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Blimp-1 Orchestrates Plasma Cell Differentiation by Extinguishing the Mature B Cell Gene Expression Program

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Summary

Blimp-1, a transcriptional repressor, drives the terminal differentiation of B cells to plasma cells. Using DNA microarrays, we found that introduction of Blimp-1 into B cells blocked expression of a remarkably large set of genes, while a much smaller number was induced. Blimp-1 initiated this cascade of gene expression changes by directly repressing genes encoding several transcription factors, including Spi-B and Id3, that regulate signaling by the B cell receptor. Blimp-1 also inhibited immunoglobulin class switching by blocking expression of AID, Ku70, Ku86, DNA-PKcs, and STAT6. These findings suggest that Blimp-1 promotes plasmacytic differentiation by extinguishing gene expression important for B cell receptor signaling, germinal center B cell function, and proliferation while allowing expression of important plasma cell genes such as XBP-1.

Introduction

Plasma cells, the final effectors of humoral immunity, are nondividing cells devoted to synthesis and secretion of immunoglobulin (Ig). A pivotal regulator of plasma cell development is B lymphocyte-induced maturation protein-1 (Blimp-1), which was cloned from the BCL1 murine lymphoma upon differentiation to a plasma cell state (Turner et al., 1994). Enforced expression of Blimp-1, in either BCL1 cells or primary mouse splenocytes (Piskurich et al., 2000; Schliephake and Schimpl, 1996) is sufficient to drive mature B cells to become antibody-secreting plasma cells. Thus, Blimp-1 is a master regulator of terminal B cell development.

The expression pattern of Blimp-1 in vivo (Angelin-Duclos et al., 2000) is consistent with its role as a master regulator of plasma cell development in vitro. Blimp-1 is present in plasma cells formed in primary and secondary responses to T cell-dependent and -independent antigens and in long-lived plasma cells in the bone marrow. Blimp-1 is not detected in memory B cells but is present in a subset of germinal center (GC) B cells with a partial plasma cell phenotype (BCL-6$^-$, CD20$^-$, IRF-4$^+$, Syndecan-1$^+$, Blimp-1$^+$), suggesting that these cells may be committed to a plasma cell fate. Of particular interest, BCL-6, a transcriptional repressor required for GC B cell formation (Chang et al., 1996; Dent et al., 1997; Fukuda et al., 1997) is not expressed in the Blimp-1$^+$ subset of GC B cells (Angelin-Duclos et al., 2000). Consistent with the finding that most GC B cells are BCL-6$^+$ and Blimp-1$^-$, microarray analyses revealed that *PRDM1*, encoding Blimp-1, is a target of BCL-6 repression (Shaffer, et al., 2000). This was further confirmed in studies showing that ectopic BCL-6 inhibited expression of Blimp-1 and plasmacytic differentiation of B cells in vitro (Reljic et al., 2000). Thus, a primary function of BCL-6 in GC B cells is to repress *Blimp-1* and thereby inhibit terminal differentiation.

How does a single transcriptional repressor drive plasma cell development? Three known targets of Blimp-1-dependent repression explain important aspects of the plasma cell phenotype. Blimp-1 represses c-*myc* transcription (Lin et al., 1997) which explains cessation of cell cycle in plasma cells as c-*myc* is required for proliferation and growth (Eilers, 1999). *CIITA* is also directly repressed by Blimp-1 (Piskurich et al., 2000), leading to the downregulation of MHC Class II genes in plasma cells (Silacci et al., 1994). Finally, Blimp-1 represses *PAX5* (Lin et al., 2002), which is required for lineage commitment and B cell development in the bone marrow (Nutt et al., 2001) as well as for isotype switching in GCs (Liao et al., 1994; Max et al., 1995). Downregulation of PAX5 is required for development of antibody-secreting cells (Lin et al., 2002; Usui et al., 1997), probably because it represses XBP-1 (Reimold et al., 1996, 2001), J chain (Rinkenberger et al., 1996; Wallin et al., 1998), and immunoglobulin heavy chain gene transcription (Singh and Birshtein, 1993).

Repression of c-*myc, CIITA*, and *PAX5* alone is unlikely to explain the entire program of plasma cell development activated by Blimp-1. Although repression of c-*myc* transcription is necessary for differentiation of BCL1 cells, removal of c-*myc* activity is not sufficient to trigger plasma cell differentiation (Lin et al., 2000). CIITA activity is primarily limited to transcription of class II MHC, invariant chain, and the DM gene in B cells (Waldburger et al., 2000). Finally, when PAX5 is deleted in mature B cells (Horcher et al., 2001), some changes associated with plasma cell differentiation occur, but these cells fail to upregulate J chain or secrete immunoglobulin. Therefore, important aspects of Blimp-1-dependent regulation remain to be discovered.

To develop an understanding of Blimp-1's control over terminal B cell differentiation, we used DNA microarrays (Alizadeh et al., 2000) to analyze gene expression changes caused by manipulation of Blimp-1 activity. We show that Blimp-1 broadly inhibits gene expression programs controlling mature B cell functions and driving proliferation. This is accomplished by direct repression

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of several transcription factor genes, including two new direct targets of Blimp-1, *Spi-B* and *Id3*.

Results

Microarray Analysis of Blimp-1 Expression in B Cell Lines

To identify genes that are the targets of Blimp-1 action, *Blimp-1* was introduced acutely or inducibly into cells which do not express Blimp-1. For acute expression, cells were transduced with *Blimp-1*-expressing or control (puromycin resistance) retroviruses. Several B cell lines were chosen for infection to improve the likelihood that a particular Blimp-1 target gene would be expressed in one of the cell lines: WI-L2, an EBV^+ mature lymphoblastoid cell line; SUDHL4, an EBV^- GC B cell-like diffuse large B cell lymphoma (DLBCL) cell line; BJAB, an EBV^- mature B lymphoma cell line; and RAJI, an EBV^+ Burkitt's lymphoma cell line.

We also developed a strategy for inducibly expressing Blimp-1, since attempts to create stable transfectants expressing Blimp-1 were unsuccessful (A.L.S., unpublished data). Neither use of a heavy metal-inducible metallothionein promoter construct (pMEP4) nor fusion of Blimp-1 to the estrogen receptor ligand binding domain (Blimp-1-ERD), a strategy used previously to circumvent the toxicity of BCL-6 (Shaffer et al., 2000), was sufficient to control Blimp-1 toxicity. We therefore created a doubly inducible system in which Blimp-1-ERD expression was controlled by the metallothionein promoter and generated stable transfectants of three B cell lines: RAJI, WI-L2, and OCI-Ly7, a GC B cell-like DLBCL cell line. Treatment of these lines with cadmium (Cd) and tamoxifen (TMX) induced expression of the Blimp-1-ERD protein, allowing us to analyze early transcriptional changes caused by Blimp-1.

The acute effects of Blimp-1 on B cell gene expression were analyzed using DNA microarrays. The change in expression for each gene was computed as a ratio of expression in Blimp-1-transduced cells versus control cells (color scale, Figure 1). Genes downregulated in the presence of Blimp-1 appear green; genes upregulated by Blimp-1 appear red. Using the doubly inducible system, gene expression analysis was performed on Blimp-1-ERD-expressing and control cells treated with Cd and TMX (Figure 1). This analysis included only named genes that were repressed or induced at least 1.8-fold (relative to control) in any of the six acute infection experiments. For time course experiments involving inducible Blimp-1, at least half of the induced samples were required to show 1.8-fold repression or induction relative to the corresponding control sample. For genes represented by more than one microarray feature, representative data is displayed. Genes meeting these criteria are referred to as Blimp-1 targets, but this is not meant to indicate whether Blimp-1 acts directly or indirectly to modulate the expression of a given gene.

Expression of a surprisingly large number of genes was affected by Blimp-1 (Figure 1A). Of the 260 target genes, 180 were targets in two or more cell systems, and 87 were targets in three or more systems (see table at http://lymphochip.nih.gov/blimp/). Three known direct targets of Blimp-1 repression, *c-Myc*, *CIITA*, and *PAX5* (Lin et al., 1997, 2002; Piskurich et al., 2000), were repressed by Blimp-1, confirming the ability of these systems to detect Blimp-1 targets. Two hundred twenty-eight genes were downregulated by Blimp-1, but Blimp-1 did not globally repress transcription since over 700 microarray elements representing named genes were unaffected by Blimp-1 expression (data not shown). A smaller set of 32 genes were induced in the presence of Blimp-1 (Figure 1B), and only 15 of these were induced in two or more cell lines. We did not, however, observe certain hallmarks of plasmacytic differentiation such as Ig secretion (Chilosi et al., 1999; Wijdenes et al., 1996), suggesting that Blimp-1 alone is insufficient to activate the complete program of plasmacytic differentiation in transformed B cells.

We confirmed the ability of Blimp-1 to downregulate many of these microarray targets by independent assays of mRNA and protein expression (see figure at http://lymphochip.nih.gov/blimp/). We observed a Blimp-1-specific decrease in the steady-state levels of target gene transcripts by RT-PCR, including *c-MYC, AID, btk, CD22, EBF*, and others. Western blotting showed reduction in levels of the target gene proteins c-Myc, PAX5, and STAT6. Blimp-1 targets encoding cell surface proteins were assessed by flow cytometry. Proteins involved in BCR signaling (CD19, CD22, CD45) lymphocyte homing, adhesion, and cell-cell interactions (CD11A, CXCR5, MHCII, etc.) were downregulated by Blimp-1.

Blimp-1 Targets in Context

The list of Blimp-1 targets is replete with genes important for B cell function and differentiation (Figure 1). To put these into a developmental context, we assembled gene expression data from several primary B cell types, including resting peripheral blood B cells, mitogenically activated peripheral blood B cells, GC B cells, and chronic lymphocytic leukemia (CLL) cells. Cell lines were also analyzed, including GC-like B cell lines and plasma cell-like multiple myeloma lines, along with several cell lines representing non-B cells. Relative gene expression was assessed by comparing each cell type to a reference mRNA pool, and genes modulated by Blimp-1 expression were organized based on hierarchical clustering (Eisen et al., 1998; Figure 2A).

This analysis revealed that Blimp-1 plays a major role in promoting B cell terminal differentiation by turning off two classes of genes. One class of Blimp-1 targets belongs to a gene expression signature of proliferation and growth (Figure 2A). These target genes were more highly expressed in dividing cells (cell lines, mitogenically activated blood B cells, and GC B cells) than in nondividing cells (resting blood B cells and CLL cells). Blimp-1 repressed its known target gene *c-myc* (Lin et al., 1997), as well as genes that are transcriptionally activated by c-*myc*, including *Rcl, DHFR, ODC*, and *LDH-A* (Coller et al., 2000; Dang, 1999; Eilers, 1999). Blimp-1 also downregulated genes involved in cell cycle progression (*CDC2*, *cdk2, PLK*, *ckshs, E2F-1*) and DNA synthesis and repair (*PCNA, Ku70, Ku86, MCM2*).

Another class of Blimp-1 targets includes genes involved in mature B cell functions (Figure 2A). These were generally highly expressed in primary B cells and GC-like B cell lines compared to multiple myeloma and non-B cell samples. Blimp-1 extinguished a gene expression program specifying B cell identity, including surface

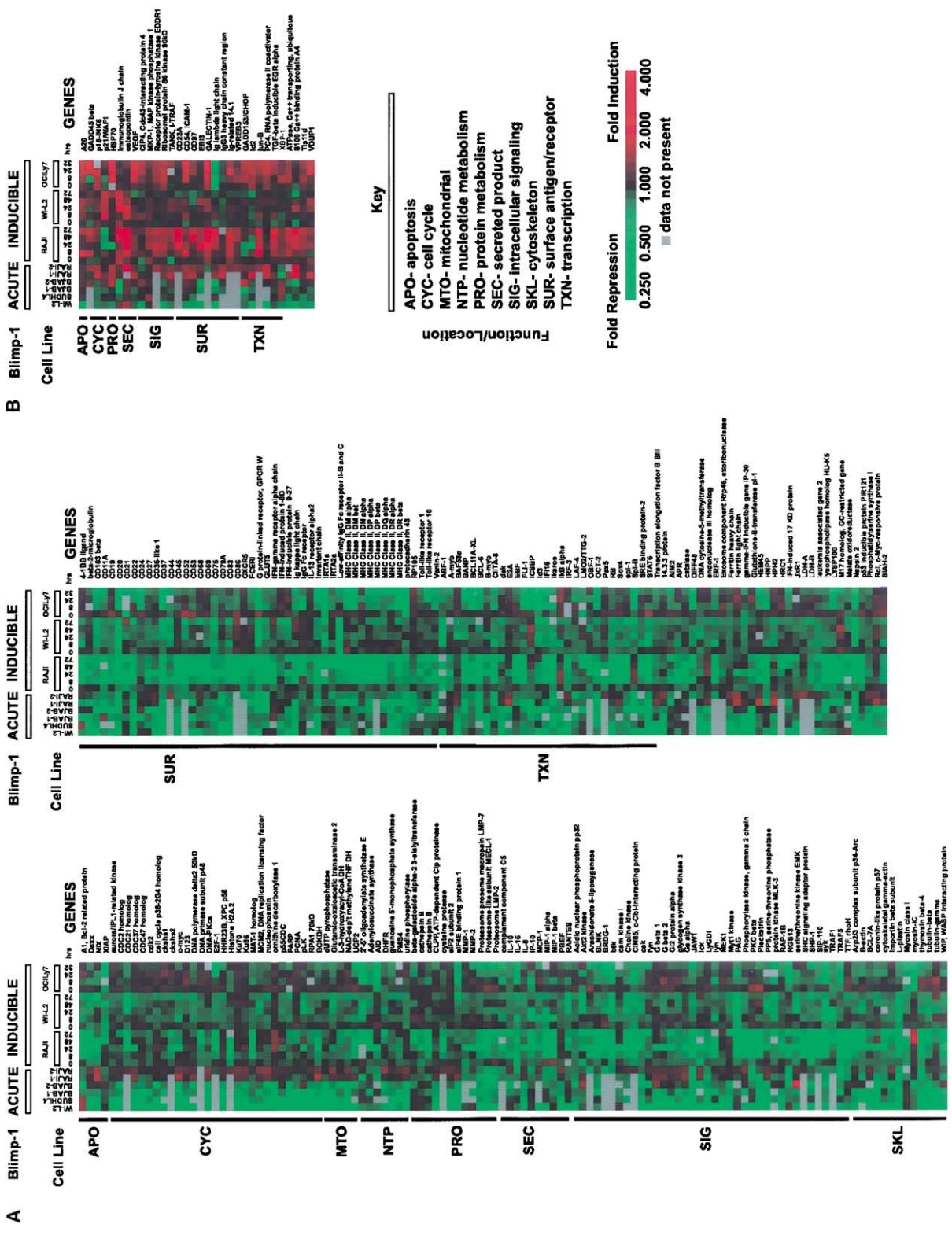
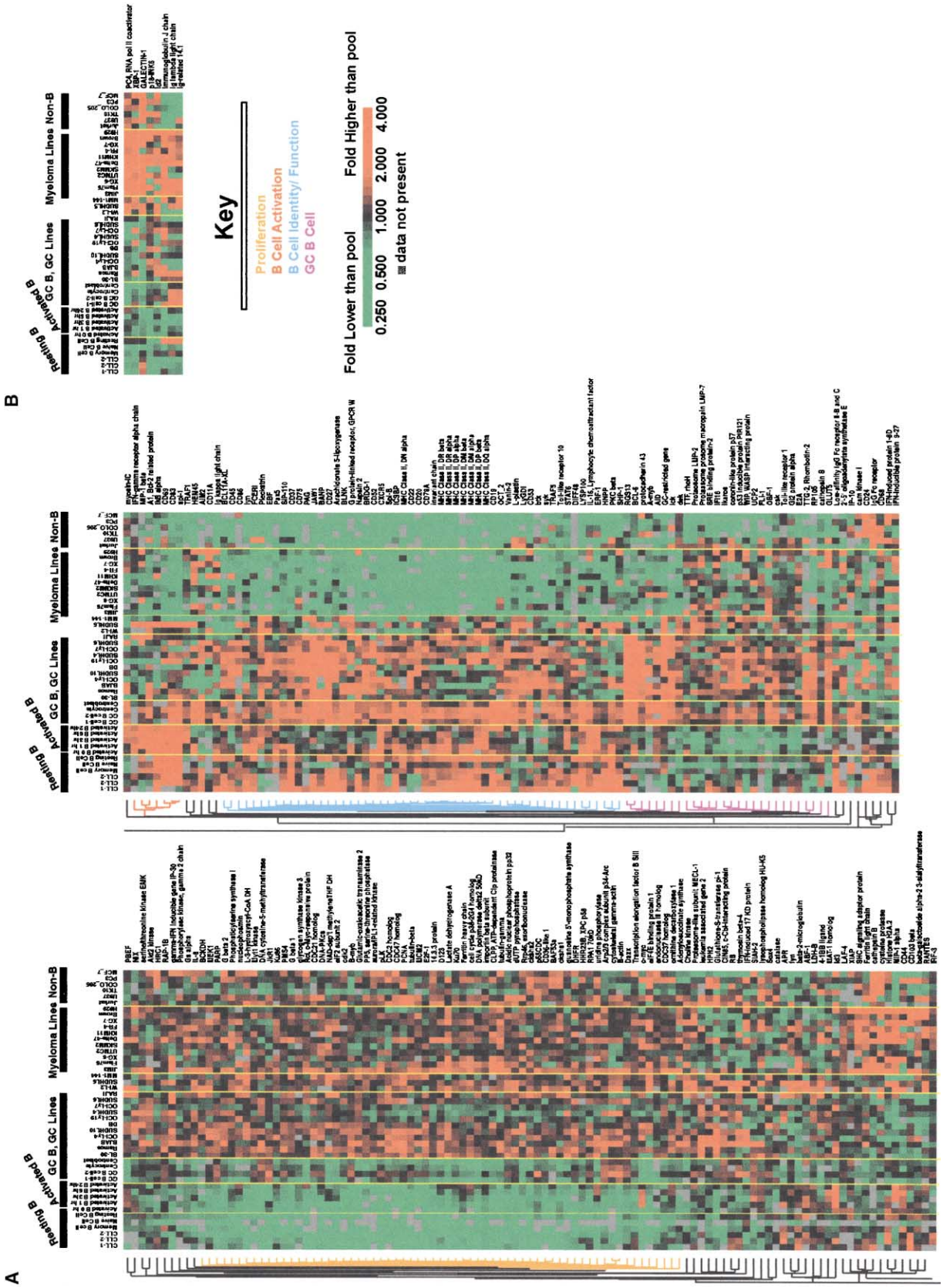


Figure 1. DNA Microarray Analysis of Blimp-1 Target Genes in Human B Cell Lines in Cells Acutely or Inducibly Expressing Blimp-1

Each column represents data from an independent infection of the indicated human B cell line. Each row represents a gene significantly induced (red) or repressed (green) following Blimp-1 expression. A color bar shows the magnitude of gene expression changes as a ratio of expression in control cells (green) versus Blimp-1-expressing cells (red). To better understand the role of Blimp-1 in controlling B cell gene expression, analysis was limited to named genes, and genes were organized into broad functional categories (see key) based on analysis of the OMIM (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi) and GO databases (http://www.geneontology.org/).

(A) Genes repressed in the presence of Blimp-1.

(B) Genes induced in the presence of Blimp-1.


A
B
Resting B
Activated B
GC B, GC Lines
Myeloma Lines
Non-B
Key
Proliferation
B Cell Activation
B Cell Identity/ Function
GC B Cell
Fold Lower than pool
Fold Higher than pool
0.250 0.500 1.000 2.000 4.000
data not present

markers (*CD19, CD20, CD45, MHCII*), B cell receptor (BCR) signaling components (*BLNK, CD79A, syk, btk*), and transcription factors (*Spi-B, PAX5, Oct-2, STAT6, EBF*). Also in this group are genes induced during B cell activation (*CD69, MIP-1β, A1*) or upregulated at the GC stage of differentiation (*AID, JAW1, A-myb)*. Among the GC-specific target genes is *BCL-6*, which encodes a transcription factor that represses *Blimp-1* and prevents plasmacytic differentiation (Reljic et al., 2000; Shaffer et al., 2000). Blimp-1 expression reduced both BCL-6 mRNA and protein levels, as assessed by RT-PCR and Western blotting, and BCL-6 DNA binding activity, as assessed by gel shift analysis (see figure at http://lymphochip.nih.gov/blimp/). The discovery of *BCL-6* as a Blimp-1 target implies a reciprocal regulatory loop in which BCL-6 and Blimp-1 antagonize each other's expression.

Among the genes induced in the presence of Blimp-1, a few showed higher expression in plasma cell-like myeloma cell lines as compared to other cell types, including genes known to be highly expressed in plasma cells (*XBP-1*, *J chain*, *Ig light chain*; Figure 2B). Of particular interest was the modest induction of *XBP-1*, which encodes a critical regulator of plasma cell differentiation (Reimold et al., 2001). We confirmed the induction of XBP-1 mRNA in RAJI cells expressing Blimp-1-ERD using a quantitative RT-PCR assay (Figure 3A) and found the induction to be in the same range as detected by microarray analysis (1.7-fold on average). This XBP-1 level was substantially lower than the XBP-1 level in a myeloma cell line (Figure 3A), indicating that Blimp-1 alone is insufficient to achieve the high *XBP-1* mRNA expression characteristic of plasma cells.

To further explore the influence of Blimp-1 on *XBP-1* expression, we studied another B cell line, SKW6.4 (SKW), which can undergo plasma cell-like differentiation when treated with IL-6 (Goldstein and Kim, 1993). Transduction of SKW cells with a Blimp-1-expressing virus induced *XBP-1* expression (Figure 3B). IL-6 treatment of these cells caused IgM secretion and induced the expression of both *Blimp-1* and *XBP-1* mRNAs (Figure 3C). To test whether induction of the endogenous *Blimp-1* gene is necessary for *XBP-1* induction, we transduced these cells with a form of Blimp-1, Tblimp, which lacks the repression domain and can dominantly interfere with Blimp-1 activity by competing for Blimp-1 DNA binding sites (Chang et al., 2000; Yu et al., 2000). Tblimp blocked both IL-6-dependent IgM secretion and induction of *XBP-1* mRNA but had no effect on the induction of *Blimp-1* mRNA (Figure 3C). Together these data argue that Blimp-1 is necessary but insufficient for complete upregulation of *XBP-1* mRNA, possibly by repressing *PAX5*, a known repressor of *XBP-1* (Reimold et al., 1996).

Gene Expression Changes Caused by Blimp-1 in Mouse Splenic B Cells

We next investigated whether the target genes identified by microarray analysis of B cell lines were similarly regulated by Blimp-1 in primary B cells. Mouse splenic B cells were purified and infected with retroviruses expressing either YFP (yellow fluorescent protein) or a bicistronic mRNA encoding Blimp-1 and YFP (Piskurich et al., 2000). Blimp-1 expression drove plasmacytic differentiation, indicated by IgM secretion (data not shown). RNA from YFP^+ cells was used for semiquantitative RT-PCR which showed a Blimp-1-dependent decrease in mRNAs for numerous microarray targets including *BCL-6*, *Spi-B*, *STAT6*, *Id3*, *CD79A*, *CD22*, *syk*, *btk*, *A1*, and *AID* (Figure 4A). These data confirm our findings in B cell lines, underscoring the physiological relevance of these targets and showing Blimp-1's functional similarity in human and mouse B cells (Piskurich et al., 2000).

We also examined the role of endogenous Blimp-1 during plasmacytic differentiation of mouse splenocytes in vitro. Semiquantitative RT-PCR showed that *Blimp-1* mRNA increased in splenic B cells after 4 days of lipopolysaccharide (LPS) treatment. Concomitantly, mRNA for *c-myc*, as well as several other targets, decreased following LPS treatment (Figure 4B). To block endogenous Blimp-1, we used a retrovirus to express Tblimp prior to LPS treatment, which prevented the downregulation of the known Blimp-1 targets c-*myc*, *CIITA*, and *PAX5* (Figure 4B). In addition, TBlimp prevented the downregulation of *BCL-6*, *Spi-B*, *ICSBP*, *STAT6*, *CD22*, *syk*, *btk*, *CXCR5*, and *A1*, although it failed to block repression of *EBF* and *CD79A* (Figure 4B, and data not shown). This provides strong evidence that endogenous Blimp-1 is necessary for the repression of these targets during LPS-driven plasmacytic differentiation.

A similar strategy was used to identify Blimp-1 targets that were repressed indirectly as a result of the repression of *PAX5* by Blimp-1. PAX5 can either activate or repress transcription from many target genes (Horcher et al., 2001; Nutt et al., 1998). Indeed, changes in expression of the PAX5 targets *CD19*, *CD79A,* and *J chain* (Kozmik et al., 1992; Nutt et al., 2001; Rinkenberger et al., 1996) were observed upon expression of Blimp-1 (Figure 1). Retroviruses were used to enforce expression of PAX5 in splenic B cells prior to LPS treatment, which interfered with the downregulation of *CD19* and the induction of *J chain* associated with differentiation (Figure 4C). *CIITA* and *STAT6* mRNAs were also higher in PAX5-expressing cells, suggesting that part of the regulation

Figure 2. Developmental Context of Blimp-1 Target Genes

(A) Repressed Blimp-1 targets in context. Expression of repressed genes from Figure 1A was analyzed comparing relative gene expression from primary purified B cells (CLL cells, representing quiescent B cells), peripheral memory ($CD19^+$, $CD27^+$) cells, peripheral naive ($CD19^+$, $CD27^-$) cells, resting peripheral B cells ($CD19^+$), peripheral B cells activated with anti-IgM/CD40L/IL-4 for the times indicated, tonsillar GC B cells (Ma and Staudt, 2001), GC centrocytes ($CD77^-$), GC centroblasts ($CD77^+$), and GC-B-like cell lines along with plasma cell-like multiple myeloma cell lines and non-B cell lines compared to a common reference mRNA pool. Data were centered, and genes were grouped by similarity in expression pattern by hierarchical clustering. Genes related to proliferation are shown in orange; genes related to B cell functions are shown in red, blue, and purple. Only genes whose data met minimum expression intensity criteria in 50% or more of the arrays are shown. (B) Induced Blimp-1 targets. Shown are those genes that are expressed at a higher level in plasma-like myeloma cell lines than in GC-B cell lines.

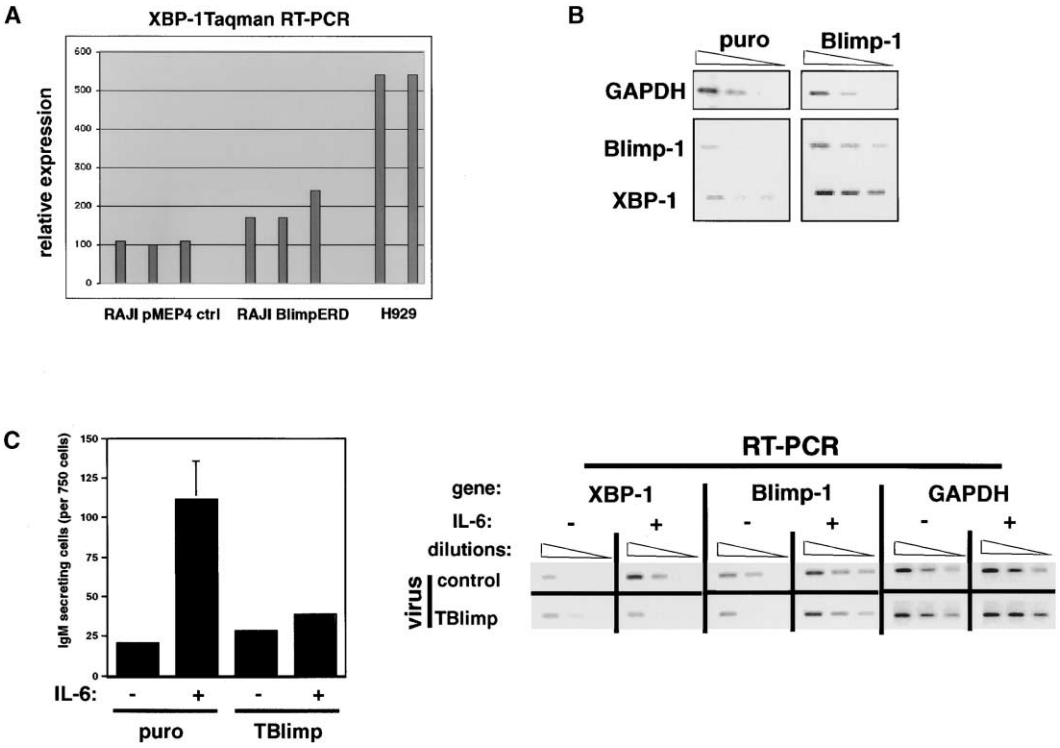


Figure 3. XBP-1 Expression Depends upon Blimp-1 Expression

(A) Quantitative RT-PCR confirms *XBP-1* mRNA increase following Blimp-1 induction. RNA from control (pMEP4, vector only) or Blimp-1-ERD-expressing RAJI cells, treated with cadmium and tamoxifen inducers for 24 hr and RNA from the H929 myeloma line were analyzed for XBP-1 (unspliced form) mRNA expression by quantitative Taqman RT-PCR using β-2-microglobulin expression as the normalization control.

(B) Blimp-1 induction of XBP-1 mRNA in SKW6.4 cells. Control or Blimp-1-expressing retroviruses were used to infect cells; RNA from transduced cells was analyzed by semiquantitative RT-PCR using 4-fold dilutions of cDNAs.

(C) Ectopic expression of dominant-negative Blimp-1 (Tblimp) is sufficient to block IgM secretion and XBP-1 mRNA expression in differentiating SKW cells. Retrovirally infected cells were treated with IL-6 (40 U/ml), and ELISPOT was used to measure IgM secretion after 3 days. Semiquantitative RT-PCR was used to measure endogenous *Blimp-1, XBP-1*, and *GAPDH* mRNA using 4-fold dilutions of cDNA.

of these genes by Blimp-1 is secondary to repression of *PAX5*. Other Blimp-1 targets, including *c-myc, BCL-6, Spi-B, Id3, AID*, and others, were mostly unaffected by PAX5, suggesting that their repression is not secondary to repression of *PAX5*.

Direct Repression of Transcription Factor Genes Important for BCR Signaling

Blimp-1's powerful ability to extinguish B cell gene expression programs may be explained by its ability to repress genes encoding other transcription factors (see table at http://lymphochip.nih.gov/blimp/). All previously identified Blimp-1 target genes encode transcription factors, as do more than 10% of the genes identified as repressed upon Blimp-1 expression in this study (Figure 1). We therefore suspected that more transcription factor genes might be direct targets of Blimp-1. Comparing the human and mouse genomic sequences of several candidate target genes, we found that two genes, *Spi-B* and *Id3*, have evolutionarily conserved sites in their transcriptional control regions that resemble known Blimp-1 binding sites (Figure 5A). Both the Spi-B and Id3 sites competed effectively in a gel mobility shift assay for Blimp-1 binding to a site from the *c-myc* gene (Figure 5B). Thus, Blimp-1 binds these sites in vitro, and their evolutionary conservation suggests the sites are functionally important.

To determine whether Blimp-1 binds the sites in vivo, we performed chromosomal immunoprecipitation (ChIP) experiments (Lin et al., 2002; Yu et al., 2000). We used WI-L2 cells expressing a FLAG epitope-tagged Blimp-1-ERD protein, which allowed us to identify genomic sites of Blimp-1 binding by immunoprecipitation (IP) with an anti-FLAG antibody. As negative controls, we used WI-L2 cells transduced with the pMEP4 vector alone or WI-L2 cells expressing a FLAG epitope-tagged form of BCL-6. Semiquantitative PCR assays were designed for the known direct Blimp-1 target, *CIITA*, for the suspected targets *Spi-B* and *Id3*, and for a control genomic locus, *CSF-1*. Each locus was equivalently represented in genomic DNA from the three cell lines prior to IP (Figure 5C). After IP, the *CIITA*, *Spi-B*, and *Id3* loci were specifically enriched in samples from cells expressing Blimp-1-ERD, but the control *CSF-1* locus was not (Figure 5C). Thus, Blimp-1 binds in vivo to conserved binding sites in the Spi-B and Id-3 genes, providing strong evidence that *Spi-B* and *Id3* are direct targets of Blimp-1 repression, extending the repertoire of transcription factor genes that are repressed by Blimp-1. Interestingly, both of these transcription factors are required for effective

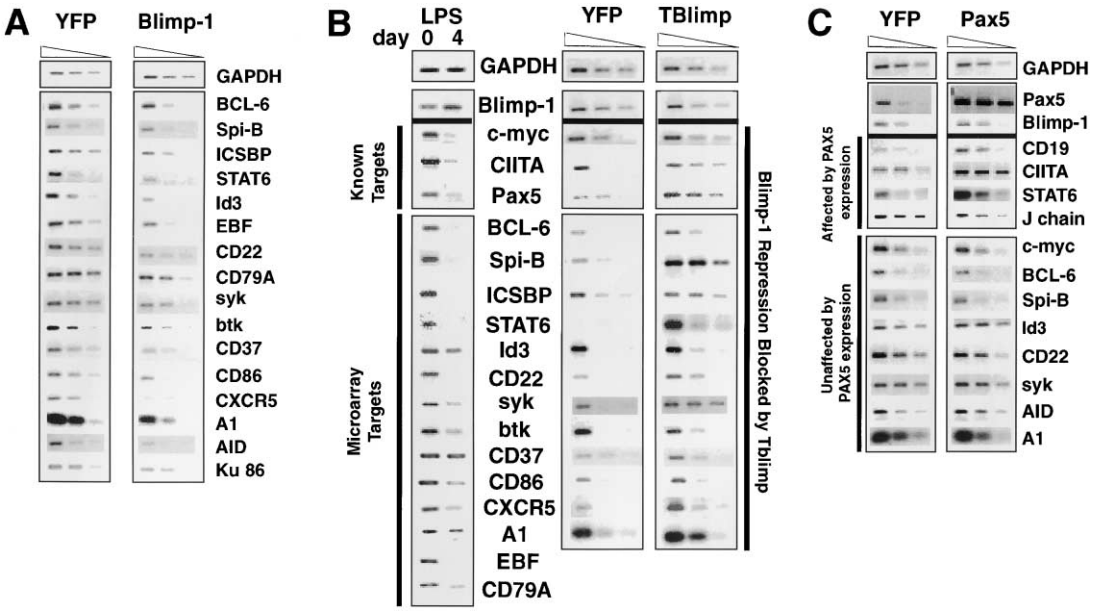


Figure 4. Blimp-1 Is Necessary and Sufficient to Repress Identified Targets during Splenic B Cell Terminal Differentiation

(A) Purified splenic B cells were treated with LPS (10 μg/ml) and anti-F(ab)′$_2$ (5 μg/ml) overnight before retrovirus infection. Semiquantitative RT-PCR was performed on RNA from sorted YFP$^+$ cells, and 4-fold serial dilutions of cDNA were tested.

(B) Target gene expression was determined by semiquantitative RT-PCR from purified splenic B cells treated with LPS (10 ug/ml) for 0 and 4 days (left panel). Semiquantitative RT-PCR (4-fold dilutions) was performed on RNA from sorted YFP$^+$ cells 4 days post-LPS stimulation (right panel).

(C) Expression of Blimp-1 targets was determined by semiquantitative RT-PCR on RNA from splenic B cells stimulated with LPS and infected with either retrovirus-expressing PAX5 or control virus as above. Signals for STAT6 and A1 in (B) and (C) were detected by blotting with internal probes.

BCR signaling (Garrett-Sinha et al., 1999, 2001; Pan et al., 1999), providing one mechanism by which Blimp-1 can attenuate this central function of mature B cells.

Blimp-1 Target Genes: A Role in Class Switching

Microarray analysis (Figure 1) showed that Blimp-1 downregulates genes required for immunoglobulin class switching (*AID, Ku70, Ku86I*, and *DNA-PKcs)* as well as *STAT6*, a transcription factor that is essential for IL-4 induction of Iγ1 transcription and switching to IgG1 (Linehan et al., 1998). Repression of these genes may account for the ability of Blimp-1 to suppress class switching induced by signaling through the BCR, CD40, and/or IL-4 receptors in mouse splenic B cells (Knodel et al., 2001). To test this possibility, mouse B cells, infected with a control or Blimp-1-expressing retrovirus, were treated with LPS and IL-4. As reported, a significant fraction of control cells switched to IgG1 (Figure 6A) whereas ectopic Blimp-1 blocked surface IgG1 (Knodel et al., 2001). Using semiquantitative RT-PCR (Figure 6B), Blimp-1 was shown to block expression of *AID, STAT6, Ku86*, and *DNA-PKcs* as well as STAT6-dependent Iγ1 and IgG1 transcripts (Figure 6B). These data support the view that Blimp-1 shuts off immunoglobulin isotype switching by reducing expression of factors required for this type of recombination and by blocking signals that activate switch region immunoglobulin transcription.

Discussion

Taking a genomic approach, we identified 228 genes that were repressed and 32 that were induced following Blimp-1 expression in multiple systems using human B cell lines. Thirty-four of these genes were confirmed as Blimp-1 targets in mouse B cells undergoing plasmacytic differentiation and other complementary systems. Repressed targets fell into two broad functional groups, one specifying B cell identity and function and the other regulating proliferation. From this perspective, it is clear that Blimp-1 directs plasmacytic differentiation by extinguishing mature B cell gene expression programs and by causing B cells to exit the cell cycle (Figure 7).

It is likely that Blimp-1 initiates this cascade of gene expression changes by directly repressing genes encoding transcription factors, including three previously identified direct targets, *c-myc, PAX5*, and *CIITA*, and two newly identified direct targets, *Spi-B* and *Id3*. Altogether, more than 25 transcription factor genes, each of which has its own set of target genes (Figure 1 and see table at http://lymphochip.nih.gov/blimp/), were downregulated in the presence of Blimp-1. Many of these transcription factors have been shown by genetic disruption to be important for B cell development and/or mature B cell function (e.g., EBF, E2A, Ikaros, Oct-2, PAX5, Spi-1/PU.1, Spi-B, Id3, Sox4, and STAT6). The downstream targets of these transcription factors are also modulated by Blimp-1. For example, the direct Blimp-1 target PAX5 activates *CD19, CD79A, BLNK*, and *CIITA* and represses *J chain* and *IgH* (Horcher et al., 2001; Nutt et al., 1998), suggesting Blimp-1-dependent regulation of these genes may be secondary to direct repression of *PAX5*. Indeed, enforced expression of PAX5 in LPS-treated differentiating B cells maintained

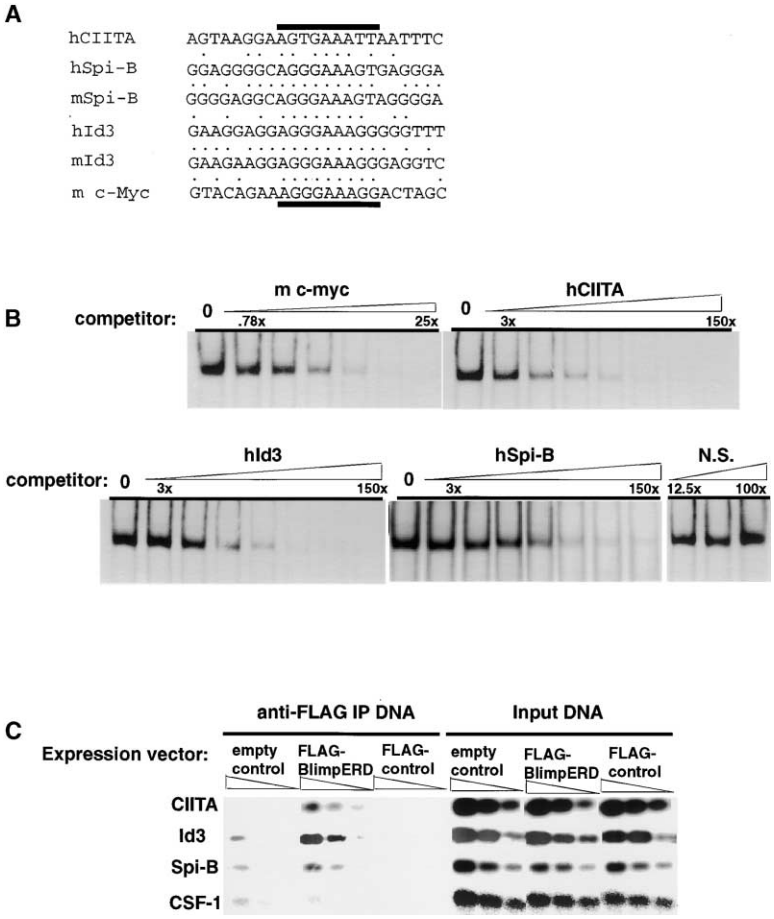


Figure 5. *Id3* and *Spi-B* Are Direct Targets of Blimp-1

(A) Putative Blimp-1 binding sites in the human and mouse *Id3* and *SpiB* genes aligned with known sites in *CIITA* and c-*myc* (Lin et al., 1997; Piskurich et al., 2000) (underlined).

(B) Competitor titrations to measure binding affinity of Blimp-1 for Id3 and Spi-B sites. EMSA was performed using nuclear extract from P3X, a Blimp-1-expressing plasmacytoma, and a Blimp-1 binding site from the mouse *c-myc* promoter (PRF). Molar equivalents of unlabeled competitor oligonucleotides were added as follows: PRF (0.78, 1.56, 3.125. 6.25, 12.5, 25), CIITA, hId3, hSpiB (3.125, 6.25, 12.5, 25, 50, 100, 150), and nonspecific, N.S. (12.5, 50, 100).

(C) ChIP showing Blimp-1 binds to *CIITA* promoter III, *hId3* promoter, and *hSpi-B* intron1 (promoter II) in vivo. Chromatin prepared from WI-L2 cells induced for the expression of FLAG-tagged Blimp-1-ERD for 20 hr was immunoprecipitated with anti-FLAG antibody, and regions encompassing the Blimp-1 sites were amplified by PCR (2-fold dilutions). Input DNA was amplified with the same primers as a control for equivalence of the starting material. Chromatin was prepared from cells with only control vector and cells expressing an irrelevant FLAG-tagged protein as specificity controls. Amplification of the CSF-1 (colony stimulating factor-1) gene was used as a negative control.

CD19 and *CIITA* mRNA expression and decreased *J chain* mRNA levels (Figure 5). Another Blimp-1 target, *STAT6*, also increased in response to enforced PAX5, suggesting its repression by Blimp-1 may be downstream of PAX5. However, many other Blimp-1 targets were insensitive to PAX5 overexpression although their expression was modulated by TBlimp, suggesting that Blimp-1 affects the expression of many genes in a PAX5-independent manner.

Blimp-1 inhibited a second gene expression program involving growth and proliferation, and this was likely due, in part, to direct repression of *c-myc* (Lin et al., 2000). In accord with this hypothesis, many known downstream targets of c-*myc* activation were also downregulated by Blimp-1, including *Rcl, ODC*, *LDH-A*, and *DHFR* (Coller et al., 2000; Dang, 1999; Eilers, 1999). However, Blimp-1 also downregulated other genes in the proliferation signature involved in DNA synthesis and repair (*PCNA, PMS4, MCM2, primase*) and mitosis (*pLK, aurora kinase, ckshs1, ckshs2*), perhaps as an indirect consequence of the cell cycle arrest that follows the loss of c-*myc* (Amati et al., 1998; Carey et al., 2000). The inhibition of these proliferation signature genes illustrates how Blimp-1 coordinates the cell cycle arrest that accompanies plasmacytic differentiation.

Mature B Cell Functions Modulated by Blimp-1

The powerful ability of Blimp-1 to alter B cell identity and function has been shown in the BCL1 line and in mouse splenic B cells (Knodel et al., 2001; Piskurich et al., 2000; Turner et al., 1994). Analysis of Blimp-1 target gene expression across a panel of primary B cells and cell lines provides insight into how Blimp-1 drives plasmacytic differentiation. Over 80 Blimp-1 target genes were expressed in mature B cells (peripheral blood and GC B cells) but were turned off in multiple myeloma cells, which represent the plasma cell stage. Proteins encoded by these target genes are critical for the function of mature B cells and include components of the BCR signaling pathway, cell surface receptors, and the host of B cell-restricted transcription factors mentioned above. Plasma cells can develop as a consequence of the germinal center reaction, probably from the small subset of Blimp-1$^+$, BCL-6$^-$ cells (Angelin-Duclos et al., 2000). A number of Blimp-1 target genes are GC B cell-restricted, including *BCL-6, A-myb, AID,* and *TTG-2* (Chang et al., 1996; Christoph et al., 1994; Ma and Staudt, 2001; Muramatsu et al., 2000; Shaffer et al., 2001), suggesting that Blimp-1 may promote plasmacytic differentiation by extinguishing the GC B cell gene expression program. Plasmacytic differentiation can also occur outside of the germinal center and is preceded by activation through the BCR. Blimp-1 downregulated a set of genes that are induced by BCR signaling (*A1, MIP-1*β*, CD69, CD83*, and *spi-1/PU.1*), which may contribute to its ability to drive plasmacytic differentiation.

Our analyses also suggest that Blimp-1 participates

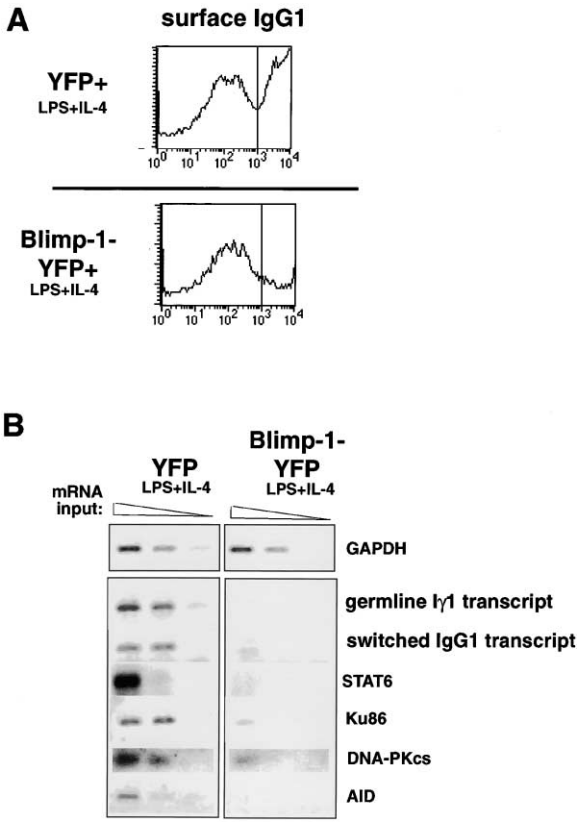


Figure 6. Blimp-1 Expression Represses Class Switching to IgG1

(A) Flow cytometric analysis of surface IgG1 expression in B cells infected with control or Blimp-1 viruses after 4 days of LPS+IL-4 treatment; the black line divides IgG^- and IgG^+ populations.

(B) Semiquantitative RT-PCR analysis of gene expression for factors involved in class switching after 4 days of LPS+IL-4 treatment of control and Blimp-1-expressing cells. STAT6 and DNA-PKcs were detected by blotting with internal probes.

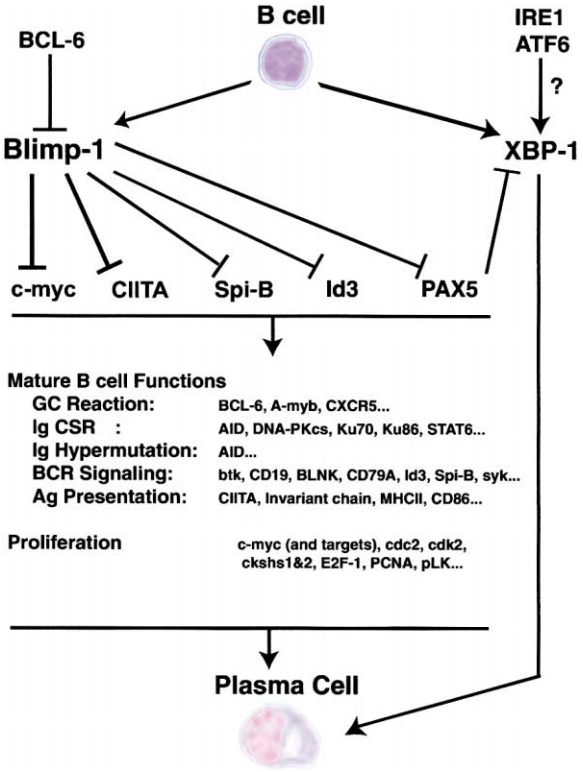


Figure 7. Blimp-1 Drives Plasma Cell Differentiation by Extinguishing Mature B Cell Gene Expression Programs

An increase in Blimp-1 expression (following relief from BCL-6 repression in GC B cells) leads to direct repression of several essential B cell transcription factors (*c-myc, CIITA, Id3, PAX5,* and *Spi-B*) and subsequent repression of several mature B cell functions as well as proliferation. In this way, Blimp-1, along with the transcription factor XBP-1, drives B cells to become terminally differentiated plasma cells.

in a feedback loop that attenuates BCR signaling. BCR signaling suppresses the terminal differentiation of mature B cells, in part by repressing Blimp-1, and ectopic expression of Blimp-1 reverses the ability of this stimulus to suppress plasma cell differentiation (Knodel et al., 2001; Schliephake and Schimpl, 1996). The ability of Blimp-1 to block BCR signaling is likely due to downregulation of genes encoding BCR signaling components (*CD79A, BLNK, btk, PKCβ, lyn, syk,* and *BRDG-1*; Katsuta et al., 1998; Kurosaki and Tsukada, 2000; Leitges et al., 1996; Ohya et al., 1999; Reth and Wienands, 1997). Moreover, both Spi-B and Id3, two likely direct targets of Blimp-1 repression, are regulators of BCR signaling (Garrett-Sinha et al., 1999, 2001; Pan et al., 1999). Spi-B cooperates with Spi-1/PU.1 in BCR signaling downstream of syk phosphorylation (Garrett-Sinha et al., 1999) and may also regulate *btk* transcription (Muller et al., 1996). Id3 is specifically required for proliferative responses to BCR signaling (Pan et al., 1999). These observations suggest an intriguing model in which initial exposure of a mature B cell to cognate antigen generates BCR signals that block Blimp-1 expression, thereby promoting clonal expansion. As these signals diminish, Blimp-1 expression may concomitantly increase, thus forming a negative regulatory loop.

Another consequence of Blimp-1 expression is inhibition of immunoglobulin class switching (Knodel et al., 1999; Figure 6). Blimp-1 downregulated *STAT6*, blocking expression of the germline Iγ1 transcript that is required for switching to IgG1 (Cunningham et al., 1998; Linehan et al., 1998). Blimp-1 also downregulated *Ku70, Ku86, DNA-PKcs,* and *AID*, four genes that are essential for class switch recombination (Manis et al., 1998; Muramatsu et al., 2000; Zelazowski et al., 1997). Furthermore, given the additional role of AID in immunoglobulin somatic hypermutation in GC B cells (Muramatsu et al., 2000), repression of AID by Blimp-1 would provide a mechanism by which this antibody diversification machinery is turned off in plasma cells.

BCL-6 and Blimp-1 Form a Developmental Feedback Loop

Blimp-1 represses *BCL-6*, a GC-restricted transcriptional repressor required for GC formation (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997). BCL-6 overexpression was previously shown to repress *PRDM1*, suppressing plasma cell differentiation (Reljic et al., 2000; Shaffer et al., 2000). The reciprocal ability of Blimp-1 to

repress *BCL-6* creates a feedback loop enforcing strict control over the B cell fate decision to become a plasma cell: as long as BCL-6 is expressed in a GC B cell, Blimp-1 expression and plasmacytic differentiation is blocked, but if Blimp-1 becomes activated, *BCL-6* will be repressed, leading to irreversible plasmacytic differentiation. Significantly, this is precisely the kind of double negative feedback loop predicted more than forty years ago by Jacob and Monod to be capable of conferring irreversibility on a biological system (Ferrell, 2002; Monod and Jacob, 1961).

This model raises the question of how BCL-6 is initially downregulated in a GC B cell. Crosslinking the BCR in mature B cells causes a dramatic drop in *BCL-6* mRNA levels (Allman et al., 1996) and in some B cell lines leads to MAP kinase-mediated phosphorylation of BCL-6 and its proteosomal degradation (Moriyama et al., 1997; Niu et al., 1998). We speculate that late in the GC response stronger BCR-mediated signals, the result of somatic mutations in Ig genes that improve BCR affinity for antigen, may exceed a threshold that leads to loss of BCL-6 and consequently to Blimp-1 expression. Once the feedback loop is initiated, Blimp-1 will attenuate BCL-6 expression even after the cell leaves the germinal center, and antigenic stimulation through the BCR ends. In B cell lymphomas, BCL-6 translocations involve substitution of the *BCL-6* promoter with a constitutively active promoter from the translocated gene (Dalla-Favera et al., 1999; Staudt et al., 1999), perhaps creating *BCL-6* alleles that are insensitive to Blimp-1-mediated repression, blocking terminal differentiation of the malignant B cell.

Blimp-1 and Other Factors Controlling Plasma Cell Differentiation

Recently, the transcriptional activator XBP-1 was shown to play a major role in plasma cell formation and immunoglobulin secretion (Ono et al., 1991; Reimold et al., 2001; Figure 7). However, Blimp-1 expression is normal in XBP-1-deficient cells, implying that Blimp-1 is developmentally upstream of XBP-1 in the plasma cell differentiation program. Since *XBP-1* is repressed by PAX5 (Reimold et al., 1996), Blimp-1-dependent repression of *PAX5* may be necessary for XBP-1 expression and full plasma cell differentiation. Consistent with this possibility, we observed a modest induction of *XBP-1* mRNA in the presence of Blimp-1, although the levels achieved were considerably lower than in multiple myeloma cells. In the SKW lymphoma cell line, we found that IL-6 treatment induced *XBP-1* mRNA, consistent with previous results identifying *XPB-1* as a target of IL-6 signaling (Wen et al., 1999). Interestingly, blockade of Blimp-1 activity by Tblimp prevented the induction of *XBP-1* mRNA by IL-6. Taken together, these results suggest that Blimp-1 repression of *PAX5* may be necessary for expression of *XBP-1* during plasmacytic differentiation but may not be sufficient to achieve the high levels of *XBP-1* expression characteristic of plasma cells. XBP-1 is also regulated under conditions of endoplasmic reticulum stress, which causes activation of *XBP-1* transcription by ATF6 and splicing of *XBP-1* mRNA by IRE1 (Calfon et al., 2002; Shen et al., 2001; Yoshida et al., 2000). Clearly, much further work is needed to fully elucidate the regulatory pathways governing *XBP-1* function during plasmacytic differentiation.

In summary, Blimp-1 plays a powerful role in plasmacytic differentiation by extinguishing mature B cell gene expression programs responsible for several B cell functions as well as for proliferation. Blimp-1 participates in regulatory loops with both BCL-6 and with BCR signaling components, suggesting that terminal plasmacytic differentiation can be viewed as an elaborate feedback and feedforward web of interacting transcription factors and signaling modules. Future studies will be needed to untangle this regulatory web and to learn how Blimp-1 synergizes with other regulators such as XBP-1 to produce the ultimate effector of humoral immunity, the plasma cell.

Experimental Procedures

Cell Culture, Viral Transductions, and Plasmids

Please refer to http://lymphochip.nih.gov/blimp/.

DNA Microarray

Microarray analysis was performed as described in Shaffer et al. (2000) using 2–5 mg of polyA$^+$ RNA (FastTrack, Invitrogen) from each sample. Arrays for each cell system (with the exception of WI-L2 and SUDHL4 acute Blimp-1 infections) were repeated two or three times with independent pools (for acute infections) or clones (for inducible Blimp-1ERD systems). For primary array data from Figures 1 and 2 in a tabular format, please see http://lymphochip.nih.gov/blimp/.

Human Cell Line RT-PCR

For nonquantitative confirmation of target genes, polyA$^+$ RNA was isolated (FastTrack, Invitrogen), and 1 mg was converted to cDNA as described (Shaffer et al., 2000). For quantitative Taqman RTPCR, serial dilutions of input mRNA (500 ng–2 ng) were analyzed in triplicate using a Taqman RT-PCR kit (ABI) and an ABI 7700 sequence detector system with primers and probes from Synthegen. Primer sequences for all RT-PCRs can be found at http://lymphochip.nih.gov/blimp/.

Primary Mouse Splenocyte RT-PCR

RNA was isolated by the Trizol method (Gibco, BRL). Subsequent RT reactions were performed as described (Piskurich et al., 2000) using 250 ng of RNA. Four-fold serial dilutions of cDNA were used in order to ensure PCR amplification within the linear range. PCR products were analyzed by electrophoresis in 1.5% agarose gel and visualized by staining with ethidium bromide or by Southern blotting with a γ-P^{32}-labeled internal probe. Primers and PCR conditions can be found at http://lymphochip.nih.gov/blimp/.

Protein Expression Analysis

Detection of target gene proteins by Western blotting was as described (Shaffer et al., 2000) using antibodies specific for Sp1, c-Myc, STAT6, and BCL-6 (Santa Cruz Biotechnology). See Lin et al. (2000) for details of the ELISPOT assay for secreted Ig. Please refer to http://lymphochip.nih.gov/blimp/ for details regarding flow cytometry.

Chromosomal Immunoprecipitation (ChIP)

For preparation of chromatin, immunoprecipitation, PCR conditions, and primers, please refer to Lin et al. (2002), Yu et al. (2000), and http://lymphochip.nih.gov/blimp/.

EMSA

Gel mobility shift for BCL-6 was performed using nuclear extracts and probes as described in Shaffer et al. (2000). EMSA for Id3 and Spi-B Blimp-1 binding was performed as described (Lin et al., 1997). The sequences of double-stranded oligonucleotides used for probe and competitors are: c-myc, CGCGTACAGAAAGGGAAAGGACTAG CGCG; CIITA, ACAGTAAGGAAGTGAAATTAATTT; hId3, GCGCTGA

GATTGCAGAAGGAGGAGGGAAAGGGGGTTTGAG; mId3, GCGCTG AGATTGCAGAAGAAGGAGGGAAAGGGAGGTCTAA; hSpiB, GGTG GGAGGGGCAGGGAAAGTGAG; mSpiB, CGCTGGGGGAGGCAGGG AAAGTAGGG; hBCL-6, GACACACCTTGCAAAGAAAAAGGGAAACT GGCAGT; nonspecific, AGGAAGCAGGTCATGTGGCAAGG.

ELISPOT
For details, please refer to Lin et al. (2002).

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