytes were isolated from IRF8−/−sHEL-Tg and IRF8+/+sHEL-Tg mice and loaded with Indo-1 Am dye to track calcium influx. Splenocytes from IRF8+/+HEL-Tg and IRF8−/−HEL-Tg mice also were analyzed. Upon the addition of HEL Ag (100 ng/ml), calcium influx was detected in B cells isolated from IRF8−/−HEL-Tg mice and IRF8+/−HEL-Tg mice (Fig. 4A, upper panel). The amplitudes of calcium influx triggered by BCR signaling were comparable between IRF8−/−HEL-Tg and IRF8+/+HEL-Tg mice, although the amplitude of calcium influx was slightly higher in B cells isolated from IRF8+/−HEL-Tg mice compared with IRF8+/+sHEL-Tg mice. This result indicates that there is no gross defect in BCR signaling in IRF8-deficient mice. Calcium influx was impaired in anergic B cells isolated from IRF8+/−HEL-Tg mice (Fig. 4A, lower panel). In contrast, calcium influx, albeit at low amplitude, could still be detected in B cells isolated from IRF8+/−HEL-Tg mice (Fig. 4A, lower panel), indicating that B cells were not anergic in those mice. We further examined Ag-induced proliferation in B cells...
isolated from IRF8+/+sHEL-Tg and IRF8−/−sHEL-Tg mice. The isolated B cells were stained with CFSE tracking dye and stimulated with different concentrations of HEL. As shown in Fig. 4B, anergic B cells from IRF8+/+sHEL-Tg mice failed to respond to Ag stimulation; intensities of CFSE dye remained unchanged between control and HEL-treated cells. In contrast, HEL treatment led to dilution of CFSE dye in B cells isolated from IRF8−/−sHEL-Tg mice, indicating that B cells in IRF8−/− HEL-Tg mice are responsive to Ag stimulation. The induction of genes that are critical for B cell proliferation, such as c-Myc, CCND2, and IRF4, is defective in anergic B cells (16). However, expression of c-Myc, cyclin D2, and IRF4 was rapidly induced by HEL in B cells isolated from IRF8−/−sHEL-Tg mice but not in anergic B cells isolated from IRF8+/+sHEL-Tg mice (Fig. 4C). Together, our results confirm that B cells in IRF8−/−sHEL-Tg mice lose their anergic state.

B cells in IRF8−/− HEL-Tg mice are more resistant to Ag stimulation–induced cell death than their counterparts in IRF8+/+ HEL-Tg mice

Our result suggests that IRF8-deficient B cells may have a survival advantage, because the numbers of splenic B cells increased significantly in IRF8−/− HEL-Tg mice and IRF8−/−sHEL-Tg mice (Fig. 2B). BAFF is critical for survival and differentiation of B cells, and overexpression of BAFF is sufficient to breach B cell tolerance (10). However, B cells isolated from IRF8+/+ HEL-Tg and IRF8−/− HEL-Tg mice responded similarly to BAFF stimulation in vitro (Fig. 5A). We further incubated splenic B cells isolated from IRF8+/+HEL-Tg and IRF8−/−HEL-Tg mice with different concentrations of HEL Ag. The effect of Ag stimulation on cell survival was examined after 60 h. Our results show that, although Ag stimulation–induced cell death was evident in B cells isolated from IRF8+/+HEL-Tg mice, this effect was attenuated in B cells isolated from IRF8−/−HEL-Tg mice (Fig. 5B). We further examined the effect of BAFF on Ag (200 ng/ml)-induced death. As shown in Fig. 5C, BAFF rescued HEL-triggered cell death, in a dose-dependent fashion, in B cells isolated from IRF8+/+HEL-Tg mice and IRF8−/−HEL-Tg mice. However, BAFF’s rescue effect was more pronounced in B cells isolated from IRF8+/+HEL-Tg mice than in B cells from IRF8−/−HEL-Tg mice. In the absence of BAFF, 20% of IRF8−/−sHEL-Tg B cells were alive; however, in the presence of BAFF (100 ng/ml), 58% were still alive, a 2.9-fold increase in survival. In contrast, 10% of IRF8+/+ HEL-Tg B cells were alive without BAFF; the percentage of living cells increased to 16% in the presence of 100 ng/ml BAFF, an increase of only 1.6-fold. Thus, in the presence of HEL, B cells from IRF8−/− HEL-Tg mice are more sensitive to BAFF than are their counterparts in IRF8+/+HEL-Tg mice.

B cells in IRF8-deficient mice are hypersensitive to Ag stimulation

To further characterize the role of IRF8 in B cell activation and anergy induction, we examined the cell response to Ag stimulation in IRF8-proficient and -deficient B cells. Splenic B cells were isolated from IRF8+/+HEL-Tg and IRF8−/−HEL-Tg mice, stained with CFSE dye, and stimulated with different concentrations of HEL for 4 d. Our results show that B cells from IRF8−/−HEL-Tg mice were more sensitive to Ag stimulation than were their
counterparts in IRF8+/+HEL-Tg mice (Fig. 6A). At a low HEL concentration (20 ng/ml), CFSE dilution was observed only in B cells isolated from IRF8+/+HEL-Tg mice. Surprisingly, B cell proliferation induced by anti-IgM cross-linking and LPS was comparable between B cells isolated from IRF8+/+HEL-Tg and IRF8−/−HEL-Tg mice (Supplemental Fig. 1). These results indicate that B cells in IRF8−/−HEL-Tg mice are hyperproliferative only to stimulation by the cognate Ag. We further examined the induction of c-Myc, cyclin D2, and IRF4 by HEL (Fig. 6B). Our results show that their induction in B cells isolated from IRF8−/−HEL-Tg mice was detected at HEL concentrations as low as 20 ng/ml. In contrast, their induction in B cells isolated from IRF8+/+HEL-Tg mice was detected only at a HEL concentration of 100 ng/ml. Together, these results show that B cells in IRF8−/−HEL-Tg mice are more sensitive to HEL stimulation.

B cells in IRF8−/− mice are hypersensitive to Ag stimulation and are resistant to Ag stimulation–induced cell death

Our results show that B cells in IRF8−/− mice were more resistant to Ag-induced cell death and were hypersensitive to Ag stimulation. However, IRF8−/− mice have defects in multiple lineages, including T cells and myeloid cells, and it is possible that the B cell defects observed in IRF8−/− mice are B cell extrinsic and caused by the pleiotropic effects of IRF8. To address this, we bred IRF8fl/fl mice with HEL-Tg mice to generate B cell–specific IRF8-knockout mice expressing HEL-BCR (IRF8fl/fl HEL-Tg). Compared with IRF8+/HEL-Tg control mice, the number of B cells was increased significantly in IRF8fl/fl HEL-Tg mice (Fig. 7A). Next we examined their response to BAFF and to Ag-induced cell death. Consistent with the results obtained from IRF8−/− mice, B cells in IRF8fl/fl HEL-Tg mice responded normally to BAFF stimulation alone (Fig. 7B) but were more resistant to HEL-induced cell death than the B cells in IRF8−/−HEL-Tg mice (Fig. 7C). Additionally, B cells in IRF8fl/fl HEL-Tg mice were more sensitive to BAFF in the presence of Ag stimulation than were their counterparts in IRF8+/−HEL-Tg mice (Fig. 7D). Finally, Ag-induced cell proliferation was also found to be more pronounced in B cells isolated from IRF8fl/fl HEL-Tg mice than in B cells from IRF8+/−HEL-Tg control mice (Fig. 7E). Together, our results indicate that IRF8

**FIGURE 5.** B cells in IRF8−/−HEL-Tg mice are more resistant to Ag stimulation–induced apoptosis than their counterparts in the IRF8+/+HEL-Tg mice. (A) Splenocytes were isolated from IRF8+/−HEL-Tg and IRF8+/+HEL-Tg mice through negative selection. The isolated cells were plated onto a 96-well plate in triplicate (5 × 10⁵ cells/well) in the presence of increasing concentrations of BAFF. After 72 h, the percentages of live cells were analyzed via PI staining. p = 0.45, linear-regression analysis. (B) The isolated splenocytes were plated onto a 96-well plate in triplicate in the presence of increasing concentrations of HEL. After 48 h, the percentages of live cells were analyzed by FACS. p = 0.02, linear-regression analysis. (C) The isolated splenocytes were incubated with HEL (200 ng/ml) in presence of increasing concentrations of BAFF. The percentages of live cells were analyzed by FACS 2 d later. p = 0.03, linear-regression analysis.

**FIGURE 6.** B cells in IRF8-deficient mice are hypersensitive to Ag stimulation. (A) B cells were isolated from spleens of IRF8+/+HEL-Tg and IRF8−/−HEL-Tg mice, stained with CFSE dye, and incubated with increasing concentrations of HEL. The cells were analyzed by FACS 4 d later. (B) The isolated B cells from IRF8+/+HEL-Tg and IRF8−/−HEL-Tg mice were treated with increasing concentrations of HEL. The treated cells were lysed after 12 h. Western blot analysis was used to detect the expression of c-Myc, cyclin D2, and IRF4. The results are representatives of at least three independent experiments.
functions in a B cell–intrinsic fashion to regulate cell survival and response to Ag stimulation.

**IRF8 is expressed at high levels in anergic B cells, and induction of IRF8 transgene triggers apoptosis in transitional B cells**

IRF8 is expressed throughout B cell development; however, its expression level in anergic B cells is unclear. We measured the expression level of IRF8 in B cells isolated from IRF8+/+HEL-Tg mice and IRF8+/+sHEL-Tg mice. Interestingly, IRF8 was expressed at a 2-fold higher level in anergic B cells compared with normal B cells (Fig. 8A). This result is consistent with our findings that IRF8 is important for anergy induction. We wanted to determine the effect of a high level of IRF8 on B cells. To this end, we generated inducible IRF8-transgenic mice in which the expression of IRF8 can be induced by Dox in B cells. CD19CreIRF8Tg mice and CD19cre control mice were administered Dox for 4 wk, and GFP+ splenic B cells were isolated by sorting. Our results show that expression levels of IRF8 were comparable between CD19creIRF8Tg and CD19cre control mice given regular water (Fig. 8B, Untreated). However, in the presence of Dox water, the expression of IRF8 in B cells was 2.5-fold higher in CD19creIRF8Tg mice than in CD19cre control mice, indicating that Dox treatment induced the expression of the IRF8 transgene (Fig. 8B, Dox). In contrast to normal B cells exhibiting decreased CD93 and elevated levels of surface IgM and CD21, these findings indicate that B cells in IRF8−/−sHEL-Tg mice are able to mature beyond the transitional stage, which eventually allows them to regain responses to Ag stimulation and the ability to secrete Abs.

**Discussion**

Engagement of cognate Ag by mature B cells leads to B cell activation and Ab secretion; however, Ag encounter by developing B cells causes developmental arrest and anergy. Anergic B cells do not respond to Ag stimulation because they are transitional B cells with defective BCR signaling (2). In this study, we demonstrate that B cell anergy is breached in IRF8-deficient mice. In contrast to anergic B cells in the wild-type background, B cells in IRF8−/−sHEL-Tg mice exhibited downregulated CD93 and elevated levels of surface IgM and CD21; these findings indicate that B cells in IRF8−/−sHEL-Tg mice are able to mature beyond the transitional stage, which eventually allows them to regain responses to Ag stimulation and the ability to secrete Abs.

Our findings indicate that IRF8 is an important regulator of B cell survival at the transitional stage. We show that expression of IRF8 was elevated in anergic B cells, and induction of the IRF8 transgene in B cells promoted apoptosis at the transitional stage. Additionally, Ag stimulation–induced cell death was attenuated in IRF8−/−HEL-Tg mice, indicating that IRF8 also regulates the survival of mature B cells. BAFF is a critical regulator of B cell survival and maturation. Although the response to BAFF stimulation alone was normal in B cells from IRF8−/−HEL-Tg mice, the
Our results show that B cells in IRF8-/- HEL-Tg mice are more sensitive to Ag stimulation than are their counterparts in IRF8+/+ HEL-Tg mice. The hypersensitivity to HEL stimulation is not due to a defect in BCR signaling, because calcium influxes triggered by HEL stimulation were comparable between B cells isolated from IRF8-/- HEL-Tg mice and IRF8+/+HEL-Tg mice. Surprisingly, our results also show that B cells in IRF8-/- HEL-Tg mice were hyperresponsive only to HEL stimulation and not to anti-IgM cross-linking or LPS. Because IRF8 expression is elevated in anergic B cells, IRF8 may function as a negative regulator of B cell activation in anergic B cells. It is conceivable that the hypersensitivity to HEL stimulation could contribute to anergy breakdown by allowing B cells in IRF8-/- sHEL-Tg mice to proliferate and secrete Abs.

Our results indicate that IRF8 functions in a B cell–intrinsic fashion to regulate B cell survival and response to Ag stimulation. IRF8 is critical for the development of several lineages of blood cells, and IRF8 germline-deficient mice exhibit defects in multiple lineages, including T, B, and myeloid cells (39). Because of this, the B cell defects observed in IRF8 germline-mutant mice could be B cell–extrinsic defects that are caused indirectly by the pleiotropic effects of IRF8. However, our study shows that B cell survival and response to Ag stimulation were similarly affected in the IRF8 conditional-mutant mice, indicating that those defects are B cell specific. Because B cell tolerance can be influenced by defects in other immune cells, such as T cells and myeloid cells, it is possible that abnormalities in other lineage cells also contribute to the tolerance breakdown in IRF8 germline-deficient mice. Our findings that B cells in IRF8 conditional-mutant mice produced lower titers of anti-dsDNA Ab and did so at later time points than did B cells in IRF8 germline-mutant mice are consistent with this scenario.

Two recent genome-wide single-nucleotide polymorphism–association studies identified IRF8 as a major susceptibility gene for systemic lupus erythematosus (SLE) (40, 41). One of the studies also showed that the single-nucleotide polymorphisms cause a high level of IRF8 in lupus patients (40). The involvement of IRF8 in SLE was linked to its function in IFN signaling and production (40, 41). Consistent with this assertion, a recent study demonstrated that lupus development was attenuated in New Zealand Black mice deficient for IRF8 (42). This study further shows that IRF8-deficient mice are lacking in plasmacytoid dendritic cells, which are the major producers of type 1 IFN in vivo (42). Together, these studies establish the requirement for IRF8 in lupus development. However, the results from this study show that lack of IRF8 in

**FIGURE 8.** IRF8 is expressed at high levels in anergic B cells, and induction of the IRF8 transgene triggers apoptosis in transitional B cells. (A) B cells were isolated from spleens of IRF8+/+HEL-Tg and IRF8+/+sHEL-Tg mice. The cells were lysed for Western blot analysis to detect the expression of IRF8 and β-actin. The intensity of the IRF8 signal in B cells of IRF8+/+sHEL-Tg mice was scanned, normalized to the signal of β-actin, and expressed as fold changes relative to its expression in IRF8+/+HEL-Tg mice. (B) To induce IRF8 transgene expression, CD19CreIRF8Tg mice and CD19Cre control mice were given Dox water for 4 wk, and splenic B cells were analyzed. Splenic B cells (GFP+) were isolated by sorting from CD19CreIRF8Tg mice and CD19Cre control mice given Dox water (Dox). Additionally, B cells were isolated from CD19CreIRF8Tg mice and CD19Cre control mice given regular water (Untreated). Real-time PCR analysis was used to measure the expression of IRF8. Data are expressed as fold changes relative to its expression in IRF8+/+HEL-Tg mice. (C) Splenic B cells in CD19CreIRF8Tg mice and CD19Cre control mice were stained with Abs against CD93, IgM, and B220 and analyzed by FACS. The number of transitional T1 (CD93+IgMhighCD23+), T2 (CD93+IgMlowCD23+), T3 (CD93+IgMlowCD23+), and mature B cells (CD93-IgM+) was enumerated. A total of five mice was used from each group. (D) Splenic B cells were stained with Abs against B220, IgM, and CD93. TUNEL+ B cells were determined. B220+IgM+CD93+ mature B cells and B220+IgM+CD93+ transitional B cells were analyzed separately. Data are mean ± SD of three independent experiments. *p < 0.05, **p < 0.01.
B cells leads to breakdown of B cell tolerance, which could also contribute to the development of autoimmune diseases. The paradoxical role that IRF8 plays in the development of autoimmune diseases mirrors that of TLRs. It has been well documented that TLR signaling is critical for IFN production and class-switching and is absolutely required for lupus development (43–45). However, it also was shown that lack of TLR signaling during B cell development leads to the breakdown of B cell tolerance (46). Like TLRs, the role of IRF8 in autoimmunity is complex and stage specific. Given the contradicting roles that IRF8 plays in SLE, our analysis core for help with data analysis.

We thank Vicki Smith at the University of Nebraska Medical Center cell analysis core for help with data analysis.

Acknowledgments
The authors have no financial conflicts of interest.

Disclosures
The authors have no financial conflicts of interest.

References