Blinp-1 Attenuates Th1 Differentiation by Repression of \textit{ifng}, \textit{tbx21}, and \textit{bcl6} Gene Expression

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Blimp-1 Attenuates Th1 Differentiation by Repression of ifng, tbx21, and bcl6 Gene Expression

Luisa Cimmino,* Gislaine A. Martins,† Jerry Liao,† Erna Magnusdottir,‡ Gabriele Grunig,† Rocio K. Perez,‡ and Kathryn L. Calame1*§

T cell-specific deletion of Blimp-1 causes abnormal T cell homeostasis and function, leading to spontaneous, fatal colitis in mice. Herein we explore the role of Blimp-1 in Th1/Th2 differentiation. Blimp-1 mRNA and protein are more highly expressed in Th2 cells compared with Th1 cells, and Blimp-1 attenuates IFN-γ production in CD4 cells activated under nonpolarizing conditions. Although Blimp-1-deficient T cells differentiate normally to Th2 cytokines in vitro, Blimp-1 is required in vivo for normal Th2 humoral responses to NP-KLH (4-hydroxy-3-nitrophenylacetyl/keyhole lymphocyte hemocyanin) immunization. Lack of Blimp-1 in CD4 T cells causes increased IFN-γ, T-bet, and Bcl-6 mRNA. By chromatin immunoprecipitation we show that Blimp-1 binds directly to a distal regulatory region in the ifng gene and at multiple sites in tbx21 and bcl6 genes. Our data provide evidence that Blimp-1 functions in Th2 cells to reinforce Th2 differentiation by repressing critical Th1 genes. The Journal of Immunology, 2008, 181: 2338–2347.

The Journal of Immunology

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differentiation of the CD4 Th cell lineages is dictated by the strength of antigenic stimulation, cytokine environment, and the complement of transcription factors activated by these processes. T-bet and GATA-3 transcription factors are master regulators of Th1 and Th2 lineage differentiation, respectively; Foxp3 is critical for regulatory T cell development and function, and, more recently, RORγt (retinoid-related orphan receptor γt) has been shown to act as a master regulator of Th17 development (1–3). Additionally, complicated regulatory pathways and feedback loops cooperate with the master regulators to establish and maintain CD4 differentiation states.

Th polarization into Th1 and Th2 lineages can be divided into hierarchical steps of initiation, reinforcement, and maintenance (1). Initiation of lineage differentiation begins upon T cell recognition of Ag presented by APCs and response to the initial cytokine environment. Reinforcement of commitment is driven by the up-regulation of cytokine production, positive feedback loops, and repression of genes necessary for the alternative fate. Maintenance of the differentiated state is conferred by heritable epigenetic changes. Transcription factors activated by TCR-signaling such as AP-1, NF-κB, and NFAT can differentially induce both IFN-γ and IL-4 expression directly or via the induction of T-bet and GATA-3, respectively (1, 4). In the case of Th1 differentiation, T-bet can be induced both by IFN-γ or IL-12 through the transcription factors STAT-1 and STAT-4, respectively, to directly reinforce expression of the IFN-γ gene while simultaneously repressing GATA-3 function (2, 5, 6). GATA-3 activates expression at the Th2 cytokine locus of IL-4, IL-5, and IL-13 genes (7) and can autoactivate its own expression both independently and as a result of IL-4-mediated STAT-6 signaling (8). How T-bet and GATA-3 compete to drive Th1 or Th2 differentiation has been intensively studied and has laid the foundation for understanding how other transcription factors influence the way CD4 lineage decisions are made.

The transcriptional repressor B lymphocyte-induced maturation protein-1 (Blimp-1)2 has recently been identified as a regulator of T cell homeostasis and function (9, 10). Encoded by the prdm1 gene, Blimp-1 is well established as a master regulator of plasma cell differentiation and maintenance (11, 12). Blimp-1 also plays a role in multiple developmental checkpoints in nonlymphoid lineages including germ cell formation during embryogenesis (13–15), myeloid differentiation (16), keratinocyte maturation (17), and sebocyte differentiation (18).

In the T cell lineage Blimp-1 is strongly induced upon TCR activation (10), with IL-2 playing an important role in the induction (19). In two independent studies, T cell-specific deletion of Blimp-1 in mice caused spontaneous colitis (10) and a multiorgan inflammatory disease (9). These autoimmune pathologies were attributed in part to altered homeostasis within the CD4 T cell lineage, including hyperproliferation in response to TCR stimulation, increased IL-2 and IFN-γ production, and decreased IL-10 production, possibly contributing to diminished regulatory T cell function in vivo (9, 10).

Blimp-1 is a transcriptional repressor. Interestingly, most of the known direct targets of Blimp-1 are also transcription regulators, explaining in part its ability to control extensive developmental programs (11). In B cells Blimp-1 directly represses myc, spiB, CIITA, id3, and pax5. In the epidermis it represses myc, fos, nfat5, and dusp16 (17). It has also been shown to repress p53 in some tumor cell lines (20).

2 Abbreviations used in this paper: Blimp-1, B lymphocyte-induced maturation protein-1; Bcl-6, B cell lymphoma-6; ChIP, chromatin immunoprecipitation; CNS, conserved noncoding sequence; IP, immunoprecipitation; MFI, mean fluorescence intensity; NP-KLH, 4-hydroxy-3-nitrophenylacetyl/keyhole lymphocyte hemocyanin.

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Direct targets for Blimp-1 in T cells have not yet been identified. However, it is known that B cell lymphoma-6 (Bcl-6) mRNA is elevated in Blimp-1-deficient CD4 T cell effectors (10). Mutual repression by Bcl-6 and Blimp-1 in B cells is important for maintaining mutually exclusive programs of germinal center B cells and plasma cells (21). In T cells, Bcl-6 repression is involved in Th1 differentiation by repressing Th2 cytokine expression. It decreases GATA-3 protein levels (22), represses IL-5 transcription (23), and competes for binding at STAT-6 recognition sequences in IL-4-responsive genes (24, 25).

The goal of the present study was to learn how Bcl-6 and Blimp-1 interact during Th1/Th2 development and to determine the molecular mechanism(s) by which Blimp-1 alters expression of cytokines important for Th1 vs Th2 differentiation and function. We show that Blimp-1 is most highly expressed in Th2 cells and that mice lacking Blimp-1 in CD4 T cells exhibit impaired humoral Th2 responses, establishing a role for Blimp-1 in Th1 differentiation. We also showed that ifng, tbx21, and bcl6 are direct targets of Blimp-1-dependent repression in CD4 T cells, providing a molecular basis for the idea that Blimp-1 helps to oppose Th1 differentiation during Th2 lineage commitment.

Materials and Methods

Mice

Prdm1 

or prdm1fl/fl mice (26) were crossed with mice expressing CD4-Cre 

(27) or Lck-Cre 

(28) to generate Cre 

or Cre 

prdm1fl/fl mice. Bcl-6 

mice were described previously (29). Mice were housed in the barrier facility of Columbia University (New York, NY). All experiments were approved by the Institutional Animal Care and Use Committee of Columbia University.

CD4+ T cell purification and CFSE labeling

Naive CD4+ T cells were purified from the spleen and lymph nodes of 4-wk-old prdm1 

or prdm1fl/fl mice expressing CD4-Cre 

or Lck-Cre 

and Bcl-6 

or Bcl-6 

littermates by flow cell-sorting for CD4+CD62L 

CD44low cells. Staining with the division-sensitive dye CFSE (Molecular Probes) was performed as previously described (10) before stimulation in vitro for 4 days with plate-bound anti-CD3 (5 

mg/ml), anti-CD28 (2.5 

mg/ml), and recombinant human IL-2 (25 U/ml).

FACS analysis and intracellular cytokine staining

FACS plots were gated on live, non-autofluorescent cells before analysis. Cells were stained with fluorochrome-conjugated Abs specific for CD3, CD4, CD8, CD25, CD44, CD62L, Thy-1.2, IFN- 

, or sorted CD4 T cell subsets, and reverse transcription was performed with SuperScript III (Invitrogen) following the manufacturer’s instructions. For quantitative PCR analysis, the cDNA was diluted 5-fold in water and 5 

l was amplified for 40 cycles using the ABI 7700 (Applied Biosystems) sequence detection system with the following primers: IFN- 

forward 5’-CCCTTTGCACTTCTCAT and reverse 5’-GTCGACCATCACCACCTTTTTGCAGT-3’; Blimp-1, forward 5’-GACGGGGTACTTCTGTTCA and reverse 5’-GGATTCTTGGGAAAGCTGAGAG and reverse 5’-CCACATCCCAAAACATCCTG; Blimp-1, forward 5’-GACGGGGTACTTCTTGTCA and reverse 5’-GGATTCTTGGGAAAGCTGAGAG and reverse 5’-CCACATCCCAAAACATCCTG; Blimp-1, forward 5’-GACGGGGTACTTCTTGTCA and reverse 5’-GGATTCTTGGGAAAGCTGAGAG and reverse 5’-CCACATCCCAAAACATCCTG; Blimp-1, forward 5’-GACGGGGTACTTCTTGTCA and reverse 5’-GGATTCTTGGGAAAGCTGAGAG and reverse 5’-CCACATCCCAAAACATCCTG; Blimp-1, forward 5’-GACGGGGTACTTCTTGTCA and reverse 5’-GGATTCTTGGGAAAGCTGAGAG.

Chromatin immunoprecipitation (ChIP) and quantitative PCR

CD4 T cells were grown for 6 days in culture and restimulated for 4 h with 20 ng/ml PMA and 1 

mg/ml ionomycin before harvesting. ChIP assays were performed as previously described (30). Briefly, 30–40 

106 cells were used per ChIP after crosslinking with 1% formaldehyde in 24-well plates coated with 5 

anti-CD3, 2.5 

anti-CD28 (25 U/ml), and recombinant human IL-2 (25 U/ml).

Quantitative RT-PCR

In vitro CD4 Th cell differentiation

For in vitro polarization, naïve CD4 T cells were seeded at 1 

106 cells/ml in 24-well plates coated with 5 

mg/l anti-CD3, 2.5 

mg/l anti-CD28 (BD Pharmingen), and 25 U/ml of recombinant human IL-2 in RPMI 1640 with the addition of murine IL-12 (10 ng/ml) and anti-IL-4 (10 ng/ml) or murine IL-4 (1000 U/ml) and anti-IFN- 

(10 

mg/ml). Western blot analysis

CD4 T cells were cultured for 2 wk under polarizing conditions described above, lysed in RIPA buffer (50 mM HEPES (pH 7.6), 1 mM EDTA, 0.7% sodium deoxycholate, 1% Nonidet P-40, and 0.5 M NaCl), and 50 

of protein from the whole-cell extract was separated by 8% SDS-PAGE and Western blotted using mouse monoclonal anti-Blimp-1 (26), anti-GATA-3, and anti- 

Abs (Santa Cruz Biotechnology).

In vitro CD4 Th cell differentiation

For in vitro polarization, naïve CD4 T cells were seeded at 1 

106 cells/ml in 24-well plates coated with 5 

mg/l anti-CD3, 2.5 

mg/l anti-CD28 (BD Pharmingen), and 25 U/ml of recombinant human IL-2 in RPMI 1640 with the addition of murine IL-12 (10 ng/ml) and anti-IL-4 (10 ng/ml) or murine IL-4 (1000 U/ml) and anti-IFN- 

(10 

mg/ml).

Results

Blimp-1 decreases the number of IFN- 

producing CD4+ cells and the amount of IFN- 

made per cell

Since our previous work showed that murine CD4+ T cells lacking Blimp-1 had elevated IFN- 

production (10) and because IFN- 

is a key component in Th1 differentiation, we wanted to explore the potential role of Blimp-1 in the commitment and/or establishment of Th1 vs Th2 cells. First, we studied the role of Blimp-1 in IFN- 

production in more detail. Naive CD4 T cells (CD4+ CD62L+) were purified from the spleen and lymph nodes of 4-wk-old Blimp CKO mice (prdm1 

with either Lck-Cre or CD4-Cre) and analyzed for intracellular IFN- 

staining with

4-Hydroxy-3-nitrophenylacetyl/kehole lymphocyte hemocyanin (NP-KLH) immunizations

Mice aged 4–6 wk were injected i.p. with 100 

of alun-precipitated NP-KLH (Biosearch Technologies) in PBS. Inject Alum was purchased from Pierce. Five to seven mice were included per genotype.

ELISA

NP-specific IgM, IgG1, and IgG2a in immunized mice for primary responses were detected by ELISA as previously described using NP-BSA (Biosearch Technologies)-coated plates (68).

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anti-CD3, anti-CD28, and IL-2 for 6 days and subsequent restimulation. As shown in Fig. 1, A and B, the fraction of cells producing IFN-γ was 2-fold higher in Blimp-1 CKO CD4 T cells compared with controls, and the IFN-γ-producing cells from the CKOs also showed a 2-fold higher mean fluorescence intensity (MFI), indicating elevated IFN-γ production on a per cell basis. The fraction of cells expressing IFN-γ increases in frequency with successive cell cycles (32); therefore, a previously observed proliferative advantage in Blimp-1 CKO CD4 T cells (10) might explain increased IFN-γ production during activation in culture. To determine the relationship between IFN-γ production and cell division in Blimp-1 CKO cells, naive CD4 T cells were stained with the division-sensitive dye CFSE before culture for 4 days under various conditions of stimulation, comparing IFN-γ production as a function of proliferation. Consistent with our previous findings, Blimp-1 CKO CD4 T cells are more proliferative than wild-type control cells under weak stimulatory conditions (10). Stimulation for 4 days with anti-CD3 alone gave rise to 6-fold more IFN-γ production in the Blimp CKO cultures compared with a 2-fold increase with anti-CD3 and anti-CD28 stimulation (Fig. 1C). However, upon stimulation with anti-CD3, anti-CD28, and IL-2, despite equivalent rates of proliferation, the frequency of IFN-γ-producing cells was 1.5-fold higher in the CKO in total culture (Fig. 1C) and, with one exception, at each round of division (Fig. 1D). These results show a cell cycle-independent effect of Blimp-1 on the number of cells producing IFN-γ. Additionally, the increased IFN-γ MFI per cell in the CKO CD4 T cells indicates that each CKO cell makes more IFN-γ. Thus, we conclude that Blimp-1 inhibits both the fraction of cells making IFN-γ and the amount of IFN-γ made per cell.

Blimp-1 is more highly expressed in Th2 cells

Increased IFN-γ production by Blimp-1-deficient CD4 T cells stimulated under neutral conditions indicates that Blimp-1 may normally play a role in Th2 differentiation. The expression pattern of Blimp-1 under Th1 and Th2 polarizing conditions was therefore analyzed in wild-type CD4 T cells to determine whether the expression pattern of Blimp-1 was consistent with a role in Th2 differentiation.

Blimp-1 steady-state mRNA, measured by quantitative RT-PCR, was 5-fold higher in Th2 cells compared with Th1 cells after 6 days in culture (Fig. 2A). Furthermore, Blimp-1 protein, analyzed by Western blotting of CD4 T cells cultured for 2 wk under Th1 and Th2 polarizing conditions, showed more Blimp-1 in Th2 cells compared with Th1 cells (Fig. 2B).

Blimp-1 mRNA was also analyzed in CD4 T cells following stimulation with different cytokines important for Th cell differentiation. Naive CD4 T cells were cultured for 6 days either with anti-CD3 and anti-CD28 stimulation alone or with added IL-2, IL-4, IL-12, or IFN-γ. Stimulation with anti-CD3, anti-CD28, and exogenous IL-2 increased Blimp-1 mRNA modestly compared with TCR stimulation alone. IL-4 induced Blimp-1 mRNA maximally under these culture conditions (Fig. 2C), while IFN-γ actually inhibited induction of Blimp-1 mRNA (Fig. 2C). Thus, Blimp-1 mRNA is induced by IL-4 and repressed by IFN-γ signaling, consistent with highest expression in Th2 cells. These data are consistent with the idea that Blimp-1 is induced in Th2 cells and plays a role in Th2 commitment or function.

Blimp-1 is required for a normal Th2 humoral response in vivo, but not for Th2 differentiation in vitro

To test if Blimp-1 deficiency alters differentiation of Th cells in vivo, naive CD4 T cells were stimulated for 6 days with plate-bound anti-CD3, anti-CD28, and IL-2 with the addition of either IL-12 and anti-IL-4 or IL-4 and anti-IFN-γ to promote Th1 or Th2 polarization, respectively. There was no significant difference in the fraction of IFN-γ-producing cells between the wild-type and CKO Th1 cultures, and, similarly, under Th2 culture conditions, equivalent fractions of IL-4-producing cells were present (Fig. 3A). However, there was a 3–5-fold decrease in the number of IL-10-producing cells upon Th2 differentiation in Blimp CKO cultures (Fig. 3B), consistent with the decreased IL-10 production observed in Blimp-1 CKO CD4 T cell effectors ex vivo and under neutral conditions of stimulation in vitro (10). Although the fraction of IFN-γ- or IL-4-producing cells was not significantly different between the wild-type and CKO Th cell cultures (Fig. 3C), the average MFI for IFN-γ was 1.6-fold higher for Blimp-1 CKO cells under Th1 conditions, showing that these cells produce more IFN-γ (supplementary Fig.

### Table I. Sequences used

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**Blimp-1 ATTENUATES Th1 DIFFERENTIATION**
Thus, we conclude that Blimp-1 is not required for Th1 or Th2 cell polarization in vitro, although in its absence, expression of IL-10 is abnormal.

Polarization in vitro provides saturating amounts of polarizing cytokines and is unlikely to reflect the more subtle signals that determine Th1/Th2 polarization in vivo. To determine whether Blimp-1 is important for a Th2 response in vivo, we immunized mice with NP-KLH in alum, which is known to cause a Th2-dependent response primarily of IgG1 (33). Sera from immunized mice were measured over the course of 21 days for NP-specific IgM, IgG1, and IgG2a by ELISA. IgG1 was decreased 2-fold in CKO mice at each time point, indicating that the Th2 responses were impaired (Fig. 4B). Interestingly, CKO mice also exhibited 2–3-fold higher titers of NP-specific IgM compared with control mice (Fig. 4A). The IgG2a response of the CKO mice was similar to controls late in the response, but 1.5–2-fold higher at days 12 and 18 (Fig. 4C). It may be that the IgM and IgG2a responses are elevated because the T cell cytokine milieu in the germinal center is not normal, but this hypothesis requires further study. These data show that Blimp-1 is required for a normal Th2 humoral response to NP-KLH in alum.

Blimp-1 represses IFN-γ and T-bet mRNA expression in activated CD4 T cells

We wanted to explore the molecular mechanisms responsible for the role of Blimp-1 in Th2 differentiation. The data showing elevated IFN-γ production in CKO CD4+ cells suggested that Blimp-1 might repress IFN-γ or other genes expressed in Th1 cells. In CD4+ cells, IFN-γ transcription is activated by TCR signaling (34, 35), and subsequent IFN-γ secretion initiates more IFN-γ production via a positive feedback loop involving the IFN-γR-STAT-1 pathway. Activated STAT-1 also induces the transcription of tbx21, encoding T-bet, and T-bet further induces ifng transcription and interferes with GATA-3 function, thus reinforcing IFN-γ production and Th1 polarization (5, 36, 37). To investigate the possible role of Blimp-1 in expression of IFN-γ and...
T-bet mRNAs, we employed quantitative RT-PCR. During activation in vitro under nonpolarizing conditions, total mRNA was collected at various times and analyzed for IFN-γ and T-bet steady-state mRNA in wild-type and CKO CD4 T cells. IFN-γ mRNA was increased 2–3-fold in Blimp-1-deficient CD4 T cells compared with wild-type cells, in naive cells, and in activated cells at each time during the 6 day stimulation and upon restimulation with PMA and ionomycin at days 3 and 6 of culture (Fig. 5A). T-bet mRNA was also elevated in the CKO, most noticeably at day 2, with 5–6-fold increased T-bet expression in CKO CD4 T cells compared with wild-type cells and upon restimulation with PMA and ionomycin after 3 days in culture (Fig. 5B). Further evidence for a requirement for Blimp-1 to repress IFN-γ expression was obtained by quantitative RT-PCR in which the levels of IFN-γ mRNA from CKO Th1 and Th2 cells were observed to be 5-fold and 3-fold increased, respectively, as compared with wild-type cells (supplementary Fig. 1B). Collectively, these data show that Blimp-1 normally represses expression of IFN-γ and T-bet mRNA. Under neutral cytokine conditions, acute stimulation of naive CD4 T cells for 24 h with PMA and ionomycin also induced 8-fold higher levels of IFN-γ and 15-fold higher levels of T-bet mRNA in Blimp-1 CKO cells (Fig. 5C). Since stimulation by PMA and ionomycin bypasses early TCR signaling events, this provides evidence that Blimp-1 regulation occurs downstream of PKC activation and Ca²⁺ mobilization and suggests that Blimp-1 might directly repress IFN-γ and T-bet expression.

**Blimp-1 and Bcl-6 repress one another in CD4 T cells**

Bcl-6 is known to play a role in repressing Th2 differentiation (22, 29, 38). Furthermore, in B cells, Bcl-6 and Blimp-1 repress one another, resulting in mutually exclusive transcriptional programs that maintain cells either in a germinal center state, where Bcl-6 is expressed, or in a plasma cell state, where Blimp-1 is present (21). In CD4⁺ T cells, Bcl-6 is induced by activated STAT-1 (39) and Bcl-6 mRNA expression is elevated in Blimp-1 CKO CD4 T cell effectors purified ex vivo (10), suggesting that Blimp-1 also represses Bcl-6 in T cells, which, we hypothesized, might also be important for Th1 vs Th2 differentiation.

We measured Bcl-6 mRNA by quantitative RT-PCR in cells stimulated in nonpolarizing conditions for 6 days. Lack of Blimp-1

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**FIGURE 2.** Blimp-1 is more highly expressed in Th2 cells and Blimp-1 mRNA expression is differentially regulated by polarizing cytokines. A, Blimp-1 steady-state mRNA levels in CD4 T cells stimulated for 6 days under Th1 and Th2 culture conditions (n = 5 with SEM). B, Western blot analysis of Blimp-1 and GATA-3 protein levels normalized to β-tubulin in CD4 T cells cultured for 2 wk under Th1 and Th2 conditions. C, Blimp-1 steady-state mRNA levels in CD4 T cells stimulated for 6 days with plate-bound anti-CD3 and anti-CD28 in the presence of IL-2, IL-4, IL-12, and IFN-γ cytokines. Data represent averages and SEM of three experiments.

**FIGURE 3.** Blimp-1-deficient T cells are able to polarize in vitro but exhibit decreased IL-10 production under Th2 conditions. Naïve CD4 T cells were cultured for 6 days under Th1 or Th2 conditions and analyzed by FACS intracellular cytokine staining for (A) IFN-γ and IL-4 production or (B) IL-10 production. Representative FACS plots are shown with percentage of IFN-γ, IL-4-, or IL-10-producing cells (inset). Bar graphs represent (C) average percentage of IFN-γ- or IL-4-producing cells in Th1 and Th2 cultures. Results are averages and SEM of four experiments. FACS data of IL-10-producing cells are representative of three experiments.
T cells following stimulation with cytokines important for Th cell differentiation. Naive CD4 T cells were cultured for 6 days either with anti-CD3 and anti-CD28 alone or in addition to IL-2, IL-4, IL-12, or IFN-γ. Stimulation with anti-CD3 and anti-CD28 in the presence of IL-2, IL-4, or IL-12 caused no change in the level of steady-state Bcl-6 mRNA (Fig. 6E). However, IFN-γ was able to increase the level of Bcl-6 mRNA ~7-fold higher than anti-CD3 and anti-CD28 stimulation alone (Fig. 6E). These data show that Blimp-1 and Bcl-6 mutually repress each other in CD4 T cells. Bcl-6 mRNA is more highly expressed in Th1 cells compared with Th2 cells and high levels are maintained upon stimulation in the presence of IFN-γ.

**Blimp-1 binds directly to the ifng, tbx21, and bcl6 genes**

To determine whether Blimp-1 directly represses *ifng, tbx21*, or *bcl6* transcription, potential Blimp-1 binding sites in these genes were identified using the Vista genomic analysis tool (40, 41) and tested for Blimp-1 binding in vivo by ChIP (Fig. 7). Candidate Blimp-1 binding sites were chosen based on their similarity to experimentally determined consensus binding site (42). For the ChIP experiments, binding to an irrelevant site on the gene *snail3* was used as a negative control, as well as an irrelevant site within each target gene. Known targets of Blimp-1 in B cells, *myc* and *id3*,

**Figure 4.** *Pدقز* CD4/LCK-Cre<sup>+</sup> (Blimp-1 CKO) mice exhibit increased IgM and decreased IgG1 production in response to immunization with alum-precipitated NP-KLH. Blimp-1 CKO and control mice were immunized with alum-precipitated NP-KLH, and serum at indicated days was analyzed by ELISA for NP-specific (A) IgM, (B) IgG1, and (C) IgG2a. For all panels, control mice are represented by open circles with averages (gray bar) and CKO mice by gray circles with averages (black bar).

**Figure 5.** IFN-γ and T-bet mRNA expression levels are elevated in Blimp-1-deficient CD4 T cells. Quantitative PCR analysis of steady-state mRNA levels of (A) IFN-γ and (B) T-bet from flow cell-sorted wild-type (open bar) and CKO (gray bar) naive CD4 T cells stimulated for 6 days with plate-bound anti-CD3/β (μg/ml), anti-CD28 (2.5 μg/ml), and IL-2 (20 U/ml) and restimulated (RS) at days 3 and 6 with PMA (20 ng/ml) and ionomycin (1 μg/ml) for 4 h. Data represent averages and SEM of four experiments and were analyzed using a Students paired t test with p values <0.05 (**) or <0.01 (***). C. Steady-state mRNA levels of IFN-γ and T-bet in CD4 T cells activated under nonpolarizing conditions for 24 h with PMA and ionomycin (Iono). Data are representative of two experiments.
were also tested but only the myc gene was bound by Blimp-1 in CD4 T cells (Fig. 7).

Seven candidate sites were tested in the ifng gene: sites 3, 4, 6, and 7 are conserved between mice and humans, whereas sites 1 and 2 are not conserved and site 5 contains an irrelevant site. None of the sites tested were found to locate to previously described proximal regulatory regions of the ifng gene (43, 44). However, sites 3–6, excluding the irrelevant site 5, fall within or near distal conserved noncoding sequence (CNS) regions 22 kb and 5 kb upstream of, and 17 kb downstream of, the ifng gene, which have been shown to regulate the expression of IFN-γ in T cells (45). Blimp-1 binding site 3 is within the region of the IFN-γ locus known as CNS-22 that was recently shown to be essential for IFN-γ expression in T cells (46) (Fig. 7A).

In tbx21, five potential Blimp-1 binding sites and one irrelevant site were tested by ChIP. Of those tested, sites 2 and 3 are conserved between mice and humans, whereas sites 4–6 are not (Fig. 8B). Blimp-1 binding on the tbx21 gene was most highly enriched for the conserved site 2, ∼600 bp upstream of the transcriptional start site, and sites 3 and 5, ∼1.5 kb and 11 kb downstream of exon 1 within the first intron of the tbx21 gene (Fig. 7A). These data, in conjunction with the expression data above, demonstrate that ifng and tbx21 are direct targets of Blimp-1 repression in CD4 T cells.

Four candidate sites in bcl6 were analyzed, including an irrelevant site 5 kb downstream of the last bcl6 exon (site 5), used as a negative control (Fig. 8C), along with an irrelevant site from snail3. Blimp-1 bound reproducibly to the bcl6 gene at site 1, located 5 kb upstream of the first exon (Fig. 7B). Thus, Blimp-1 directly represses bcl6 in T cells.

It has been previously shown that Blimp-1 can repress Bcl-6 when ectopically expressed in activated B cells (21), but direct repression of bcl6 by Blimp-1 has not been demonstrated in B cells. To determine whether bcl6 was also a direct target of Blimp-1 repression in B cells, ChIP was performed for the Blimp-1 binding sites identified in T cells using P3X plasmacytoma cells. In these B cells, both myc and id3 were reproducibly bound by endogenous Blimp-1 at their known binding sites, as was site 1 on the bcl6 gene (Fig. 7C). These data show that bcl6 is a direct target of Blimp-1 repression in both CD4 T cells and in B cells.
FIGURE 8. Schematic representation of sites investigated within the (A) ifng, (B) tbx21, and (C) bcl6 murine genomic loci. Blimp-1 recognition sequences are shown for the site most greatly enriched by ChIP within each gene. Conserved noncoding sequences (CNS) are indicated by an asterisk and locations are shown in bases.

Discussion

We have shown that the transcriptional repressor Blimp-1 is most highly expressed in Th2 cells and directly represses transcription of three genes that encode proteins with critical roles in Th1 differentiation: IFN-γ, T-bet, and Bcl-6. Furthermore, the Th2 IgG1 response to immunization with NP-KLH in alum is defective in mice lacking Blimp-1 in their T cells.

Blimp-1 repression of ifng

IFN-γ is a critical cytokine for commitment to the Th1 lineage and for establishment of feedback loops that reinforce Th1 lineage commitment. Many activators but few repressors are known for ifng, which is induced by both TCR stimulation and cytokines IL-12 and IL-18. Th1 selective expression of IFN-γ is regulated by T-bet binding in both the proximal promoter (43, 44) and in distal regulatory elements of the ifng gene (46–48). Many activators of the ifng gene have been identified in the proximal promoter region, including STATs, NFAT, NF-κB, AP-1, Oct-1, and T-bet (43, 44, 49–51), but only Yin-Yang 1 (YY-1) has been shown to directly repress ifng gene expression (52). Later, however, this was found to result in transcriptional activation of IFN-γ expression in partnership with nearby NFAT sites (49). Foxo3a and Foxj1 can indirectly repress IFN-γ expression by directly repressing NF-κB (53, 54). Thus, Blimp-1 is one of the first direct repressors of ifng to be identified. Interestingly, the site recognized by Blimp-1, ~22 kb upstream of the ifng transcription start site, is in a conserved region that was shown to have histone 3 K4 methylation in Th1 cells but histone 3 K27 methylation in Th2 cells (55). Furthermore, this region has been shown to be required for IFN-γ expression in CD4 T cells, CD8 T cells, and NK cells (46), suggesting that Blimp-1 regulation of Th1 differentiation could extend beyond the setting of Th cells.

Blimp-1 also increases IL-10 production, evidenced by lower IL-10 production in CKO CD4 T cells (Fig. 3) (10). However, this probably involves regulatory as well as nonregulatory CD4 cells and is predicted to proceed by an indirect mechanism, since Blimp-1 is a transcriptional repressor in all situations studied thus far. Although we have not explored the mechanism of Blimp-1-dependent IL-10 regulation in this study, it is likely that in addition to direct repression by Blimp-1, increased IL-10 is another mechanism by which Blimp-1 attenuates IFN-γ production.

Blimp-1-dependent repression of tbx21, encoding T-bet

T-bet is the master transcription factor initiating and maintaining Th1 differentiation by driving high level of IFN-γ expression. In addition to ifng, T-bet also induces additional genes in the Th1 program, including those encoding IL-12Rβ2, HLX, and T-bet itself. When ectopically expressed in Th2 cells, T-bet reprograms them to a Th1 phenotype (56) both by inducing a Th1 program and by interfering with the Th2 program. Interference with the Th2 program occurs via a tyrosine phosphorylated form of T-bet that interferes with the ability of the master Th2 transcription factor GATA-3 to bind its cognate sites (57). Thus, by repressing tbx21 and ifng, Blimp-1 attenuates expression of both the master Th1 transcription factor and the critical Th1 cytokine.

Both STAT-1 and NF-κB can induce tbx21 expression; however, studies in T cells have not yet confirmed their cis-activating potential (6, 54, 58). Notch1 signaling activated by DCs can convert a basal repressor of T-bet, the recombination signal binding protein Jκ (RBJκ), into an activator that will drive T-bet expression (59, 60). Additionally, the forkhead transcription factor Foxj1 can indirectly repress T-bet induction and Th1 differentiation by repressing NF-κB activation (54). While GATA-3 can reverse Th1 differentiation and block T-bet activity, it also does so indirectly by downregulating STAT-4 (61). We have shown that Blimp-1 binds directly to a region containing a conserved consensus binding site in the proximal promoter of the tbx21 gene and to regions containing conserved and nonconserved consensus sites in the first intron. Binding of Blimp-1 to the tbx21 gene provides a mechanism by which this repressor can directly regulate Th1/Th2 polarization; however, future studies using retroviral transduction could provide further insight into the role of Blimp-1 in this process.

Mutual repression by Blimp-1 and Bcl-6

Data in this paper, in conjunction with previous reports (21, 31), provide strong evidence that Blimp-1 and Bcl-6 mutually repress one another by direct repression in both T and B cells. In B cells, this is important for mutually exclusive establishment of germinal center vs plasmacytic states of B cell differentiation (62, 63). Although it was known that Bcl-6 directly repressed prdm1 in B cells (31, 64), the data presented herein provide the first evidence that Blimp-1 directly represses bcl6 in B cells.
In CD4 T cells, our data support the notion that Blimp-1-dependent repression of bcl6 is important for mutually exclusive Th1 vs Th2 commitment. Bcl-6 is more highly expressed in Th1 cells than in Th2 cells (65, 66) and plays a role in Th cells of attenuating the expression or function of Th2 genes. Bcl-6-deficient mice have eosinophilic inflammation caused by elevated Th2 cytokines (29), and Bcl-6 competes with STAT-6 for binding to Th2 genes (24, 25). Bcl-6 also functions by other mechanisms in Th1 cells. It binds to a silencer region in i5 and directly represses its transcription (23). It induces degradation of GATA-3 by a poorly understood mechanism, leading to decreased expression of IL-10 (22). Finally, it directly represses prdm1, and we show in this work that Blimp-1 represses Th1 genes ifng and tshr1. Thus, repression of bcl6 by Blimp-1 would block the ability of Bcl-6 to repress Th2 gene expression. Reciprocally, repression of prdm1 by Bcl-6 blocks the ability of Blimp-1 to repress Th1 gene expression. This negative feedback loop between Blimp-1 and Bcl-6 in CD4 T cells is reminiscent in some ways of the feedback loop between T-bet and GATA-3, which also reinforces Th1 or Th2 differentiation states. Further studies using retroviral transduction of Blimp-1 or Bcl-6 in CD4 T cells will help to determine the extent to which Th lineage choice depends on direct mutual repression by these antagonistic transcription factors.

Importance of Blimp-1 in Th2 differentiation

We and others have shown that IL-4 signaling increases Blimp-1 mRNA expression (Fig. 2) (19). These data, along with the finding that Blimp-1 mRNA is highest in Th2 cells (Fig. 2A), support a particular role for Blimp-1 in Th2 cells.

Blimp-1-dependent repression of ifng, tshr1, and bcl6 provides a molecular mechanism for how Blimp-1 may function during Th2 polarization to repress Th1 genes. Indeed, an important role for Blimp-1 in Th2 responses is supported by our demonstration that the response of CKO mice to immunization with NP-KLH in alum, an adjuvant causing a predominantly IgG1 Th2 response (33), is defective, showing reduced IgG1 and increased IgM. It is also consistent with the acute colitis the CKO mice develop, because colitis is usually an aberrant Th1 response. However, since CKO mice show abnormally activated T cells as early as 4–5 wk it is possible that they were already predisposed to a Th1-like response, having an environment that disfavors Th2 responses. However, Blimp-1 is not strictly required for Th2 differentiation because Blimp-1-deficient CD4 cells develop normally into IL-4-producing cells when stimulated with strongly polarizing conditions (IL-4 and anti-IFN-γ) in vitro (Fig. 3A). Rather, we think that the role of Blimp-1 is to attenuate Th1 responses, thereby enhancing the commitment and establishment of Th2 responses in vivo. In this way its role is similar to that of Bcl-6. Bcl-6 KO CD4 T cells (67) polarize normally in vitro in contrast to their spontaneous cytokine skewing toward Th2 cell differentiation in vivo. In vitro polarization bypasses the effect of imbalances in endogenous cytokine production; therefore, unlike the obligatory requirement for STAT-1 and STAT-6 or T-bet and GATA-3 to potentiate cytokine signaling, Blimp-1 and Bcl-6 are repressors that attenuate Th differentiation during differentiation in vivo when signals for Th1 vs Th2 polarization are likely to be more subtle.

In summary, we have shown that Blimp-1 attenuates Th1 differentiation by directly repressing transcription of the signature cytokine, IFN-γ, and the master regulator of Th1 cell development, T-bet. Additionally, Blimp-1 also represses Bcl-6, thus expanding from B cells to T cells the settings in which these two transcriptional repressors can antagonize one another to promote alternative cell fates.