Differential requirement for OBF-1 during antibody-secreting cell differentiation

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Resting B cells can be cultured to induce antibody-secreting cell (ASC) differentiation in vitro. A quantitative analysis of cell behavior during such a culture allows the influences of different stimuli and gene products to be measured. The application of this analytical system revealed that the OBF-1 transcriptional coactivator, whose loss impairs antibody production in vivo, has two effects on ASC development. Although OBF-1 represses early T cell–dependent (TD) differentiation, it is also critical for the completion of the final stages of ASC development. Under these conditions, the loss of OBF-1 blocks the genetic program of ASC differentiation so that *Blimp-1/prdm1* induction fails, and *bcl-6*, *Pax5*, and *AID* are not repressed as in control ASC. Retroviral complementation confirmed that OBF-1 was the critical entity. Surprisingly, when cells were cultured in lipopolysaccharide to mimic T cell–independent conditions, *OBF-1–null* B cells differentiated normally to ASC. In the *OBF-1*$^{-/-}$ ASC generated under either culture regimen, antibody production was normal or only modestly reduced, revealing that Ig genes are not directly dependent on OBF-1 for their expression. The differential requirement for OBF-1 in TD ASC generation was confirmed in vivo. These studies define a new regulatory role for OBF-1 in determining the cell-autonomous capacity of B cells to undergo terminal differentiation in response to different immunological signals.

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Abbreviations used: ASC, antibody-secreting cell; CFSE, carboxyfluorescein diacetate succinimidyl ester; GC, germinal center; TD, T cell–dependent.

Antibody-secreting plasma cells represent the culmination of the B cell differentiation program, which is a multistep process that is tightly regulated by both extrinsic and intrinsic factors. Because serum antibody arises from complex molecular and cellular interactions (migration, cellular interactions, and cell-autonomous responses to receptor-mediated signals), it is often difficult to determine the mechanism underlying a functional defect. An important example is the transcription factor OBF-1 (also known as OCA-B or Bob.1; references 1, 2). OBF-1 is a transcriptional coactivator that binds, with Oct-1 or Oct-2, to the octamer DNA element in the regulatory regions of B cell–restricted target genes (2, 3). Such binding sites occur in the promoters of most Ig variable region genes, though mice lacking OBF-1 and/or Oct-2 exhibit largely normal B cell development and express normal levels of Ig on the surface of peripheral B cells (4–8). In vivo studies of *OBF-1–null* mice revealed that B cell numbers in peripheral lymphoid organs were near normal, but that humoral responses to the antigen were severely diminished; this was particularly evident for the Ig of switched isotypes (5–7). *OBF-1–null* mice lack both germinal centers (GCs) and marginal zone B cells, and they have a paucity of fully mature B cells in the periphery (9, 10), which implies that OBF-1 has diverse influences on B cell development.

Kim et al. (5) proposed that the poor antibody response of OBF-1–deficient mice was a consequence of reduced Ig gene transcription by an antibody-secreting cell (ASC) expressing switched isotypes. Indirect effects have also been suggested, as OBF-1 modulates the expression of the *CXCR5* gene that encodes the receptor for CXCL13, which is a chemokine that positions B cells in lymphoid follicles (11). Furthermore, Casellas et al. (12) found that a subset of Igκ gene promoters were particularly dependent on OBF-1 for efficient expression. In the face of these manifold influences, the precise mechanism underlying the poor humoral response in *OBF-1*$^{-/-}$ mice is unclear.

We recently developed a quantitative culture system for T cell–dependent (TD) B cell

activation in vitro, leading to proliferation, isotype switching, and the generation of dividing, antibody-secreting "plasmablast" cells (13, 14). Using this analytical system, cell behavior can be monitored in relation to the cell division number, and even subtle changes in differentiation rates, proliferation, or survival can be measured. The ability to measure multiple properties simultaneously and quantitatively provides a powerful tool for identifying B cell–intrinsic defects arising from genetic alterations. We applied this analysis to B cells lacking OBF-1 and revealed a critical role for this factor in division-linked ASC differentiation. OBF-1 deletion was found, paradoxically, to accelerate the initial rate of B cell differentiation while blocking the final stages of ASC development. OBF-1 was found to lie upstream of *Blimp-1/prdm1*, an essential regulator of ASC differentiation, during T cell–driven ASC differentiation in vitro and in vivo. In contrast, OBF-1 was found to be dispensable for ASC differentiation induced in vitro or in vivo using LPS, a T cell–independent B cell activator. These results give novel insights into the role of OBF-1 in determining serum Ig levels and serve to illustrate how quantitative in vitro methods may prove useful for exploring the mechanisms of action of other B cell function regulators.

RESULTS

OBF-1–deficient B cells display a cell-autonomous defect in antibody production

OBF-1$^{-/-}$ mice, both naive and immunized, display abnormally low levels of serum Ig. IgM levels are modestly reduced, whereas Ig levels of switched isotypes such as IgG1 are markedly lower (5–8). As the absence of GCs in OBF-1–null mice (5–7) would have a strong indirect influence on Ig titers in vivo, we wanted to determine the intrinsic antibody-producing capacity of *OBF-1–null* B cells in our model system of TD ASC differentiation (14). To do this, we purified small resting *OBF-1–null* B cells, cultured them in the presence of the T cell stimuli CD40L and IL-4, and measured the Ig secreted into the medium over 7 d. B cells from similarly purified C57BL/6 (B6) control mice were used as controls. Representative data, shown in Fig. 1 A, indicate that IgM and IgG1 production were differentially affected by the loss of OBF-1 (reduced by 3.6 ± 2.5-fold and 38.9 ± 11.8-fold, respectively; mean ± SD of five determinations). IgE is also produced under these conditions and was reduced in *OBF-1*$^{-/-}$, compared with control cultures, by 38- and 49-fold in two independent assessments. Therefore, OBF-1–deficient B cells have an intrinsically reduced capacity to produce Ig in response to T cell–derived signals when cultured under optimal conditions, and switched isotypes are most strongly affected.

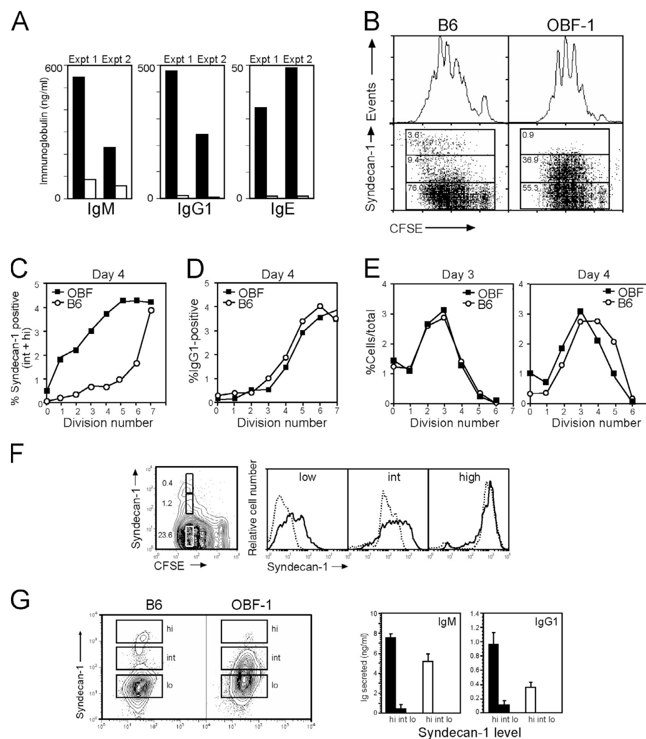

Figure 1. OBF-1 is required for normal ASC differentiation. (A) Ig levels in day 7 culture supernatants of small resting B cells, purified and pooled from four B6 or *OBF-1*$^{-/-}$ mice, stimulated with CD40L/IL-4. Data from two independent experiments are shown (shaded bars, B6; open bars, *OBF-1*$^{-/-}$). (B) B cells prepared, CFSE labeled, and cultured as described previously (14) were harvested after 4 d and stained for Syndecan-1 expression. Histograms demonstrate the CFSE profile (top) and the dot plots show Syndecan-1 expression. (C) Graph showing the proportion of cells present in each cell division peak that was Syndecan-1^{+} at day 4 of the culture (as in B; open circles, B6; closed squares, *OBF-1*$^{-/-}$). (D) Graph showing the proportion of IgG1^{+} cells present in each cell division peak at day 4 of the culture (as in B). (E) Graph showing the proportion of live cells present in each cell division peak at days 3 and 4 of the culture (as in B). (F) The cell division linked differentiation of Syndecan-1^{-} cells to Syndecan-1^{int} and Syndecan-1^{hi} cells by control B6 B cells. CFSE-labeled B6 B cells were cultured for 3 d, and then stained for Syndecan-1 expression. Cells from division 3 were sorted by Syndecan-1 level (high, int, and low; left, boxes indicate sorting gates and include the percentage of cells in each sorting gate). Postsort purity is shown by the dotted line histograms (right). Cells were returned to the culture for a further 26 h and restained for Syndecan-1 (continuous line histograms). (G) B cells were stained after 4 d of CD40L/IL-4 stimulation and sorted on the basis of Syndecan-1 expression (left, sorting gates shown as boxes). There is no stain in the x axis channel, but the gain is set to enable easy visualization of rare Syndecan-1^{hi} cells. Cells were recultured in the absence of mitogens and cytokines. The antibody-secreting capacity of the cells, based on the Syndecan-1 level, was determined. Ig secreted into supernatant by 2,000 sorted cells during 4 h of culture is shown ([right] shaded bars, B6; open bars, *OBF-1*$^{-/-}$). Values are mean ± SD of triplicate cultures.

OBF-1 is required for normal ASC differentiation in vitro

B cells cultured in CD40L/IL-4 over several days differentiate to dividing ASC (plasmablasts) that express high levels of the surface marker Syndecan-1 (14). ASC generated in vivo, or produced in vitro under other culture conditions, can be polymorphic with respect to their Syndecan-1 expression (15, 16). However, we found that all antibody-secreting functions lie within the Syndecan-1^{hi} population of CD40L/

IL-4 cultures and have used this marker here to identify ASC generated in vitro. Functional confirmation was performed in parallel (Fig. 1 F).

The poor antibody yield from *OBF-1*$^{-/-}$ cell cultures suggested that OBF-1 is required either for B cells to differentiate to ASC under these conditions, or for the ASC generated to secrete normal amounts of antibody. To monitor the rate of proliferation and the development of ASC per division, B6 and *OBF-1*$^{-/-}$ B cells were labeled with the cell division–tracking dye carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured with CD40L/IL-4. Cells were harvested after 4 d and analyzed for Syndecan-1 expression. The CFSE histograms show that cells in both cultures proliferated strongly (Fig. 1 B, top). Although control cultures yielded significant numbers of Syndecan-1^{hi} cells in later divisions, there was a clear deficit in the *OBF-1*$^{-/-}$ cultures of the Syndecan-1^{hi} cells that marked the ASC population (Fig. 1 B, bottom). Instead, cells expressing an intermediate level of Syndecan-1 were found in markedly increased numbers (Fig. 1 C). The proportion of isotype-switched cells per division was similar in both cultures (Fig. 1 D); however, the development of both IgM^{+} and isotype-switched IgG1^{+} Syndecan-1^{hi} ASC was blocked as a result of the *OBF-1* mutation (not depicted). The cell survival and average division profiles of control and mutant B cells were identical on day 3 of the culture (Fig. 1 E and not depicted), but by day 4, control cultures contained a slightly higher number of cells in later divisions. We recently demonstrated that Syndecan-1^{hi} plasmablasts, which arise in later divisions, divide more rapidly than undifferentiated B cell blasts (14). This would contribute to the difference in division profiles on day 4.

We were interested in the derivation of cells that bore an intermediate level of the ASC marker Syndecan-1 (Fig. 1 B). Therefore, we subjected normal mouse B cells to a kinetic analysis and monitored the fate of the Syndecan-1 marker. B6 B cells were purified, CFSE labeled, and cultured as described previously (13, 14). After 3 d, cells from a single cell division were sorted by their Syndecan-1 levels (Fig. 1 F) and returned to culture. After 24 h, cells were reanalyzed for Syndecan-1 expression. We found no evidence for the shedding or internalization of surface Syndecan-1, as expression at 6 h was equal to or higher than the level at the time of sorting (not depicted). The Syndecan-1^{hi} cells remained stable after 26 h and many Syndecan-1^{int} cells had become Syndecan-1^{hi}, whereas some Syndecan-1^{lo} cells became Syndecan-1^{int}, which was consistent with a progression towards the increased expression of this marker.

We determined the antibody-producing capacity of cells sorted by Syndecan-1 level to confirm that only the Syndecan-1^{hi} cells were ASC and to directly measure the requirement for OBF-1 in antibody production by preformed plasma cells. The loss of OBF-1 dramatically reduces the number of Syndecan-1^{hi} cells produced in culture, but rare Syndecan-1^{hi} cells are generated in *OBF-1*$^{-/-}$ cultures. B6 and *OBF-1–null* B cells, cultured for 4 d in CD40L/IL-4, were sorted into low, intermediate, and high Syndecan-1 populations (Fig. 1 F). Equal numbers of each were cultured for a further 4 h in the absence of stimuli. ASC activity in each culture was determined using ELISA to measure the amount of antibody secreted during this time. Only the Syndecan-1^{hi} cells secreted antibody effectively, regardless of genotype (Fig. 1 F). *OBF-1–null*, Syndecan-1^{hi} ASCs secreted almost normal levels of IgM. In contrast, IgG1 secretion was reproducibly reduced in the *OBF-1*$^{-/-}$ cultures compared with controls. Collectively, these data show that OBF-1 is required for efficient ASC differentiation under TD conditions, but it is dispensable for IgM production by terminally differentiated ASCs. However, optimal IgG1 production from ASCs requires OBF-1, which is consistent with previous results (5).

OBF-1 regulates the rate of ASC differentiation

We previously showed that Syndecan-1^{hi} ASCs are generated in this culture system at an increasing frequency with

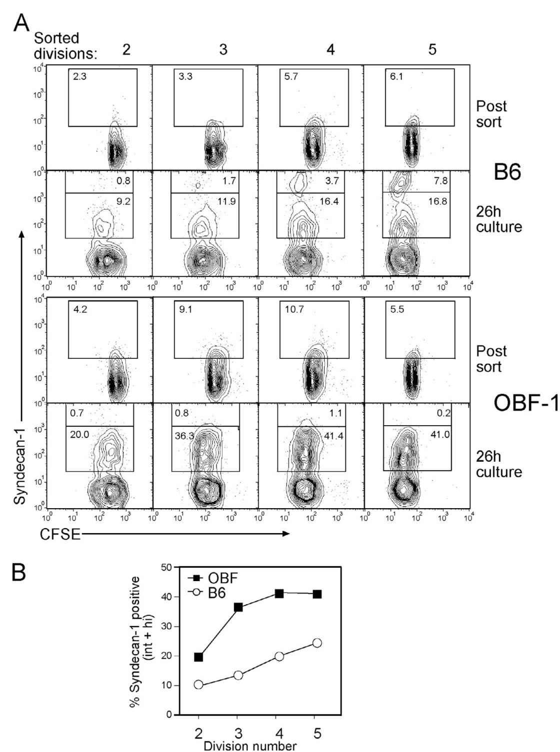

Figure 2. Accelerated differentiation of *OBF-1*$^{-/-}$ B cells. (A) Comparison of differentiation rates for B6 and *OBF-1*$^{-/-}$ B cells. CFSE-labeled B cells were cultured with CD40L/IL-4 for 3 d, and then stained for Syndecan-1 expression. Syndecan-1^{-} cells sorted from divisions 2, 3, 4, and 5 are shown. Postsort purity is shown (top). Cells were returned to culture for a further 26 h and restained for Syndecan-1 (bottom). The percentages of cells in the indicated regions are given. Note that Syndecan-1^{hi} cells largely fail to form in the *OBF-1*$^{-/-}$ cultures. (B) Quantitative summary of the differentiation depicted in the contour plots of A. The percentage of Syndecan-1^{-} cells that became Syndecan-1^{+} during 26 h of culture after sorting each cell division peak is shown.

successive divisions (14), and Fig. 1 F shows that cells pass through a Syndecan-1^{int} phase during this differentiation. The higher proportion of Syndecan-1^{int} cells in *OBF-1*$^{-/-}$ cultures (Fig. 1, B and C) suggested that this process is initiated more rapidly in cells lacking OBF-1, but the data might also be explained by the preferential growth or survival of *OBF-1*$^{-/-}$ Syndecan-1^{int} cells. To directly assess whether OBF-1 loss alters the rate of generation of Syndecan-1^{int} from Syndecan-1^{-} cells, using the division number as a reference, control and mutant B cells were purified, CFSE labeled, and cultured. On day 3, individual cell division peaks were sorted. Syndecan-1^{-} cells from each cell division were purified (Fig. 2 A, postsort plots) and placed back into the culture (with CD40L/IL-4) for a further 26 h. Although control cells differentiated to Syndecan-1^{int} and then to Syndecan-1^{hi} cells, *OBF-1*$^{-/-}$ B cells differentiated to Syndecan-1^{int} cells much more rapidly per division than control B cells (Fig. 2, A and B), implying that a normal function of OBF-1 is to restrict this transition. There was little progression of *OBF-1*$^{-/-}$ cells to the Syndecan-1^{hi} state, confirming a second critical role for OBF-1 in the differentiation of Syndecan-1^{hi} ASC in this system.

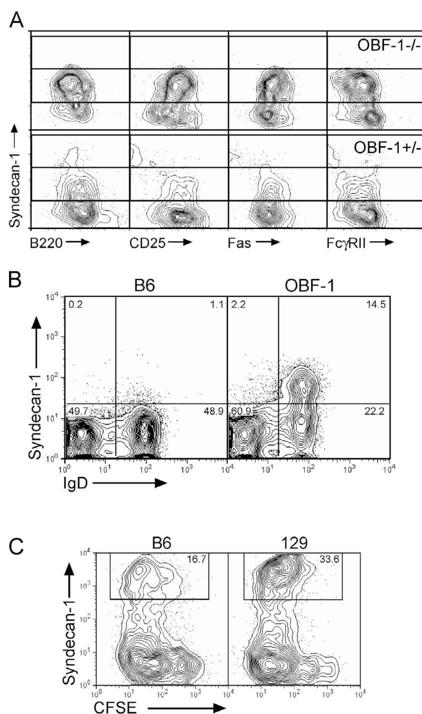

Figure 3. Loss of OBF-1 blocks differentiation at a Syndecan-1^{int} stage. (A) Comparison of the phenotypes of B cells cultured for 4 d in CD40L/IL-4. (B) Ex vivo staining of splenocytes from B6 and *OBF-1–null* mice. Syndecan-1 and IgD expression are shown. The percentage of cells in each quadrant is shown. (C) Analysis of the differentiation of day 4 cultures of stimulated B cells from B6 and 129/Sv control mice, using Syndecan-1 surface staining on CFSE-labeled cells. The percentage of Syndecan-1^{hi} cells is shown.

We examined the cell-surface phenotypes of *OBF-1*$^{-/-}$ and heterozygous control cells in more detail, staining simultaneously for Syndecan-1 and other markers known to change during ASC differentiation. In control cultures, Syndecan-1^{hi} ASC lost the expression of CD25, Fas, and FcγRII, but Syndecan-1^{int} cells retained these markers (Fig. 3 A). Syndecan-1^{int} cells in *OBF-1*$^{-/-}$ cultures displayed a similar phenotype. When freshly isolated splenic B cells from naive mice were examined, few B6 control cells expressed Syndecan-1, but a significant proportion of *OBF-1–null* B cells bore an intermediate level of this marker. Staining for IgD confirmed that the Syndecan-1^{int} cells were mature and not pre–B cells (Fig. 3 B). This suggests that Syndecan-1^{int} cells are generated more readily in vivo in *OBF-1*$^{-/-}$ mice because they are in the in vitro system. We propose that Syndecan-1 levels cells rise incrementally during ASC differentiation and that the loss of OBF-1 blocks the acquisition of the high Syndecan-1 levels that reflect terminal differentiation.

The *OBF-1*$^{-/-}$ mice used here were generated on a 129/Sv mouse background (7) and were backcrossed several generations to the B6 strain. Both 129/Sv and B6 B cells differentiated readily to Syndecan-1^{hi} ASC in our culture system (Fig. 3 C and not depicted). Furthermore, serum Ig levels in 129/Sv mice were comparable to those of B6 mice (17). Finally, the ASC differentiation defect persists in ninth generation backcross animals (unpublished data). Thus, the ASC differentiation block we observed in *OBF-1*$^{-/-}$ B cells is not linked to the genetic background of the mice. Collectively, the results show that the differentiation of B cells to the Syndecan-1^{hi} plasmablast stage, in response to CD40L and IL-4 signaling, depends critically on OBF-1 and its target gene(s).

Isotype switching continues in Syndecan-1^{int} cells and is independent of OBF-1

CD40L/IL-4–stimulated B cells undergo isotype switching with an increasing frequency per division. However, once B cells become committed to the ASC differentiation program under these culture conditions, they no longer switch isotypes, even though they continue to divide (14). As Syndecan-1^{int} cells did not secrete antibody (Fig. 1 G), we assessed their capacity to undergo isotype switching. IgG1^{-} cells were sorted by cell division peak (Syndecan-1^{-} and Syndecan-1^{int}) from B6 and *OBF-1*$^{-/-}$ cultures (Fig. 4 A). After a reculture period of 26 h, cells were labeled for surface IgG1 to assess the isotype switching rate. Complete results are shown for the sorted Syndecan-1^{-} populations (Fig. 4 A), whereas results for the Syndecan-1^{int} cells are shown only for the division 5 sort (Fig. 4 B). We found no difference between the isotype switching rates of control and *OBF-1–null* B cells; this result agrees with Kim et al. (5), who also looked at isotype-switched cells ex vivo. Interestingly, the switching rate of Syndecan-1^{int} cells was even higher than that of the Syndecan-1^{-} cells for both controls and mutants (Fig. 4 C), implying that these cells are not yet committed to becoming ASC.

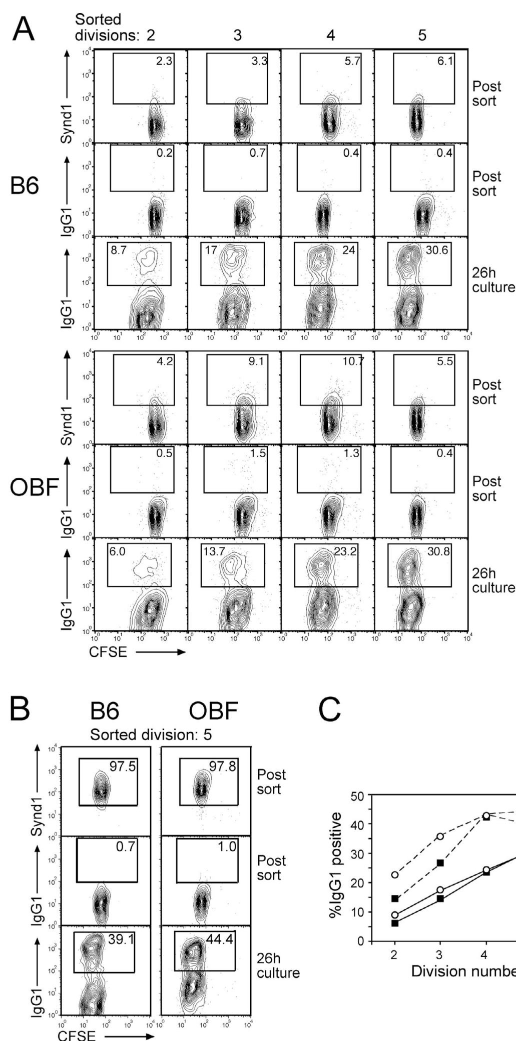

Figure 4. In vitro isotype switching frequency by Syndecan-1$^-$ and Syndecan-1^{int} cells is normal in the absence of OBF-1. (A) Cell division–linked isotype switching from IgM to IgG1 in cells from CD40L/IL-4–stimulated cultures is shown. IgG1$^-$, Syndecan-1$^-$ cells were sorted by cell division number on day 3 and returned to culture (with complete medium) for an additional day. The percentage of cells that became IgG1$^+$ during this time is shown. (B) Same procedure as for A, but switching in sorted Syndecan-1^{int} cells from division 5 only is shown. (C) Quantification of the rate of isotype switching. The percentage of cells from each division that had switched to IgG1 expression in the reculture period is graphed (open circle, B6 controls; closed squares, *OBF-1$^{-/-}$*; continuous lines, switching rates for Syndecan-1$^-$cells; dotted lines, data for Syndecan-1^{int} cells).

Retroviral reconstitution of *OBF-1$^{-/-}$* B cells restores ASC phenotype and function

It was possible that *OBF-1$^{-/-}$* B cells performed aberrantly in our culture system because they were not equivalent to B cells from control mice, despite their normal proliferative responses and isotype-switching capacity. To formally demonstrate that the phenotype described in Figs. 1 and 2 could be attributed directly to the absence of OBF-1, we restored the *OBF-1* expression in primary *OBF-1$^{-/-}$* B cells using retroviral-mediated gene delivery and assessed the consequences in terms of cell-surface phenotype and antibody-secreting functions.

B cells were infected one day after CD40L/IL-4 activation and monitored over 4 d. On day 2, neither the B6 nor the *OBF-1$^{-/-}$* cultures contained Syndecan-1^{hi} cells (Fig. 5 A), but by day 4, such cells were evident in the control cultures (Fig. 5 B). Both infected (GFP positive) and noninfected control cells displayed similar differentiation properties, regardless of whether the retrovirus expressed OBF-1. In *OBF-1$^{-/-}$* cultures infected with the control vector, the characteristic Syndecan-1^{int} cells appeared by day 4, but few Syndecan-1^{hi} cells were present. Significantly, in *OBF-1$^{-/-}$* cultures infected with an *OBF-1*–expressing retrovirus, Syndecan-1^{hi} cells appeared in the GFP$^+$ cell gate but not in the uninfected cell gate (Fig. 5 B). These were primarily cells that expressed the highest levels of GFP and, presumably, the highest levels of OBF-1. If puromycin was included in the cultures, there was an enrichment for infected cells to >90% of viable cells by day 4 (Fig. 5, C and D). Here the Syndecan-1^{hi} cells were more clearly discernable and, in the *OBF-1$^{-/-}$* cultures, reached ~60% of the number produced in control cultures (Fig. 5 C). A Western blot analysis of puromycin-selected cells showed that OBF-1 was expressed in reconstituted *OBF-1$^{-/-}$* B cells, albeit at a significantly lower level than endogenous OBF-1 (Fig. 5 D). Nevertheless, the level was sufficient enough to drive a considerable proportion of cells to differentiate to the more mature Syndecan-1^{hi} state.

In addition to the attainment of a differentiated surface phenotype, the reconstituted *OBF-1$^{-/-}$* B cells acquired an increased capacity for antibody secretion (Fig. 5 E). Cells from the puromycin-selected cultures at day 4 were harvested and washed, and equal numbers were returned to the culture for another 18 h in a medium lacking stimuli. Ig secretion by B6 control B cells was unaltered by infection with either retrovirus, but IgM and, more dramatically, IgG1 production was increased when *OBF-1–null* B cells were infected with the *OBF-1*–expressing retrovirus (3–4-fold and 10–20-fold, respectively; Fig. 5 E). These studies confirm that OBF-1 is necessary for the differentiation of B cells to Syndecan-1^{hi} ASC in this system.

Loss of OBF-1 disrupts the genetic program regulating ASC differentiation

We measured mRNA levels for genes that are differentially regulated during ASC differentiation to discover whether the loss of OBF-1 would affect their expression. CFSE-labeled B6 and *OBF-1$^{-/-}$* B cells were cultured in CD40L/IL-4 for 4 d and sorted by their level of Syndecan-1 (the gates used were similar to those shown in Fig. 1 B). Five populations were recovered, including low, intermediate, and high Syndecan-1 for B6, and low and intermediate Syndecan-1 for *OBF-1$^{-/-}$*. cDNA was prepared from sorted cells and titrated to give equivalent β-actin signals on RT-PCR. The data in Fig. 6 depict a representative experiment

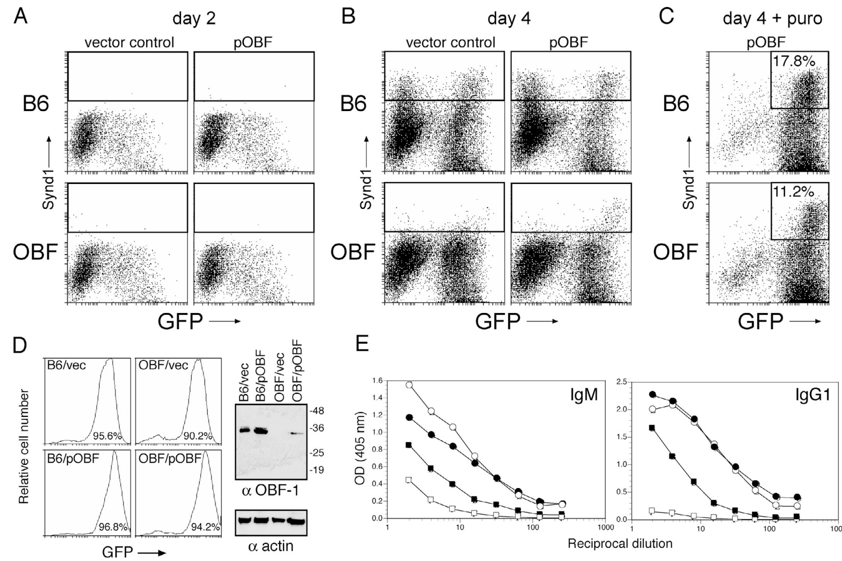

Figure 5. Reconstitution of OBF-1 expression restores ASC differentiation to *OBF-1$^{-/-}$* B cells. (A) CD40L/IL-4–activated B cells infected with a control (vector) or an OBF-1–expressing retrovirus (pOBF) are analyzed on day 2 after infection for Syndecan-1 expression. The boxes highlight Syndecan-1^{hi} cells. Infected cells express GFP. (B) Analysis of the cultures on day 4, in the absence of drug selection. (C) Analysis of parallel day 4 cultures after puromycin selection. The cultures infected with the OBF-1–expressing retrovirus are shown, as is the percentage of live cells in the Syndecan-1 ^{hi}, GFP^{+} gate. Note that only the brightest GFP^{+} cells in B and C, which likely express the highest amounts of OBF-1, have differentiated to Syndecan-1^{hi} cells. (D) Histograms show the percentage of live cells that express GFP on day 4 of puromycin-containing cultures (left). B6/vec indicates B6 B cells infected with the control retroviral vector; OBF/pOBF indicates *OBF-1$^{-/-}$* B cells infected with the OBF-1–expressing virus. Western blot analysis of *OBF-1* and *actin* expression in puromycin-selected cells on day 4 after infection (right). Marker sizes are in kiloDaltons. (E) ELISA assay on serial dilutions of 18-h culture supernatants of day 4 puromycin-selected cells (circles, B6 B cells; squares, *OBF-1$^{-/-}$* B cells). Open symbols indicate an infection with the control retrovirus and closed symbols indicate an infection with the OBF-1–expressing retrovirus.

(A) and the averages of normalized expression values from several experiments (B).

Several genes behaved as expected in control B cells with differentiation. Indeed, changes characteristic of ASC differentiation were already apparent in the Syndecan-1^{int} population. Regulation of c-myc, CstF-64, a polyadenylation factor mediating the transition from membrane-bound to secreted forms of IgH chains (18), and the transcription factor Xbp-1, a regulator of the unfolded protein response that is essential for plasma cell development (19, 20), were not affected by the loss of OBF-1. Strikingly, the gene encoding Blimp-1, a key factor driving ASC differentiation (21), was induced strongly in control cells, but was not induced in *OBF-1$^{-/-}$* cells (Fig. 6, A and B). Similarly, genes encoding J chain—a mediator of polymeric IgM and IgA transport (22)—the transcription factor IRF-4 (23, 24), and the CDK inhibitor p18(INK4c), which is required for the generation of functional plasma cells (25), required OBF-1 for full induction (Fig. 6 and not depicted). When we looked at the expression of mRNAs for the membrane-bound or secreted isoforms of Cμ and Cγ1, the loss of OBF-1 dramatically reduced the level of the mRNA encoding secreted isoforms only. Consistent with our kinetic assessment and previous studies of isotype switching by *OBF-1$^{-/-}$* B cells (5), the levels of Iγ1 sterile transcripts were not affected by the loss of OBF-1.

As expected from the existing model of ASC differentiation (26), some genes were repressed during normal differentiation in vitro (Fig. 6 A), including those encoding AID, a mediator of somatic hypermutation and isotype switching (27), Bcl-6, a repressor required for GC formation (28), and Pax5, both a positive and negative regulator of genes in B lineage cells (29). However, these genes were not repressed in *OBF-1$^{-/-}$* cells and were even expressed at somewhat higher levels than in the controls. Together, these data reveal an unexpected and critical role for OBF-1 in determining the proper regulation of genes driving the terminal differentiation of B cells to antibody-secreting plasma cells in vitro.

Even though the loss of OBF-1 severely restricted the capacity of cells to differentiate to ASC under these conditions, rare Syndecan-1^{hi} ASC were reproducibly generated (Fig. 1). When these cells were sorted and compared with controls, the expression of genes required for ASC differentiation, including *Blimp-1, IRF-1*, and *J chain,* appeared normal (Fig. 6 C), which is consistent with their antibody-secreting capacities (Fig. 1 F). Therefore, OBF-1 is not absolutely required for the ASC differentiation program of gene expression to be elaborated in response to T cell signals, but it dramatically improves the efficiency with which ASC differentiation proceeds.

To confirm that OBF-1 is required for *Blimp-1* expression during ASC differentiation, we made use of a novel

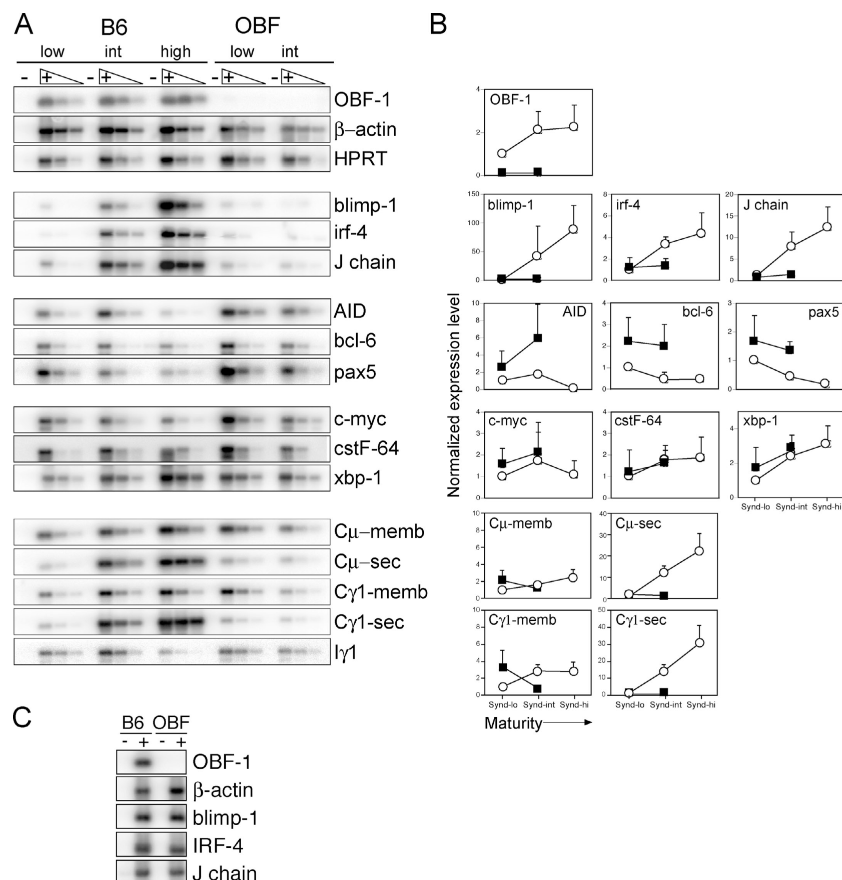

Figure 6. Gene expression changes in cells differentiating in vitro and the influence of OBF-1 loss. (A) Semiquantitative RT-PCR measuring the mRNA levels for *β-actin, HPRT, OBF-1*, and 11 other genes implicated in ASC differentiation or function. Serial titrations of cDNA (+), and controls were prepared without reverse transcriptase (−) from cells cultured and sorted on day 4 by their Syndecan-1 level. (B) Quantification and normalization of RT-PCR products. The x axes represent increasing maturity (from Syndecan-1^{lo} to intermediate and high). All values are expressed relative to the level in the B6 Syndecan-1^{lo} population (set to 1.0) for each assay. Values are the means (+SD) of multiple measurements, including multiple PCR assays and template concentrations, at least two independent biological samples, and normalization using both *β-actin* and *HPRT.* (C) Same procedure as in A, but sorted Syndecan-1^{hi} cells are assessed.

mouse strain that bears a *GFP* cDNA integrated into the endogenous *Blimp-1* locus to report *Blimp-1* expression while disabling the gene. In heterozygous mice, GFP faithfully reveals all ASC (16). We crossed this *Blimp*$^{gfp/+}$ mouse with the *OBF-1*$^{-/-}$ strain to look for the influence of OBF-1 on the expression of the endogenous *Blimp*^{gfp} allele. B cells from mice of all possible genotypes were purified, cultured in CD40L/IL-4, and examined for differentiation to Syndecan-1^{+}, GFP^{+} cells (Fig. 7 A). As homozygous *Blimp*$^{gfp/gfp}$ mice died during gestation, B cells of this genotype were obtained from *RAG-1*$^{-/-}$ mice reconstituted with fetal liver stem cells as previously described (4). An analysis of cultured cells confirmed earlier findings that *Blimp-1* expression is required for differentiation to Syndecan-1^{hi} ASC (30). It also confirmed the OBF-1 dependence of *Blimp-1* expression. Indeed, *Blimp*$^{gfp/gfp}$ and *OBF-1*$^{-/-}$ B cells both seem to be blocked at a Syndecan-1^{int} state. A larger number of these cells accumulated in the *OBF-1*$^{-/-}$ culture, which was consistent with the accelerated differentiation we observed earlier (Fig. 2). This indicates a role for OBF-1 at this stage of development that is independent of Blimp-1. These data confirm that OBF-1 is required for *Blimp-1* expression in B cells stimulated under conditions that mimic TD activation.

The *Blimp*$^{gfp/+}$ mouse model enabled us to test whether OBF-1 is required during ASC differentiation induced under other conditions and in vivo. We previously found that stimulating control *Blimp*$^{gfp/+}$ B cells with LPS, a T cell–independent B cell activator, induced ASC universally expressing GFP, but with heterogeneous Syndecan-1 levels (reference 16; Fig. 7 B). Strikingly, ASC differentiation occurred normally in the absence of OBF-1 under these culture conditions, as did switching to IgG3 (unpublished data). Indeed, GFP^{+} cells sorted from both control and *OBF-1*$^{-/-}$ cultures secreted equal amounts of IgM during a short reculture period, with GFP^{+}, Syndecan-1^{hi} cells producing more Ig per cell than GFP^{+}, Syndecan-1^{-} cells. These studies show that

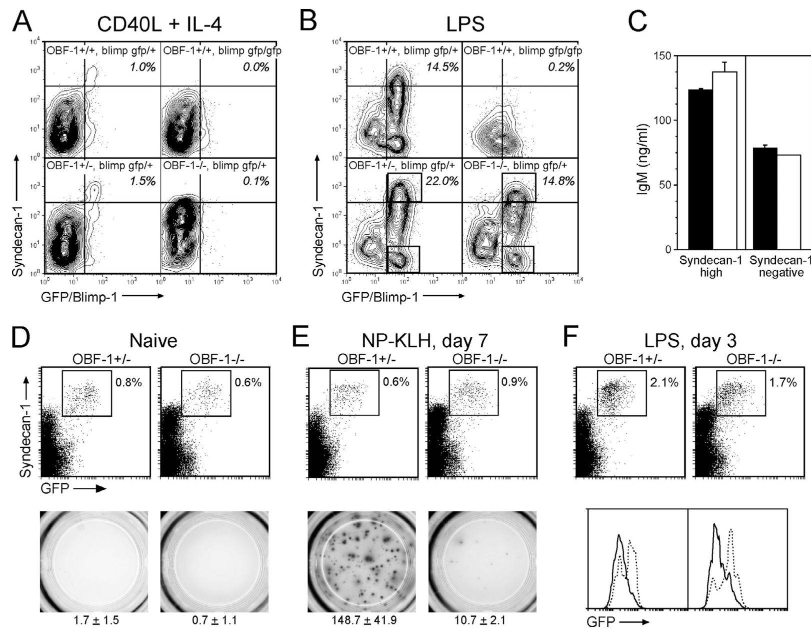

Figure 7. OBF-1 is required for ASC differentiation and the expression of a *Blimp-1* reporter gene under T cell–dependent, but not T cell–independent conditions. (A) Syndecan-1 and GFP expression (reporting an endogenous *Blimp-1* gene expression), in B cells cultured for 4 d in CD40L/IL-4. Percentages of cells that have the phenotype of ASC (top right, Synthetic-1^{hi} and GFP^{+}) are shown. Genotypes are shown in each plot. (B) Analyzed as in A, but cells were stimulated with 20 μg/ml LPS. Percentages of cells that have the phenotype of ASC (top right, Syndecan-1^{hi} and GFP^{+}) are shown. (C) Antibody secreted from 6,000 Syndecan-1^{-} or Syndecan-1^{hi} cells (B, boxed regions) sorted from LPS cultures of control (shaded bar) or OBF-1–deficient (open bar) cells during 6 h reculture without stimuli. (D) Syndecan-1 and GFP expression in splenocytes from naive *Blimp*$^{gfp/+}$/*OBF-1*$^{+/-}$ and *Blimp*$^{gfp/+}$/*OBF-1*$^{-/-}$ mice (top). ASC (boxed) percentages are given. ELISPOT plate and count (mean ± SD) of NP-specific IgG1 ASC among 10,000 splenocytes plated (bottom). (E) Same procedure as in D, but spleens were from mice immunized 7 d earlier with NP-KLH. (F) Same procedure as in D, but from mice immunized 3 d earlier with LPS (top). The histograms are of GFP levels on ASCs (boxed, bottom). Dotted lines represent unimmunized mice and solid lines represent LPS-immunized mice.

ASC differentiation in response to a model T cell–independent activator proceeds in the absence of OBF-1. Importantly, they reveal differential molecular requirements for ASC differentiation induced through T cell–dependent and –independent means

To confirm these findings, *Blimp*$^{gfp/+}$ mice that were either heterozygous or homozygous for the OBF-1 mutation were immunized with the T cell–independent antigen, LPS, or with the TD antigen (4-hydroxy-3-nitrophenyl)acetyl (NP)–KLH. Spleens of unimmunized control and OBF-1–deficient mice contained small populations of ASCs (Blimp-1/GFP^{+} and Syndecan-1^{hi}; Fig. 7 D). Although the proportion of ASC remained unchanged 7d after NP-KLH immunization, NP-specific IgG1 ASCs, as detected by ELISPOT, were only generated in the control animals. *OBF-1*$^{-/-}$ mice made almost no anti-NP IgG1 ASCs (Fig. 7 E). The vast majority of ASCs present at this time reside in foci, as the GC reaction had not yet reached fruition (31).

When ASC numbers peaked 3 d after LPS immunization (16), ASCs had nearly tripled in both control and *OBF-1–null* spleens (Fig. 7 F). The majority of LPS-induced ASC in both control and *OBF-1*$^{-/-}$ spleens expressed the intermediate level of Blimp^{gfp} that is characteristic of newly generated, rapidly expanding plasmablasts (16). These studies extend our conclusions drawn from in vitro analyses, indicating that OBF-1 is required specifically for ASC differentiation in response to TD antigens both in vitro and in vivo.

DISCUSSION

Defining the specific influences of OBF-1 on late B cell differentiation

We have used a quantitative in vitro culture system (13, 14) to examine B cells that lack OBF-1, a transcriptional regulator that strongly influences antibody levels in vivo, and to establish the underlying cause of the low serum Ig levels in *OBF-1–null* mice. Although B cells from *OBF-1*$^{-/-}$ mice proliferated in response to CD40L/IL-4 to the same extent as wild-type cells and displayed a normal rate of isotype switching, they exhibited a significantly increased rate of differentiation per division, which generated an unusually high proportion of Syndecan-1^{int} cells in culture and in vivo. We propose that OBF-1 normally represses the division-based rate of differentiation, thereby restraining the development of the transitional Syndecan-1^{int} cell. The basic helix-loop-helix protein Mitf has recently been shown to serve a similar role in B cells (32), and future studies will investigate a functional relationship between these two factors. In addition to this early role, OBF-1 is later required to promote ASC dif-

ferentiation in vitro to generate Syndecan-1^{hi} cells characterized by high rates of Ig secretion. The differentiation block is evident in both IgM^{+} and isotype-switched cells. This requirement for OBF-1 is manifest specifically in response to CD40L and IL-4, but not LPS stimulation.

During the differentiation of ASC, a genetic hierarchy has been described whereby two transcriptional repressors, Bcl-6 and Blimp-1, appear to be critical (26, 33, 34). Bcl-6 maintains the GC phenotype of cells, and Blimp-1 drives ASC differentiation through the repression of genes such as *Pax5*. In the model, Bcl-6 and Pax5 inhibit differentiation, allowing sufficient time for affinity maturation and class switch recombination to occur in response to antigen and T cell signals (35, 36). Once the differentiating cell reaches some functional sufficiency, the B cell receptor signals the MAPK-mediated phosphorylation and degradation of Bcl-6 (37). Bcl-6 has been shown to repress *Blimp-1* expression (34, 38, 39), so that once Bcl-6 is lost, *Blimp-1* is derepressed and ASC differentiation ensues. Blimp-1 repression of the *bcl-6* and *Pax5* promoters ensures that the process is irreversible (33). OBF-1, like Bcl-6 and Mitf, may be necessary for the inhibition of differentiation that enables the GC reaction to proceed successfully. This property may contribute to the lack of GC in *OBF-1*$^{-/-}$ mice, but other OBF-1 activities, such as the regulation of B cell receptor–signaling components (PM and LMC; unpublished data) would also contribute.

OBF-1 appears to play an important role in the fulfilment of the ASC program through a dramatic effect on the transcription of the *Blimp-1* gene. Two nonconsensus octamer motifs are present in the *Blimp-1* promoter, but we could find no evidence for a direct effect of OBF-1 on *Blimp-1* transcription using EMSA and chromatin immunoprecipitation (unpublished data). Nevertheless, in the absence of OBF-1, *Blimp-1* is not expressed in vitro in response to CD40L and IL-4. Consequently, genes normally repressed by Blimp-1, including *bcl-6, Pax5*, and *oct-2* (33), are expressed at abnormally high levels in differentiating *OBF-1*$^{-/-}$ cells. Similarly, the Pax5 target gene encoding the J chain is not fully activated in *OBF-1*$^{-/-}$–Syndecan-1^{int} cells (Fig. 6), and AID, a presumptive Pax5 target (40), is overexpressed, suggesting that affinity maturation may be affected in maturing *OBF-1*$^{-/-}$ B cells.

The model of TD B cell differentiation (Fig. 8) indicates the points at which OBF-1 appears to exert its influence. OBF-1 regulates the rate of generation of Syndecan-1^{int} cells, but two scenarios may exist at later stages of differentiation. In the first (Fig. 8, dashed arrow), Syndecan-1^{int} cells are a homogeneous group of cells that simultaneously exhibit phenotypic and genetic features of both ASC and less differentiated "GC-like" cells. OBF-1 restricts the differentiation of these precursor cells, so that ASCs are not efficiently made, and the blocked cells express an aberrant gene expression profile. We prefer a second scenario (Fig. 8, solid arrow) in which the Syndecan-1^{int} populations are developmentally heterogeneous, with some cells already committed to ASC

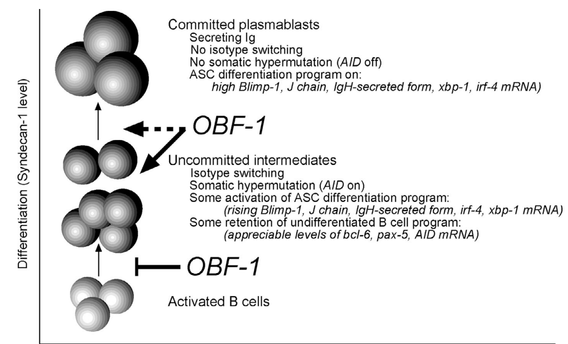

Figure 8. Model of OBF-1 regulation during T cell–induced B cell differentiation in vitro. The scheme depicts the differentiation of resting B cells under the influence of CD40L and IL-4, indicating points at which OBF-1 loss influences the process.

differentiation (upregulating; e.g., *Blimp-1*, *IRF-4*, and mRNAs for secreted IgH chains), while others remain in a less differentiated state that can undergo switching and somatic hypermutation (e.g., expressing *bcl-6, Pax5,* or *AID*). The loss of OBF-1 specifically influences the fate, or the survival, of the committed ASC "precursor," thus blocking differentiation. Kinetic and single cell analyses should distinguish between these possibilities.

It is clear that the immune phenotypes of *OBF-1*$^{-/-}$ mice and *Blimp-1*$^{-/-}$ mice are different, and that the impact of Blimp-1 loss on ASC differentiation and antibody titers in vivo is more severe (16, 30). ASC differentiation is universally Blimp-1 dependent, whereas it is OBF-1 dependent only under particular circumstances. We have shown that Blimp-1^{+} ASCs are generated inefficiently in the absence of OBF-1 in CD40L/IL-4 cultures and in vivo upon immunization with a TD antigen. In contrast, much of the IgM-producing capacity of an animal is OBF-1 independent (5–7, and this study). Thus, an effective TD response requires OBF-1, both before involvement of the GC, and for the GC reaction itself.

OBF-1 regulates antibody production indirectly, with only minor influences on Ig gene expression

Both in vitro and in vivo, OBF-1 loss results in decreased antibody production, and IgG production is affected more severely than IgM production. Several factors have been proposed to modulate the types and final titers of Ig in the serum of *OBF-1*$^{-/-}$ mice. For instance, because *OBF-1*$^{-/-}$ mice lack GCs, they would be expected to lack high titers of switched antibody isotypes and memory responses.

Our data suggest that the poor production of IgG1 by *OBF-1*$^{-/-}$ B cells is inherent and is independent of the GC setting. By examining the rare ASC produced in vitro from *OBF-1*$^{-/-}$ B cells cultured with CD40L/IL-4, we found that IgM production is OBF-1 independent, as secretion was similar to controls. In contrast, IgG1 secretion was moderately sensitive to OBF-1 loss. Consistent with this, the ec-

topic expression of OBF-1 enhanced IgM secretion from *OBF-1–null* ASCs, but IgG1 secretion was even more dramatically improved (Fig. 5 E). Kim et al. (5) proposed a requirement for OBF-1 in the transcription of switched forms of IgH genes to explain the differential effects of OBF-1 deletion on isotype-switched antibodies and went on to show that the 3′ IgH enhancer was less active in *OBF-1*$^{-/-}$ cells (41). Importantly, we found that OBF-1 is critical only for TD *Blimp-1* induction and ASC differentiation (Figs. 6 and 7), whereas a T cell–independent inducer of ASC differentiation works through an OBF-1–independent pathway. The differential requirement for OBF-1 would thereby severely, but specifically, affect only a subset of antibody responses.

We have identified a new and important role for OBF-1 in the generation of antibodies. Further analysis of how OBF-1 both positively and negatively regulates key aspects of ASC differentiation is underway. Our model for OBF-1 function in late B cell differentiation is consistent with the poor humoral responses of *OBF-1*$^{-/-}$ mice to viral infections (42), as well as with the observation that the loss of OBF-1 prevents the generation of autoantibodies and the SLE-like disease of *Aiolos*-null mice (43). Conceivably, the loss of *OBF-1* expression from some human B cell malignancies (44, 45) might reflect the importance of OBF-1 in mediating normal terminal differentiation. Use of this in vitro model of cell behavior in the context of isotype switching and ASC differentiation will be extremely useful in the characterization of the abnormal behavior of B cells from designed or spontaneous mouse mutants, or from immunodeficient patients displaying aberrations in the humoral arm of the immune response.

MATERIALS AND METHODS

Mice. OBF-1–deficient mice (7) and age- and sex-matched control C57BL/6 and 129/SV mice were bred at the Walter and Eliza Hall Institute of Medical Research (WEHI). *Blimp*$^{gfp/+}$ mice, generated and bred at WEHI, have been described elsewhere (16). Animal studies were approved by the Animal Ethics Committee of Royal Melbourne Hospital. Mice were immunized, and ASCs were characterized and enumerated, by ELISPOT as described previously (16).

Reagents and antibodies. Cell membranes expressing the murine ligand for CD40 (CD40L) were prepared as previously described (46). Recombinant mouse IL-4 was provided by R. Kastelein (DNAX Research Institute, Palo Alto, CA). Biotinylated anti–mouse IgG1 (A85.1), anti–mouse Fas (Jo2), anti–mouse CD25 (7D4), anti–mouse FcγRII (2.4G2), and PE-conjugated anti–Syndecan-1 antibody were purchased from BD Biosciences. Mouse IgM-specific b.7.6 mAb was biotinylated using NHS-biotin purchased from Sigma-Aldrich. Streptavidin-Tricolor (SA-TC) was purchased from Caltag Laboratories. FITC-conjugated anti–mouse IgD (11-26c.2a) was purchased from BD Biosciences.

Cell culture and flow cytometry. Small resting B cells were prepared and labeled for division tracking with CFSE (Molecular Probes Inc.) as described elsewhere (47, 48). B cells were cultured and labeled for flow cytometric analysis as described previously (4, 14). The analysis was performed on FACScan or LSR flow cytometers (Becton Dickinson) using CELLQuest (Becton Dickinson) or Flow-Jo (Tree Star Inc.) software. Cell sorting was performed on a FACStar+ (Becton Dickinson) or MoFlo (Cytomation Inc.) cytometer.

CFSE peaks were individually gated and the proportion of Ig- or Syndecan-1–positive cells within each division round was calculated as described previously (49). In some experiments, the proportion of live cells per culture was estimated by reference to a known number of CaliBRITE beads (unlabeled; Becton Dickinson) run simultaneously with cells on flow cytometer (13, 49).

ELISA. Small resting B cells were cultured at 2×10^5 cells/ml for 7 d, at which time supernatants were harvested. For Syndecan-1–sorted cell populations, cells were returned to culture for the stated period, without mitogens, before harvesting supernatants. Goat anti–mouse IgM or IgG1 antibodies to coat the plates and biotinylated goat anti–IgM or IgG1 antibodies as a secondary detection step were purchased from Southern Biotechnology Associates, Inc. Purified mouse monoclonal IgM (TEPC 183) and IgG1 (MOPC 21) purchased from Sigma-Aldrich were used for the quantitative determination of Ig concentrations.

RT-PCR analysis of plasma cell markers. Semiquantitative RT-PCR was performed on cDNA made from sorted cell populations as previously described (14).

The following primers were used: β-actin, 5′-GTG GGC CGC TCT AGG CAC CAA-3′, 3′-CTC TTT GAT GTC ACG CAC GAT TTC-5′; J chain, 5′-GTC TTC ACT GGG GAG TCC TAG CC-3′, 3′-GGG TGC AAA TGG AGA GCC TCT AAG G-5′; *Blimp-1*, 5′-CAT TCC TGT CCC CAA CGC ATC AAC TG-3′, 3′-GGT GCC CAA GCA CCA AAG TCA TAG C-5′; xbp-1, 5′-GCT GGA GCA GCA AGT GGT GGA TTT GG-3′, 3′-GGC TTC CAG CTT GGC TGA TGA GGT CC-5′; AID, 5′-CCG GCA CGT GGC TGA GTT T-3′, 3′-GAT GCG CCG AAG TTG TCT GGT TAG-5′; bcl-6, 5′-CAG CAC CTT CCT CTT CTC TGA TGA GGA GCT CC-3′, 3′-CTG GCG GAG AGC CAG AGG CCT GAA GGA TGC-5′; Pax5, 5′-CCT ACC CTA TTG TCA CAG GCC-3′, 3′-CCT CTG TCT GTC TCA GGG GGT T-5′; CXCR5, 5′-CCA CTA ACC CTG GAC ATG GGC TCC-3′, 3′-GGG CCT GCA GTA GCC TGT GGA CC-5′; c-myc, 5′-GAC CTC GAC TAC GAC TCC GTA CAG CCC-3′, 3′-CAG GCT GGT GCT GTC TTT GCG CGC AGC C-5′; cstF-64, 5′-CCA CCT CTG ATG CAG ACC TCT ATC CAG GGA GG-3′, 3′-CTG GGA TCT GCT GCC AAT GGT CCT CCT CTC-5′; irf-4, 5′-GAA GCC CCA AAG CCC TCA GTC GTT G-3′, 3′-GGG GCA TGT AAT TAA ACC TTG TGT G-5′; obf-1, 5′-CGG TGT TGA CCT ATG CTT CTC CAC C-3′, 3′-GAG GGG CGC CTG GTG CTC GGG ACC C-5′; Cμ-common, 5′-CCC ACA GCA TCC TGA CTG TGA CAG AGG AG-3′; Cμ-membrane isoform, 3′-GAT GAA GGT GGA GGC AGT GGT CCA CAG G-5′; Cμ-secreted isoform, 3′-CAG GTG CCG CCT GTG TCA GAC ATG ATC AGG-5′; Cγ1-common, 5′-CAC CAG GAC TGG CTC AAT GGC AAG GAG-3′; Cγ1-membrane isoform, 3′-GTC CTG GGC CTC AGC ACA GGT CTC GTC C-5′; Cγ1-secreted isoform, 3′-GTA GGA CCA GAG GGC TCC AAG GAC AC-5′; and Iγ1, 5′-AGC ACG CAT CTG TGG CCC TTC CAG ATC T-3′, 3′-CAG GTC ACT GTC ACT GGC TCA GGG AAA T-5′.

Retroviral complementation and in vitro differentiation of primary B cells. B cells were purified from red- and dead cell–depleted spleen cell suspensions using anti–B220-coupled magnetic beads (Miltenyi Biotec) and cultured for 18–24 h with 5 μg/ml anti-CD40 antibody (FGK45.5) and 100 U/ml IL-4 (complete medium). Cells were then infected with retroviral supernatants using a modified spin infection protocol (50). Cells were washed 1 d after infection, and a new complete medium was applied, with or without 2 μg/ml puromycin. Cells were monitored for GFP and Syndecan-1 expression for another 3–4 d. Retroviral supernatants were prepared from the Phoenix packing cell line (51) transfected (FuGENE 6; Roche Diagnostics) with the pMXpie retroviral vector (gift of D. Krebs, Walter and Eliza Hall Institute, Parkville; Australia, reference 52), or with a vector encoding full-length murine OBF-1.

Western blot. Total cell lysates of puromycin-resistant (retrovirally infected) B cells were prepared on day 4 of the culture and analyzed by Western blot using a monoclonal antibody raised against the NH_2-terminal 45

amino acids of murine OBF-1. A goat antiactin antiserum (Santa Cruz Biotechnology, Inc.) was used as a loading control.

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REFERENCES

1. Gstaiger, M., L. Knoepfel, O. Georgiev, W. Schaffner, and C.M. Hovens. 1995. A B-cell coactivator of octamer-binding transcription factors. *Nature*. 373:360–362.
2. Strubin, M., J.W. Newell, and P. Matthias. 1995. OBF-1, a novel B cell-specific coactivator that stimulates immunoglobulin promoter activity through association with octamer-binding proteins. *Cell*. 80:497–506.
3. Luo, Y., and R.G. Roeder. 1995. Cloning, functional characterization, and mechanism of action of the B-cell-specific transcriptional coactivator OCA-B. *Mol. Cell. Biol.* 15:4115–4124.
4. Corcoran, L.M., and M. Karvelas. 1994. Oct-2 is required early in T cell-independent B cell activation for G1 progression and for proliferation. *Immunity*. 1:635–645.
5. Kim, U., X.F. Qin, S. Gong, S. Stevens, Y. Luo, M. Nussenzweig, and R.G. Roeder. 1996. The B-cell-specific transcription coactivator OCA-B/OBF-1/Bob-1 is essential for normal production of immunoglobulin isotypes. *Nature*. 383:542–547.
6. Nielsen, P.J., O. Georgiev, B. Lorenz, and W. Schaffner. 1996. B lymphocytes are impaired in mice lacking the transcriptional co- activator Bob1/OCA-B/OBF1. *Eur. J. Immunol.* 26:3214–3218.
7. Schubart, D.B., A. Rolink, M.H. Kosco-Vilbois, F. Botteri, and P. Matthias. 1996. B-cell-specific coactivator OBF-1/OCA-B/Bob1 required for immune response and germinal centre formation. *Nature*. 383:538–542.
8. Schubart, K., S. Massa, D. Schubart, L.M. Corcoran, A.G. Rolink, and P. Matthias. 2001. B cell development and immunoglobulin gene transcription in the absence of Oct-2 and OBF-1. *Nat. Immunol.* 2:69–74.
9. Hess, J., P.J. Nielsen, K.D. Fischer, H. Bujard, and T. Wirth. 2001. The B lymphocyte-specific coactivator BOB.1/OBF.1 is required at multiple stages of B-cell development. *Mol. Cell. Biol.* 21:1531–1539.
10. Samardzic, T., D. Marinkovic, P.J. Nielsen, L. Nitschke, and T. Wirth. 2002. BOB.1/OBF.1 deficiency affects marginal-zone B-cell compartment. *Mol. Cell. Biol.* 22:8320–8331.
11. Wolf, I., V. Pevzner, E. Kaiser, G. Bernhardt, E. Claudio, U. Siebenlist, R. Forster, and M. Lipp. 1998. Downstream activation of a TATA-less promoter by Oct-2, Bob1, and NF-kappaB directs expression of the homing receptor BLR1 to mature B cells. *J. Biol. Chem.* 273:28831–28836.
12. Casellas, R., M. Jankovic, G. Meyer, A. Gazumyan, Y. Luo, R. Roeder, and M. Nussenzweig. 2002. OcaB is required for normal transcription and V(D)J recombination of a subset of immunoglobulin kappa genes. *Cell*. 110:575–585.
13. Hasbold, J., A.V. Gett, J.S. Rush, E. Deenick, D. Avery, J. Jun, and P.D. Hodgkin. 1999. Quantitative analysis of lymphocyte differentiation and proliferation in vitro using carboxyfluorescein diacetate succinimidyl ester. *Immunol. Cell Biol.* 77:516–522.
14. Hasbold, J., L.M. Corcoran, D.M. Tarlinton, S.G. Tangye, and P.D. Hodgkin. 2004. Evidence from the generation of immunoglobulin G-secreting cells that stochastic mechanisms regulate lymphocyte differentiation. *Nat. Immunol.* 5:55–63.
15. Underhill, G.H., K.P. Kolli, and G.S. Kansas. 2003. Complexity within the plasma cell compartment of mice deficient in both E- and P-selectin: implications for plasma cell differentiation. *Blood*. 102: 4076–4083.
16. Kallies, A., J. Hasbold, D.M. Tarlinton, W. Dietrich, L.M. Corcoran, P.D. Hodgkin, and S.L. Nutt. 2004. Plasma cell ontogeny defined by quantitative changes in Blimp-1 expression. *J. Exp. Med.* 200:967–977.
17. Corcoran, L.M., and D. Metcalf. 1999. IL-5 and Rp105 signaling defects in B cells from commonly used 129 mouse substrains. *J. Immunol.* 163:5836–5842.
18. Takagaki, Y., and J.L. Manley. 1998. Levels of polyadenylation factor CstF-64 control IgM heavy chain mRNA accumulation and other events associated with B cell differentiation. *Mol. Cell.* 2:761–771.
19. Iwakoshi, N.N., A.H. Lee, P. Vallabhajosyula, K.L. Otipoby, K. Rajewsky, and L.H. Glimcher. 2003. Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. *Nat. Immunol.* 4:321–329.
20. Reimold, A.M., N.N. Iwakoshi, J. Manis, P. Vallabhajosyula, E. Szomolanyi-Tsuda, E.M. Gravallese, D. Friend, M.J. Grusby, F. Alt, and L.H. Glimcher. 2001. Plasma cell differentiation requires the transcription factor XBP-1. *Nature*. 412:300–307.
21. Turner, C.A., Jr., D.H. Mack, and M.M. Davis. 1994. Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. *Cell*. 77:297–306.
22. Hendrickson, B.A., L. Rindisbacher, B. Corthesy, D. Kendall, D.A. Waltz, M.R. Neutra, and J.G. Seidman. 1996. Lack of association of secretory component with IgA in J chain-deficient mice. *J. Immunol.* 157:750–754.
23. Mittrucker, H.W., T. Matsuyama, A. Grossman, T.M. Kundig, J. Potter, A. Shahinian, A. Wakeham, B. Patterson, P.S. Ohashi, and T.W. Mak. 1997. Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function. *Science*. 275:540–543.
24. Gupta, S., A. Anthony, and A.B. Pernis. 2001. Stage-specific modulation of IFN-regulatory factor 4 function by Kruppel-type zinc finger proteins. *J. Immunol.* 166:6104–6111.
25. Tourigny, M.R., J. Ursini-Siegel, H. Lee, K.M. Toellner, A.F. Cunningham, D.S. Franklin, S. Ely, M. Chen, X.F. Qin, Y. Xiong, et al. 2002. CDK inhibitor p18(INK4c) is required for the generation of functional plasma cells. *Immunity*. 17:179–189.
26. Calame, K.L., K.I. Lin, and C. Tunyaplin. 2003. Regulatory mechanisms that determine the development and function of plasma cells. *Annu. Rev. Immunol.* 21:205–230.
27. Muramatsu, M., K. Kinoshita, S. Fagarasan, S. Yamada, Y. Shinkai, and T. Honjo. 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell*. 102:553–563.
28. Dent, A.L., A.L. Shaffer, X. Yu, D. Allman, and L.M. Staudt. 1997. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science*. 276:589–592.
29. Nutt, S.L., D. Eberhard, M. Horcher, A.G. Rolink, and M. Busslinger. 2001. Pax5 determines the identity of B cells from the beginning to the end of B-lymphopoiesis. *Int. Rev. Immunol.* 20:65–82.
30. Shapiro-Shelef, M., K.I. Lin, L.J. McHeyzer-Williams, J. Liao, M.G. McHeyzer-Williams, and K. Calame. 2003. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity*. 19:607–620.
31. Kelsoe, G. 1995. In situ studies of the germinal center reaction. *Adv. Immunol.* 60:267–288.
32. Lin, L., A.J. Gerth, and S.L. Peng. 2004. Active inhibition of plasma cell development in resting B cells by microphthalmia-associated transcription factor. *J. Exp. Med.* 200:115–122.
33. Shaffer, A.L., K.I. Lin, T.C. Kuo, X. Yu, E.M. Hurt, A. Rosenwald, J.M. Giltnane, L. Yang, H. Zhao, K. Calame, and L.M. Staudt. 2002. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity*. 17:51–62.
34. Tunyaplin, C., A.L. Shaffer, C.D. Angelin-Duclos, X. Yu, L.M. Staudt, and K.L. Calame. 2004. Direct repression of prdm1 by Bcl-6 inhibits plasmacytic differentiation. *J. Immunol.* 173:1158–1165.
35. Reljic, R., S.D. Wagner, L.J. Peakman, and D.T. Fearon. 2000. Suppression of signal transducer and activator of transcription 3–dependent B lymphocyte terminal differentiation by BCL-6. *J. Exp. Med.* 192:1841–1848.
36. Rinkenberger, J.L., J.J. Wallin, K.W. Johnson, and M.E. Koshland. 1996. An interleukin-2 signal relieves BSAP (Pax5)-mediated repression of the immunoglobulin J chain gene. *Immunity*. 5:377–386.

37. Niu, H., B.H. Ye, and R. Dalla-Favera. 1998. Antigen receptor signaling induces MAP kinase-mediated phosphorylation and degradation of the BCL-6 transcription factor. *Genes Dev*. 12:1953–1961.
38. Shaffer, A.L., X. Yu, Y. He, J. Boldrick, E.P. Chan, and L.M. Staudt. 2000. BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. *Immunity*. 13:199–212.
39. Vasanwala, F.H., S. Kusam, L.M. Toney, and A.L. Dent. 2002. Repression of AP-1 function: a mechanism for the regulation of Blimp-1 expression and B lymphocyte differentiation by the B cell lymphoma-6 protooncogene. *J. Immunol*. 169:1922–1929.
40. Gonda, H., M. Sugai, Y. Nambu, T. Katakai, Y. Agata, K.J. Mori, Y. Yokota, and A. Shimizu. 2003. The balance between Pax5 and Id2 activities is the key to AID gene expression. *J. Exp. Med*. 198:1427–1437.
41. Stevens, S., J. Ong, U. Kim, L.A. Eckhardt, and R.G. Roeder. 2000. Role of OCA-B in 3′-IgH enhancer function. *J. Immunol*. 164:5306–5312.
42. Fehr, T., C. Lopez-Macias, B. Odermatt, R.M. Torres, D.B. Schubart, T.L. O'Keefe, P. Matthias, H. Hengartner, and R.M. Zinkernagel. 2000. Correlation of anti-viral B cell responses and splenic morphology with expression of B cell-specific molecules. *Int. Immunol*. 12:1275–1284.
43. Sun, J., G. Matthias, M.J. Mihatsch, K. Georgopoulos, and P. Matthias. 2003. Lack of the transcriptional coactivator OBF-1 prevents the development of systemic lupus erythematosus-like phenotypes in Aiolos mutant mice. *J. Immunol*. 170:1699–1706.
44. Hertel, C.B., X.G. Zhou, S.J. Hamilton-Dutoit, and S. Junker. 2002. Loss of B cell identity correlates with loss of B cell-specific transcription factors in Hodgkin/Reed-Sternberg cells of classical Hodgkin lymphoma. *Oncogene*. 21:4908-4920.
45. Re, D., M. Muschen, T. Ahmadi, C. Wickenhauser, A. Staratschek-Jox, U. Holtick, V. Diehl, and J. Wolf. 2001. Oct-2 and Bob-1 deficiency in Hodgkin and Reed Sternberg cells. *Cancer Res*. 61:2080–2084.
46. Kehry, M.R., and B.E. Castle. 1994. Regulation of CD40 ligand expression and use of recombinant CD40 ligand for studying B cell growth and differentiation. *Semin. Immunol*. 6:287–294.
47. Hodgkin, P.D., J.H. Lee, and A.B. Lyons. 1996. B cell differentiation and isotype switching is related to division cycle number. *J. Exp. Med*. 184:277–281.
48. Lyons, A.B., and C.R. Parish. 1994. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods*. 171:131–137.
49. Lyons, A.B., J. Hasbold, and P.D. Hodgkin. 2001. Flow cytometric analysis of cell division history using dilution of carboxyfluorescein diacetate succinimidyl ester, a stably integrated fluorescent probe. *Methods Cell Biol*. 63:375–398.
50. Bahnson, A.B., J.T. Dunigan, B.E. Baysal, T. Mohney, R.W. Atchison, M.T. Nimgaonkar, E.D. Ball, and J.A. Barranger. 1995. Centrifugal enhancement of retroviral mediated gene transfer. *J. Virol. Methods*. 54:131–143.
51. Grignani, F., T. Kinsella, A. Mencarelli, M. Valtieri, D. Riganelli, L. Lanfrancone, C. Peschle, G.P. Nolan, and P.G. Pelicci. 1998. High-efficiency gene transfer and selection of human hematopoietic progenitor cells with a hybrid EBV/retroviral vector expressing the green fluorescence protein. *Cancer Res*. 58:14–19.
52. Krebs, D.L., Y. Yang, M. Dang, J. Haussmann, and M.R. Gold. 1999. Rapid and efficient retrovirus-mediated gene transfer into B cell lines. *Methods Cell Sci*. 21:57–68.