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High Rate of Antibody Secretion Is not Integral to Plasma Cell Differentiation as Revealed by XBP-1 Deficiency

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During B cell terminal differentiation, a complex set of transcription factors interact to drive the phenotypic and functional changes leading to the development of Ab-secreting cells (ASCs). The transcription factor X-box binding protein 1 (XBP-1) is an essential part of one of the branches of the unfolded protein response (UPR). The UPR is induced when a cell has to handle large amounts of proteins, as is the case in ASCs. Although XBP-1 was initially also ascribed an indispensable function in plasma cell development, later studies of B cell-specific deletion reported a much milder consequence of XBP-1 deficiency. Our interest was to determine whether XBP-1 was integral for the differentiation of plasma cells. Using both in vitro and in vivo assays, we found efficient generation of ASCs in the absence of XBP-1. ASCs were present at normal frequencies in resting and immunized mice and displayed a pattern of surface markers typical for plasma cells. The absence of XBP-1 resulted in a reduction but not ablation of Ab secretion.

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The differentiation of B cells to Ab-secreting cells (ASCs) requires the regulated interplay of several transcription factors (1). In this process, factors that sustain a B cell in an undifferentiated state, such as Pax5, are downregulated (2, 3), whereas factors promoting ASC differentiation are activated. Master regulators such as Blimp-1 or IFN regulatory factor 4 (IRF4) belong to this latter group and are responsible for repressing transcription of B cell genes or actively promoting the expression of ASC genes, respectively (4–9). X-box binding protein 1 (XBP-1) is a transcription factor also implicated in plasma cell differentiation, although its mechanism of action is less well defined (10–13). XBP-1 has a role in the unfolded protein response (UPR) that is induced when the endoplasmic reticulum (ER) is overloaded with unfolded proteins (14, 15). This may result in a translational blockade to reduce the amount of new unfolded proteins (16, 17) and an increased expression of chaperones that facilitate protein folding (18). An inability of the UPR to resolve ER stress can further result in the initiation of apoptosis in some circumstances (18, 19). XBP-1 is a critical downstream player in the IRE1 branch of the UPR (14). After activation, the bifunctional transmembrane kinase/endoribonuclease IRE1 oligomerizes and thereby autoactivates its RNase domain. Activated IRE1 then splices out a small fragment of the mRNA transcript of XBP-1. This creates a frameshift, which generates the functional version of the XBP-1 protein, containing both a transactivation and a DNA-binding domain (14).

Plasma cells are specialized cells that are able to secrete large amounts of Ig and therefore rely on a functional UPR (15). It is thus not surprising that defective expression of XBP-1 impacts on the normal operation of a plasma cell. The role of XBP-1 in the development of plasma cells is, however, proposed to extend beyond its function in the UPR (10, 20). Although XBP-1 is expressed at a low level throughout B cell development, expression is induced dramatically during plasma cell differentiation in response to cytokines such as IL-4 (11). Early studies using chimeric mice demonstrated that the generation of plasma cells is highly impaired in the absence of XBP-1 (12). This led to the conclusion that XBP-1 is required for the differentiation of plasma cells themselves, not only for ensuring their proper secretory function. The developmental block in plasma cell differentiation of these chimeras occurred between B cell activation and terminal differentiation (12), and XBP-1 was attributed a key role in the signaling events during this process, including the upregulation of the plasma cell survival factor IL-6 (11).

In more recent studies, the generation of conditional, B cell-specific knockout mice has allowed examination of the impact of XBP-1 deficiency specifically on B cell differentiation (10, 13). These studies confirmed that XBP-1 was required for high rates of Ig secretion but surprisingly not for the formation of plasma cells. These two studies differed, however, in important aspects of their conclusions, with Todd et al. (13) concluding that XBP-1 controlled a novel developmental stage after the expression of the canonical plasma cell marker, Syndecan-1 (CD138), whereas Hu et al. (10) proposed broader functions of XBP-1 in signal transduction through

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N.T. performed most of the experiments and wrote the manuscript; S.C. and L.M.C. performed experiments; and D.M.T., P.D.H., and S.L.N. provided input into experimental design and manuscript preparation.

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Abbreviations used in this article: ASC, Ab-secreting cell; ER, endoplasmic reticulum; IRE4, IFN regulatory factor 4; KLH, keyhole limpet hemocyanin; m, membrane-bound; NP, 4-hydroxy-3-nitrophenylacetyl; s, secreted; UPR, unfolded protein response; WT, wild-type; XBP-1, X-box binding protein 1.

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the BCR, controlling the appropriate expression of IRF4 and Blimp-1 and homing to the bone marrow niche.

We have had a long-standing interest in defining the cellular and molecular pathways involved in late B cell differentiation (1, 5, 21, 22). Thus, the purpose of the current study was to more precisely dissect the different functions of XBP-1 and to clarify whether it is involved in development of plasma cells independent of its function in UPR. Using quantitative analysis of in vitro B cell cultures, we have demonstrated a striking relationship between cell division history and ASC differentiation, with the proportion of B cells that undergo either of these differentiation events typically increasing with each consecutive division (22). In this study, we have used this quantitative approach as a tool to investigate how XBP-1 deficiency impacted on the development and function of ASCs. We find that XBP-1 plays no role in the division-linked differentiation of B cells to ASCs in vitro, in the expression of IRF4 or Blimp-1, or in the generation of plasmablasts and plasma cells in vivo. Thus, XBP-1 is not a major player in the gene regulatory network controlling ASC development. We show, however, that XBP-1 is specifically required for high-level expression of intracellular Ig and the resulting morphological changes that enable the plasma cell to secrete large amounts of Ab.

Materials and Methods

Mice

Xbp1fl/flCd19Cre/+ mice have been previously described (10, 13, 23), and Xbp1fl/flCd19Cre/+ mice were also crossed to Blimp1GFP/+ reporter mice (7). All procedures involving animals were approved by the Animal Ethics Committee of the Walter and Eliza Hall Institute.

B cell purification and cell culture

Small resting B cells were purified out of splenic single-cell suspensions by Percoll density gradient. Naive B cells were then isolated with the B cell purification kit (MACS; Miltenyi Biotec), and for some experiments, cells were labeled with Cell Trace Violet (Molecular Probes), according to the manufacturer’s protocol. Cells were cultured in B cell medium as previously described (22) and harvested at the specified time points for further analysis. Concentrations of stimuli were 20 μg/ml LPS (Sigma-Aldrich), 500 U/ml IL-4 (a gift from R. Kastelein, DNAX Research Institute, Palo Alto, CA), and 5 ng/ml IL-5 (a gift from A. Hapel, John Curtin School of Medical Research, Canberra, ACT, Australia). Mouse CD40L was expressed on the Sf21 insect cell line infected with a mouse CD40L- Medical Research, Canberra, ACT, Australia). Mouse CD40L was produced by Qiagen (Hilden, Germany) RNasey kit, and cDNA was prepared using an Invitrogen (Carlsbad, CA) SuperScript III First-Strand Synthesis System, both according to the manufacturers’ protocols. Expression of all genes analyzed was quantified and normalized to expression of the housekeeping gene H3bns using the following primer sequences. The primers used for analysis of Xbp1 expression detected all isoforms. Primer sequences were as follows: Xbp1, fwd (ex2), 5′-AGCAGCAAGAGTTGTTGTTGTCAC-3′; Xbp1, rev (ex3), 5′-CCAGCCGTTCTTCATTACCTCTTT-3′; H3bns, fwd, 5′-GACCTGGCTTACTCACCCTTTGAG-3′; H3bns, rev, 5′-AAACAGAGCATCAAGAGTTTCCCT-3′; Csf1r, fwd, 5′-CACCTTGAGCAACCTCTTATACCGAGGAG-3′; Csf1r, rev, 5′-CTGGATCTGCAGGCTTGTCTCTTCTCTCTC-3′; C1y, membrane bound, 5′-GTCCTGGGCTCTACAGCAGTCTGGTC-3′; C1y secreted, 5′-CCAAGAGGCTGTCAGAGCAC-3′; CAAGAGGCTGTCAGAGCAC-3′; CAAGAGGCTGTCAGAGCAC-3′; C4a, membrane bound, 5′-GATGAACTGTGAGCTGTCAGAGCAC-3′; C4a secreted, 5′-CAGGGTCGCCCTGTGTCAGAGCAC-3′; and Cm, common, 5′-CTCAGGATCGGCCATGGCAAGGAG-3′. Reactions were run and products analyzed using a Bio-Rad (Hercules, CA) C1000 Thermal Cycler.

Preparation of cells for transmission electron microscopy

Cells were pelleted in microcentrifuge tubes, the supernatant was discarded, and the intact cell pellet was fixed in 1% osmium tetroxide in PBS for 2 h. The cell pellets were rinsed three times in fresh PBS for 10 min each before being dehydrated in increasing concentrations of ethanol consisting of 10, 30, 50, 70, 90, 100, and 100% anhydrous ethanol for 15 min each step. Following dehydration, the cell pellets were infiltrated with increasing concentrations of LR-White Resin (Sigma-Aldrich) in ethanol consisting of 25, 50, 75, and 100% resin for 6 h each step. After a second change of 100% resin, the cell pellets were embedded in fresh resin in gelatin capsules. The gelatin capsules were capped to exclude air, and the resin was polymerized in an oven at 60°C for 24 h.

Embedded cells in blocks were sectioned with a diamond knife on a Leica Ultracut R microtome, and ultrathin sections (90 nm) were collected onto pioloform-coated 100 mesh hexagonal copper grids. The sections on grids were sequentially stained with 2% uranyl acetate for 10 min and Triple Lead Stain for 5 min (26) and viewed in a Phillips CM120 Biotwin transmission electron microscope at 120 kV.

Results

XBP-1 deficiency does not affect B cell proliferation or survival

The amount of Ab produced following an immune response can be affected by genetic changes that influence cell behavior at numerous levels, including altering proliferation, survival, or differentiation rate. Because plasma cell differentiation is tightly linked to cell division number, we first examined the proliferation kinetics of B cells lacking XBP-1. It has been previously described that the deletion of XBP-1 in B cells does not affect cell viability and proliferation, when stimulated with LPS (13). We wanted to expand this analysis by performing a more detailed investigation of the proliferation of XBP-1 deficient B cells using different stimulation conditions and analyzing the proliferation profiles based on the distribution of cells per division. For these experiments we isolated naive B cells that were wild-type (WT), heterozygous (Het), and XBP-1 deficient (Xbp−/−).
homozygous (Xbp1fl/+Cd19Cre/+) or XBP-1 deficient (Xbp1fl/fl Cd19Cre/+). As expected, conditional deletion of XBP-1 in mature B cells expressing CD19-Cre was very efficient, with little residual full-length Xbp1 mRNA detected under all conditions tested (Supplemental Fig. 1). Stimulation of the naive B cells with CD40L+IL-4 for various times lead to comparable proliferation in B cells deficient or heterozygous for XBP-1 as compared with WT B cells (Fig. 1A). Moreover, the cells were distributed over the same spectrum of division numbers at each of the time points, irrespective of their XBP-1 expression (Fig. 1B). Similar results were observed for cells stimulated with LPS (Supplemental Fig. 2A). Viability of the cells, as determined by the exclusion of nucleosomes.
Annexin V-positive cells, was identical at the different time points (Supplemental Fig. 2B). Thus, this quantitative analysis demonstrates that neither proliferation nor survival of B cells under different stimulation conditions is affected by XBP-1 deficiency.

**XBP-1 is dispensable for plasma cell differentiation in vitro**

We investigated the capacity of XBP-1–deficient B cells to differentiate into plasma cells by analyzing in vitro-stimulated B cells for the expression of proteins that are linked to plasma cell differentiation. We first crossed the Xbp1<sup>fl/fl</sup> Cd19<sup>Cre/+</sup> mice to Blimp1<sup>GFP/+</sup> reporter mice to detect plasmablasts that have upregulated Blimp-1 by their expression of GFP (7). Using a range of stimuli such as CD40L+IL-4+IL-5, LPS, LPS+IL-4, and CpG+IL-4 or CpG+IL-4+IL-5 (Fig. 2; data not shown), we observed little difference between B cells that lacked XBP-1 and WT B cells in the induction of Blimp-1 expression, as measured by total percentage of GFP<sup>+</sup> cells in culture or as the proportion of differentiated cells per division. This result indicates that XBP-1 is not required for the coordinated, division-linked expression of Blimp-1 in activated B cells.

Terminal B cell differentiation is further accompanied by changes in expression of many other proteins, such as upregulation of IRF4, Syndecan-1, or CD43, isotype switching and downregulation of B220. Because XBP-1 deficiency was proposed to affect IRF4 expression (10) and plasma cell development downstream of Syndecan-1 expression (13), we determined whether these changes could occur in the absence of XBP-1. To do this, we measured the expression of each of these proteins after stimulation with CD40L+IL-4+IL-5 or LPS+IL-4, conditions that strongly induce plasma cell differentiation and isotype switching in vitro. Under both conditions, the upregulation of IRF4 and the downregulation of B220 were independent of XBP-1 (Fig. 3). Similarly, isotype switching to IgG<sub>1</sub> under both stimulation conditions occurred normally in the absence of XBP-1 (Fig. 3). The proportion of Syndecan-1<sup>+</sup> cells in later divisions was reduced by ~25% in XBP-1–deficient B cells, when stimulated with CD40L+IL-4+IL-5 but not with LPS+IL-4. In contrast, CD43, a protein expressed on terminally differentiated B cells in vitro (27), was strongly dependent on XBP-1 for its expression after stimulation with both CD40L+IL-4+IL-5 and LPS+IL-4 (Fig. 3).

**Development of ASC in the absence of XBP-1**

The previous results showed that, in the absence of XBP-1, the capacity of B cells to assume a plasma cell phenotype remained virtually intact. It was thus interesting to see whether in vitro-generated XBP-1–deficient Syndecan-1<sup>+</sup> cells secreted Ab at the same rate as WT cells. We sorted Syndecan-1<sup>+</sup> cells from day 4 cultures of different stimulation conditions and analyzed the proportion of IgG<sub>1</sub>-secreting cells via ELISPOT. In comparison with WT, XBP-1–deficient B cells produced fewer IgG<sub>1</sub>-secreting cells, and these produced ELISPOTs that were uniformly of small size (Fig. 4A, 4B). Furthermore, XBP-1–deficient B cells secreted lower amounts of IgM and IgG<sub>1</sub> when stimulated with CD40L+IL-4, CD40L+IL4+IL-5, or LPS+IL-4 (Fig. 4C).

Closer examination of the ASC population that develops after stimulation with LPS+IL-4 showed that while the proportion of cells that differentiated to IgG<sub>1</sub> Syndecan-1<sup>+</sup> cells was comparable to WT B cells (Fig. 4D), the level of IgG<sub>1</sub> expression in this population, as detected by intracellular staining, was markedly lower in B cells that lacked XBP-1 (Fig. 4E). We investigated whether this was due to a defective splicing of Ig transcripts by analyzing the expression of the mRNA encoding the membrane-bound (m) and the secreted (s) forms of IgM and IgG<sub>1</sub> (Fig. 4F).

Because the polyadenylation factor Cstf64 is required for the switch from the membrane-bound to the secreted form of IgM (28), we wanted to determine whether the reduced frequency of sIgG<sub>1</sub> in XBP-1–deficient B cells resulted from reduced expression of Cstf64. This was not the case because the expression of Cstf64 was if anything higher in the absence of XBP-1. The reduced amount of intracellular IgG<sub>1</sub> in XBP-1–deficient ASCs was consistent with the...
Because the in vitro activation of B cells might not reflect their full plasma cell development and Ab secretion. (Supplemental Fig. 3), indicating significant XBP-1–independent Syndecan-1 expression on the Blimp-1 hi population (Fig. 5A, the absence of XBP-1, however, we consistently observed higher in any of these subsets with or without XBP-1 (Fig. 5A, 5B). In isons revealed no significant difference in the proportion of cells lived plasma cells in the spleen and bone marrow (7). Compar-

To analyze the capacity of the cells to secrete Abs ex vivo, we sorted Syndecan-1+Blimp1-GFP+ cells of the different subsets and determined the number of ASC, and the rate of Ig secretion per hour on a per cell basis. The total number of Igk-secreting cells in Xbp1fl/flCd19Cre/+ mice did not differ significantly from WT mice (Fig. 5F), but in both splenic and bone marrow Blimp1-GFP+ cells, the absence of XBP-1 caused a significant reduction in Igk secretion per cell (Fig. 5G). We further found significantly lower numbers of IgM- and IgA-secreting cells among the Blimp1-GFP hi subset of the spleen in Xbp1fl/flCd19Cre/+ mice (Fig. 5C, 5E), whereas the numbers of IgG-secreting cells was significantly reduced only in the Blimp1-GFP hi subset of the bone marrow (Fig. 5D). The reduced Ab secretion of XBP-1–deficient B cells was also apparent from the much smaller spots in the ELISPOT assay (Supplemental Fig. 4). Given the similar frequencies of Igk-secreting and Blimp1-GFP+ cells in control and Xbp1fl/flCd19Cre/+ mice (Fig. 5A, 5B, 5F), we conclude that the decreased ASC number recorded for some Ig isotypes was the result of the very low Ig production and not an actual decrease in ASC number. In comparison, cells with intermediate Blimp-1 expression from the spleen showed a normal representation of ASCs of all isotypes and did not differ in the amount of Ig secreted per cell when XBP-1 was absent (Fig. 5C-G). These defects in Ab secretion paralleled those observed in vitro.

Because the in vitro-generated plasmablasts had abnormal ER levels, we sought to examine the ultrastructures of the different plasma cell subsets acquired ex vivo. As shown in Fig. 6A, all XBP-1–deficient cells displayed an abnormal pattern, lacking the multiple dense ER layers that were observed in WT plasma cells. All ASCs had increased amounts of cytosol, which is typical for plasma cells, but in most of the cells lacking XBP-1, large vesicle-like structures were present that were not characteristic of WT ASCs. Many of the XBP-1–deficient plasma cells contained enlarged and disorganized ER (Fig. 6B), with higher magnification revealing the presence of ribosomes around the vesicles indicative of rough ER (Fig. 6C).

To further analyze the in vivo generation of ASCs, we examined B cell differentiation during a T cell-dependent immune response by immunizing mice i.p. with the hapten NP coupled to the carrier protein KLH in alum (Fig. 7). Development of NP-specific B cells and ASCs was assessed by FACS or ELISPOT, and secretion of anti-NP Abs was measured by ELISA. At days 7 and 28, Ag-specific B cells, identified as NP-binding IgG1+B220+ and negative for IgD, IgM, and Gr-1, were found in the spleen at equal frequency in WT and Xbp1fl/flCd19Cre/+ mice (Fig. 7A). Thus, GC-dependent expansion of NP-specific B cells was unimpaired in the absence of XBP-1. The numbers of NP-specific ASCs detected by ELISPOT did not differ in spleens from WT and Xbp1fl/flCd19Cre/+ mice on day 7 (Fig. 7B), suggesting a normal extrafollicular plasmablast response, but showed a significant reduction in both the spleen and bone marrow on day 28 (Fig. 7C). Affinity maturation did not seem to be affected by the absence of XBP-1, be-cause the ratio of high-affinity anti–NP-secreting cells over total NP-specific ASCs was equivalent to the WT controls (Fig. 7D).

Once again, the most striking difference between WT and XBP-1–deficient mice was in the amount of sIg. Serum was taken from the mice on days 7, 21, and 28 after immunization, and the concentration of NP-specific IgG1 was determined. At all three time points, the titers of NP-specific IgG1 were significantly lower (p < 0.005) in Xbp1fl/flCd19Cre/+ sera than in control sera (Fig. 7E). This was further confirmed by analyzing the NP-IgG1 secretion per
FIGURE 4. In vitro analysis of Ab secretion in XBP-1-deficient B cells. (A and B) In vitro-stimulated B cells from Xbp1+/+ (WT) and Xbp1fl/+Cd19Cre/+ (fl/+)) and Xbp1fl/flCd19Cre/+ (fl/fl) mice were cultured for 4 d with different stimuli before being sorted for Syndecan-1 expression and analyzed by ELISPOT for the presence of IgG1-secreting cells. (A) shows the percentage of cells secreting IgG1 ± SEM (n = 6), with a representative well of cells cultured in CD40L+IL-4 shown in (B). Original magnification ×2.7. (C) IgM and IgG1 concentration in the supernatant from cells stimulated for 5 d in the indicated conditions as determined by ELISA. Each dot represents cultures derived from an individual mouse of the indicated genotypes. The p values for (A) and (C) compare the indicated genotypes using a t test. ns, p > 0.05. (D and E) B cells from mice of the indicated genotypes were stimulated for 4 d with LPS+IL-4 and analyzed by intracellular FACS for the expression of Syndecan-1 and IgG1. (E) shows the IgG1 expression of gated Syndecan-1+IgG1+ cells (WT, solid line, Xbp1fl/flCd19Cre/+ dashed line). (F) Expression of the mRNA encoding mIgM and mIgG1 as well as expression of the polyadenylation factor Cstf64 were determined by quantitative PCR. RNA was generated from B cells of WT or fl/fl mice cultured for 5 d with the indicated stimuli. (G) Syndecan-1+ cells were sorted from a day 5 culture with CD40L+IL-4+IL-5 and analyzed by transmission electron microscopy. Three representative cells of the indicated genotypes are shown.
cell. This rate was highly reduced in XBP-1–deficient ASCs on day 7 and sank below the ELISA detection level on days 21 and 28 (Fig. 7E). Thus, these in vivo studies align with our in vitro observations of XBP-1–deficient B cells. Although mutant B cells can develop and differentiate into ASCs and home to bone marrow, their capacity to secrete Ab is strongly reduced in all tissue sites.

**Discussion**

The aim of this study was to gain a deeper understanding of the function and importance of XBP-1 in the terminal differentiation of B cells to plasma cells. Because of the embryonic lethality of XBP-1 deficiency, the initial report on the role of XBP-1 in plasma cells used complementation of Rag2<sup>−/−</sup> blastocysts with Xbp1<sup>−/−</sup> embryonic stem cells to generate chimeric animals where all lymphocytes were XBP-1 deficient (12). Besides low Ig in the serum, these mice showed a strong reduction in the number of plasma cells, pointing toward a role for XBP-1 as a key player in the transcriptional control of plasma cell development. Later studies using B cell-specific Xbp1<sup>−/−</sup> knockout mice, however, cast doubt on these conclusions because they showed the presence of XBP-1–deficient plasma cells at numbers equal to those of WT mice. The analysis of a B cell-specific Xbp1<sup>−/−</sup> knockout mouse by Todd et al. (13) revealed the normal development of Ag-specific plasma cells during the course of a T cell-dependent immune response. The numbers of Ag-specific cells and ASCs were comparable to WT mice, with reduced serum Ig levels being the only difference. These authors concluded that XBP-1 plays a role in a novel developmental stage where ASCs are Syndecan-1 + yet have not differentiated to a high Ig-secreting stage. Conversely, Hu et al. (10) analyzed the same conditional mouse model and proposed...
a more profound function of XBP-1 in the differentiation process, with XBP-1 controlling the outcome of signaling through the BCR, IRF4 expression level, and bone marrow homing. As we aim to fully understand the transcriptional program underlying the steps leading to plasma cell formation, we considered it important to refine our understanding of XBP-1. In particular, we wanted to investigate whether XBP-1–deficient B cells were not only impaired in their capacity to secrete Ig but were also affected in other ways. To answer this question, in this study, we analyzed the generation and function of plasma cells in steady-state after in vitro stimulation or after T cell-dependent immunization.

A number of proteins are differentially regulated during the development of plasma cells. We investigated whether the expression of these proteins was affected by the lack of XBP-1. Although previous studies reported stronger expression of Blimp-1 or IRF4 protein after stimulation of XBP-1–deficient B cells with LPS (10, 13), our in vitro studies revealed normal induction of these transcription factors that act both as definitive markers of ASCs and as key regulators of the differentiation process. The appropriate acquisition of IRF4 and Blimp-1 demonstrates that terminal B cell differentiation proceeds relatively normally in the absence of XBP-1. Similarly, the proportion of cells that upregulate the plasma cell marker Syndecan-1 was relatively normal. Interestingly, whereas the intensity of the Syndecan-1 expression was not different in vitro, XBP-1–deficient plasma cells in vivo showed higher expression compared with WT plasma cells. One possible explanation for this observation is that XBP-1–deficient plasma cells have altered membrane turnover kinetics because of the decreased Ig secretion rates observed.

A striking phenotypic difference we found was the loss of CD43 expression in the absence of XBP-1 in vitro. The function of CD43 is poorly understood. It is a sialoglycoprotein expressed on
most leukocytes and has been proposed to regulate proliferation and adhesion of T cells (29–31). CD43 is expressed on early developing B cells until the pre-B cell stage and is upregulated after stimulation in vitro with LPS and other stimuli (27, 32). In our in vitro studies, we found the induction of CD43 to be a terminal event in differentiation, being expressed after the induction of Blimp-1 and Syndecan-1 (data not shown). From these results, CD43 appears to be a late marker of in vitro-generated ASCs and is a potential XBP-1 target gene at this stage. However, we saw only low CD43 expression levels on plasma cells ex vivo from both the bone marrow and spleen. It is therefore unclear whether CD43 has an important function on plasma cells in vivo or whether its reduced expression on XBP-1–deficient B cells in vitro is related to the defective Ig secretion of Xbp1fl/flCd19Cre/+ mice.

The induction of the transcriptional repressor Blimp-1 is undoubtedly a major event in plasma cell differentiation (7–9). Using B cells from Blimp1GFP/+ reporter mice, we saw little difference in the Blimp-1 expression between WT and XBP-1–deficient B cells on both a population- and a division-based level (Fig. 2). In vivo, plasma cells in the spleen and bone marrow can be separated into short-lived plasmablasts and long-lived, established plasma cells, according to their increasing levels of Blimp-1 expression (7). The analysis of plasma cells in the spleen and bone marrow of naive mice or mice immunized with NP-KLH showed that the development of both Blimp1-GFPint and Blimp1-GFPint cells was not affected by the absence of XBP-1. In contrast to earlier reports (10), our studies furthermore demonstrate that plasma cells are not dependent on XBP-1 for migration to the bone marrow. The discrepancy between the findings of Hu et al. (10) and that reported in this study are surprising because both studies used identical Xbp1fl/flCd19Cre/+Blimp1GFP/+ mice on a C57BL/6 background. However, an important difference between the studies is that we have used T-dependent immunization to elicit an immune response from the normal polyclonal B cell repertoire, whereas Hu et al. (10) used hen egg lysozyme-specific MD4-transgenic B cells. It is conceivable that the repeated immunization of MD4 cells with

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**FIGURE 7.** T cell-dependent immune responses in Xbp1fl/flCd19Cre/+ mice. Xbp1fl/+ (WT) or Xbp1fl/flCd19Cre/+ (fl/fl) mice were immunized with NP-KLH in alum. Mice were sacrificed on day −7 or −28 and analyzed for the proportion of NP IgG1+ cells in the spleens of WT (black circles) and fl/fl (open circles) mice (A). Data are the mean ± SEM of six mice per genotype (B–D). Splenocytes (days −7 and −28) as well as bone marrow cells (day −28 only) were analyzed for the presence of NP-specific IgG1+ ASCs by ELISPOT. ELISPOT wells were coated with either high (NP14) or low (NP2) haptenated proteins to detect total or high-affinity NP-specific IgG1 ASCs as indicated on the graphs. The p values in (A) and (C) compare values from the indicated genotypes. (B) Original magnification ×3.2. (D) Affinity maturation was determined by measuring the ratio of high-affinity NP2-IgG1 to total NP14-IgG1. Data are the mean of this ratio ± SEM. (E) Serum was collected on days −7, −21, and −28, and the titer of NP14-IgG1 was determined by ELISA. The secretion of NP14-IgG1 on a per cell basis was analyzed by cultivating splenocytes or bone marrow cells ex vivo for 6.5 h and performing ELISA of the supernatant and then normalizing the data for the number of NP14-IgG1+ ELISPOTs as determined in (B) and (C). Note the NP14-IgG1 secretion rate per cell per hour of samples from day −28 spleen and bone marrow from XBP-1–deficient were below the detection rate in this assay. Each dot is representing a single mouse. WT (black circles) and fl/fl (open circles).
a high-affinity Ag such as hen egg lysozyme results in energy and the reduced plasma cell homing to the bone marrow that was not observed in this study using a polyclonal response.

Both our in vitro and in vivo analyses thus show development of XBP-1–deficient plasma cells that, according to their expression of the typical surface markers, are virtually indistinguishable from WT plasma cells. In contrast, XBP-1–deficient plasma cells are functionally defective in that they secrete much less Ig and fail to remodel their ER in a manner typical of a highly secretory cell type. Thus, despite Ig secretion being the sole function of ASCs, it is not required for the relatively normal B cell terminal differentiation. Surprisingly, analysis of the transition from the mIg to sIg forms of Ig mRNA and intracellular staining for IgG1 indicated that the loss of XBP-1 resulted in a defect prior to the high production of Ig. This finding agrees with the observation that XBP-1 promotes IgM synthesis (33) and is inconsistent with the idea of an accumulation of abnormal amounts of Ig in plasma cells because of the absence of XBP-1 and thus a functional UPR. How XBP-1 controls the switch to the mRNA coding for the secreted form of Ig is at present unclear. A complex of the polyadenylation factor, Cstf64 and the elongation factor ELL2 with RNA polymerase II, is known to promote sIgM and sIgG (34). At present, we have no evidence that XBP-1 directly controls the expression of either of those genes because ELL2 is a known target of IRF4 (35) and Blimp-1 (36, 37), whereas we found that the expression of Cstf64 was unchanged in the absence of XBP-1. Future work will address the mechanism by which XBP-1 promotes the transition from mIg to sIg.

The inability to upregulate XBP-1 in plasma cells was not without consequence because the electron microscopic analysis of XBP-1–deficient plasma cells ex vivo shows the presence of large intracellular vesicles instead of the typical structure with layers of ER. These vesicles appear to be the end product of enlarged ER structures that can be observed in many of the XBP-1–deficient plasma cells. Further evidence that the vesicles emerge from rough ER is the presence of ribosomes on their membrane, whereas the lack of a second enveloping membrane suggested that these structures were not autophagic vesicles. Given that one of the branches of the UPR is defective in XBP-1–deficient cells, it was conceivable that the ER expands as a consequence of the accumulation of large amounts of intracellular Ig. We believe that this is unlikely, because immunofluorescent confocal microscopy of the different plasma cell subsets did not reveal aberrant structures containing large amounts of Ab, when stained for IgG1, or Igκ (data not shown). Because Ig represents the vast majority of the total secreted protein in a WT ASC, one explanation is that the aberrant ER structures form as a consequence of the lack of available Ig in XBP-1–deficient cells (33). This scenario is in keeping with the previous findings that the early enlargement of the ER precedes the increased Ig production and is XBP-1 independent (38) and would explain the specific appearance of these structures in vivo, because these ASCs have a much higher Ig secretion rate than their in vitro-produced counterparts.

Although the development of plasma cells in steady-state conditions was relatively unaffected by the loss of XBP-1, we have also investigated whether XBP-1–deficient B cells showed impaired phenotypic or functional differentiation in the course of a T cell–dependent immune response. In this situation, it was possible that cells would be subject to more acute ER stress in the absence of XBP-1. Analysis of the Ab responses at early and later time points after immunization revealed that XBP-1–deficient B cells could undergo relatively normal extrafollicular and germinal center responses, as judged by the frequency of Ig class-switched Ag-binding ASCs and the normal rate of affinity maturation. Again, the major defect was in Ig secretion, with XBP-1–deficient ASCs secreting much lower amounts of Ig on a per cell basis than WT cells. These experiments did show some loss of NP-specific ASCs at 4 wk after immunization; however, because of the lower sensitivity of ELISPOT compared with ELISA and the fact that Ab production decreases during the immune response, it is likely that this simply reflects the fact that XBP-1–deficient secretion rate had dropped below a threshold where spots could be detected and not an actual loss of ASCs.

The data presented in this paper show that XBP-1 does not function as a key transcription factor in the gene regulatory network that governs the differentiation of mature B cells into plasma cells. Although our data are broadly in agreement with that of Todd et al. (13), the conclusions differ because the authors of that study propose that XBP-1 regulates a late yet-to-be-defined stage in plasma cell differentiation that occurs after the acquisition of Blimp-1 and Syndecan-1 expression but before full Ig secretion. Although it is clear that the activation of XBP-1 is an event that is triggered by differentiation (39), we propose a different interpretation of the data, where the function of XBP-1 is predominantly, if not exclusively, enabling high Ig expression and the morphological changes that are associated with B cell terminal differentiation.

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Disclosures
The authors have no financial conflicts of interest.

References
High rate of antibody secretion is not integral to plasma cell differentiation as revealed by XBP-1 deficiency

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Supplemental Figure 1. Xbp-1 expression under different stimulation conditions. Splenic B cells from WT, Xbp1<sup>fl/fl</sup>Cd19<sup>Cre/+</sup> and Xbp1<sup>fl/fl</sup>Cd19<sup>Cre/+</sup> mice were stimulated <em>in vitro</em> for 4 days under different conditions. RNA was obtained from resting and stimulated B cells and Xbp-1 expression analyzed by qPCR.
Supplemental Figure 2. Proliferation and apoptosis of B cells from WT and Xbp1<sup>fl/fl</sup>Cd19<sup>Cre/+</sup> mice. Splenic B cells were labeled with Cell Trace Violent and stimulated <em>in vitro</em> with LPS or CD40L+IL4 for the indicated times. (A) Absolute number of live cells per well at different time points and division distribution of cells at each time point. Data shows mean ± SEM of three replicates per genotype per time point. (B) Proportion of viable (Annexin V-negative) cells at the indicated timepoint after stimulation with CD40L+IL4 or LPS. Data shows the mean ± SEM of three replicates per genotype per time point.
Supplemental Figure 3. Serum Ig levels of naïve WT, Xbp1<sup>fl/fl</sup>Cd19<sup>Cre/+</sup>, Xbp1<sup>fl/fl</sup>Cd19<sup>Cre/+</sup> and Rag2<sup>−/−</sup> mice. Serum levels of IgG<sub>1</sub>, IgG<sub>2b</sub>, IgG<sub>3</sub>, IgM and IgA were determined by ELISA. Significance was determined by student t-test. ns=not significant (P > .05), * P < .05, ** P < .01, *** P < .005, **** P < .001.
Supplemental Figure 4. ELISPOT of plasma cells sorted from WT and Xbp1^{RIP} Cd19^{Cre/+} mice. Blimp-GFP_{int} and Blimp-GFP_{hi} cells from the spleen as well as Blimp-GFP_{hi} cells from the bone marrow were sorted and the number of cells secreting Igk, IgM, IgA or IgG determined by ELISPOT.