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 bel–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 null 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.

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−/− B cells in our model system of TD ASC differentiation (14). To do this, we purified small resting OBF-1−/− 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 in OBF-1−/−, compared with control cultures, by 38.9 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.

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+ population of CD40L/...
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-1hi cells in later divisions, there was a clear deficit in the *OBF-1*−/− cultures of the Syndecan-1hi 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-1hi 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-1hi 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-1hi cells remained stable after 26 h and many Syndecan-1int cells had become Syndecan-1hi, whereas some Syndecan-1hi cells became Syndecan-1lo, 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-1hi 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-1hi cells produced in culture, but rare Syndecan-1hi cells are generated in OBF-1−/− cultures. B6 and *OBF-1*−/− 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-1hi cells secreted antibody effectively, regardless of genotype (Fig. 1 F). *OBF-1*−/−, Syndecan-1hi 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).

**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-1hi cells largely fail to form in the OBF-1−/− cultures. (B) Quantitative summary of the differentiation depicted in the contour plots of A. The percentages of Syndecan-1− cells that became Syndecan-1hi 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\textsuperscript{int} phase during this differentiation. The higher proportion of Syndecan-1\textsuperscript{int} cells in OBF-1\textsuperscript{-/-} 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\textsuperscript{-/-} Syndecan-1\textsuperscript{int} cells. To directly assess whether OBF-1 loss alters the rate of generation of Syndecan-1\textsuperscript{int} from Syndecan-1\textsuperscript{-} 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\textsuperscript{-} 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\textsuperscript{int} and then to Syndecan-1\textsuperscript{hi} cells, OBF-1\textsuperscript{-/-} B cells differentiated to Syndecan-1\textsuperscript{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\textsuperscript{-/-} cells to the Syndecan-1\textsuperscript{hi} state, confirming a second critical role for OBF-1 in the differentiation of Syndecan-1\textsuperscript{hi} ASC in this system.

We examined the cell-surface phenotypes of OBF-1\textsuperscript{-/-} 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\textsuperscript{hi} ASC lost the expression of CD25, Fas, and FcγRII, but Syndecan-1\textsuperscript{int} cells retained these markers (Fig. 3 A). Syndecan-1\textsuperscript{int} cells in OBF-1\textsuperscript{-/-} 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\textsuperscript{-null} B cells bore an intermediate level of this marker. Staining for IgD confirmed that the Syndecan-1\textsuperscript{int} cells were mature and not pre–B cells (Fig. 3 B). This suggests that Syndecan-1\textsuperscript{int} cells are generated more readily in vivo in OBF-1\textsuperscript{-/-} 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\textsuperscript{-/-} 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\textsuperscript{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\textsuperscript{-/-} 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\textsuperscript{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\textsuperscript{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\textsuperscript{int} cells did not secrete antibody (Fig. 1 G), we assessed their capacity to undergo isotype switching. IgG1\textsuperscript{-} cells were sorted by cell division peak (Syndecan-1\textsuperscript{-} and Syndecan-1\textsuperscript{int}) from B6 and OBF-1\textsuperscript{-/-} 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\textsuperscript{-} populations (Fig. 4 A), whereas results for the Syndecan-1\textsuperscript{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\textsuperscript{-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\textsuperscript{int} cells was even higher than that of the Syndecan-1\textsuperscript{-} cells for both controls and mutants (Fig. 4 C), implying that these cells are not yet committed to becoming ASC.

![Figure 3. Loss of OBF-1 blocks differentiation at a Syndecan-1\textsuperscript{int} stage.](https://jem.rupress.org/)

(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\textsuperscript{-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\textsuperscript{hi} cells is shown.
Retroviral reconstitution of OBF-1/

Figure 4. In vitro isotype switching frequency by Syndecan-1int and Syndecan-1hi 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-linked isotype switching from IgM to IgG1 in cells from CD40L/IL-4–expressing retrovirus, Syndecan-1int cells from division 5 only is shown. (C) Quantification of the rate of isotype switching. The percentage of cells that became IgG1 during this time is shown. (B) Same procedure as for A, but switching in sorted Syndecan-1int cells from division 5 only is shown. (C) Quantification of the rate of isotype switching. The percentage of cells that became IgG1 during this time is shown. (B) Same procedure as for A, but switching in sorted Syndecan-1int cells from division 5 only is shown. (C) Quantification of the rate of isotype switching. The percentage of cells that became IgG1 during this time is shown.

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-1hi cells (Fig. 5A), but by day 4, such cells were evident in the control cultures (Fig. 5B). 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-1hi cells appeared by day 4, but few Syndecan-1hi cells were present. Significantly, in OBF-1−/− cultures infected with an OBF-1–expressing retrovirus, Syndecan-1hi cells appeared in the GFP+ cell gate but not in the uninfected cell gate (Fig. 5B). 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-1hi cells were more clearly discernable and, in the OBF-1−/− cultures, reached ~60% of the number produced in control cultures (Fig. 5C). 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. 5D). Nevertheless, the level was sufficient enough to drive a considerable proportion of cells to differentiate to the more mature Syndecan-1hi 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. 5E). 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−/− B cells were infected with the OBF-1–expressing retrovirus (3–4-fold and 10–20-fold, respectively; Fig. 5E). These studies confirm that OBF-1 is necessary for the differentiation of B cells to Syndecan-1hi 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. 1B). 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...
Several genes behaved as expected in control B cells with differentiation. Indeed, changes characteristic of ASC differentiation were already apparent in the Syndecan-1\(^{hi}\) 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 C\(/\text{H}9262\) and C\(/\text{H}9253\), 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 IgY1 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\(^{hi}\) cells 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
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\textsuperscript{gfp/+} mouse with the OBF-1\textsuperscript{-/-} strain to look for the influence of OBF-1 on the expression of the endogenous Blimp\textsuperscript{gfp} allele. B cells from mice of all possible genotypes were purified, cultured in CD40L/IL-4, and examined for differentiation to Syndecan-1\textsuperscript{hi} ASC (Fig. 7 A). As homozygous Blimp\textsuperscript{gfp/gfp} mice died during gestation, B cells of this genotype were obtained from RAG-1\textsuperscript{-/-} 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\textsuperscript{hi} ASC (30). It also confirmed the OBFS-1 dependence of Blimp-1 expression. Indeed, Blimp\textsuperscript{gfp/gfp} and OBFS-1\textsuperscript{-/-} B cells both seem to be blocked at a Syndecan-1\textsuperscript{int} state. A larger number of these cells accumulated in the OBFS-1\textsuperscript{-/-} culture, which was consistent with the accelerated differentiation we observed earlier (Fig. 2). This indicates a role for OBFS-1 at this stage of development that is independent of Blimp-1. These data confirm that OBFS-1 is required for Blimp-1 expression in B cells stimulated under conditions that mimic TD activation.

The Blimp\textsuperscript{gfp/+} mouse model enabled us to test whether OBFS-1 is required during ASC differentiation induced under other conditions and in vivo. We previously found that stimulating control Blimp\textsuperscript{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\textsuperscript{+} cells sorted from both control and OBF-1\textsuperscript{-/-} cultures secreted equal amounts of IgM during a short reculture period, with GFP\textsuperscript{+}, Syndecan-1\textsuperscript{hi} cells producing more Ig per cell than GFP\textsuperscript{+}, Syndecan-1\textsuperscript{-} cells. These studies show that
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, Blimpflp/flp 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 7 d after NP-KLH immunization, NP-specific IgG1 ASCs, as detected by ELISPOT, were only generated in the control animals. OBF-1/flp/flp 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-1 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-1int cells (31). The vast majority of ASCs present at this time reside in foci, as the GC reaction had not yet reached fruition (31).

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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 fulfillment 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 immuno-precipitation (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 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 Aiobos–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-1–/– 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 FcRn antid 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′-GTC GCC TCT AGG CAC CAA-3′, 3′-CTC TTT GAT GTC ACG CAC GAT TTC-5′; J chain, 5′-GTC TTC TCT ACT GGG TGG GAC TAC TAG CC-3′, 3′-GGG TGC AAA TGG AGA GCC TCT AAG G-5′; Blimp-1, 5′-CAT TCT TGG CCC CAA GCC AAT TGG TGC TCA TGG C-3′, xbp-1, 5′-GCT GGA GCA GCA AGT GGT GGA TTT GG-3′, 3′-GGG TTC CAG CTT GAG TGA TGG CC-5′; AID, 5′-CCG GCA GTC GGG TGA GTT T-3′, 3′-GAT GCG CAG CAG AAG TGG TCT GCT TCT TAG-5′; bcl-6, 5′-CAG CAC CTT CCT CTT CTC TGA TGA GGA CCC TCT CC-3′, 3′-CTG CGG GAM AGC AGG CAG CCT GAA GGA TGC-5′; Pax5, 5′-CCT ACC CTA TTG CAG ACC TGA GGA TGC GGC TCT-3′, 3′-CTG CGT TCT GCA GAT CGG AGG AGC AGG CCT-5′; c-myc, 5′-CAG GTC TCC GAC TAC GAC TCC GTA GCC TGT TGA CC-3′, c-mye, 5′-GAC GTC TCC GAC TAC TCC GCT TGA CAG CCC-3′, 3′-CAG GCT GGT GCT GTC TTT CCC TGG CGC AGC C-5′; cstF-64, 5′-CCA CTT CCT ATG ACG ACC TGC CAG GAA GGA TGC-3′, 3′-CCA CTA ACC CTG GAG TGG CCC TGC TGG C-5′; irf-4, 5′-GAA GCA CCA AAG CCC TCA GTC GTT G-3′, 3′-GGG GCA TGT AAT TAA ACC TTG TGT G-5′; obf-1, 5′-CCG TGT TGA CCT ATG CTT CTC CAC C-3′, 3′-GAG GGG CCC CGC CGT GTC CTC GTC GGG ACC C-5′; CstF-64, 5′-CCA CTT CCT ATG ACG ACC TGC CAG GAA GGA TGC-3′, 3′-CCA CTA ACC CTG GAG TGG CCC TGC TGG C-5′; irf-4, 5′-GAA GCA CCA AAG CCC TCA GTC GTT G-3′, 3′-GGG GCA TGT AAT TAA ACC TTG TGT G-5′; obf-1, 5′-CCG TGT TGA CCT ATG CTT CTC CAC C-3′, 3′-GAG GGG CCC CGC CGT GTC CTC GTC GGG ACC C-5′; CstF-64, 5′-CCA CTT CCT ATG ACG ACC TGC CAG GAA GGA TGC-3′, 3′-CCA CTA ACC CTG GAG TGG CCC TGC TGG C-5′; irf-4, 5′-GAA GCA CCA AAG CCC TCA GTC GTT G-3′, 3′-GGG GCA TGT AAT TAA ACC TTG TGT G-5′; obf-1, 5′-CCG TGT TGA CCT ATG CTT CTC CAC C-3′, 3′-GAG GGG CCC CGC CGT GTC CTC GTC GGG ACC C-5′; CstF-64, 5′-CCA CTT CCT ATG ACG ACC TGC CAG GAA GGA TGC-3′, 3′-CCA CTA ACC CTG GAG TGG CCC TGC TGG C-5′; irf-4, 5′-GAA GCA CCA AAG CCC TCA GTC GTT G-3′, 3′-GGG GCA TGT AAT TAA ACC TTG TGT G-5′; obf-1, 5′-CCG TGT TGA CCT ATG CTT CTC CAC C-3′, 3′-GAG GGG CCC CGC CGT GTC CTC GTC GGG ACC C-5′; CstF-64, 5′-CCA CTT CCT ATG ACG ACC TGC CAG GAA GGA TGC-3′, 3′-CCA CTA ACC CTG GAG TGG CCC TGC TGG C-5′; irf-4, 5′-GAA GCA CCA AAG CCC TCA GTC GTT G-3′, 3′-GGG GCA TGT AAT TAA ACC TTG TGT G-5′; obf-1, 5′-CCG TGT TGA CCT ATG CTT CTC CAC C-3′, 3′-GAG GGG CCC CGC CGT GTC CTC GTC GGG ACC C-5′; CstF-64, 5′-CCA CTT CCT ATG ACG ACC TGC CAG GAA GGA TGC-3′, 3′-CCA CTA ACC CTG GAG TGG CCC TGC TGG C-5′; irf-4, 5′-GAA GCA CCA AAG CCC TCA GTC GTT G-3′, 3′-GGG GCA TGT AAT TAA ACC TTG TGT G-5′; obf-1.

**Western blot.** Total cell lysates of murine-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 NH2-terminal 45
amino acids of murine OBF-1. A goat antiactin antiserum (Santa Cruz Bio-
technology, Inc.) was used as a loading control.

The authors thank P. Newton for critical input into this work, K. Daven and colleagues for mAb production, and Dr. S. Tangye for comments on the manuscript. This work was supported by the Australian National Health and Medical Research Council (grants 257526, 305513, and 356202).

The authors have no conflicting financial interests.

Submitted: 12 November 2004
Accepted: 8 March 2005

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