Continuous inhibitory signaling by both SHP-1 and SHIP-1 pathways is required to maintain unresponsiveness of anergic B cells

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Many autoreactive B cells persist in the periphery in a state of unresponsiveness called anergy. This unresponsiveness is rapidly reversible, requiring continuous BCR interaction with self-antigen and resultant regulatory signaling for its maintenance. Using adoptive transfer of anergic B cells with subsequent acute induction of gene deletion or expression, we demonstrate that the continuous activities of independent inhibitory signaling pathways involving the tyrosine phosphatase SHP-1 and the inositol phosphatase SHIP-1 are required to maintain anergy. Acute breach of anergy by compromise of either of these pathways leads to rapid cell activation, proliferation, and generation of short-lived plasma cells that reside in extrafollicular foci. Results are consistent with predicted/observed reduction in the Lyn–SHIP-1–PTEN–SHP-1 axis function in B cells from systemic lupus erythematosus patients.

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An estimated 70% of newly formed B cells express autoreactive B cell antigen receptors BCRs (Wardemann et al., 2003). To avoid autoimmunity, these B cells must be silenced. Three major tolerance mechanisms are in place to achieve silencing: clonal deletion, receptor editing, and anergy (Goodnow et al., 1988; Nemazee and Bürki, 1989; Gay et al., 1993; Tiegs et al., 1993). Whereas all of these mechanisms operate during B cell development, B cell anergy is the major mechanism operating in the periphery. Available evidence indicates that in the normal peripheral repertoire, ∼5–7% of B cells are anergic (Merrell et al., 2006; Duty et al., 2009; Quách et al., 2011). Based on this frequency and reports that anergic B cells have a much shorter half-life (∼5 d) than their naive counterparts (∼40 d), it has been estimated that up to 50% of newly formed, autoreactive B cells are silenced by anergy.

Anergy is not an absolute state. Maintenance of B cell unresponsiveness requires constant occupancy of 20–40% of their BCR (Goodnow et al., 1991). Removal of self-antigen results, within minutes, in restoration of BCR signaling function (Gauld et al., 2005). As a consequence of this reversibility and presence of anergic cells in the periphery, where they may encounter high levels of locally produced inflammatory mediators, anergy is fragile and compromised anergic cells are therefore likely to contribute to autoimmunity.

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Abbreviations used: HEL, hen egg lysozyme; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; SHIP-1, SH2-containing inositol 5-phosphatase 1; SHP-1, SH2-containing tyrosine phosphatase 1; SLE, systemic lupus erythematosus; SRBC, sheep RBC.

The rapid reversibility of anergy indicates that it is maintained by nondurable mechanisms, such as inhibitory signaling (Goodnow et al., 1991; Gauld et al., 2005). Such mechanisms are suggested by reported chronic immunoreceptor tyrosine-based activation motif (ITAM) monophosphorylation, as well as increased phosphorylation of SH2-containing inositol 5-phosphatase 1 (SHIP-1) and its adaptor docking protein 1, in anergic cells (Merrell et al., 2006; O'Neill et al., 2011). However, the causality of these events in maintaining anergy has not been demonstrated. A significant proportion of thus far identified systemic lupus erythematosus (SLE) risk alleles encode proteins that function in regulation of BCR signaling (Cambier, 2013). Toward eventual development of personalized therapies based on risk allele genotype, it is of critical importance to understand the molecular mechanisms that underlie maintenance of anergy, and their interplay.

The earliest defined event in BCR signaling is the phosphorylation of one or both tyrosines in the ITAM motif of CD79a (Igα) and CD79b (Igβ) receptor subunits by Src-family tyrosine kinases, e.g., Lyn or Fyn. This leads to the recruitment, via SH2 binding, and activation of Lyn. Upon dual phosphorylation, ITAMs become docking sites for the kinase Syk that, in turn, is activated by phosphorylation, leading to phosphorylation of several downstream targets and culminating in B cell activation (Packard and Cambier, 2013). Whereas Lyn plays a role in B cell activation, it also propagates activity of regulatory signaling pathways by, for example, phosphor-

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ylation of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in inhibitory receptors, such as CD22 and CD32B. Phosphorylated ITIMs mediate recruitment and activation of the SH2-containing tyrosine phosphatase-1 (SHP-1) and the inositol phosphatase SHIP-1. These phosphatases can act in negative feedback loops controlling the magnitude and duration of the initial response to antigen (Ono et al., 1997).

We previously reported that in anergic B cells CD79a and b ITAMs are monophosphorylated, and that further stimulation of BCR on these cells leads to additional monophosphorylation but not dual phosphorylations (O'Neill et al., 2011). While Syk recruitment to BCR and Syk function requires that both ITAM tyrosines be phosphorylated, Lyn engagement requires that only one tyrosine be phosphorylated (Pao et al., 1998). These data suggest that in anergic B cells the balance between Lyn and Syk activation shifts, leading to a bias toward inhibitory signaling. Indeed, in cell lines that contain receptors that can only be monophosphorylated, we observe no Syk phosphorylation, whereas the SHIP-1 and its adaptor docking protein 1 are strongly phosphorylated (O'Neill et al., 2011).

Inhibitory signaling pathways have long been recognized to be of importance in B cell tolerance. B cell–targeted loss of Lyn (Lamagna et al., 2014), SHIP-1 (Maxwell et al., 2011; O'Neill et al., 2011), and SHP-1 (Pao et al., 2007) all lead to a lupus-like B cell–driven autoimmunity. However, these studies have not revealed which tolerance mechanisms are compromised by such mutations. Deletion of negative regulators of BCR signaling leads to increased signal strength, resulting in increased clonal deletion (Cyster and Goodnow, 1995; Cornall et al., 1998; O'Neill et al., 2011; Leung et al., 2013) and, potentially, receptor editing (Kraus et al., 1999; Hippen et al., 2005). This increase in central tolerance suggests that the observed loss of tolerance in mice lacking negative regulators that have a wild-type BCR repertoire is caused by a loss of peripheral tolerance. Paradoxically, however, although loss of SHP-1 does not lead to autoimmunity in a transgenic model of B cell anergy in which BCR affinity for autoantigen is very high (Cyster and Goodnow, 1995), it does lead to autoimmunity in normal mice with a polyclonal BCR repertoire (Pao et al., 2007). Interpretation of these studies is further confounded by the fact that signal strength affects the lineage fate decision of B cells (Casola et al., 2004). Thus, the role of these regulatory signaling proteins in the maintenance of anergy is unclear.

We have hypothesized that BCR ITAM monophosphorylation maintains the unresponsiveness of anergic B cells via activation of Lyn and downstream targets such as SHIP-1 and SHP-1. The requirement for continuous receptor engagement to maintain unresponsiveness suggests that continuous activation of this pathway is required to maintain anergy. In this study, we specifically address the role SHIP-1 and SHP-1 in maintenance of B cell anergy by adoptive transfer of anergic B cells, followed by induction of acute deletion or activation of gene expression. Using this approach, we were able to circumvent confounding effects of such manipulations on B cell development and central tolerance. Thus, the specific roles of SHP-1 and SHIP-1 on induction versus maintenance of B cell anergy could be delineated. We found that both SHIP-1 and SHP-1 are required for the maintenance of anergic B cell unresponsiveness, and that they appear to function in independent pathways. Tracking clonal fate of compromised anergic B cells revealed that they generate short-lived, extrafollicular autoantibody responses.

RESULTS

SHIP-1 is required for the maintenance of B cell anergy

To assess the role of signaling intermediaries in the maintenance of B cell anergy, we developed an adoptive transfer strategy for use in conjunction with acute induction of B cell–targeted gene deletion or expression (Fig. 1 A). Donor Ars/A1 B cells express a BCR that is reactive with the hapten *p*-azophenylarsonate and with DNA (Benschop et al., 2001). Presumably as a consequence of their DNA reactivity, B cells in this mouse are anergic (Benschop et al., 2001). For these studies, Ars/A1 mice were further engineered to contain genes encoding human CD20 promoter-driven, and therefore B cell–targeted, TamCre (Khalil et al., 2012). In addition, they contained a ROSA-26 floxed-stop-YFP reporter of Cre activity, with or without floxed genes of interest. Isolated anergic Ars/A1 B cells were loaded with CellTracker Violet to allow monitoring of proliferation, and then adoptively transferred into low-dose irradiated C57BL/6 recipients. Transferred populations were homogeneous, expressing phenotypic markers consistent with anergy, and were unresponsive to BCR stimulation, as shown previously (Benschop et al., 2001; andnot depicted). 24 and 48 h after cell transfer, recipients were treated with tamoxifen to induce Cre activity and consequent gene deletion (or activation); treatment included marking affected cells by induction of YFP expression. Use of adoptive transfer allowed monitoring of the fate of these clonal anergic B cells in the presence of niche competitors, while avoiding the confounding effects of continuous influx of newly formed DNA/chromatin-reactive Ars/A1 B cells.

We first used this approach to determine the need for continuous inhibitory signaling mediated by SHIP-1 to maintain the anergy. Intracellular staining of SHIP-1 confirmed elimination of SHIP-1 protein in YFP^+ Ars/A1 cells by 7 d after tamoxifen treatment (Fig. 1 B). At this time point, staining was equivalent to that of B cells lacking SHIP-1 due to mb1-cre–driven knockout (mb1-cre SHIP-$1^{fl/fl}$). As expected, transferred and tamoxifen-treated SHIP-1–sufficient Ars/A1 B cells ($SHIP^{WT}$ Ars/A1; Fig. 1 C) and SHIP-$1^{fl/fl}$ Ars/A1 B cells in mice that were not tamoxifen treated (not depicted) did not proliferate or differentiate to become plasmablasts at any time after transfer and tamoxifen treatment (Figs. 1 C and 2, A and B). In contrast, Ars/A1 B cells that became SHIP-1 deficient after tamoxifen treatment made robust responses ($SHIP^{fl/fl}$ Ars/A1; Figs. 1 C and 2, A and B). By 11 d after tamoxifen treatment, a significant proportion of Ars/A1 B

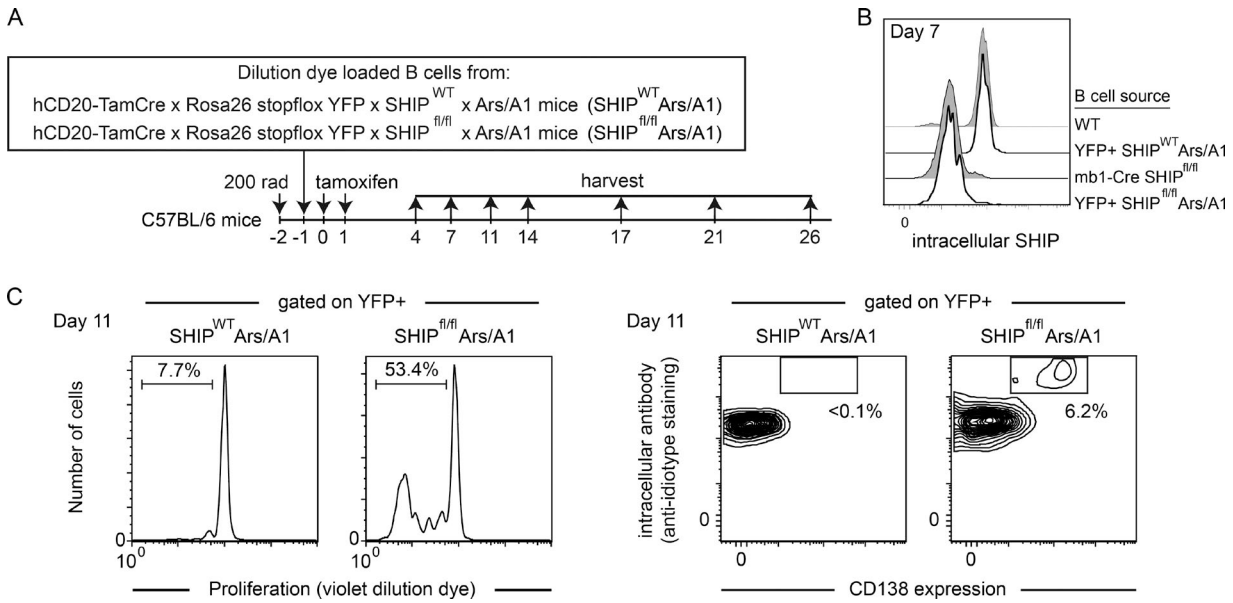

Figure 1. **SHIP-1 is required for the maintenance of anergy in autoreactive B cells.** (A) A schematic representation of the experimental protocol. (B) Intracellular SHIP-1 protein expression in splenic YFP$^+$ cells 7 d after tamoxifen treatment. B220$^+$ C57BL/6 recipient cells (WT) and B220$^+$ mb1-cre x SHIP-1$^{fl/fl}$ cells are used as controls (n = 5/group, representative plots shown). (C) Proliferation (left) and differentiation (right) of splenic YFP$^+$ cells to plasmablasts 11 d after tamoxifen treatment of recipients (n = 5/group, representative plots shown). Data shown are representative of at least two replicate experiments.

cells that had lost SHIP-1 proliferated as indicated by dye dilution and expressed CD138 as well as increased intracellular antibody (idiotype), indicating differentiation to plasmablasts (Fig. 1 C). To determine if this response was merely a result of the acute loss of SHIP-1 or was autoantigen driven, we generated mice in which SHIP-1 deletion could be induced in transferred B cells specific for hen egg lysozyme (HEL), a foreign antigen. These MD4 B cells did not proliferate or differentiate into plasmablasts upon acute SHIP-1 deletion, unless recipients (that did not express HEL) were immunized with sheep RBC (SRBC)–conjugated HEL (SRBC-HEL; Fig. 2, C and D). Together, these results show that SHIP-1 is required for the maintenance of unresponsiveness of DNA-reactive B cells that are silenced by anergy, and suggest that loss of SHIP-1 converts the response to continuous self-antigen from B cell anergy to activation.

Upon acute loss of SHIP-1, anergic B cells become responsive to BCR stimulation, are activated, and make short-lived extrafollicular antibody responses

We next determined whether the acute loss of SHIP-1 by Ars/A1 B cells affects their ability to mobilize calcium upon BCR cross-linking. Despite lower surface IgM expression, Ars/A1 B cells that had not yet proliferated after SHIP-1 deletion mobilized calcium more robustly than SHIP-1–sufficient anergic Ars/A1 B cells, indicating relief of anergy-associated suppression of BCR signaling (Fig. 3 A). Next, we monitored in vivo changes in activation markers on Ars/A1 B cells after acute SHIP-1 deletion, to determine whether those changes resemble changes in surface phenotype that are normally associated with responses of naive B cells to antigen. We first analyzed marker expression along a time continuum from 4–14 d, analyzing only those cells that had not yet proliferated. Loss of SHIP-1 (Fig. 3 C) was associated with acquisition of an activated phenotype as indicated by down-regulation of antigen receptors and CD23, and up-regulation of MHC class II and CD86 (Fig. 3 D). CD80, normally up-regulated on anergic B cells, was down-regulated (Fig. 3 D). CD80 has been reported to have an inhibitory function on B cells (Suvas et al., 2002), thus its down-regulation is consistent with activation.

We then evaluated changes in marker expression as a function of progressive steps of activation (undiluted violet label; red quadrant), proliferation (dye diluted; green quadrant), and differentiation (intracellular Ig^{hi}, B220^{lo}, and CD138^{hi}; blue quadrant) of genetically compromised anergic cells (Fig. 3 E). As can be seen, CD95 was down-regulated by compromised cells before proliferation, and then increased with proliferation. Loss of CD95 may enable these cells to escape CD95-mediated mechanisms that promote death of anergic cells (Rathmell et al., 1995). Conversely, reacquisition of CD95 by plasmablasts may be responsible for the clonal extinction seen at 20–25 d (Fig. 3 B). As expected, CD138 was expressed only by plasmablasts, and many of the plasmablasts exhibited reduced expression of B220 consistent with an advanced state of differentiation. Finally, the plasmablasts had a CXCR4hi, CXCR5lo chemokine receptor profile, consistent with extrafollicular localization (Hargreaves et al., 2001).

Plasmablasts detected based on CD138 expression and high levels of cytoplasmic antibody were functional, as Ars/A1 B cell–derived IgM^a anti-Ars was detected in the serum

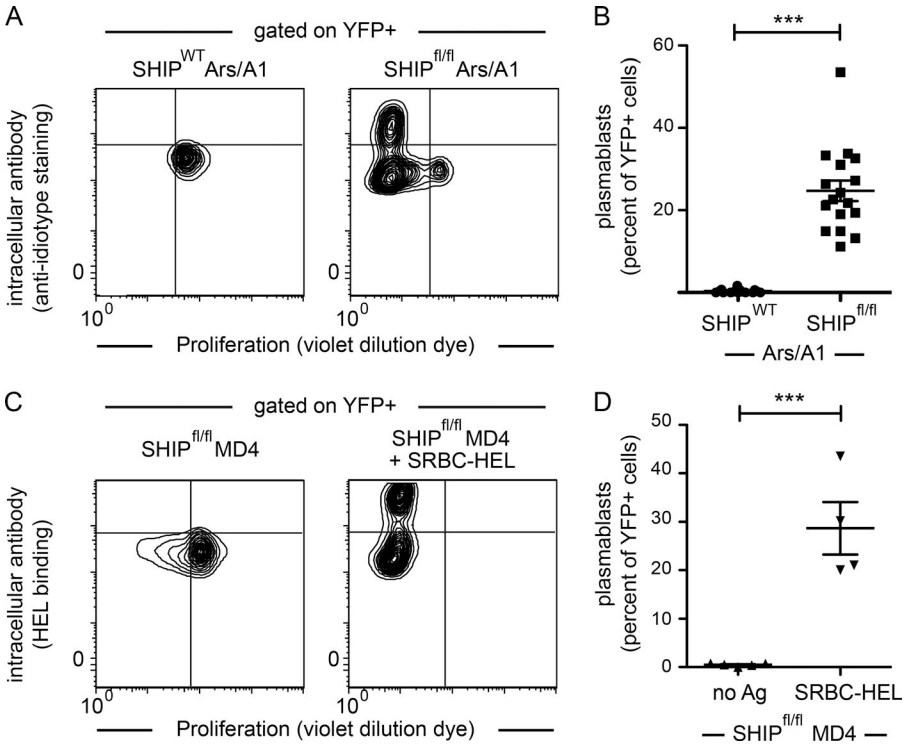

Figure 2. **Proliferation and differentiation of B cells after SHIP-1 deletion is antigen dependent.** (A) Proliferation and differentiation to plasmablasts of acutely SHIP-1–deleted or SHIP-1–sufficient splenic Ars/A1 B cells 14 d after tamoxifen treatment. Cells were gated on YFP$^+$ cells (n = 5/group, representative plots shown). (B) Statistical analysis of pooled data from four independent experiments (n = 16/group) comparing the percentage of splenic YFP$^+$ cells that were plasmablasts (intracellular anti-idiotype high, dilution dye low, top left quadrant of plots depicted in A) 14 d after tamoxifen treatment. (C) hCD20-Tam-Cre x Rosa26 stopflox YFP x SHIP-1$^{fl/fl}$ x MD4 B cells were adoptively transferred according to the schematic representation in Fig. 1 A (n = 4–5/group). 9 d after tamoxifen treatment, one group was immunized with 5% SRBC-HEL i.p., and the other group was left unimmunized. 14 d after tamoxifen treatment, splenic YFP$^+$ cell proliferation and differentiation to plasmablasts was determined. (D) Statistical analysis of the plasmablasts (top left quadrant of C) as percentage of total YFP$^+$ cells in C. Data shown are representative of at least two replicate experiments. Bars in B and D represent mean ± SEM. ***, $P < 0.005$ using an unpaired Student's t test.

(Fig. 3 F) and IgM^a anti-Ars secreting cells were detected by ELISPOT (Fig. 3 G). Consistent with initial activation of these B cells by endogenous antigen and previous reports that Ars/A1-encoded antibodies react with DNA/chromatin, antibody-secreting cells were also detected as IgM^a anti-chromatin ELISPOTs (Fig. 3 H). Histological examination demonstrated that these antibody-secreting cells localize at the red pulp/T cell border (Fig. 3 I), a site previously shown to harbor autoreactive antibody-producing cells (Herlands et al., 2007; Nickerson et al., 2013a). We did not observe YFP$^+$ Ars/A1 B cell–containing germinal centers in any time point examined (unpublished data). Although the frequency of plasmablasts increased in time after tamoxifen treatment, they became undetectable at ∼21 d after tamoxifen treatment (Fig. 3 B). They were also undetectable in LNs and the bone marrow at this time, suggesting clonal extinction had occurred (unpublished data). Together, these results show that upon loss of SHIP-1, anergic B cells become responsive to autoantigen, and subsequently are activated, proliferate, and differentiate into short-lived autoantibody-secreting plasmablasts that localize in extrafollicular sites.

Loss of tolerance by transferred compromised anergic B cells is not dependent on irradiation of recipient mice

In the aforementioned experiments, we irradiated the recipients with 200 rad before adoptive transfer, assuming a need to make space for the transferred cells. However, this irradiation could have additional effects on the environment in recipients, including increased release of self-antigen, production of inflammatory cytokines, and a decrease in competition for B cell–activating factor (BAFF). To exclude roles for these effects of irradiation in loss of anergy, we compared the behavior of Ars/A1 B cells after induced loss of SHIP-1 in irradiated and nonirradiated recipients. Although responses terminated earlier and cell recoveries were lower in nonirradiated recipients (Fig. 4, A–C) we observed no qualitative differences in the autoantibody response (Fig. 4, C and D). These results demonstrate that acute SHIP-1 loss is sufficient to break tolerance. In further experiments, we observed two quantitative differences after cell transfer to irradiated versus nonirradiated recipients. First, when fewer Ars/A1 B cells were transferred (6×10^{-5} vs. 2.5×10^{-6}), cells transferred into irradiated recipients made significantly larger responses after SHIP deletion than those transferred into nonirradiated recipients (not depicted). Second, transferred control anergic B cells (which remained SHIP-sufficient, and thus anergic) remained constant in number between day 7 and 14 in irradiated recipients, whereas rapidly decreasing in nonirradiated recipients (Fig. 4 E). These results suggest that in the slightly lymphopenic environment of irradiated recipients anergic B cells survive longer, likely due to reduced competition for B cell–activating factor (Lesley et al., 2004; Thien et al., 2004). This would increase the number of precursors available to engage in autoantibody responses after breach of tolerance by SHIP-1 deletion.

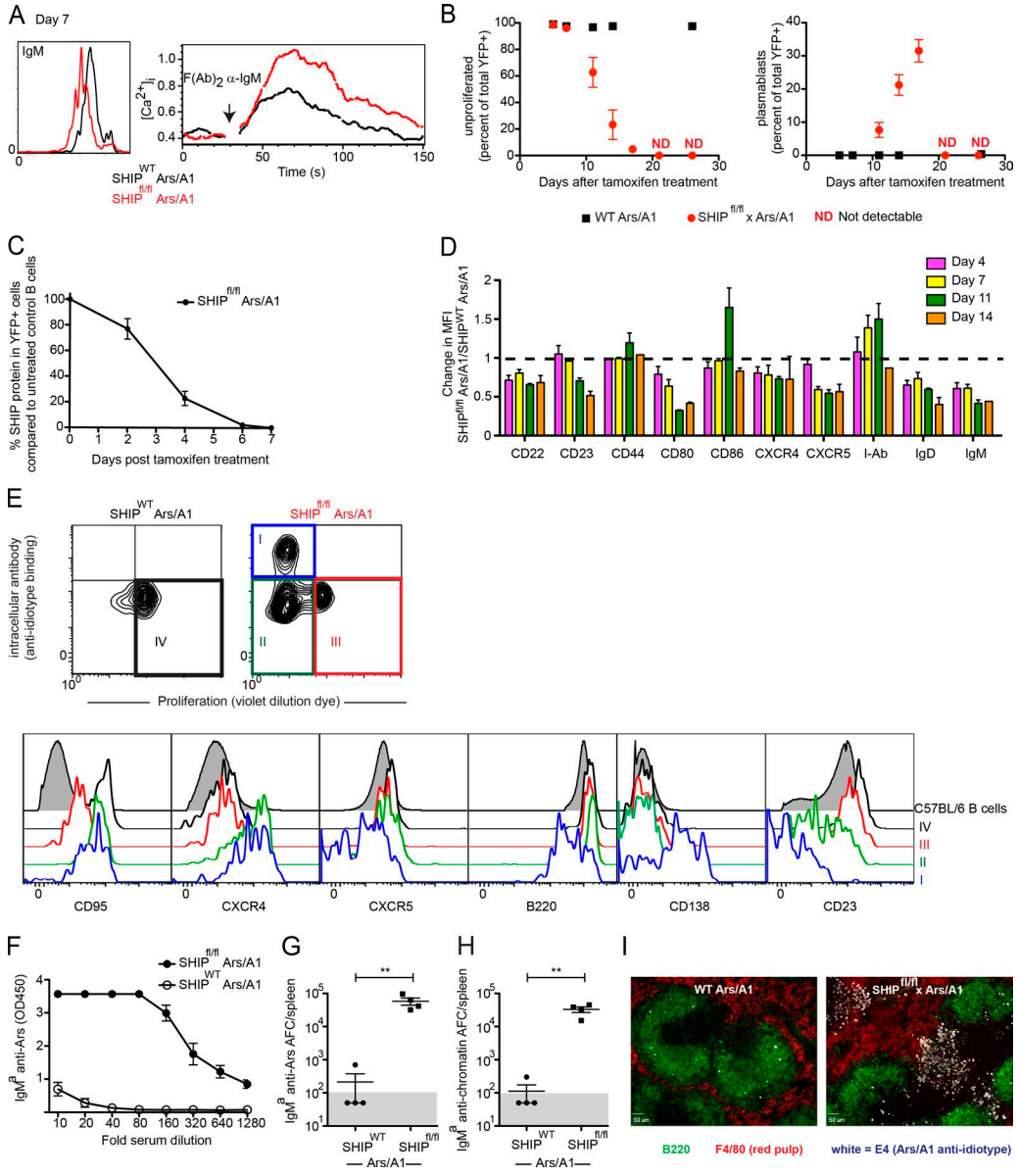

Figure 3. **Upon loss of SHIP-1, autoreactive B cells become responsive to antigen receptor stimulation ex vivo, express activation markers in vivo, and make short-lived extrafollicular plasma cell responses.** (A) Surface IgM expression by (left) and anti-IgM induced calcium mobilization responses (right) of undivided, acutely SHIP-1–deleted anergic B cells 7 d after tamoxifen treatment. (n = 5/group, representative plots shown). (B) Kinetics of proliferation and differentiation into plasmablasts of YFP$^+$ cells from the experiment described in Fig. 1 A (n = 5/group, data shown from two pooled experiments). ND, nondetectable. (C) Relative SHIP-1 expression by transferred B cells after treatment of recipients with tamoxifen (n = 4/group). (D) Fold changes in cell surface marker expression by nondivided YFP$^+$ cells, comparing SHIP-1$^{fl/fl}$ Ars/A1 to WT Ars/A1 cells at the indicated times after tamoxifen treatment. At the time of adoptive transfer, the SHIP-1$^{fl/fl}$ Ars/A1 and WT Ars/A1 B cells expressed equivalent levels of surface markers represented by a black line (n = 3/group). (E) Cell surface expression of the indicated markers by nondivided (red line, representing quadrant III in SHIP$^{fl/fl}$ Ars contour plot), proliferated (green line, representing quadrant II in SHIP$^{fl/fl}$ Ars contour plot), and differentiated (blue line, representing quadrant I in SHIP$^{fl/fl}$ Ars contour plot) B cells 14 d after tamoxifen treatment. For comparison, the cell surface expression of SHIP-sufficient Ars/A1 (black line, representing quadrant IV in SHIP WT Ars contour plot) and cell surface expression of the recipients C57BL/6 B cells (gray shaded histogram) are shown (n = 4/group, representative plot shown). (F) Ars/A1-derived IgM^a anti-Ars antibody detected in serum 14 d after tamoxifen treatment (n = 5/group). (G and H) Enumeration of antibody-producing cells producing IgM^a anti-Ars (G) and IgM^a anti-chromatin (H). ELISPOTs were analyzed on the same spleen cells from mice treated with tamoxifen 14 d earlier (n = 4/group). Gray area represents the limit of detection (100 spots/spleen). (I) Representative spleen section from mice treated with tamoxifen 14 d earlier stained for B cells (B220, green), red pulp (F4/80, red), and Ars/A1 B cells (E4, white; n = 4/group, representative slides shown). Data shown are representative of at least two replicate experiments. Bars in B, C, D, F, G, and H represent mean ± SEM. **, P < 0.01 using an unpaired Student's t test.

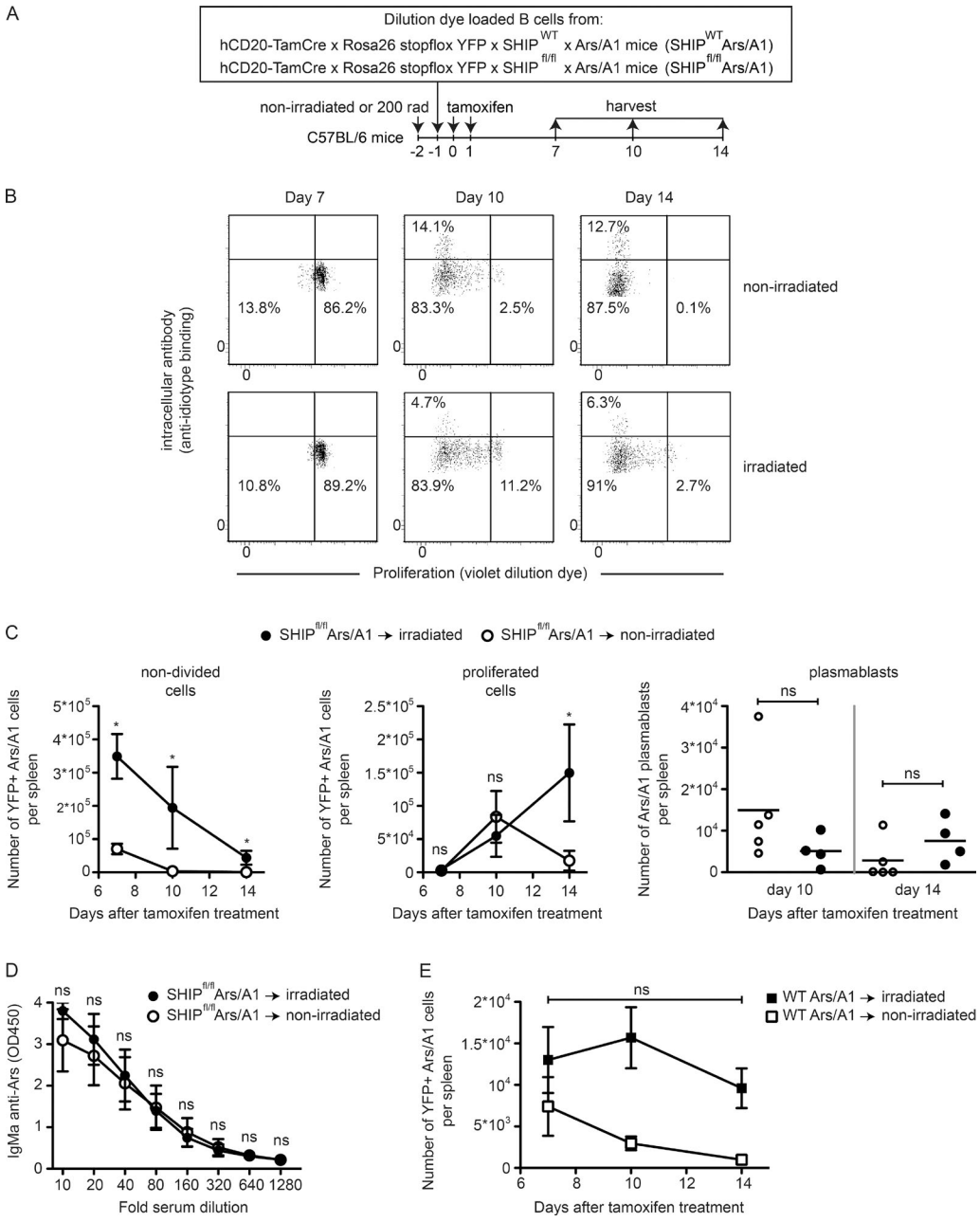

Figure 4. **Autoimmune responses of compromised anergic B cells are independent of irradiation of recipient mice.** (A) Schematic representation of the experimental protocol. In the experiment shown (A–D) 2.5×10^{-6} $SHIP^{fl/fl}$ Ars/A1 B cells were transferred to each recipient. (B) Proliferation and differentiation to plasmablasts of splenic YFP^+ cells transferred into nonirradiated or irradiated hosts at the indicated days after tamoxifen treatment (n = 4–5/group, representative plots shown). Note that in some of the nonirradiated recipients, no YFP^+ events were detected at day 14. (C) Enumeration of the number of nondivided, proliferated, and plasmablast YFP^+ cells per spleen in the experiment shown in B. Open symbols indicate cell transfer into nonirradiated hosts and filled symbols indicate transfer into irradiated hosts. The number of plasmablasts per spleen were calculated based on the top left quadrant of panels in B. (D) Ars/A1-derived IgM^a anti-Ars antibody detected in serum 14 d after tamoxifen treatment (n = 4–5/group) of nonirradiated and irradiated recipients. (E) The total number of control YFP^+ SHIP-sufficient Ars/A1 B cells present in the spleens of nonirradiated and irradiated recipients after transfer of 6×10^{-5} SHIP WT Ars/A1 B cells. None of the YFP^+ SHIP-sufficient Ars/A1 B cells had proliferated at any time point. Data shown are representative of at least two replicate experiments. Bars in C–E represent mean ± SEM. ns, $P > 0.05$; *, $P < 0.05$ using an unpaired Student's t test.

Efficiency of recruitment of compromised anergic B cells into the immune response is comparable to that of naive B cells responding to immunization

Although the aforementioned results demonstrate that loss of SHIP-1 can lead to loss of anergy, they do not report the efficiency of this response, i.e., what proportion of cells actually participate in the response upon loss of SHIP-1. We addressed this question in irradiated hosts because they allow us to assume that the starting population remains constant in size (Fig. 4 E). Before analyzing the response of rogue anergic B cells, we first determined the response of naive B cells under comparable conditions (Fig. 5, A–G). CellTracker Violet-labeled MD4 B cells were adoptively transferred into low-dose irradiated C57BL/6 recipients, and their response to two doses of SRBC-HEL was followed. As expected, higher antigen dose resulted in larger responses as measured by serum antibody titers (Fig. 5 B), as well as proliferated and differentiated cell numbers (Fig. 5, C and D). In unimmunized controls, the MD4 B cell population remained constant in size over the duration of the experiment (Fig. 5 D, left). Thus, we could calculate the proportion of precursor cells that responded based on the decrease in number of undivided cells (Fig. 5 E). As expected, more B cells responded at higher antigen doses and the proportion of cells that responded remained constant over time after a single antigen pulse. Alternatively, enumeration of cells in which dye was diluted allowed back-calculation of the approximate number of precursor MD4 B cells that seeded the proliferative response (see Materials and methods; Fig. 5 F). The fact that fewer cells can be accounted for using the latter method suggests that a significant portion of the responding MD4 B cells died after activation (Fig. 5, E vs. F). Back-calculation to determine the approximate frequency of precursor MD4 B cells that seed the plasmablast responses suggested that only a small proportion of cells ultimately seed this response (Fig. 5 G).

A similar analysis was done to determine the frequency of compromised anergic B cells that participate in the autoimmune response (Fig. 5, H–M). The number of YFP^+ Ars/A1 B cells per spleen present at day 7 after tamoxifen treatment, a point at which these cells had not begun to proliferate (Figs. 3 B and 5 I), was used as the denominator precursor population size. Based on loss of undivided cells, as in the response of naive B cells to antigen, it appeared that the majority of compromised anergic cells engaged in the autoimmune response (Fig. 5, J and K). This degree of participation is comparable to that observed in the MD4 B cell response to high antigen dose (Fig. 5 F), although the proportion of cells recruited increased over time in the case of the autoimmune response. This likely reflects the continuous exposure to self-antigen versus a pulse of exogenous antigen. It should be noted that it is not possible to exclude the possibility that loss of SHIP-1 affects the half-life of the precursor population, thereby potentially inflating this calculation. Back-calculation from proliferated cell frequency to determine the number of precursor cells that seeded the proliferative response and the plasmablast response suggested that although relatively more activated cells die before they can make a successful response (compare Fig. 5, K vs. L) to (Fig. 5, E vs. F), the frequency of cells that give rise to plasmablasts (Fig. 5, M vs. G) is comparable between naive B cells and anergic B cells that have lost tolerance. Collectively, these results suggest that once anergic B cells break tolerance, they respond to autoantigen comparably to naive B cells responding to immunogen.

Continuous suppression of the PI3K pathway is required for the maintenance of B cell anergy

We next set out to determine whether the effect of acute SHIP-1 deletion reflects a need to suppress PtdIns(3,4,5)P3 levels to maintain anergy, or if it involves some other SHIP-1 function. SHIP-1 has been reported to function as an adaptor protein, interacting with several proteins that could affect signaling independent of its phosphatase activity (Tridandapani et al., 1999; Condé et al., 2012). In addition, the product of SHIP-1 hydrolysis of PtdIns(3,4,5)P3, PtdIns(3,4)P2, has been shown to recruit TAPP1/2 proteins that play a role in maintaining B cell tolerance (Landego et al., 2012). To determine if reduction of PtdIns(3,4,5)P3 levels is required to maintain anergy, we took two approaches. Using a similar adoptive transfer strategy as described earlier for analysis of SHIP-1 function, we either induced expression of a constitutively active phosphoinositide 3-kinase (PI3K; Srinivasan et al., 2009) or induced deletion of the inositol phosphatase and tensin homologue (PTEN). Whereas PTEN is up-regulated in the MD4.ML5 model of anergy, Ars/A1 express naive cell levels of PTEN (Browne et al., 2009; O'Neill et al., 2011). Both manipulations were predicted to compensate for SHIP-1 hydrolysis of PtdIns(3,4,5)P3 in anergic cells. Indeed both approaches caused Ars/A1 B cells to gain responsiveness and proliferate and differentiate into autoantibody-producing plasmablasts (Fig. 6, A–D). These results demonstrate that SHIP-1 and PTEN enforce anergy by reduction of PtdIns(3,4,5)P3 levels.

Deletion of SHP-1 affects B cell development and disrupts B cell anergy

Src-family kinases have been implicated in activation of the regulatory tyrosine phosphatase SHP-1, as well as SHIP-1 (Nishizumi et al., 1998). Although targeted deletion of SHP-1 early in B cell development leads to a lupus-like autoimmunity (Pao et al., 2007), its role in maintenance of B cell anergy is unclear (Cyster and Goodnow, 1995). Because we hypothesize that monophosphorylation of CD79a/b ITAMs leads to a preferential activation of Lyn, thereby driving activation of SHIP-1, we reasoned that SHP-1 may also be activated and play a role in maintenance of anergy. To address this question, we crossed SHP-$1^{fl/fl}$ mice with Ars/A1 mice in which cre is expressed constitutively from early B cell development onward by virtue of the mb1 promoter or can be acutely induced using the hCD20 promoter driven TamCre and tamoxifen (as shown diagrammatically in Fig. 1 A).

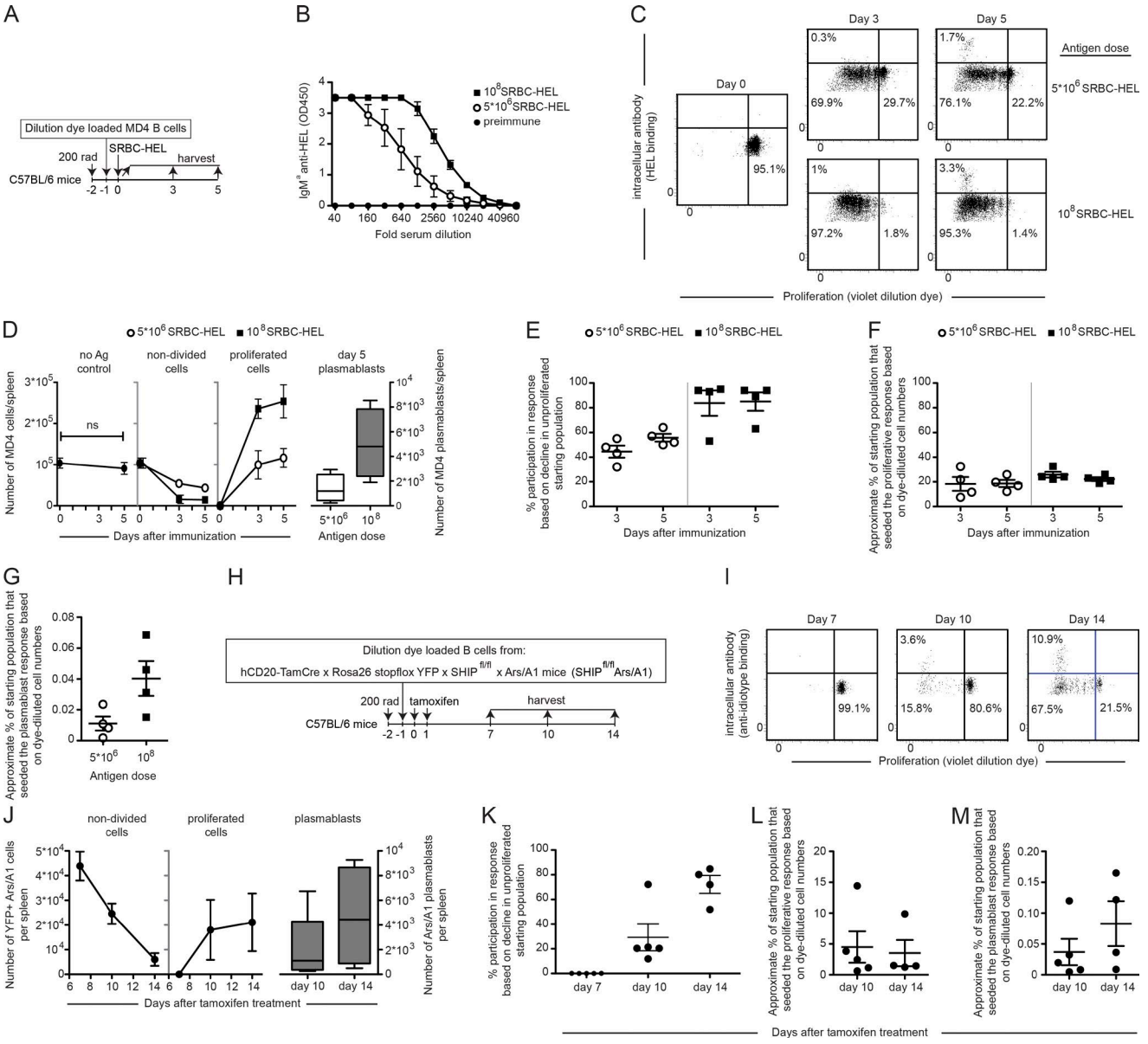

Figure 5. **Efficiency of recruitment of compromised anergic B cells into the immune response is comparable to that of naive B cells responding to immunization.** (A) Schematic representation of the MD4 experimental protocol. In the experiment shown, 6×10^{-5} MD4 B cells were transferred to each recipient. (B) IgM^a anti-HEL antibody detected in serum 5 d after immunization of adoptive MD4 recipients with the indicated doses of SRBC-HEL (n = 4–5/group). (C) Proliferation and differentiation to plasmablasts of splenic B220$^+$ anti-HEL$^+$ cells at the indicated days after immunization (n = 4–5/group, representative plots shown). (D) Enumeration of nondivided, proliferated, and plasmablast B220$^+$ anti-HEL$^+$ cells in spleen from the experiment shown in C). Plasmablast number was calculated based on the cells in the top left quadrant of cytograms in C. These numbers were correlated with antibody-secreting cell numbers determined by ELISPOT (not depicted). (left) Total number of B220$^+$ anti-HEL$^+$ cells per spleen in unimmunized mice at the indicated time points. (E) The percentage of starting population of MD4 B cells that responded to immunization based on the decrease in number of nondivided MD4 B cells per spleen. (F) The approximate percentage of the starting population of MD4 B cells that seeded the proliferative response seen at the indicated time points after immunization. (G) The approximate percentage of the starting population of MD4 B cells that seeded the plasmablast response 5 d after immunization with indicated antigen dose. (H) Schematic representation of the Ars/A1 experimental protocol. In the experiment shown, 6×10^{-5} SHIP$^{fl/fl}$ Ars were transferred into each recipient. (I) Proliferation and differentiation to plasmablasts of splenic YFP$^+$ cells at the indicated days after tamoxifen treatment (n = 4–5/group, representative plots shown). (J) Enumeration of the total number of nondivided, proliferated, and plasmablast YFP$^+$ cells per spleen of the experiment shown in I). The number of plasmablasts was calculated based on the upper left quadrant. These numbers were correlated with antibody-secreting cell numbers determined by ELISPOT (not depicted). (K) The percentage of starting YFP$^+$ E4$^+$ B cells that responded to self-antigen based on the decrease in number of nondivided YFP$^+$ E4$^+$ B cells per spleen. (L) The approximate percentage of the starting population of YFP$^+$ E4$^+$ B cells that seeded the proliferative response

First, we confirmed that constitutive B cell–targeted deletion of SHP-1 leads to autoimmunity in mice with a wild-type repertoire as indicated by antichromatin IgG levels in serum and ANA staining (Fig. 7, A and B; Pao et al., 2007). We also confirmed that in these mice, B cells preferentially differentiate into B-1 cells (Fig. 7 C). Both SHP-1–deficient B1 and B2 cells expressed reduced surface IgM and, when normalized to IgM expression level, displayed accelerated and increased calcium mobilization upon BCR cross-linking, indicating loss of regulatory signaling (Fig. 7 D).

We next crossed Ars/A1 B cell antigen receptor transgenic mice with mb1-cre SHP-$1^{fl/fl}$ mice to determine the effect of SHP-1 deficiency on the fate of chromatin-reactive B cells. As reported for the MD4.ML5 line (Cyster and Goodnow, 1995), we observed a significant decrease in the number of SHP-1–deficient Ars/A1 B cells that reached the periphery, suggesting an increase in central tolerance (Fig. 7 E). Deletion of negative regulators of B cell receptor signaling can be expected to increase BCR signal strength, thus increasing central tolerance, which in these mice results in clonal deletion because receptor editing cannot rescue these B cells that harbor a randomly inserted immunoglobulin transgene. Despite apparent increased deletion, Ars/A1 B cells that reached the periphery under these circumstances exhibited a higher frequency of autoantibody producing cells, suggesting that peripheral tolerance was not properly maintained (Fig. 7 F). Interestingly the Ars/A1 B cells that developed without SHP-1 acquired a B-1-like phenotype (Hardy, 2006; Fig. 7 G), confirming previous studies (Casola et al., 2004; Diz et al., 2008) showing that BCR signal strength controls B-1 B cell differentiation.

To avoid these confounding effects of SHP-1 deletion on B cell development and specifically assess SHP-1 requirements for maintenance of B cell anergy, we crossed these mice with hCD20 TamCre mice. Because of the proximity of the ptpn6 (SHP-1) locus to rosa26 gene, we could not use the YFP reporter in this experiment, so instead we used CD45.1 congenic mice as adoptive recipients. Using this approach, we could distinguish adoptively transferred cells (CD45.2) from host B cells (CD45.1). Using the adoptive transfer strategy described earlier for analysis of SHIP-1 function, we observed that like acute loss of SHIP-1, acute loss of SHP-1 resulted in a loss of anergy, resulting in proliferation and differentiation into autoantibody-producing plasmablasts (Fig. 8, A–C). Collectively, these results show that although a loss of SHP-1 promotes central tolerance, likely by increasing signal strength, SHP-1 is required to maintain anergy.

Genetic complementation analysis indicates that SHIP-1 and SHP-1 function in parallel pathways, both of which are required for maintenance of B cell anergy

Although at some level, all signaling pathways are interconnected, SHP-1 and SHIP-1 have distinct substrates and affect distinct pathways initiated after B cell receptor cross-linking (Ono et al., 1997). To gain insight into the relationship between the SHP-1 and SHIP-1 inhibitory pathways in maintenance of anergy we used a genetic complementation approach (Cornall et al., 1998). This experiment built on the observation that acute induction of SHIP-1 or SHP-1 haploinsufficiency in Ars/A1 B cells does not compromise anergy (Fig. 9, A and B). Similarly, induced haploinsufficiency of PTEN had no effect on the ability of Ars/A1 B cells to maintain unresponsiveness. However, acute induction of haploinsufficiency of both SHIP-1 and PTEN caused loss of anergy, presumably due their combined effects to elevate PtdIns(3,4,5)P3 levels. Both PTEN and SHIP-1 reduce PtdIns(3,4,5)P3 levels, attacking inositol 3- and 5-phosphate, respectively. Importantly, however, acute induction haploinsufficiency of both SHIP-1 and SHP-1 did not result in loss of anergy. These results suggest that risk-conferring alleles that act in the same pathway, e.g., SHIP-1 and PTEN may, by complementing each other, have a superadditive effect on risk, whereas risk-conferring alleles encoding proteins that act in different pathways do not.

DISCUSSION

Induction and maintenance of unresponsiveness of B cells that recognize low valency autoantigens presents a challenging biological problem. Such antigens, even if they have high affinity for the BCR, are likely to induce relative weak signals because they do not aggregate receptors efficiently. Available evidence suggests that when autoantigen avidity and, consequently, ability to induce signaling is not sufficient to induce editing or clonal deletion, autoreactive B cells may be rendered anergic. Chronic stimulation by such antigens leads to changes in intracellular signaling circuitry that makes the cell unresponsive to a variety of signals, including aggregation of previously unoccupied BCR. This unresponsiveness is not durable, as would be expected if it were mediated by genetic reprogramming. Removal of autoantigen from BCR can lead to restoration of responsiveness within minutes, suggesting maintenance by activation of labile regulatory signaling pathways (Gauld et al., 2005). Definitive testing of this concept and elucidation of operative pathways requires the ability to manipulate candidate regulators in already anergic cells. Availability of mice carrying acutely activable cre recombinase and loxP-flanked candidate regulatory genes have, in the context of immunoglobulin transgenesis, made it possible to address this question.

observed at the indicated time points after tamoxifen treatment. (M) The approximate percentage of the starting population of YFP^+ $E4^+$ B cells that seeded the plasmablast response at the indicated time points after tamoxifen treatment. Data shown are representative of at least two replicate experiments. Bars in B, D, E, F, G, J, K, L, and M represent mean ± SEM. ns, $P > 0.05$ using an unpaired Student's *t* test.

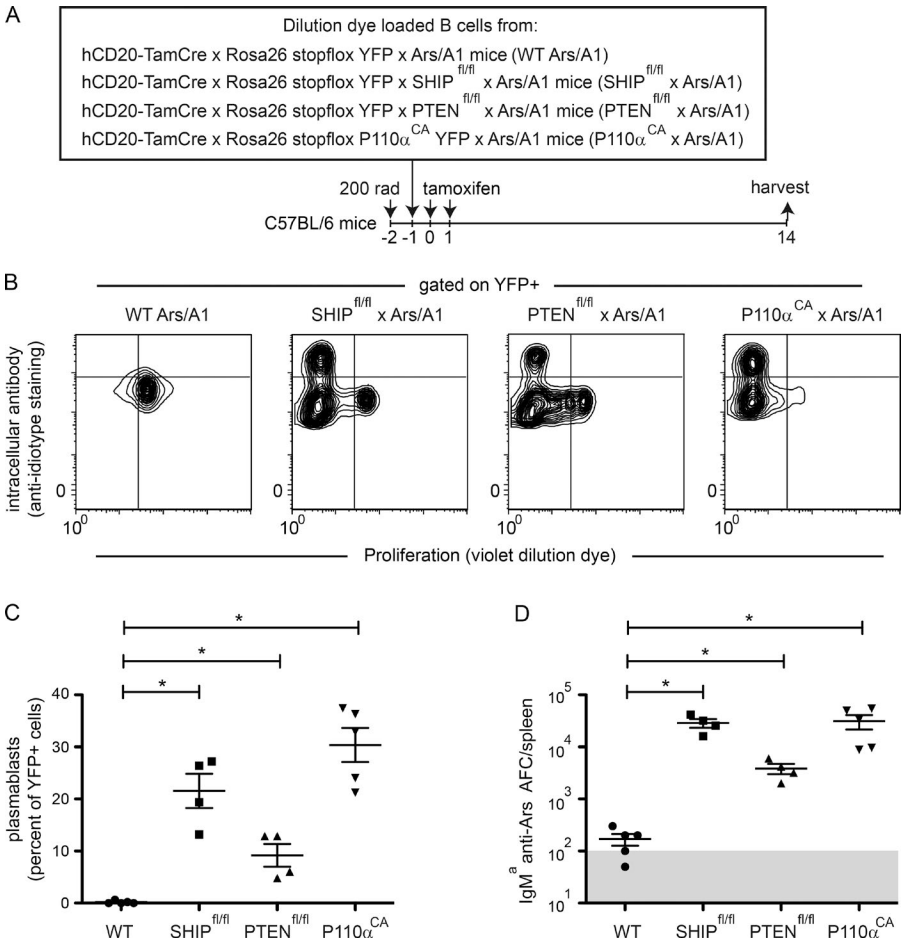

Figure 6. **Continuous suppression of the PI3K pathway is required for maintenance of B cell anergy.** (A) Schematic representation of the experimental protocol. (B) Proliferation and differentiation to plasmablasts of splenic YFP+ cells 14 d after tamoxifen treatment (n = 4–5/group, representative cytograms shown). (C) Statistical analysis of plasmablast generation based on data from top left quadrants of cytograms in B. (D) Quantification of antibody-producing cells (IgM^a anti-Ars) by ELISPOT of spleen cells from B). Gray area delineates the limit of detection (100 spots/spleen). Data shown are representative of at least two replicate experiments. Bars in C and D represent mean ± SEM. *, $P < 0.05$ using an unpaired Student's t test.

In this study, we used an experimental system in which mature anergic B cells were adoptively transferred into a normal environment, and cre was then activated to modulate inhibitory signaling in the transferred cells. We demonstrate that continuous inhibition of the PI3K pathway by the inositol 5-phosphatase SHIP-1 is required to maintain B cell anergy (Figs. 1, 2, 4, 5, and 6). We further demonstrate that tyrosine phosphatase SHP-1 is also required for the maintenance of B cell anergy (Figs. 7 and 8). Genetic complementation studies suggest that these phosphatases function in distinct pathways (Fig. 9). Finally, we demonstrate that anergic B cells that break tolerance, up-regulate activation markers and mount short-lived extrafollicular antibody responses (Figs. 3 and 4).

The approach used has clear advantages over those using conventional knockouts. Besides allowing more definitive analysis of the requirements for maintaining anergy, use of anergic B cells that were allowed to develop normally before acute induction of alterations in inhibitory signaling eliminated influences of confounding developmental effects. Although our findings regarding the role of SHIP-1 in anergy are consistent with previously published work using mb-1cre–driven deletion of SHIP-1 (O'Neill et al., 2011; Akerlund et al., 2015), they pinpoint the function of SHIP-1 to maintenance of anergy. Although SHP-1 was shown previously to be essential for maintenance of tolerance (Pao et al., 2007), its role in B cell anergy was unclear (Cyster and Goodnow, 1995). Results reported here demonstrate a critical role for SHP-1 in maintenance of anergy of chromatin-reactive B cells.

Experiments described here reveal other nonredundant effects of SHIP-1 and SHP-1 on B cell biology. Whereas a previous study using B cell–targeted deletion of SHP-1 only reported an increase in calcium mobilization in B1 cells (Pao et al., 2007), here we demonstrated that when corrected for differences in surface IgM, all SHP-1–deficient B cells have an accelerated and increased calcium mobilization response to BCR stimulation (Fig. 7 D). In contrast, SHIP-1 deficiency results in a delayed contraction of the calcium response (Liu et al., 1998). These differences indicate that these regulators likely affect distinct phases of B cell receptor signaling. In agreement with increased initial signal strength (Casola et al., 2004), we observed an increase in differentiation into B1 B cells after SHP-1 loss, even when the B cell expressed a transgenic BCR (Fig. 7 G).

How do SHP-1 and SHIP-1 maintain B cell unresponsiveness? Although SHIP-1 can modulate signaling in more ways than by its phosphatase activity (Tridandapani et al.,

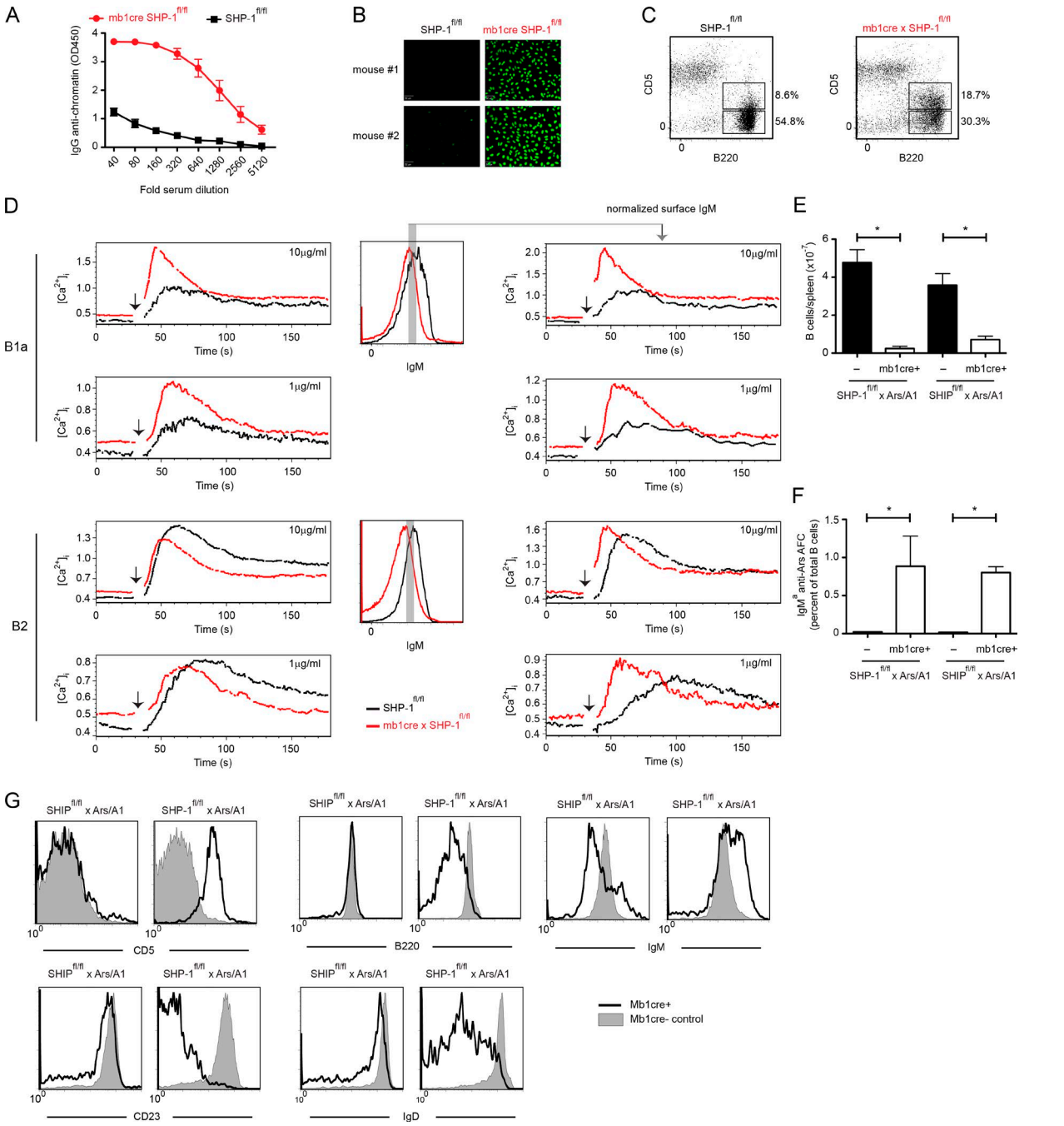

Figure 7. **Deletion of SHIP-1 and SHP-1 during early B cell development directs development of distinct peripheral B cell populations.** (A) Serum IgG anti-chromatin antibody detected by ELISA in 3-mo-old mb1cre SHP-1$^{fl/fl}$ and SHP-1$^{fl/fl}$ control mice (n = 5/group). (B) ANA staining by serum antibody from 3-mo-old mb1cre SHP-1$^{fl/fl}$ (diluted 1:100) and SHP-1$^{fl/fl}$ control mice (diluted 1:40). (C) Cell surface expression of CD5 and B220 by splenocytes from SHP-1$^{fl/fl}$ and mb1cre x SHP-1$^{fl/fl}$ mice (n = 5/group, two representative slides shown). (D) Calcium mobilization after stimulation with F(ab')$_2$ anti-IgM using the indicated final concentration of stimulus for SHP-1$^{fl/fl}$ (black line) and mb1cre x SHP-1$^{fl/fl}$ (red line) B cells. Splenic B1a cells were defined as B220$^+$ CD5$^+$, B2 cells as B220$^+$ CD5$^-$. Cytograms on the right were gated on IgM expression (gray-boxed) to analyze responses of cells expressing equivalent IgM levels (middle; n = 4/group, representative plots shown). (E) Enumeration of B cells in the spleens of mb1cre-driven B cell–targeted Ars/A1 mice deficient in SHIP-1 or SHP-1 (n = 5/group). (F) Frequency of autoantibody producing Ars/A1 cells, as measured by ELISPOT, among the total splenic B lineage cells in each mouse (n = 5/group). (G) Cell surface marker expression by B cells (B220$^+$ or CD19$^+$) from mb1cre-driven B cell targeted Ars/A1 mice deficient in SHIP-1 or SHP-1 (n = 4/group, representative plots shown). Data shown are representative of at least two replicate experiments. Bars in A, E, and F represent mean ± SEM. *, P < 0.05 using an unpaired Student's t test.

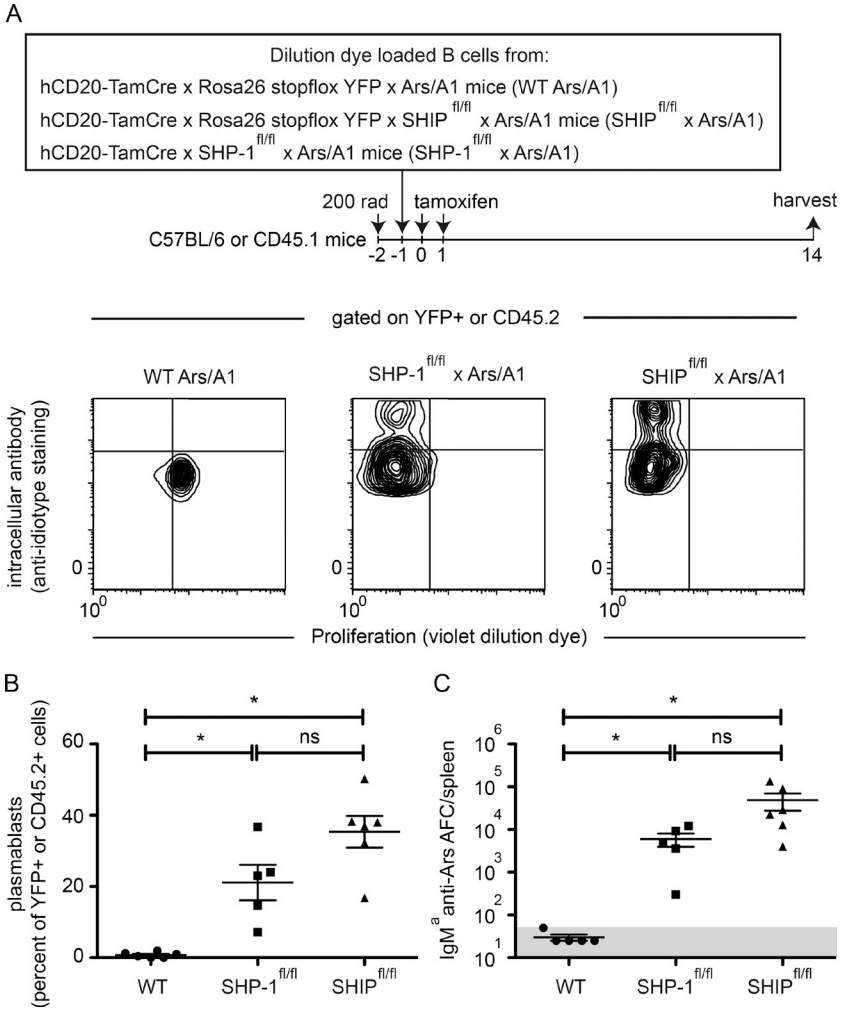

Figure 8. **Acute deletion of SHP-1 disrupts the maintenance of tolerance of anergic B cells.** (A) Schematic representation of the experimental protocol (top). (bottom) Proliferation and differentiation to plasmablasts of splenic YFP$^+$ cells (WT and SHIP-1$^{fl/fl}$) or CD45.2$^+$ cells (SHP-1$^{fl/fl}$) Ars/A1 B cells 14 d after tamoxifen treatment (n = 5/group, representative plot shown). (B) Statistical analysis of plasmablast (top left quadrant) percentage of total YFP$^+$ cells or CD45.2$^+$ cells in A. (C) Enumeration of antibody-producing cells (IgM^a anti-Ars), by ELISPOT, among spleen cells from A. Gray area delineates the limit of detection (50 spots/spleen). Data shown are representative of at least two replicate experiments. Bars in B and C represent mean ± SEM. ns, P > 0.05; *, P < 0.05 using an unpaired Student's t test.

1999; Condé et al., 2012), we demonstrated that SHIP-1–mediated suppression of the PI3K pathway is sufficient for the maintenance of B cell anergy (Fig. 6). This is consistent with a reported disability of anergic B cells to accumulate PI(3,4,5)P3 in their membrane upon BCR stimulation (Browne et al., 2009), the observed increase in SHIP-1 activity (O'Neill et al., 2011; Akerlund et al., 2015), and increased PTEN expression (Browne et al., 2009) in anergic B cells. The fact that the PI3K pathway promotes plasma cell differentiation (Omori et al., 2006) fits with the observed autoantibody response seen when inhibition of the PI3K pathway is relieved. In vitro, SHP-1 dephosphorylates several proteins important in B cell signaling, such as the Igα/β ITAMs, Syk, and BLNK (Mizuno et al., 2000; Adachi et al., 2001). Syk activation and it subsequent stimulation of downstream pathways involving proteins such as BLNK, PLCγ2, and Btk, are required for B cell activation (Takata et al., 1994). The observed 75% reduction in Syk phosphorylation seen upon receptor cross-linking in anergic B cells (Feuerstein et al., 1999; and unpublished data) is likely attributable to increased SHP-1 activity. Other SHP-1 targets of obvious importance are the ITAM tyrosines of Igα/β. SHP-1 has been reported to dephosphorylate the two tyrosines in the ITAM of the Fcγ receptor chain at different rates (Yamashita et al., 2008) suggesting a potential mechanism that may mediate the biased ITAM monophosphorylation observed in anergic B cells. The combined activity of SHP-1 and SHIP-1 are likely responsible for other downstream signaling characteristics, for example, a deficiency in CARD11 activation (Jun and Goodnow, 2003). This could occur via negative regulation of PCLγ2 resulting in failed PKCβ activation. As we have shown, both phosphatases are critical for the maintenance of unresponsiveness, functioning in a nonredundant manner.

The experimental system used in these studies allows monitoring of the fate of compromised anergic B cells. As reported previously (Nickerson et al., 2013b), anergic anti-DNA B cells that break tolerance form extrafollicular foci in which autoantibody-secreting cells reside. Studies of other models (William et al., 2002) have also demonstrated that extrafollicular responses, instead of germinal center–driven responses, are an important source for autoantibody generation. These responses are reportedly TLR dependent (Herlands et al.,

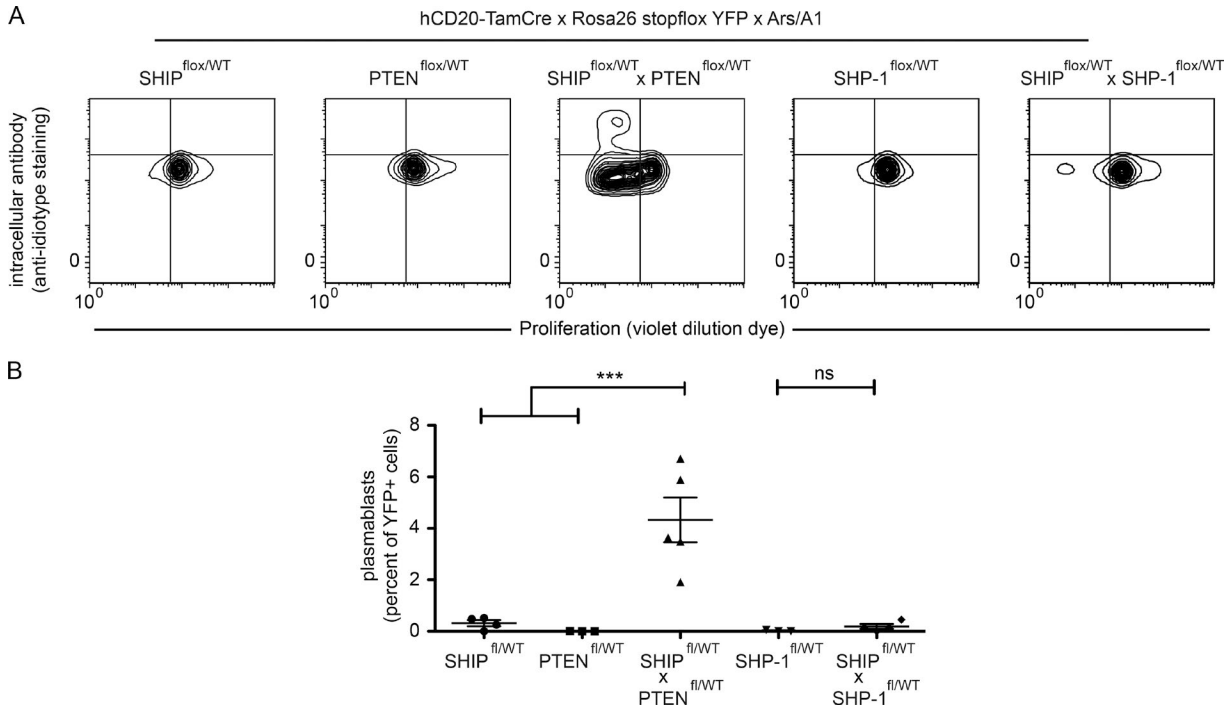

Figure 9. **Lack of genetic complementation suggests that SHIP-1 and SHP-1 maintain tolerance by functioning in independent regulatory signaling pathways.** (A) Effects of acute induction of haploinsufficiency of SHIP-1, PTEN, or SHP-1 alone or combination in Ars/A1 B cells. Proliferation and differentiation to plasmablasts of splenic YFP$^+$ cells 14 d after tamoxifen treatment (n = 4–5/group, representative plot shown). (B) Statistical analysis of the percent of plasmablasts (top left quadrant) among total YFP$^+$ cells shown in A. Data shown are representative of at least two replicate experiments. Bars in B represent mean ± SEM. ns, $P > 0.05$; ***, $P < 0.005$ using an unpaired Student's t test.

2008) and give rise to predominantly short-lived plasmablast responses (William et al., 2005). The observed short lifespan of the plasmablast response in our study (Fig. 3 B) resembles those seen in patients (Grammer and Lipsky, 2003), suggesting that in patients pathogenic autoreactive clones could have an anergic origin. Combined, available data support the concept that continuous replenishment of corrupt clones is required to sustain autoimmunity. This is perhaps the reason why Rituxan depletion of B cells has been effective therapeutically in several autoimmune diseases despite sparing plasma cells (Edwards et al., 2004; Hauser et al., 2008; Pescovitz et al., 2009).

Several studies have suggested that T cell help can prompt anergic B cells to participate in autoantibody responses (Fulcher et al., 1996; Seo et al., 2002). However, it is widely assumed that under normal conditions autoimmunity does not occur because T cells of relevant autoantigen specificity are deleted in the thymus (Adelstein et al., 1991). It is intriguing that immediately before proliferation, SHIP-1–deficient Ars/A1 B cells express elevated levels of MHC class II and CD86 consistent with subsequent productive interaction with CD4 T cells. Indeed, it has been suggested that one of the reasons interactions between CD4 T cells and anergic B cells is unproductive is because the latter do not up-regulate CD86 (Ho et al., 1994; Rathmell et al., 1998). Our own preliminary studies bearing on this point suggest that although acute SHIP-1 deletion breaks anergy leading to B cell activation, CD4 T cells are required for subsequent proliferation and differentiation into plasmablasts (unpublished data). The nature of this relationship is currently under investigation, but almost unquestionably must indicate that T cells that are competent to provide help for autoreactive B cells must exist in the periphery of normal mice. Our findings also suggest that upon becoming antigen responsive, formerly anergic B cells become competent to present cognate autoantigen antigen to T cells. This could have implications for autoimmune diseases such as diabetes, in which the role for B cells in pathogenesis is likely as antigen-presenting cells (Serreze et al., 1998; Wong et al., 2004).

Our studies to determine the extent of participation of corrupt autoreactive clones in autoantibody responses (Figs. 4 and 5, I–M) suggest that in individuals with genetic predisposition that weakens the inhibitory pathways of anergic cells, the likelihood that an individual cell instigates autoimmunity is very low. Multiple factors, either immune system-intrinsic or environmental, likely including availability of survival factors (Lesley et al., 2004; Thien et al., 2004), autoantigen, T cell help, and genetic predisposition (Fig. 9 A vs. 2 A), conspire to prompt autoimmunity.

How do our findings relate to initiation of B cell–mediated autoimmune diseases such as SLE? Although regulators

of the PI3K pathway have not yet been identified in autoimmune GWAS studies, the relevance of suppression of the PI3K pathway in human autoimmunity has recently been demonstrated. Wu et al. (2014) reported decreased expression of PTEN in B cells from patients with SLE that directly correlated with disease severity. This was a result of increased expression of microRNA miR-7. Similarly SHIP-1 has been shown to be reduced in B cells from Fas^{lpr} lupus-prone mice because of increased miR-155 expression (Thai et al., 2013). Deletion of miR-155 increases SHIP-1 expression and protects from autoimmunity. MiR-155 levels are elevated in B cells but not T cells of SLE patients and this is correlated with disease activity (Stagakis et al., 2011), suggesting that both negative regulators of the PI3K pathway may be compromised in B cells and instigate autoimmunity in certain SLE patients. SHP-1 protein levels are also reduced in B cells from a proportion of lupus patients (Huck et al., 2001). Our data suggest that these changes in protein levels destabilize the anergic state, increasing the likelihood that autoreactive cells become activated. Upstream of SHIP-1 and SHP-1, certain alleles of Lyn and the kinase Csk, and the phosphatase PTPN22 that regulate Lyn activity all predispose for development of SLE and are predicted to decrease Lyn activity (Cambier, 2013). These risk alleles may destabilize anergy by indirectly reducing SHIP-1 and SHP-1 activation in anergic cells.

Collectively, the results presented here demonstrate a mechanism by which autoreactive B cells that are silenced by anergy can become activated and drive the development of autoimmunity in individuals that possess certain risk alleles. Future work will examine further the relationship between genotype and the stability of anergy. Recently described changes that occur in the anergic population preceding development of autoimmune disease in diabetes (Smith et al., 2015) and SLE patients (Quách et al., 2011) suggest that loss of B cell anergy plays an important role in the development of autoimmunity.

MATERIALS AND METHODS

Mice. Except where otherwise indicated, 6–16-wk-old mice were used in all experiments. hCD20-TamCre animals (Khalil et al., 2012) were intercrossed with mice carrying the rosa26-flox-STOP-YFP allele (Srinivas et al., 2001), generating mice in which YFP is expressed in B cells upon Cre activation. These mice were crossed with Ars/A1 (Benschop et al., 2001) and MD4 (Goodnow et al., 1988) B cell antigen receptor transgenic mice to generate hCD20-TamCre x rosa26-flox-STOP-YFP x Ars/A1 and hCD20-TamCre x rosa26-flox-STOP-YFP x MD4 mice. B cells from these mice will be referred to as WT Ars/A1 and MD4. To generate mice in which SHIP-1 deletion can be induced in B cells, these mice were crossed with SHIP-$1^{flox/flox}$ mice (gift from J. Ravetch and S. Bolland, The Rockefeller University, New York, NY; Karlsson et al., 2003). hCD20-TamCre x rosa26-flox-STOP-YFP x Ars/A1 were also crossed to PTEN$^{flox/flox}$ mice (gift from R. Rickert, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA; Anzelon et al., 2003) and SHP-$1^{flox/flox}$ mice (Pao et al., 2007) to generate mice in which PTEN and SHP-1 deletion can be induced in anergic B cells. To generate mice in which anergic B cells can be induced to express a constitutively active PI3K pathway, hCD20-TamCre x Ars/A1 mice were crossed with Rosa26-flox-STOP-P110α^{CA}-YFP mice (gift from K. Rajewsky, Max Delbrück Center for Molecular Medicine, Berlin, Germany; Srinivasan et al., 2009). These mice have a cassette encoding a constitutively active form of P110α, the catalytic subunit of PI3K, followed by an IRES and GFP gene and preceded by a *loxP*-flanked STOP cassette, knocked into rosa26 locus. Mice in which SHP-1 deficiency is constitutively restricted to the B cell lineage were generated by crossing SHP-$1^{flox/flox}$ mice with mb1cre mice (gift from M. Reth, Max Planck Institute for Immunobiology and Epigenetics, Freiburg, Germany; Hobeika et al., 2006). These lines were crossed with Ars/A1 to generate SHP-$1^{flox/flox}$ x Ars/A1 mice and mb1cre x SHP-$1^{flox/flox}$ x Ars/A1 mice. Mice in which SHIP-1 deficiency is constitutive and restricted to the B cell lineage, mb1-Cre x SHIP-$1^{flox/flox}$, SHIP-$1^{flox/flox}$ x Ars/A1, mb1-Cre x SHIP-$1^{flox/flox}$ x Ars/A1 have been described previously (O'Neill et al., 2011). Except where otherwise indicated in adoptive transfer experiments C57BL/6 mice were used as recipients. Mice were housed and bred in the Biological Resource Center at National Jewish Health or at the University of Colorado Denver Anschutz Medical Center Vivarium (Aurora, CO), with the exception of C57BL/6 mice and CD45.1 mice (B6.SJL-*Ptprca Pepcb*/BoyJ) which were purchased from The Jackson Laboratory. All experiments with mice were performed in accordance with the regulations and with approval of the National Jewish Health (Denver, CO) and University of Colorado Denver Institutional Animal Care and Use Committee.

Adoptive transfers and tamoxifen induction. 4–24 h before adoptive transfer, C57BL/6 recipient mice were irradiated with 200 rads. Alternatively, recipients were used without prior irradiation. B cells from donor mice were enriched for by depletion of $CD43^+$ cells with anti-CD43-conjugated magnetic beads (MACS anti–mouse CD43; Miltenyi Biotec). Resultant populations were >97% B cells based on B220 staining and FACS analysis. In most experiments, the donor B cells were labeled with CellTrace Violet (Molecular Probes) at 5 μM for 4 min at room temperature before transfer. 0.5–2.5 $\times 10^6$ B cells in 200 μl PBS were adoptively transferred by IV injection. 24 h after transfer, Cre activity was induced by tamoxifen treatment. Tamoxifen (T-5648; Sigma-Aldrich) was dissolved in 10% ethanol (Decon Laboratories) and 90% corn oil (Sigma-Aldrich) at 20 mg/ml. 100 μl (2 mg) was injected IP on two consecutive days.

Antigens and immunization. To produce antigen for experiments with MD4 B cells, HEL was chemically coupled to SRBCs. SRBCs were purchased from the Colorado Serum

Company and stored in sterile Alsever's solution at 4°C. The cells were washed three times in PBS before use. 1 ml packed SRBC was resuspended in 15 ml PBS containing 5 mg/ml HEL (Sigma-Aldrich). To cross-link, 1 ml of 50 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma-Aldrich) in PBS was added, mixed, and incubated for 1 h at room temperature, with occasional agitation. Cells were then washed four times in PBS and conjugation was confirmed by flow cytometry by staining with Dylight650-D1.3 anti-HEL. Mice were immunized with 200 µl 5% SRBC-HEL in PBS by IP injection. For some experiments, we counted the SRBC-HEL with a hemocytometer and immunized with 5×10^6 or 10^8 SRBC-HEL in 200 µl PBS by IP injection.

Phenotypic analysis by staining and FACS. Single-cell suspensions of splenic cells were prepared, and red blood cells were lysed using ammonium chloride TRIS. Cells were resuspended in PBS containing 1% FCS and 0.05% sodium azide and incubated with an optimal amount of antibodies. For analysis of cell surface markers, all cells were stained with B220-PerCP (RA3-6B2; BioLegend) and biotinylated antibodies were directed against the following molecules: CD5 (53-7.3; BD), CD22 (Cy34.1), CD45.2 (104; BioLegend), CD80 (16-10A1; BD), CD95 (Jo2; BD), CXCR4 (2B11; BD), CXCR5 (2G8; BD), IgM (b-7-6), IgD (JA12.5), I-Ab (AF6-120.1; BD), and B220 (RA3-6B2). This was followed by SA-PE-Cy7 (BioLegend) staining or stained directly with PE-Cy7–conjugated antibodies directed against the following: CD23 (B3B4; BioLegend), CD44 (IM7; BioLegend), CD69 (H1.2F3; BioLegend), CD86 (GL-1; BioLegend); CD138 (281–2, BioLegend). Cy34.1, b-7-6, JA12.3, and RA3-6B2 were produced in our own laboratory and were biotinylated using EZ-Link Sulfo-NHs-Biotin (Thermo Fisher Scientific) according to the manufacturer's protocol. After cell surface staining, the cells were fixed and permeabilized with Cytofix/Cytoperm (BD) and stained with Dylight650-E4 anti-Ars/A1 idiotype (produced and conjugated in our laboratory) and Alexa Fluor 488–anti-GFP (rabbit polyclonal; Life Technologies). For detection of intracellular SHIP-1, splenocytes were first stained with PE–anti-B220, fixed, and permeabilized with Cytofix/Cytoperm (BD) and stained with affinity-purified rabbit Alexa Fluor 647–anti–SHIP-1 (raised against murine SHIP-1 909–959 peptide; Tamir et al., 2000) and Dylight450-E4–anti-Ars/A1 idiotype (both produced and conjugated in our laboratory). To determine the kinetics of SHIP protein depletion after tamoxifen treatment, we stained peripheral blood B cells intracellularly for SHIP at the indicated time points. The mean fluorescent intensity of SHIP staining of YFP^+ B cells was compared with that of control C57BL/6 B cells (100%) and mb1-cre $SHIP^{fl/fl}$ B cells (0%). Events were collected on a CyAn ADP (Dako) analyzed using FlowJo software (Tree Star).

Calculations to determine participation in the immune response. To determine Ars/A1 B cell localization in spleen, we determined the total number of YFP^+ Ars/A1 B cells per spleen by multiplying the total splenocyte count (using a hemocytometer) with the percentage of YFP^+ $E4^+$ cells (as determined by flow cytometry). The CellTrace Violet dye dilution profile was determined for the YFP^+ $E4^+$ population. Using FlowJo, we were able to detect 8 peaks of violet dye fluorescence intensity, peak 1 (undivided cells), peaks 2–7 (1–6 divisions) and peak 8 (≥7 divisions). The total number of cells in each division state was calculated by determining their frequency among the YFP^+ $E4^+$ population. The mean number of YFP^+ $E4^+$ cells present at day 7 (at this time there was no proliferation [Fig. 3 B]) was assumed to reflect the starting population (X). Participation in the immune response was determined in two ways. Because a control population of anergic B cells remained constant in size during the duration of the experiment, we measured the number of undivided YFP^+ $E4^+$ cells per spleen (Y) and calculated the relative decrease of this population $(X - Y)/X$ assuming that the decrement had divided, participating in the response. We also estimated the number of cells (S) that seeded the proliferative response observed by dividing total number of cells in each dye-diluted peak by the number of divisions undergone by cells of that intensity: (Peak 2/2) + (Peak 3/4) + (Peak 4/8) + (Peak 5/16) + (Peak 6/32) + (Peak 7/64) + (Peak 8/128). These data are presented as proportion of the starting population S/X. To estimate the frequency of cells (P) that seeded the plasmablast population we divided the total number of plasmablast (YFP^+, intracellular $E4^{hi}$) by 128 because all YFP^+E4^{hi} cells fell in peak 8, having divided at least seven times. These data were presented as proportion of the starting population P/X. The frequency of MD4 B cells participating in a response was calculated similarly by gating on $B220^{hi/int}$ intracellular high HEL $binding^+$ population.

Analysis of calcium mobilization. For measurements of intracellular free calcium concentration ($[Ca^{2+}]_i$), splenocytes were simultaneously stained with APC-RA3-6B2 anti-B220 and loaded with Indo-1 acetoxymethyl (Indo1-AM; Molecular Probes), as described previously (Gauld et al., 2005). In some experiments, PE-RA3-6B2 anti-B220 and Dylight650-b76 Fab anti-IgM (made in-house) was used instead of APC-RA3-6B2 anti-B220. For analysis of $[Ca^{2+}]_i$, cells were resuspended at 10^7 cells/ml in warm IMDM with 2% FCS in a 500 µl volume. After analysis for 30 s to establish the baseline, cells were stimulated with 5 µg $F(ab')_2$ rabbit anti–mouse IgM (µ chain; Jackson ImmunoResearch Laboratories, Inc.; or the dose indicated in the figure) for 2.5 min. Mean relative $[Ca^{2+}]_i$ was monitored over time using an LSR II flow cytometer (BD) with analysis using FlowJo software (Tree Star).

Enzyme linked immunosorbent assay. For detection of IgM^a anti-p-Azophenylarsonate (Ars) antibodies, microtiter plates were coated with 10 µg/ml Ars-BSA_{16} in PBS and blocked with 2% BSA in PBS 0.05% Tween-20. For detection of IgM^a anti-HEL antibodies, microtiter plates were coated with 10

μg/ml HEL in PBS and blocked with 2% BSA in PBS 0.05% Tween-20. Serial dilutions of mouse serum in PBS were added and incubated overnight at 4°C. Ars/A1-derived IgM anti-Ars antibodies were detected with biotinylated RS3.1 anti-IgM^a, followed by Streptavidin-HRP (Thermo Fisher Scientific). Between all steps, the plates were washed four times with PBS-0.05% Tween-20. The ELISA was developed with TMB single solution (Invitrogen) and the reaction was stopped with 1N H_2PO_4 (Sigma-Aldrich). The OD was determined at 450 nm using a VERSAMax plate reader (Molecular Devices), and the data were analyzed with Softmax software.

ELISPOT. For detection of IgM^a anti–p-Azophenylarsonate (Ars) antibodies, microtiter plates were coated with 10 μg/ml Ars-BSA_{16} in PBS and blocked with 2% BSA in PBS 0.05% Tween-20. For detection of IgM^a anti-chromatin antibodies, microtiter plates were coated with 10 μg/ml calf chromatin in PBS with 1 mM EDTA, followed by incubation with blocking buffer (2% BSA in PBS 0.05% Tween-20, and 1 mM EDTA). Before use, the plates were washed twice with PBS and once with complete medium (IMDM supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml Pen/Strep, 50 mg/ml gentamicin, and 0.1 mM 2-Me.). Spleens were disrupted in complete medium and red blood cells were lysed. Two-fold serial dilutions were made starting at 1/100 of a spleen in the first well. The plates were incubated at 37°C for 6 h or overnight. Ars/A1-derived antibodies were detected with biotinylated RS3.1 (anti-IgM^a), followed by Streptavidin-AP (SouthernBiotech). Between all steps, the plates were washed four times with PBS–0.05% Tween-20. The plates were developed by incubating with ELI SPOT development buffer (25 μM 5-bromo-chloro-3-indolyl phosphate p-toluidine, 100 mM NaCl, 100 mM Tris, and 10 mM $MgCl_2$, pH 9.5) for 1 h. The reaction was stopped by washing the plate three times with double-distilled H_2O. The number of spots at a cell dilution in the linear range was determined, and the number of ASCs was calculated.

ANA detection. Mouse sera were diluted to 1:40 (SHP-$1^{flox/flox}$) or 1:100 (mb1cre x SHP-$1^{flox/flox}$) in PBS and incubated on HEp-2 cell slides (BION) for 1 h at room temperature. The slides were then washed in PBS, stained with Alexa Fluor 488 anti–mouse IgG (Molecular Probes) for 1 h at room temperature, washed, and mounted in Fluoromount G (SouthernBiotech). The slides were analyzed using a Leica DMRXA microscope (ZEISS) under a 10× objective and further analyzed using Slidebook software.

Immunohistochemistry. Spleens were flash frozen in Tissue-Tek OCT embedding media (Sakura) and 6 mm sections were cut using a cryostat and thaw-mounted on Superfrost Plus glass slides (Thermo Fisher Scientific), air dried for >3 h, and kept at −80°C. Frozen slides were fixed in ice-cold acetone for 5 min, air-dried, rehydrated in PBS, and blocked with 5% FCS in PBS. The sections were stained with FITC-RA3-6B2 anti-B220 (BD), Dylight650-E4 anti-Ars/A1 idiotype, and PE-BM8 anti-F4/80 (BioLegend). The slides were washed twice and mounted in fluoromount G (SouthernBiotech). Images were obtained using an Inverted 200M microscope (ZEISS) under a 10× objective and further analyzed using Slidebook software.

Statistics. Statistical analyses were performed using the unpaired Student's *t* test. P-values <0.05 (*) were considered statistically significant. P-values <0.01 are represented by (**), and p-values <0.005 are represented by (***).

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