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Graded Expression of Interferon Regulatory Factor-4 Coordinates Isotype Switching with Plasma Cell Differentiation

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Summary

Molecular mechanisms underlying the coordination of isotype switching with plasma cell differentiation are poorly understood. We show that interferon regulatory factor-4 (IRF-4) regulates both processes by controlling the expression of the *Aicda* and *Prdm1* genes, which encode AID and Blimp-1, respectively. Genome-wide analysis demonstrated that *Irf4*$^{-/-}$ B cells failed to induce the entire Blimp-1-dependent plasma cell program. Restoration of AID or Blimp-1 expression in *Irf4*$^{-/-}$ B cells promoted isotype switching or secretion, respectively. IRF-4 was expressed in a graded manner in differentiating B cells and targeted *Prdm1*. Higher concentration of IRF-4 induced *Prdm1* and consequently the transition from a germinal center gene expression program to that of a plasma cell. We propose a gene-regulatory network in which graded expression of IRF-4 developmentally coordinates isotype switching with plasma cell differentiation.

Introduction

Upon antigen challenge, B lymphocytes undergo affinity maturation, isotype switching, and terminal differentiation into plasma cells that secrete antibodies (McHeyzer-Williams, 2003). Considerable progress has been achieved in the analysis of signaling molecules and transcription factors that regulate these processes (Shapiro-Shelef and Calame, 2005). Although many important components have been identified and genetically characterized, an understanding of their interconnectivity in current regulatory networks is incomplete. Furthermore, the molecular mechanisms underlying the developmental coordination of isotype switching with immunoglobulin secretion are poorly understood.

Plasma cell differentiation is regulated by the zinc-finger transcription factor Blimp-1 (encoded by the *Prdm1* gene) and involves a dramatic expansion of the endoplasmic reticulum (ER) and increased protein synthesis, both of which are necessary for high levels of antibody production and secretion (Shapiro-Shelef and Calame, 2005; Turner et al., 1994). Blimp-1 represses the expression of *Pax5*, which results in the derepression of *Xbp1*, a gene that encodes a member of the CREB/ATF transcription factor family (Lin et al., 2002; Reimold et al., 1996). Xbp1 directly activates transcription of the genes encoding chaperones and enzymes that function in the secretory apparatus (Shaffer et al., 2004). In addition, Blimp-1 regulates, in an Xbp1-independent manner, the differential 3′ end processing of the immunoglobulin heavy chain pre-mRNA, which results in a transcript that encodes the secreted polypeptide (Sciammas and Davis, 2004; Shapiro-Shelef et al., 2003). Blimp-1 represses the germinal center program, including the expression of *Bcl6* and *Aicda* (Sciammas and Davis, 2004; Shaffer et al., 2002). Blimp-1 expression also results in upregulation of both the chemokine receptor CXCR4 and α4 integrin that enable homing and maintenance of long-lived plasma cells (Sciammas and Davis, 2004). Expression of Blimp-1 after B cell activation is considered to be the primary trigger for plasma cell differentiation and the generation of antibody-secreting cells (ASC). *Prdm1* expression is positively regulated by Stat3 and negatively regulated by Bcl6 (Fujita et al., 2004; Reljic et al., 2000; Shaffer et al., 2000; Tunyaplin et al., 2004). Thus, Bcl6 and Blimp-1 appear to represent a pair of counteracting mutual repressors that play a pivotal role in regulating the transition between alternative B cell states (Staudt, 2004).

AID (activation-induced deaminase; encoded by the *Aicda* gene) is a cytidine deaminase that is essential for SHM (somatic hypermutation) and isotype switching/class switch recombination (CSR) (Muramatsu et al., 2000). In vivo, expression of AID is generally restricted to activated B cells undergoing affinity maturation and CSR in germinal centers. AID is also expressed upon polyclonal activation of B cells in vitro and results in CSR in the absence of affinity maturation. *Aicda* expression is positively regulated by the transcription factors E2A and Pax5 (Gonda et al., 2003; Sayegh et al., 2003).

IRF-4/Pip is a member of the interferon regulatory factor family of transcription factors characterized by a DNA binding domain containing five conserved tryptophan repeats (Escalante et al., 2002; Taniguchi et al., 2001). In contrast to other IRF family members, expression of IRF-4 is not regulated by type I or type II interferons. Its expression is limited to lymphoid and myeloid cell types (Eisenbeis et al., 1995; Marecki et al., 1999). *Irf4* is an immediate early gene in B as well as T lymphocytes whose expression is induced by antigen receptor or mitogen stimulation. It is constitutively and highly expressed in plasma cells (Eisenbeis et al., 1995; Matsuyama et al., 1995). IRF-4 is most closely related to IRF-8/ICSBP in terms of primary structure and pattern of cellular expression. High-affinity DNA binding by IRF-4 has been observed only when interacting with partners of the Ets family (PU.1 or SpiB) or the helix-loop-helix family (E2A) of transcription factors (Brass et al., 1999; Eisenbeis et al., 1995; Nagulapalli and Atchison, 1998).

IRF-4 functions redundantly with IRF-8 to regulate pre-B cell cycling and differentiation into immature sIgM+ B cells (Lu et al., 2003). In contrast, IRF-4 functions in

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a nonredundant manner to regulate B and T cell activation as well as terminal differentiation (Mittrucker et al., 1997). *Irf4*$^{-/-}$ mice display low serum immunoglobulin, and immunization fails to elicit antigen-specific antibodies. This is despite the presence of normal numbers of mature B cells in the secondary lymphoid organs. While *Irf4*$^{-/-}$ B lymphocytes produce low levels of immunoglobulins in vivo and in vitro, the nature of the molecular pathways by which IRF-4 regulates isotype switching, Ig secretion, and/or plasma cell differentiation remain to be elucidated.

We find that *Irf4*$^{-/-}$ B cells are severely compromised for isotype switching and plasma cell generation as a consequence of the failure to properly induce expression of the *Aicda* and *Prdm1* genes, respectively. Genome-wide expression analysis demonstrates that IRF-4 regulates the induction of the entire Blimp-1-dependent plasma cell program. Restoration of AID and Blimp-1 expression promotes isotype switching and antibody secretion, respectively, indicating that these regulatory molecules are limiting in *Irf4*$^{-/-}$ B cells. IRF-4 is expressed in a stepwise manner during B cell differentiation, and its higher concentration functions to induce the *Prdm1* gene, an IRF-4 target. We propose a gene-regulatory network that can account for the developmental coordination of CSR and plasma cell differentiation based on graded expression of IRF-4.

Results

Irf4$^{-/-}$ B Lymphocytes Are Defective in the Development of ASC as Well as CSR

To analyze the differentiation defects of the *Irf4*$^{-/-}$ B cells, we employed quantitative in vitro assays that measure the capacity for plasma cell differentiation, Ig secretion, and isotype switching. These included flow cytometric detection of Syndecan-1, IgG3, and IgG1 expression, as well as ELISA and ELISpot assays. Purified splenic B cells were stimulated with LPS, which leads to Syndecan-1 expression and isotype switching to IgG3, or with LPS and IL-4 (LPS+IL-4), which leads to isotype switching to IgG1 (Snapper and Paul, 1987). Because the capacity to undergo isotype switching and to secrete is correlated with the number of cell divisions, we monitored the latter via CFSE (Tangye and Hodgkin, 2004). In all experiments, we compared splenic B cells from *Irf4*$^{+/-}$ with *Irf4*$^{-/-}$ littermates because the heterozygous mice are phenotypically normal (Mittrucker et al., 1997). We observed a marked reduction in the generation of Syndecan-1-expressing cells in LPS-stimulated cultures of *Irf4*$^{-/-}$ B cells (Figure 1A). Syndecan-1 is a marker of ASC (Hasbold et al., 2004). In addition, a severe defect was observed in CSR to IgG3 or IgG1 (Figure 1A). Similar results were obtained when *Irf4*$^{-/-}$ B cells were stimulated with CD40 ligand and IL-4 (CD40L+IL-4, data not shown). Antibody production by *Irf4*$^{-/-}$ B cells was analyzed by ELISA. *Irf4*$^{-/-}$ B cells secreted IgM at 1% of the levels observed with their heterozygous counterparts (Figure 1B). *Irf4*$^{-/-}$ B cells also generated 95% fewer antibody-secreting precursor cells as measured by ELISpot analysis, an assay that enumerates antibody-secreting clones directly (Figure 1C). Furthermore, we observed that the mean spot size of the *Irf4*$^{-/-}$ ASC was about 3-fold reduced, suggesting a decreased secretory output (10.8 ± 2.2 μm^2 versus 3.5 ± 1 μm^2) (Hasbold et al., 2004). Collectively, these results demonstrate that IRF-4 regulates both isotype switching and plasma cell differentiation in a cell-autonomous manner.

Failure of *Irf4*$^{-/-}$ B Cells to Switch or Secrete Ig Is Not Due to a Defect in Undergoing Multiple Cell Divisions

It should be noted that *Irf4*$^{-/-}$ B cells properly regulate the cell-surface markers CD25, CD69, CD62L, CD23, CD80, CD86, and MHCII after acute stimulation (see Figure S1A in the Supplemental Data available with this article online) and CD44, CD62L, and CD23 upon prolonged stimulation (Figure S1B). Curve fitting analysis of the CFSE histograms of LPS-activated cells revealed that while many of the *Irf4*$^{-/-}$ B cells progress through multiple cell divisions normally, the number of cells present in later divisions (division five through seven) are reduced as compared to *Irf4*$^{+/-}$ B cells (Figure S2A). This was borne out by cell yield (Figure S2B). In contrast, many more *Irf4*$^{-/-}$ B cells progressed through late cell divisions, and the cell yields were improved when the LPS stimulations were supplemented with IL-4 (Figures S2A and S2B). To rigorously examine the cell-cycle dynamics and proliferative capacity of the *Irf4*$^{-/-}$ B cells, we compared DNA synthesis and cell-cycle distributions in LPS and LPS+IL-4 cultures. Purified splenic B cells were pulsed with bromodeoxyuridine (BrdU) at days 1, 2, and 3 of stimulation, and incorporation was measured by flow cytometry of fixed cells and analyzed in conjunction with DNA content by propidium iodide (PI) staining. Although the overall cell-cycle profiles of the mutant cells were similar to their control counterparts, *Irf4*$^{-/-}$ B cells consistently incorporated less BrdU at day 2 (70% ± 12%, n = 7, $p < 0.01$, Figure S2C). We also observed an increase in the number of *Irf4*$^{-/-}$ B cells containing less than 2N DNA content (sub-G1 population) during the late phase of stimulation (day 3 sub-G1, 2.2- ± 0.8-fold, n = 7, $p < 0.02$). The sub-G1 population is characteristic of apoptotic cells. These data along with lower cell yields suggest that *Irf4*$^{-/-}$ B cells may be more prone to apoptosis during the expansion phase. Nevertheless, these data demonstrate that IRF-4 is neither required for the induction of activation-associated cell-surface markers nor for multiple cell divisions. Thus, the failure of *Irf4*$^{-/-}$ B cells to generate ASC or isotype-switched B cells is not due to an inability to undergo multiple cell divisions.

IRF-4 Regulates *Aicda* and *Prdm1* Expression

Since CSR is regulated by the induction of AID expression, the levels of *Aicda* mRNA were analyzed with quantitative RT-PCR. *Aicda* mRNA expression was impaired in *Irf4*$^{-/-}$ B cells (Figure 2A). To determine whether the failure in CSR was specifically due to diminished *Aicda* expression, we analyzed IgM and IgG1 germline transcripts by RT-PCR, after LPS+IL-4 stimulation (Figure 2B). The relative expression levels of IgM and IgG1 germline transcripts (GLT) were unaffected in the *Irf4*$^{-/-}$ B cells. In contrast, there was a marked reduction in expression of the IgG1-switched transcripts in the absence of IRF-4, suggesting that a step downstream of locus accessibility, namely AID-dependent DNA recombination, was impaired. Importantly, *Irf4*$^{-/-}$ B cells were not impaired for the expression of other CSR genes

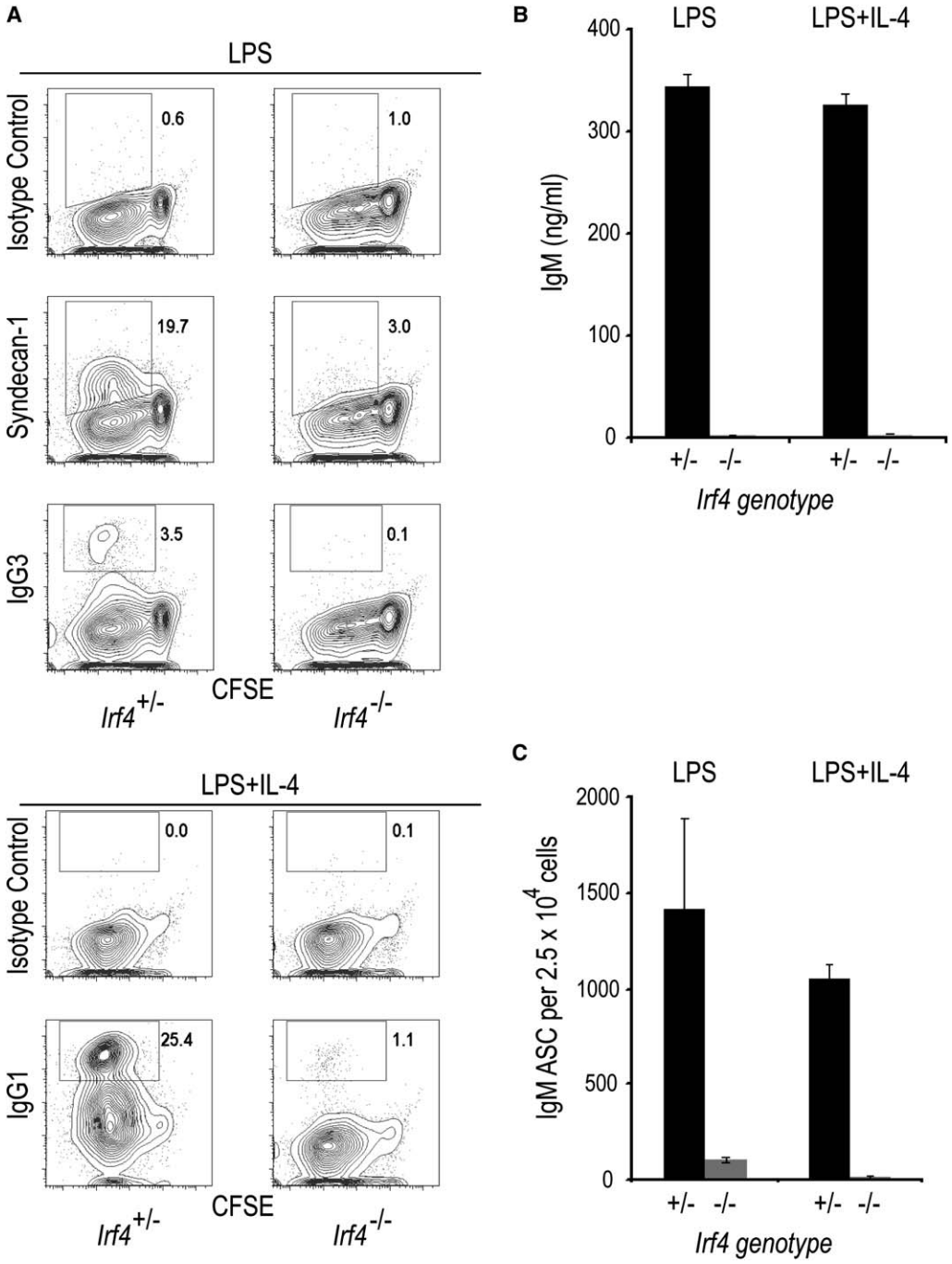

Figure 1. *Irf4*$^{-/-}$ B Cells Are Defective in Isotype Switching and in the Generation of Antibody-Secreting Cells

Splenic B cells from *Irf4*$^{+/-}$ and *Irf4*$^{-/-}$ mice were purified, labeled with CFSE, and stimulated with LPS or LPS+IL-4 for 4 days.

(A) Flow cytometric analysis of division-linked differentiation monitored by the expression of Syndecan-1 and IgG3 in LPS-stimulated cells (top) or IgG1 in LPS+IL4-stimulated cells (bottom).

(B) Analysis of IgM secretion. Day 4 stimulated cells were harvested, washed, and cultured for 24 hr prior to isolating cell-free supernatants. Secreted IgM was measured with sandwich ELISA.

(C) ELISpot analysis. Day 4 stimulated cells as described above were cultured for 24 hr on anti-IgM-coated membranes prior to ELISpot processing. These experiments were repeated four times and representative results are shown; error bars correspond to the SEM of the replicates in the ELISA and ELISpot assays.

(*Ung, Msh2*), and they exhibited higher expression of transcription factors required for CSR (Figure S3). Thus, the defect in CSR manifested by *Irf4*$^{-/-}$ B cells appears to be due to the selective impairment in *Aicda* expression.

Since the plasma cell differentiation program is regulated by the induction of Blimp-1 expression, the levels of *Prdm1* mRNA and Blimp-1 protein were analyzed. There was a striking failure to induce *Prdm1* expression in the absence of IRF-4 (Figures 2A and 2C). Consistent

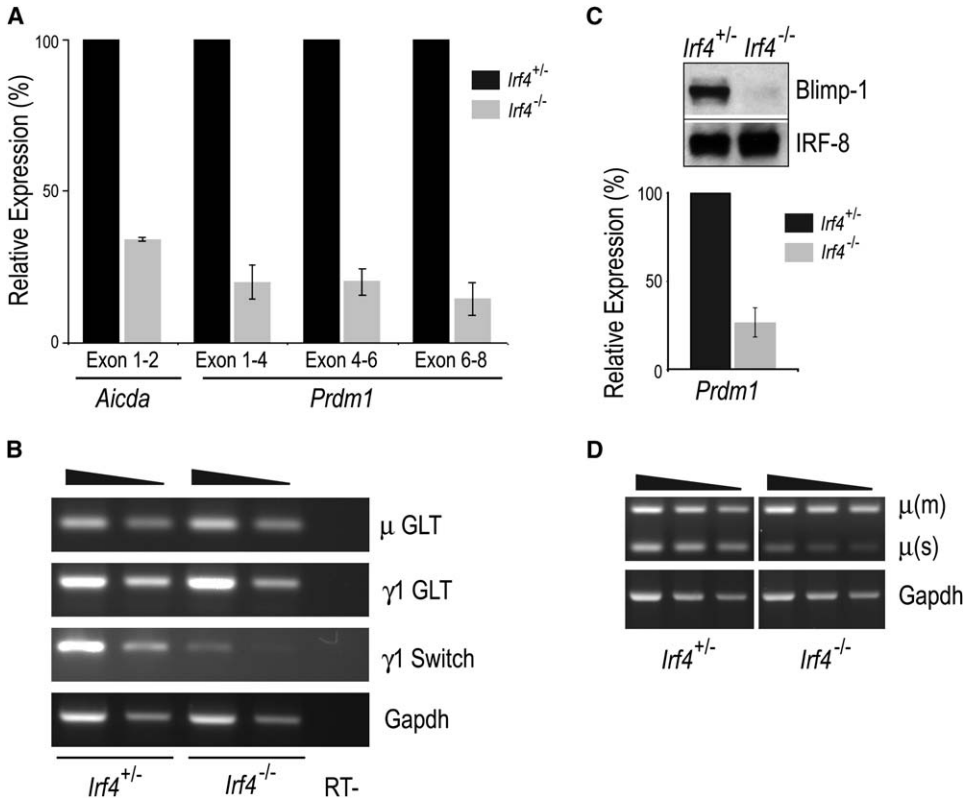

Figure 2. *Prdm1* and *Aicda* Expression Are Impaired in *Irf4*$^{-/-}$ B Cells

Splenic B cells from *Irf4*$^{+/-}$ and *Irf4*$^{-/-}$ mice were purified and stimulated with LPS. RNA was isolated and used for quantitative RT-PCR.

(A) Relative expression of *Aicda* and *Prdm1* transcripts at 2 and 4 days after stimulation, respectively. Three different primer pairs spanning the indicated exons of the *Prdm1* gene were used. *Oct-1* transcripts were used for normalization. Data are from three independent experiments, and error bars correspond to the SEM of the averages.

(B) RT-PCR analysis of μ and γ1 germline transcripts (GLT) and the post-switch γ1 transcript from the IgG1 locus via serially diluted cDNAs (5-fold).

(C) Expression of Blimp-1 protein (top) or *Prdm1* mRNA (bottom) at 2 days after LPS stimulation. The immunoblot of nuclear extracts from stimulated B cells of the indicated genotype was probed with Blimp-1 and IRF-8 antibodies and is representative of three independent experiments. The average expression of *Prdm1* transcripts by qPCR from three independent experiments is quantitated below; error bars correspond to the SEM.

(D) RT-PCR analysis of membrane μ(m) and secretory μ(s) immunoglobulin transcripts with serially diluted cDNAs (3-fold). Results in (B) and (D) are representative of three independent experiments.

with the impaired expression of Blimp-1, *Irf4*$^{-/-}$ B cells did not efficiently process IgH pre-mRNA to generate transcripts encoding the secreted polypeptide, μ(s) (Figure 2D).

Genome-Wide Analysis Demonstrates that IRF-4 Regulates the Blimp-1-Dependent Plasma Cell Differentiation Program

To analyze how loss of IRF-4 affects the transcriptional program underlying B cell differentiation, we performed genome-wide expression analysis with mouse lymphochips (Shaffer et al., 2004). Purified *Irf4*$^{+/-}$ or *Irf4*$^{-/-}$ B cells were cultured in the presence of LPS for 24, 48, or 96 hr (Figure 3). Genes induced by at least 1.5-fold in heterozygous B cells over untreated controls were organized by hierarchical clustering. *Irf4*$^{-/-}$ B cells showed no apparent defect in the induction of proliferation genes (Figure 3A). Second, the loss of IRF-4 did not impair the induction or sustained expression of LPS-responsive genes (Figure 3B).

As expected by the impaired expression of Blimp-1, a prominent subcluster of genes associated with plasma cell differentiation was not expressed in *Irf4*$^{-/-}$ B cells. Genes that were differentially expressed by greater than 2-fold ($p < 0.015$) between *Irf4*$^{+/-}$ and *Irf4*$^{-/-}$ B cells at day four of LPS stimulation are shown (Figure 3C). These genes include immunoglobulins (both heavy and light chains), as well as genes required for the elaboration of the endoplasmic reticulum and secretory apparatus (*Xbp1* and its targets). Importantly, five genes dependent on Blimp-1 but not Xbp1 also failed to be induced in *Irf4*$^{-/-}$ B cells. In fact, by comparing these profiles with the list of genes previously identified as requiring Xbp1 and/or Blimp-1 for induction (Shaffer et al., 2004), we find that the entire plasma cell gene expression program is defective in *Irf4*$^{-/-}$ B cells (Figure S4). Overall, the gene-expression analysis of *Irf4*$^{-/-}$ B cells demonstrates that whereas the induction of proliferation and LPS-responsive genes occurs normally, the initiation of the Blimp-1-dependent plasma cell program is severely compromised.

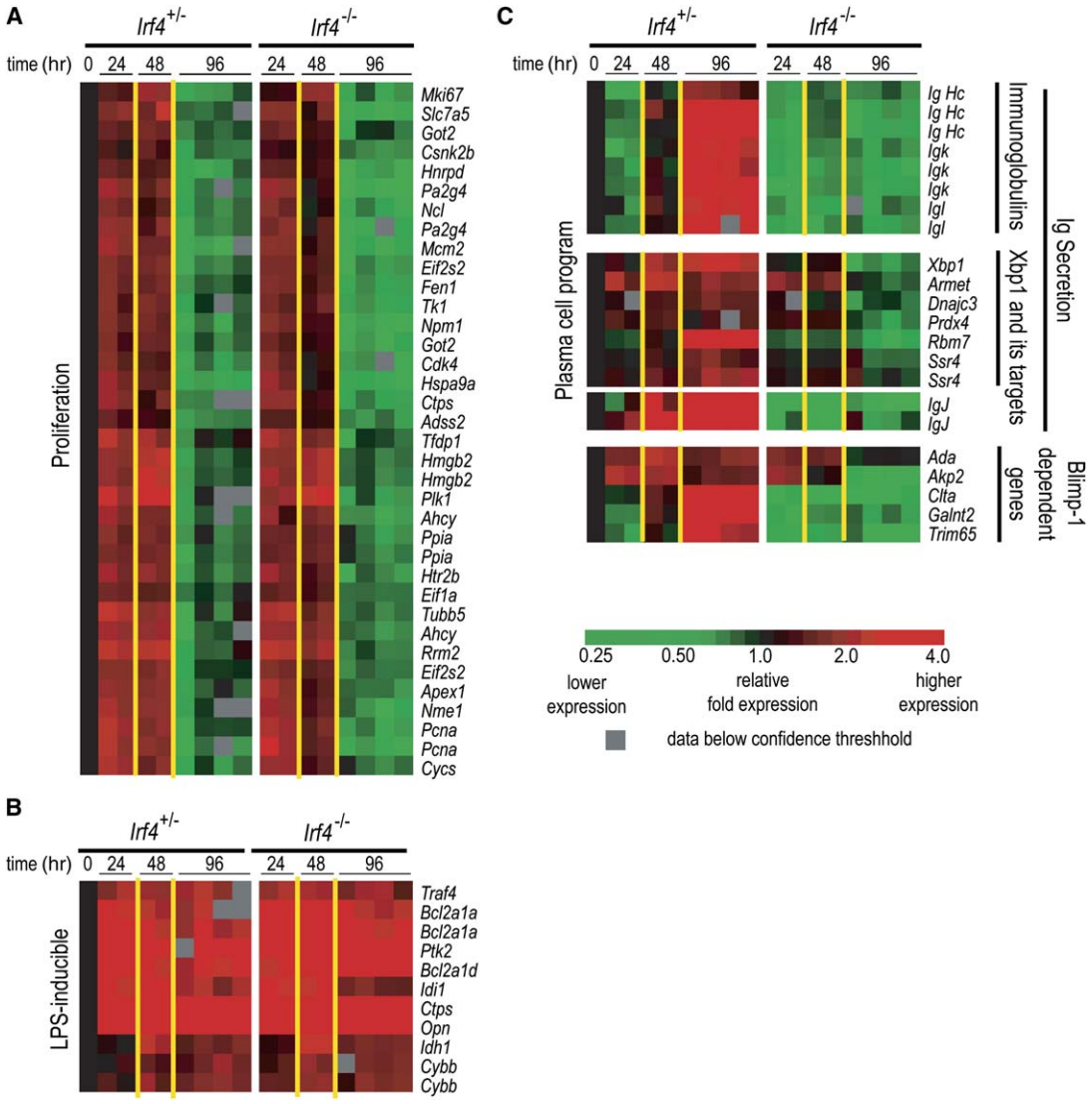

Figure 3. *Irf4*$^{-/-}$ B Cells Fail to Activate the Plasma Cell Gene-Expression Program

Purified splenic B cells from *Irf4*$^{+/-}$ and *Irf4*$^{-/-}$ mice were stimulated with LPS for the indicated times to induce plasma cell differentiation. Microarray results from sequence-verified elements representing genes that were induced at least 1.5-fold in heterozygous B cells. These elements meet spot confidence criteria and are present on >90% of the arrays. Relative expression changes are indicated by the color bar. Data were normalized to the average expression in purified, untreated (0 hr) B cells from both genotypes and analyzed by hierarchical clustering. Subclusters of this hierarchy are shown.

(A) Proliferation cluster.

(B) LPS-inducible gene cluster.

(C) Plasma cell program genes expressed differentially by 2-fold or more, with a statistically significant difference (Student's t test, $p < 0.015$) between *Irf4*$^{+/-}$ and *Irf4*$^{-/-}$ B cells at 96 hr post LPS induction.

The Expression of Blimp-1 and AID Are Limiting for Antibody Secretion and Isotype Switching in *Irf4*$^{-/-}$ B Cells

To directly test whether the impaired induction of Blimp-1 and AID accounts for the Ig secretion and switching defects in *Irf4*$^{-/-}$ B cells, we transduced these cells with retroviruses (with an IRES-GFP segment) expressing Blimp-1 or AID. An IRF-4 retrovirus was used as a control to complement the *Irf4*$^{-/-}$ B cells. As shown in Figure 4A, IRF-4 efficiently restored the capacity of the *Irf4*$^{-/-}$ B cells to secrete IgM, as measured by ELISA from supernatants of sorted GFP^{+} cells. Importantly, Blimp-1 also induced a significant increase in secretion (6.4- ± 4.6-fold, $n = 3$, $p < 0.02$) by the *Irf4*$^{-/-}$ B cells (Figure 4A). Although the complementation with Blimp-1 was less efficient (see Discussion), this result nevertheless demonstrates that Blimp-1 can functionally bypass the secretion defect in *Irf4*$^{-/-}$ B cells. As shown in Figure 4B, restoration of IRF-4 expression greatly increased the ability of the GFP^{+} *Irf4*$^{-/-}$ B cells to switch to IgG1. Importantly, expression of AID in the *Irf4*$^{-/-}$ B cells also resulted in a substantial increase in IgG1 (2.6- ± 0.6-fold, $n = 5$, $p < 0.03$) generated by isotype switching. As expected, IRF-4 expression in the mutant B cells induced the expression of *Aicda* and *Prdm1* genes (Figure S5). Collectively, these results demonstrate that AID and

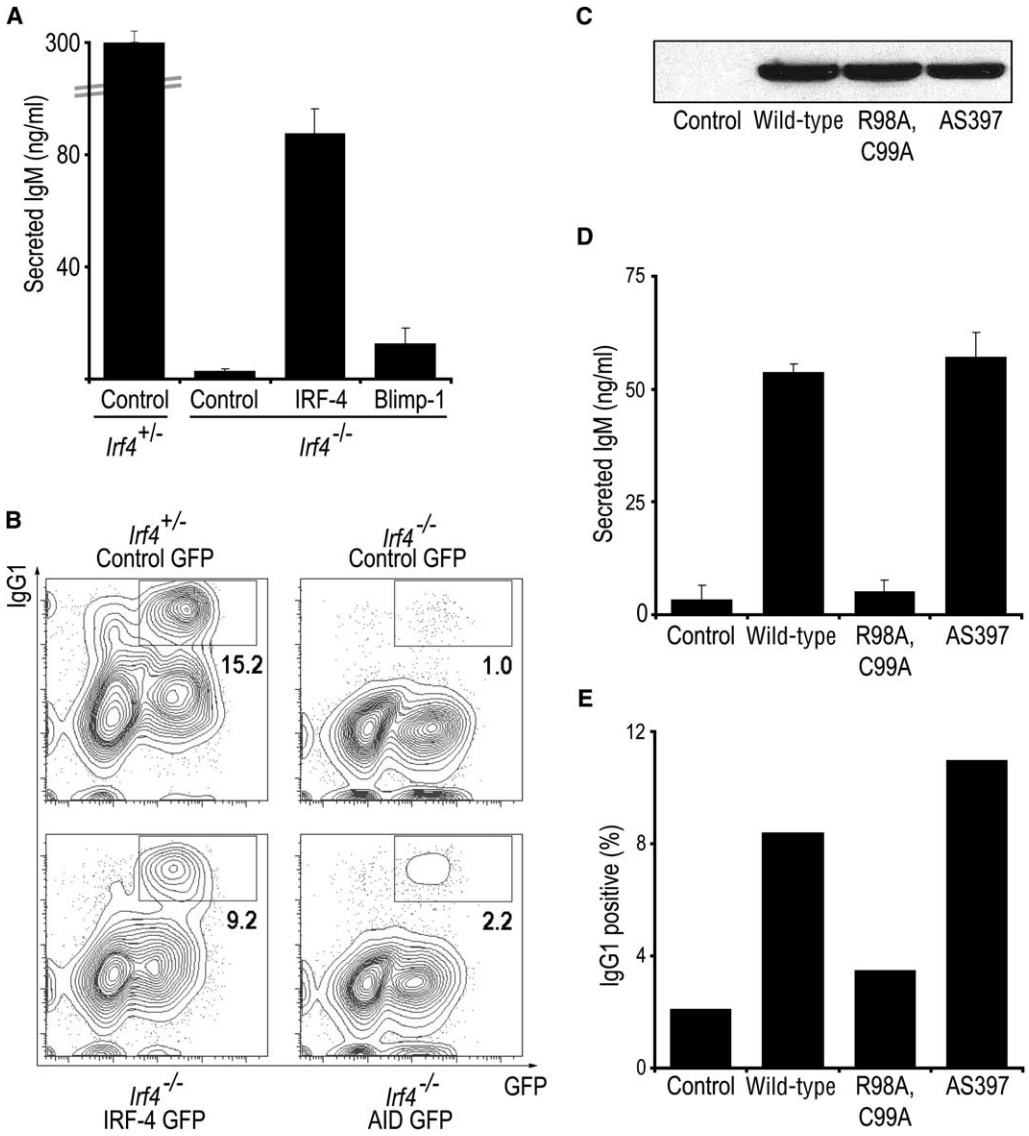

Figure 4. Restoration of Blimp-1 or AID Expression in *Irf4*$^{-/-}$ B Cells Promotes Ig Secretion or Isotype Switching, Respectively

Splenic B cells from *Irf4*$^{-/-}$ mice were purified and stimulated with LPS+IL-4. After activation for 24 hr, the cells were transduced with retroviruses encoding the indicated cDNAs fused to an IRES-GFP segment. Two days posttransduction, the GFP^{+} cells were analyzed.

(A) Ig secretion by sorted GFP^{+} cells. After sorting, cells were cultured for 24 hr, and secreted Ig from supernatants was quantitated with sandwich ELISA. Results are representative of three independent experiments; error bars correspond to the SEM of the replicates in the ELISA.

(B) Flow cytometric analysis of isotype switching to IgG1. Representative results from five independent experiments are shown.

(C) Immunoblot analysis of HA-tagged IRF-4 proteins expressed in 293T cells.

(D and E) Complementation of *Irf4*$^{-/-}$ B cells with wild-type or mutant IRF-4 proteins. *Irf4*$^{-/-}$ B cells were transduced with retroviruses encoding the indicated proteins and analyzed for Ig secretion as described in (A) and isotype switching as described in (B), respectively. The wild-type and AS397-transduced cells exhibited an average of 80- and 105-fold induction of Ig secretion in four independent experiments. Both the wild-type and AS397-transduced cells displayed an 8-fold increase in IgG1 expression (averaged over five experiments). The R98A,C99A mutant is defective in DNA binding. The AS397 mutant is defective for interaction with PU.1 or SpiB. Results in (D) and (E) are representative of four and five experiments, respectively; error bars in (D) correspond to the SEM.

Blimp-1 expression are limiting in *Irf4*$^{-/-}$ B cells and therefore substantially account for the defects in CSR and plasma cell differentiation, respectively.

The DNA Binding Domain of IRF-4 but Not Its PU.1/SpiB Interaction Surface Is Required for CSR and Ig Secretion

To analyze the mechanism of IRF-4 function in B cells, two mutant IRF-4 constructs were tested in the complementation assay. IRF-4 R98A,C99A does not bind DNA, and IRF-4 AS397 has a 4 residue mutation that disrupts IRF-4's interaction with PU.1 or SpiB (Brass et al., 1999). Each of the constructs expressed IRF-4 protein at similar amounts as judged by immunoblotting of transiently transfected 293T cells (Figure 4C). When transduced into *Irf4*$^{-/-}$ B cells stimulated with LPS+IL-4, the DNA binding mutant failed to induce secretion or isotype switching (Figures 4D and 4E). In contrast, the PU.1/SpiB binding mutant was able to restore both secretion and isotype switching (Figures 4D and 4E). These results

suggest that DNA binding by IRF-4, but not its interaction with PU.1 or SpiB, is required for induction of Ig secretion and isotype switching.

Graded Expression of IRF-4 Functions to Regulate CSR and Plasma Cell Differentiation

Paradoxically, IRF-4 positively regulates both *Aicda* and *Prdm1* expression even though they are key components of antagonistic developmental states. We reasoned that varied expression of IRF-4 might differentially regulate the expression of *Aicda* and *Prdm1* genes. According to this model, a higher concentration of IRF-4 is required for *Prdm1* upregulation and thus Blimp-1-dependent repression of the germinal center program, including the *Aicda* gene. To explore this possibility, we analyzed the expression of IRF-4 protein in primary wild-type cells during the course of LPS- or CD40L+IL-4-induced B cell activation and differentiation by performing intracellular flow cytometry. After induction, IRF-4 expression was sustained through multiple cell divisions (Figure 5A). Strikingly, we observed a division-linked appearance of an IRF-4 high-expressing population. In fact, costaining with Syndecan-1 revealed that the Syndecan-1-positive cells also exhibited uniformly high IRF-4 expression (Figure 5B). These results demonstrate that the differentiation of activated B cells into plasma cells is associated with a substantial increase in IRF-4 protein.

To test whether overexpression of IRF-4 can augment plasma cell differentiation, wild-type splenic B cells, stimulated with CD40L+IL-4, were transduced with control or IRF-4-encoding retroviral vectors. IRF-4 augmented the generation of Syndecan-1-expressing cells (Figure 5C) and Ig secretion (Figure 5D). In fact, the amounts of secreted Ig achieved by IRF-4 transduction were similar to that attained by transduction of Blimp-1 expression vector (Figure 5D). Importantly, as predicted by our model, elevated expression of IRF-4 resulted in upregulation of the *Prdm1* and the downregulation of *Aicda* genes (Figure 5D). To further test whether high concentrations of IRF-4 expression are essential for plasma cell differentiation, we complemented *Irf4*$^{-/-}$ cells with the IRF-4 retroviral vector and analyzed GFP expression as a surrogate for IRF-4 in the resulting Syndecan-1^{+} plasma cells. Strikingly, the rescued Syndecan-1^{+} cells exclusively expressed very high amounts of GFP, indicating that a low concentration of IRF-4 is insufficient to initiate the plasma cell program (Figure 5C, bottom). It should be noted that IRF-4 transduction of wild-type B cells resulted in a broader range of GFP expression in Syndecan-1^{+} cells (Figure 5C, top). Overexpression of IRF-4 increased the relative proportion of Syndecan-1^{+} cells in the bottom 75% of the GFP^{+} distribution. This ratio changed from 1.8 ± 0.05 to 1.5 ± 0.02 (n = 2). We suggest that these cells require lower concentration of ectopically expressed IRF-4 to initiate the plasma cell program due to endogenously expressed IRF-4 and the amplification provided by a positive regulatory loop with Blimp-1 (see model below). Finally, in rescue experiments with the same IRF-4 vector, and examining Ig class switching (Figure 4B, bottom), we note that an intermediate concentration of IRF-4 appears sufficient for inducing AID expression and isotype switching to IgG1. Collectively, these experiments support our model that proposes graded expression of IRF-4 functions to differentially regulate *Aicda* and *Prdm1* gene expression and consequently CSR and plasma cell differentiation.

Elevated Expression of IRF-4 Induces a Germinal Center to Plasma Cell Transition in a Human B Cell Line

To test the role of IRF-4 in initiating a transition from the germinal center to the plasma cell program of gene expression, we examined the consequences of elevating the expression of IRF-4 in a human germinal center B cell (GCB)-derived cell line, Oci-Ly7 (Shaffer et al., 2002). IRF-4 transduction induced the expression of CD38, which is expressed on human plasma cells (Figure 5E). Increased IRF-4 expression also resulted in the induction of the key regulators of plasma cell differentiation, Blimp-1 and Xbp1 and many of their target genes (Figure 5F, Figure S6). Consistent with the induction of Blimp-1, we observed the repression of a large cohort of genes expressed in germinal center B cells, including *Bcl6*, *Pax5*, *SpiB*, and *Aicda* (Figure 5F, Figure S6; Shaffer et al., 2002). The expression of the Blimp-1 target *c-MYC* (Lin et al., 1997) was not substantially changed, likely due to a chromosomal abnormality at the *c-MYC* locus in this tumor line that relieves *c-MYC* from its proper regulation. These findings demonstrate that high concentration of IRF-4 can induce *Prdm1* expression and the transition from a germinal center B cell state to a plasma cell program of gene expression.

IRF-4 Interacts with a Conserved Region in the *Prdm1* Gene

To determine whether the *Aicda* and *Prdm1* genes are directly regulated by IRF-4, we surveyed conserved regions of these genes by chromatin crosslinking and immunoprecipitation (ChIP). In these experiments involving naive and activated B cells, IRF-4 crosslinking to the Ig lambda 3′ enhancer was used as a positive control (Figure 6; Lu et al., 2003). We did not observe crosslinking of IRF-4 to three conserved noncoding sequences (CNS) of the *Aicda* gene, including the promoter (Lee et al., 2006) and an intronic enhancer (Figure S7, data not shown; Sayegh et al., 2003). Importantly, IRF-4 crosslinking was detected to CNS 9 between exons 5 and 6 of the *Prdm1* gene (Figure 6, Figure S7) but not to CNS 1-8. This interaction was dependent upon B cell activation, and the efficiency of crosslinking was similar to that observed with the Ig lambda enhancer. These data demonstrate that the *Prdm1* gene is a direct IRF-4 target.

Discussion

The defect in CSR manifested by *Irf-4*$^{-/-}$ B cells is due to a failure to induce sufficient levels of *Aicda* expression. Ectopic expression of AID in the mutant cells promotes CSR. Consistent with this observation, *Irf4*$^{-/-}$ B cells, upon activation, express wild-type levels of germline transcripts from the Cμ and Cγ1 regions of the IgH locus, indicating that the failure to undergo CSR is not due to impaired accessibility of the switch regions to AID. Furthermore, expression of other transcription factors required for CSR (e.g., Pax5, Bach2, E2A) is not affected

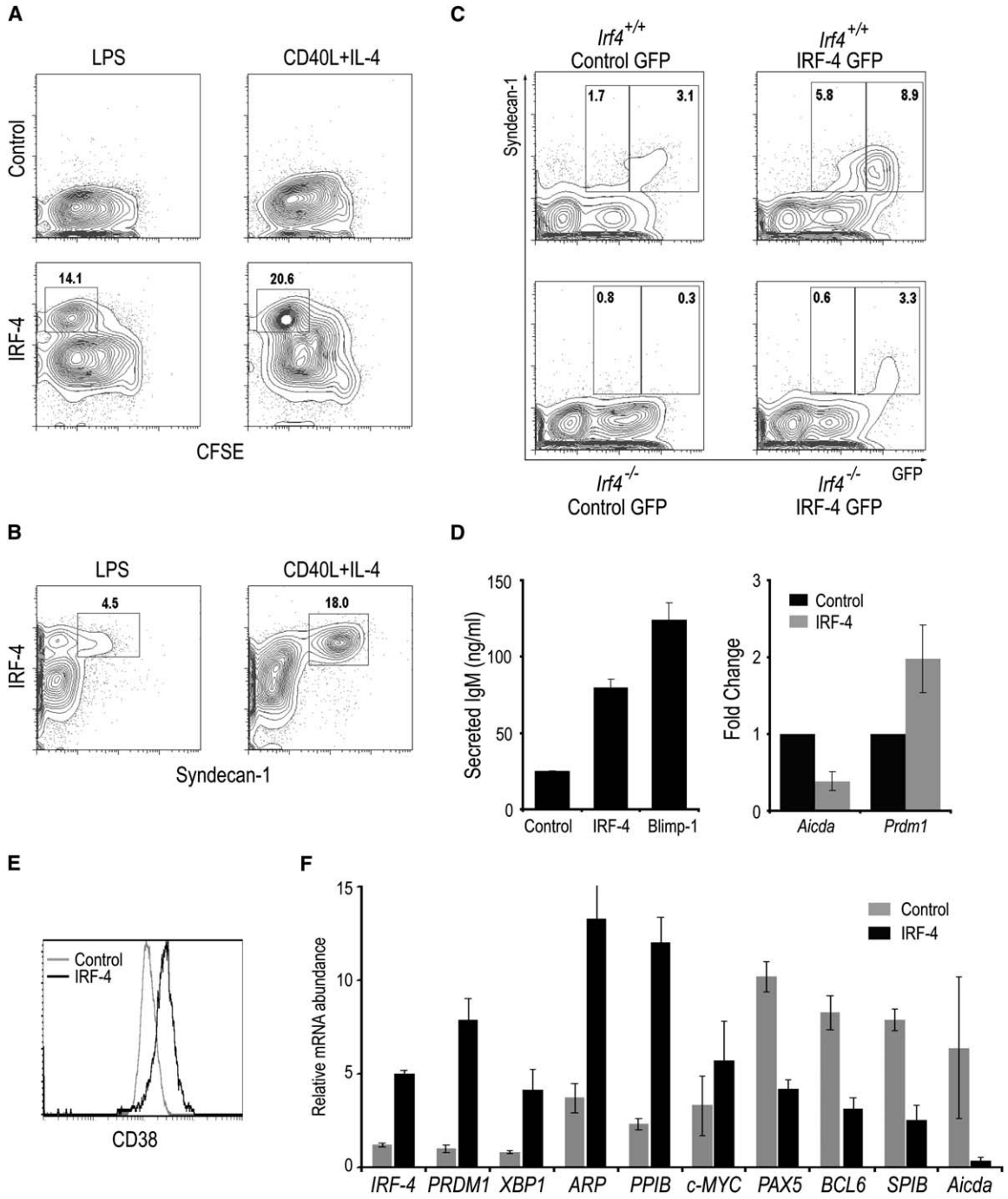

Figure 5. IRF-4 Is Expressed in a Biphasic Manner in B Cells and High Levels Function to Induce Plasma Cell Differentiation

Splenic B cells from wild-type mice were purified and labeled with CFSE prior to stimulation with LPS or CD40L+IL-4.

(A) 4 days after stimulation, cells were processed for intracellular staining with IRF-4 and goat IgG (control) antibodies and then analyzed by flow cytometry. Specific staining by the IRF-4 antibodies was confirmed with *Irf4*$^{-/-}$ B cells (data not shown).

(B) IRF-4-stained cells were costained with Syndecan-1 in order to analyze the plasma cell population. Results in (A) and (B) are representative of four experiments.

(C) Purified splenic B cells from *Irf4*$^{+/+}$ and *Irf4*$^{-/-}$ mice were stimulated with CD40L+IL-4 and transduced with retroviruses encoding the indicated cDNAs fused to an IRES-GFP segment. 2 days posttransduction, the cells were analyzed for GFP and Syndecan-1 expression by flow cytometry. IRF-4 transduction of wild-type B cells resulted in a 3.1- ± 0.5-fold increase in Syndecan-1-expressing cells (n = 3). Indicated gates represent the 75th percentile and below of the GFP^{+} cells.

(D) Splenic B cells were activated and transduced as above. 2 days posttransduction, the GFP^{+} cells were sorted and cultured for 24 hr to analyze IgM secretion (left) and *Aicda* and *Prdm1* transcripts (right) as described in Figures 1 and 2.

(E) Flow cytometric analysis of CD38 expression in the human Oci-Ly7 germinal center-like DLBCL cell line transduced with control or human IRF-4 retroviruses.

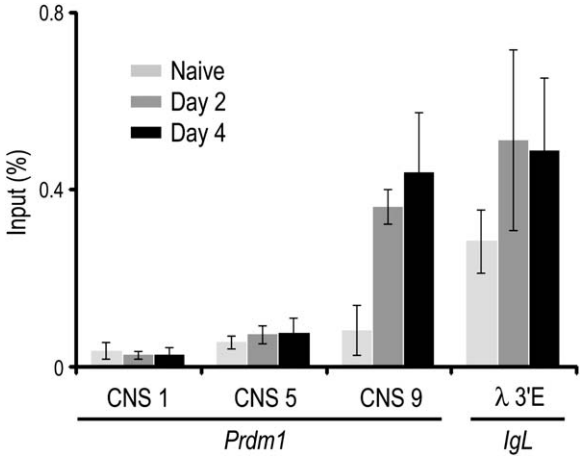

Figure 6. IRF-4 Crosslinks to a Conserved Region in the *Prdm1* Gene

Splenic B cells were purified and processed for chromatin immunoprecipitation before and after LPS stimulation. Immunoprecipitated DNA corresponding to conserved noncoding sequences (CNS) of the *Prdm1* locus (Figure S7A) was analyzed by quantitative PCR and the results are displayed as percent of input. The lambda light chain 3′ enhancer was used as a positive control. Data are from two independent experiments and the error bars represent the SEM of the averages.

by the loss of IRF-4. Our results strongly suggest that the primary cause of the CSR defect in *Irf4*$^{-/-}$ mice (Mittrucker et al., 1997) is the impaired expression of *Aicda*. It remains to be determined whether *Aicda* is an IRF-4 target gene.

The *Prdm1* gene is bound by IRF-4 upon B cell activation. Of nine CNSs interrogated by ChIP, only CNS 9 in the fifth intron of the *Prdm1* gene was efficiently crosslinked by IRF-4. Interestingly, Bcl6 has also been shown to crosslink to this region of the *Prdm1* gene (Tunyaplin et al., 2004). Two putative IRF sites exist in this region, and they are not composite elements that could be recognized additionally by PU.1 or Spi-B. This is in keeping with the observation that the PU.1 interaction-defective mutant of IRF-4 (AS397) is capable of rescuing the plasma cell defect manifested by *Irf4*$^{-/-}$ B cells. The molecular mechanism by which IRF-4 regulates the *Prdm1* gene via this CNS remains to be elucidated.

We demonstrate that the failure of the *Irf4*$^{-/-}$ B cells to undergo plasma cell differentiation is due to impaired induction of the *Prdm1* gene. Multiple lines of experimental evidence support the conclusion that IRF-4 positively regulates Blimp-1 expression. (1) *Irf4*$^{-/-}$ B cells fail to induce the entire plasma cell gene-expression program, which is dependent on Blimp-1. In fact, the molecular signature of LPS-activated *Irf4*$^{-/-}$ B cells resembles that of *Prdm1*$^{-/-}$ B cells and not *Xbp1*$^{-/-}$ B cells (Shaffer et al., 2004). (2) *Irf4*$^{-/-}$ B cells do not efficiently process the IgH pre-mRNA to generate the secretory μ(s) transcript. Blimp-1 has been previously shown to specifically regulate this RNA-processing function, although the mechanism remains elusive (Sciammas and Davis, 2004; Shapiro-Shelef et al., 2003). (3) Syndecan-1 induction is defective in LPS-stimulated *Irf4*$^{-/-}$ B cells. (4) Ectopic expression of IRF-4 in primary murine B cells upregulates *Prdm1* expression and Ig secretion. (5) Ectopic expression of IRF-4 in a human B lymphoblastoid cell line upregulates *Prdm1* expression and the plasma cell gene-expression program. (6) IRF-4 crosslinks to a conserved noncoding sequence in the *Prdm1* gene. Our data and conclusion concerning IRF-4 regulation of Blimp-1 expression differs from that in a recent report (Klein et al., 2006). Although Klein et al. also observed profound defects in CSR and plasma cell differentiation via a newly generated Irf4 mutant allele, they attributed the latter defect to a failure to express Xbp1 and not Blimp-1. Our conclusion is based not only on expression analysis of *Prdm1* transcripts and Blimp-1 protein in *Irf4*$^{-/-}$ B cells but also on the multiple lines of evidence cited above. Finally, our conclusion is strongly complemented by the findings that the transcription factor Mitf, which is highly expressed in naive B cells, represses the expression of *Irf-4* and precocious plasma cell differentiation (Lin et al., 2004). Mutation of the *Mitf* gene results in the derepression of *Irf-4* expression, along with spontaneous B cell activation, antibody secretion, and autoantibody production. Inhibition of IRF-4 expression with antisense oligonucleotides suppresses the spontaneous antibody-secretion phenotype of *Mitf*$^{Mi/Mi}$ B cells. Thus, precocious expression of IRF-4 likely results in premature induction of Blimp-1 and the onset of plasma cell differentiation.

In spite of IRF-4 regulating *Prdm1* expression, it appears that not all of the functions of IRF-4 in plasma cells can be attributed to Blimp-1. Although Blimp-1 can complement the Ig secretion defect of *Irf4*$^{-/-}$ B cells, it does so inefficiently. We note that the Blimp-1 expression vector used in these experiments induces efficient Ig secretion in B cell lines and primary B cells (Sciammas and Davis, 2004). Therefore, IRF-4 could regulate Ig secretion independently of, or in a synergistic manner with, Blimp-1.

The plasma cell phenotype of *Irf4*$^{-/-}$ B cells is similar to that of *Prdm1*$^{-/-}$ B cells (Shapiro-Shelef et al., 2003) but distinct from those manifested by mutations in the *Pou2f2* (*Oct-2*), *Pou2af1* (*Obf1/OcaB/Bob1*), or *Cdkn2c* (*p18*) genes (Corcoran et al., 2005; Corcoran and Karvelas, 1994; Tourigny et al., 2002). *Pou2f2*$^{-/-}$ B cells are blocked at the G1 to S phase transition, fail to properly upregulate activation markers, and undergo exaggerated cell death (Corcoran and Karvelas, 1994). OBF-1 appears to play a selective role in regulating the development of ASC, because fewer Blimp-1-positive plasma cells are generated in the context of T-dependent but not T-independent responses in *Pou2af1*$^{-/-}$ mice (Corcoran et al., 2005). *Cdkn2c*$^{-/-}$ B cells fail to exit the cell cycle and consequently are impaired for Ig secretion (Tourigny et al., 2002). In contrast, IRF-4 appears to represent an obligate and intimate regulator of plasma cell differentiation.

IRF-4 functions redundantly with IRF-8 to limit the clonal expansion of pre-B cells, which is driven by signaling through the pre-BCR (Lu et al., 2003). In the

(F) Quantitative RT-PCR analysis of selected genes from the cells described in (E). Average values from three independent experiments, transcripts of each gene are normalized to the expression of *USF2*; error bars correspond to SEM. (C), (E), and (F) are representative of three independent experiments, whereas (D) represents data from two independent experiments.

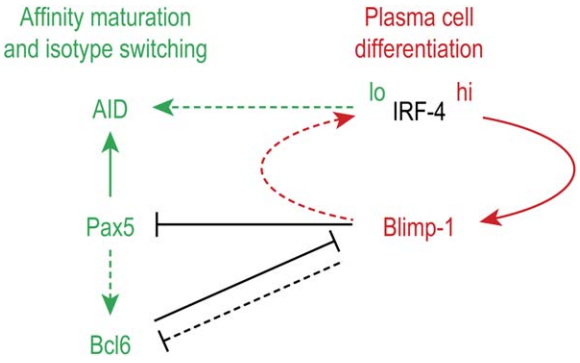

Figure 7. Proposed Gene-Regulatory Network Orchestrating the Germinal Center to Plasma Cell Transition

The network depicts regulatory connections between four genes encoding transcription factors and the *Aicda* gene. Arrows represent positive regulation and barred lines represent repression; dashed lines indicate that a relationship could be indirect. As indicated, the circuit architecture allows IRF-4 to differentially regulate AID and Blimp-1 in competing cellular states. Importantly, the graded expression of IRF-4, generated during the course of antigen-dependent B cell differentiation, is proposed to promote the transition of a germinal center B cell to the plasma cell state.

absence of IRF-4 and IRF-8, pre-B cells fail to undergo cell-cycle arrest and do not differentiate into immature B cells. By analogy, we propose that IRF-4 functions in a nonredundant manner to limit the clonal expansion of activated B cells by inducing cell-cycle arrest and terminal differentiation into nondividing plasma cells. Consistent with this proposal, IRF-4 is expressed in a biphasic manner, with the highest concentration being reached in plasma cells. The molecular pathways by which IRF-4 inhibits the cell cycle in pre-B or plasma cells remain to be elucidated. In plasma cells, IRF-4 may function to inhibit cell-cycle progression in part via the induction of Blimp-1, which represses *c-myc* and other proliferation-associated genes (Sciammas and Davis, 2004; Shaffer et al., 2002).

How does IRF-4 regulate temporally distinct patterns of *Aicda* and *Prdm1* gene expression and therefore coordinate isotype switching with plasma cell generation? Based on earlier analyses and the findings herein, we assemble a minimal gene regulatory network in which IRF-4 functions as a critical node in regulating the developmental transition from a germinal center B cell to a plasma cell (Figure 7). During the onset of B cell differentiation when IRF-4 concentrations are low, we propose that it activates the *Aicda* gene in combination with the transcription factor Pax5 (Gonda et al., 2003). We note that the *Aicda* gene may be indirectly regulated by IRF-4. Pax5 expression at this stage prevents Blimp-1 induction by promoting the expression of Bcl6 (Nera et al., 2006). Increased expression of IRF-4 induces *Prdm1*, which, in turn, feeds back to augment expression of the *Irf4* gene. In addition to our own data, two lines of evidence support the positive autoregulatory loop between Blimp-1 and IRF-4. First, deletion of the *Prdm1* gene in B cells leads to a failure to sustain IRF-4 expression upon LPS activation (Kallies et al., 2004). Second, ectopic expression of Blimp-1 in a B lymphoma (M12) that results in expression of the plasma cell program and Ig secretion also leads to a 4-fold induction of *Irf4* expression (Sciammas and Davis, 2004). A key feature of the model is that Bcl6 represses *Prdm1* (Fujita et al., 2004; Reljic et al., 2000; Shaffer et al., 2000; Tunyaplin et al., 2004). In turn, Blimp-1 represses *Bcl6* (Sciammas and Davis, 2004; Shaffer et al., 2002) and *Pax5* (Lin et al., 2002; Shaffer et al., 2002) and thus the germinal center program of gene expression, which includes the *Aicda* gene. Thus, the proposed autoregulatory loop between IRF-4 and Blimp-1 is suggested to overcome Bcl6-mediated repression of the *Prdm1* gene. This mechanism would temporally delay the activation of the *Prdm1* gene in relation to the *Aicda* gene by IRF-4. An attractive feature of our regulatory network involving a positive autoregulatory loop between IRF-4 and Blimp-1 is that it enables the generation of a stable differentiated state (plasma cell) that requires inducing signals (antigen receptor, TLR, cytokines) for its establishment but not for its maintenance.

Experimental Procedures

Detailed methods are described in Supplemental Data.

Mice

The *Irf4*$^{-/-}$ mice have been previously described (Mittrucker et al., 1997). Mice were housed in specific pathogen-free conditions and were used and maintained in accordance with the Institutional Animal Care and Use Committee guidelines.

Cells and Culture Conditions

Splenic B cells were isolated by sequential hypotonic lysis of RBC and magnetic bead separation of non-B cells with a biotin-coupled cocktail of T, NK, macrophage, and granulocyte-specific antibodies, followed by adsorption to Streptavidin microbeads (Miltenyi Biotec Inc.). The unbound cells (typically greater than 90% B220^{+}) were washed and placed into culture with LPS, IL-4, or CD40L. Cell divisions were monitored by labeling the purified B cells with 1.5 μM CFSE (Molecular Probes). Retroviral infections were performed 24 hr after stimulation, and GFP^{+} cells were sorted 48 hr after infection. The germinal center B cell line Oci-Ly7 was cultured and transduced as described (Shaffer et al., 2002).

DNA Constructs

The murine IRF-4 retroviruses were constructed by subcloning HA-tagged cDNA segments into the MigR1 retrovirus that contains an IRES-GFP segment. The Blimp-1 retrovirus has been previously described (Sciammas and Davis, 2004), and the AID retrovirus was a kind gift from F. Livak (University of Maryland). The HA-IRF-4 mDBD binding mutant was constructed by changing four nucleotides to confer insertion of a restriction site and codons for two alanine mutations at positions 98 and 99 of the protein with the Quickchange site-directed mutagenesis kit (Stratagene). The HA-IRF-4 AS397 mutation consists of a linker scanning mutation changing four amino acids to alanines starting at position 397 of the protein and has been previously described (Brass et al., 1999). The human IRF-4 retrovirus consists of a FLAG-tagged IRF-4 cDNA expressed by the vector Vxy-puro (Shaffer et al., 2004).

ELISA and ELISpot

Secreted IgM was analyzed by harvesting and washing cells from day 3 or 4 stimulated cultures and plating at 1 × 10^{6} cells/ml for an additional day, prior to analyzing supernatants by ELISA as described (Sciammas and Davis, 2004). Antibody-secreting cells were enumerated with an ELISpot technique by preparing cells as above and plating serial dilutions of cells in triplicate on anti-IgM-coated ELISpot plates (Millipore). After washing, the spots were detected with a horseradish peroxidase-coupled anti-IgM and finally developed with AEC substrate (Sigma). The spots were counted with the CTL Analyzer powered by ImmuoSpot Software (Cellular Technology, Ltd.). All ELISA and ELISpot antibodies were purchased from Jackson Immunoresearch.

Retrovirus Production

The PlatE packaging cells were transiently transfected with retroviral constructs with the Fugene6 reagent (Roche) to produce viral supernatants.

Flow Cytometry

Cells were washed in staining buffer, PBS containing BSA (0.5%, w/v) and sodium azide (0.05%, w/v), and blocked with anti-FcR (clone 2.4G2) hybridoma supernatant. Cells were stained with biotin- or fluorochrome-coupled antibodies in staining buffer according to standard procedures. Antibodies specific for murine Syndecan-1, IgG3, IgG1, B220, CD23, CD62L, CD44, CD25, CD80, CD86, BrdU, and isotype controls, as well as antibodies specific for human CD10 and CD38 were purchased from BD Pharmingen. Data were collected with either the LSR II or FACSVantage. Dead cells were excluded by DAPI. Data were analyzed with the FlowJo software (Tree Star, Inc.). Intracellular detection of IRF-4 was performed by sequential labeling with Live/Dead Fixable Blue (UV excitable) Stain (Molecular Probes), followed by staining for extracellular markers and fixation. The fixed cells were permeabilized with Saponin (Sigma) and stained with anti-IRF-4 or goat IgG (Santa Cruz Biotech.) followed by a Cy5-coupled anti-goat secondary.

Cell Lysate Preparation and Immunoblotting

Whole-cell lysates were prepared after washing cells with PBS and resuspending cells in detergent lysis buffer containing protease inhibitors. Preparation of nuclear extracts was performed as described (Sciammas and Davis, 2004). Proteins were resolved by denaturing SDS-PAGE and transferred to nitrocellulose. The blots were probed with Blimp-1 (Kallies et al., 2004), IRF-8 (Santa Cruz), or HA antibodies (Covance) and visualized with appropriate HRP-coupled secondary antibodies.

RNA Analysis by RT-PCR

Total RNA was isolated with Trizol (Invitrogen), quantitated, and used to make cDNA. Quantitative PCR was performed in duplicate with a SYBR green kit (Stratagene) or a TaqMan RT-PCR kit (ABI). Gene-specific and intron-spanning primers were validated for the generation of unique products. Semiquantitative PCR was used to analyze the expression of germline and post-switch transcripts as described (Muramatsu et al., 2000) or for the membrane and the secretory forms of IgM transcripts (Reimold et al., 2001).

Microarray Analysis

For microarray analysis, experimental RNAs were converted to Cy5-labeled cDNA and cohybridized on mouse lymphochip arrays with RNA from a reference pool labeled with Cy3 (see http://lymphochip.nih.gov/ShafferPCfactors/). Microarray experiments with the control and IRF-4-transduced human cell line Oci-Ly7 involved cohybridization of the differentially labeled RNA samples on human lymphochip arrays (Shaffer et al., 2002). Microarray data analysis was performed with the Cluster and Treeview programs. Only data from sequence-verified elements are displayed.

Chromatin Immunoprecipitation

ChIP was perfomed on 2.5×10^6 cells with conditions optimized for lymphocytes (Lu et al., 2003). DNA was sonicated with three pulses of 45 s each, and DNA fragmentation was monitored by agarose gel electrophoresis and was typically in the range of 0.5 to 1 kb. Immunoprecipitation was performed with 1 μg of anti-IRF-4 or goat IgG (Santa Cruz) and Protein G Sepaharose. SYBR green-based quantitative PCR reactions were set up in duplicate to analyze the precipitated DNA for 40 cycles with primer sets known to generate unique products. Standard curves were generated for each DNA segment analyzed in each run. The relative IP efficiency was determined by dividing the value of the immunoprecipitated sample with that of the input sample and is displayed as percent of input. The background from control IgG samples averaged 0.05% $\pm$ 0.01%.

Supplemental Data

Supplemental Data include seven figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://www.immunity.com/cgi/content/full/25/2/225/DC1/.

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