Blimp-1 directly represses *Il2* and the *Il2* activator *Fos*, attenuating T cell proliferation and survival

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Mice with a T cell–specific deletion of *Prdm1*, encoding Blimp-1, have aberrant T cell homeostasis and develop fatal colitis. In this study, we show that one critical activity of Blimp-1 in T cells is to repress IL-2, and that it does so by direct repression of *Il2* transcription, and also by repression of *Fos* transcription. Using these mechanisms Blimp-1 participates in an autoregulatory loop by which IL-2 induces *Prdm1* expression and thus represses its own expression after T cell activation, ensuring that the immune response is appropriately controlled. This activity of Blimp-1 is important for cytokine deprivation–induced T cell death and for attenuating T cell proliferation in antigen-specific responses both in vitro and in vivo.

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The terminal differentiation of effector lymphocytes after encounter with antigen is a complex and tightly regulated process that ensures rapid, but limited, immune responses. Transcription factors play crucial regulatory roles in the differentiation processes that culminate in the formation of effector and memory B and T cells (1, 2).

The transcriptional repressor B lymphocyte–induced maturation protein-1 (Blimp-1) is one of the few transcription factors known to be crucial for regulating B lymphocyte terminal differentiation. Blimp-1 is required (3) and sufficient (4) for the formation of fully functional antibody-secreting plasma cells and for maintenance of long-lived plasma cells in the bone marrow (5).

Blimp-1 is a SET domain and a zinc finger–containing transcriptional repressor encoded by the *Prdm-1* gene. Transcriptional repression by Blimp-1 is mediated by repressive modifications in chromatin structure, through recruitment of Groucho family transcriptional corepressors, and chromatin-modifying enzymes (for review see [6]). In addition to its crucial role in the differentiation of plasma cells, Blimp-1 has critical functions in embryonic development (7, 8), and targeted deletion of the *Prdm1* gene in the mouse is embryonically lethal (9). Blimp-1 is also required for terminal differentiation of several nonlymphoid cell lineages in adult organisms (for review see [6]).

Recently, a role for Blimp-1 in T cell differentiation was demonstrated in two laboratories (10, 11). After T cell receptor (TCR) stimulation, both $CD4^+$ and $CD8^+$ T lymphocytes express Blimp-1 mRNA in amounts comparable to that in fully mature plasma cells. Blimp-1 mRNA is also expressed at high levels in $Foxp3^+CD4^+$ regulatory T cells. Conditional deletion of Blimp-1 in T cells results in profound alterations of T cell homeostasis and function and culminates in the spontaneous development of fatal colitis (10, 11). Blimp-1–deficient $CD4^+$ regulatory T cells (T reg cells) are partially dysfunctional, as they perform properly in in vitro immunosupression assays and in one colitis model in vivo (11), but fail to inhibit chemically induced colitis in WT mice (10).

TCR stimulation of Blimp-1–deficient $CD4^+$ T cells in vitro results in hyperresponsiveness

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The online version of this article contains supplemental material.

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 205 No. 9 1959-1965
www.jem.org/cgi/doi/10.1084/jem.20080526

that is revealed by robust proliferation in suboptimal stimulatory conditions and increased numbers of IL-2 producers, indicating that Blimp-1 is a negative regulator of IL-2 production (10). This idea is also reinforced by two recent observations: (a) Blimp-1 and IL-2 expression upon TCR stimulation are inversely correlated, and (b) enforced expression of Blimp-1 in T cells represses IL-2 production upon TCR stimulation (12, 13).

IL-2 was initially identified as an autocrine cytokine necessary for in vitro expansion of T cells (14). Subsequent studies indicated a role for IL-2 in promoting T cell expansion, survival, effector differentiation, and memory cell survival via promotion of IL-7R expression (15). Indeed, the stimulatory properties of IL-2 make it a therapeutic target, especially in AIDS and cancer, where IL-2 administration promotes T cell expansion in vivo (15, 16). IL-2 has also been shown to participate in the contraction of inflammatory responses, by programming activated $CD4^+$ T cells for apoptosis (17, 18) and promoting the growth and survival of the innate $CD4^+FoxP3^+$T reg cells (15). Because of these effects, IL-2– or IL-2R–deficient mice exhibit a multifaceted autoimmune phenotype characterized by multiorgan inflammation, absence of T reg cells, and accumulation of autoreactive T cells (for review see [15]).

Interestingly, IL-2 represses its own expression in a classical negative-feedback loop that functions in a STAT-5–dependent manner (19). This finding, together with the observation that IL-2 is a potent inducer of Blimp-1 expression in T cells (12), led to the suggestion that Blimp-1 plays important roles in IL-2 autoregulation.

We have investigated this hypothesis, and report that Blimp-1 directly represses the *Il2* gene and indirectly represses *Il2* by repressing *Fos*, which encodes Fos; Fos is a component of AP-1, a strong activator of *Il2*. As a consequence of the increased production of IL-2 in the absence of Blimp-1, $CD4^+$ T cell proliferate more upon antigen-specific stimulation and are more resistant to cytokine deprivation–induced cell death. Attenuation of IL-2 production by Blimp-1 plays a role in an antigen-specific response in vivo. Thus, one important function of Blimp-1 in T cells is to attenuate IL-2 production upon antigen stimulation, by both direct and indirect gene repression.

RESULTS AND DISCUSSION

Blimp-1 attenuates IL-2 expression in the primary response

We have previously reported that lack of Blimp-1 results in increased proliferation and IL-2 production after polyclonal TCR stimulation (10). To further understand the mechanisms by which Blimp-1 regulates IL-2 production, we evaluated the kinetics of IL-2 production in an antigen-specific context. Blimp-1 conditional KO (CKO) mice were bred to OT2 TCR transgenic mice to generate antigen-specific, Blimp-1–sufficient (Ctrl) and –deficient (CKO) $CD4^+$ T cells. When naive ($CD44^{low}$) OT2 $CD4^+$ cells were stimulated in vitro with APCs and cognate antigen, the percentage of IL-2–producing cells was significantly higher in the CKO than in the Ctrl cultures in all time points evaluated. In agreement with results from polyclonal stimulation (10), TCR restimulation 3 d after primary activation also resulted in more IL-2–producing cells in the CKO cultures (Fig. 1 A). CKO cells proliferated more robustly, especially when antigen was provided in lower doses (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20080526/DC1). Thus, Blimp-1 attenuates proliferation and the number of IL-2–producing cells upon antigen-specific stimulation, and attenuation of IL-2 production by Blimp-1 can be observed at the time of primary stimulation.

IL-2 is known to induce Blimp-1 in B cells (4), and most of the induction of Blimp-1 mRNA and protein upon T cell activation has been shown to depend on IL-2 production (12). Consistent with our observation of increased IL-2 production in Blimp-1 CKO T cells (Fig. 1 A) (10), forced expression of Blimp-1 in T cells decreases expression of IL-2 (12, 13), and IL-2 causes its own down-regulation (12, 19). Given these complicated interactions, we investigated the kinetics of Blimp-1 and IL-2 expression in normal T cells, measuring steady-state mRNA levels. At days 1 and 2 after stimulation of naive $CD4^+$ T cells in vitro, IL-2 steady-state mRNA is more strongly induced than Blimp-1 mRNA. However, by day 3, Blimp-1 mRNA increases significantly and IL-2 mRNA decreases (Fig. 1 B). To study this at the single-cell level, we used a mouse in which Blimp-1 mRNA expression is reported by EGFP (7). Total spleen cells were stimulated in vitro and analyzed for IL-2 and GFP expression. 1 d after stimulation, <10% of the $CD4^+$ T cells expressed GFP, whereas ~5% of cells expressed IL-2. 3 d after stimulation, GFP expression increased to 17%, whereas IL-2 expression decreased to <3% (Fig. 1 C, top right). Restimulation on day 3 resulted in increased expression of both IL-2 protein and Blimp-1 mRNA, and addition of IL-2 amplified Blimp-1 expression, while decreasing IL-2 production (Fig. 1 C, bottom). Strikingly, in all time points, very few cells were double-positive for GFP and Blimp-1.

Previous studies show that IL-2 production is tightly regulated (20, 21), and that even under optimal conditions not all T cells in a population will acquire the competence to transcribe the *Il2* gene and synthesize IL-2 upon primary stimulation (22). The negative correlation between Blimp-1 expression and IL-2 production at the single-cell level (Fig. 1, B and C), along with the observation that more cells make IL-2 when Blimp-1 is absent (Fig. 1 A), provide evidence that expression of Blimp-1 is important in the exclusion of IL-2 production upon TCR stimulation. These results confirm and expand previous data (10, 12), supporting the model that upon T cell activation, most induction of Blimp-1 occurs secondary to IL-2 production and that the induced Blimp-1 participates in a regulatory loop to repress IL-2 expression.

Blimp-1 is required for cytokine deprivation–induced cell death after activation

IL-2 also has important effects on T cell apoptosis (18). After T cell activation, IL-2 production decreases, triggering passive cell death and limiting T cell numbers at the conclusion

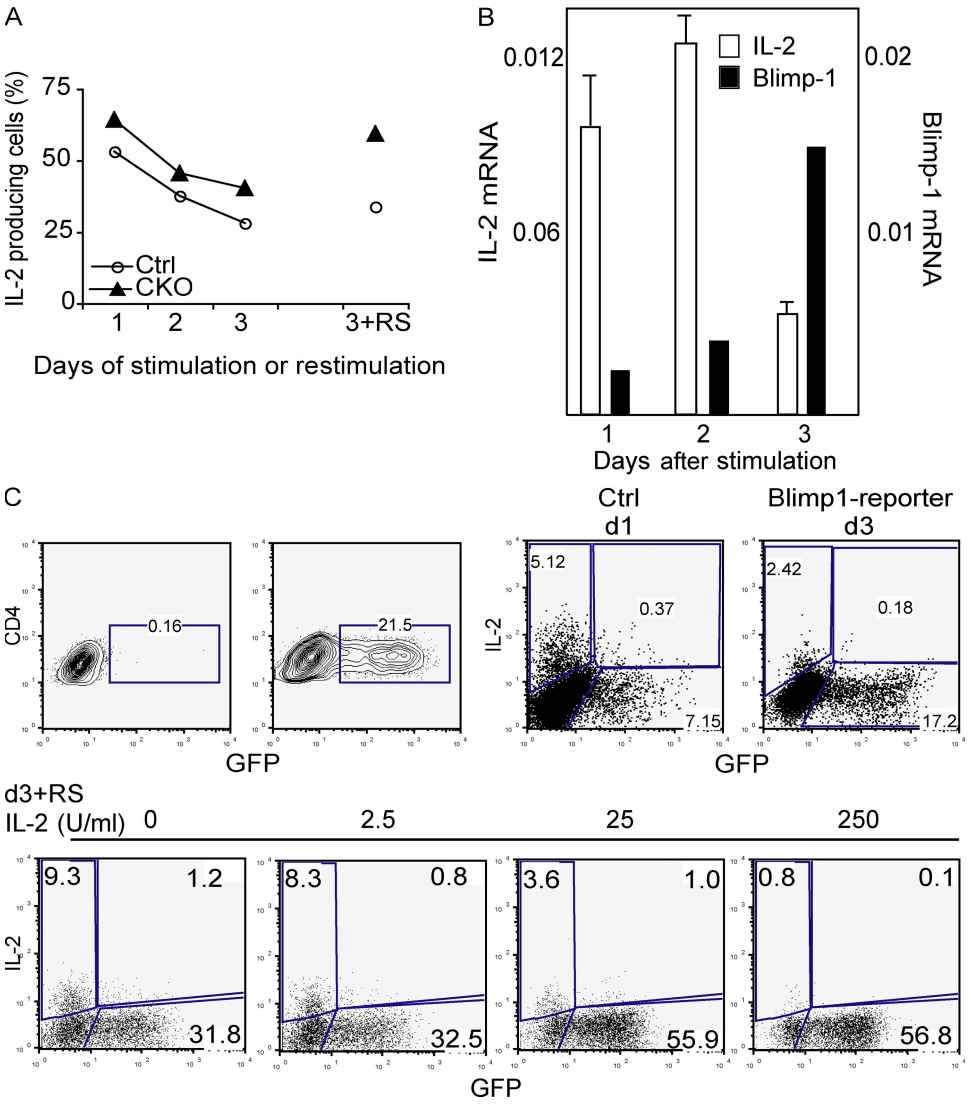

Figure 1. Expression of Blimp-1 and IL-2. (A) Production of IL-2 by naive CD4+ OT2 Blimp-1–sufficient (Ctrl) and –deficient (CKO) cells stimulated with 0.1 μM OVA and APC (determined as described in Materials and methods). Data shown is the percentage of IL-2–producing cells in the live, CD4+ cell gate. (B) Steady-state Blimp-1 and IL-2 mRNA (determined by quantitative real-time PCR) in naive WT CD4+ T cells stimulated as described in Materials and methods. (C) Few, if any, Blimp-1–expressing cells produce IL-2. Lymph node and spleen cells from Blimp-1-GFP reporter mice were stimulated (top) or restimulated (bottom) and stained for IL-2 and GFP. Plots show GFP and IL-2 in the CD4+ cells. Specificity of GFP staining is shown on the top left.

of an immune response. Because our data (Fig. 1) implicated Blimp-1 in postactivation down-regulation of IL-2, we next investigated whether Blimp-1 also plays a role in passive cell death after IL-2 withdrawal.

Naive CD4+ T cells from Ctrl or CKO mice were stimulated for 5 d, in optimal conditions, so IL-2 production was maximal and Blimp-1 expression was high in the Ctrl cells. 5 d after stimulation, cells were extensively washed and replated with or without exogenous IL-2. Blimp-1 CKO cells survived better in the absence of exogenous IL-2, and there were two- to threefold more live cells in CKO cultures compared with Ctrl (Fig. 2). Addition of IL-2 at the time of replating abrogated the differences between Ctrl and CKO cells, indicating that the increased IL-2 production in the absence of Blimp-1 was responsible for the increased survival of effector cells in this assay (Fig. 2). Thus, Blimp-1–dependent repression of IL-2 is important for passive cell death at the conclusion of an immune response.

Lack of Blimp-1 results in increased IL-2 and Fos mRNA

We wished to understand the molecular mechanism by which Blimp-1 controls IL-2 production. Although the production of IL-2 is regulated transcriptionally and posttranscriptionally (20, 21) because Blimp-1 is a transcriptional repressor, it probably regulates the transcription of the *Il2* gene, either directly and/or indirectly. To test this hypothesis, we first analyzed steady-state levels of IL-2 mRNA in cells from Ctrl and CKO mice. Naive CD4+ cells from CKO mice had significantly

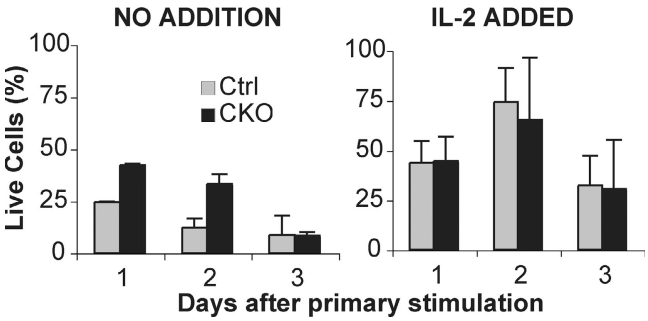

Figure 2. Attenuation of IL-2 production by Blimp-1 promotes susceptibility to IL-2 deprivation–induced cell death. Control and CKO naive CD4+ T cells were stimulated with plate-bound αCD3, αCD28, and IL-2 for 5 d (primary stimulation), washed, and replated in medium only (left) or in the presence of IL-2 (right). Cell death was determined by staining with Annexin V and 7-AAD. Results shown (mean and SEM from two independent experiments) are the percentage of Annexin V− and 7-AAD− cells.

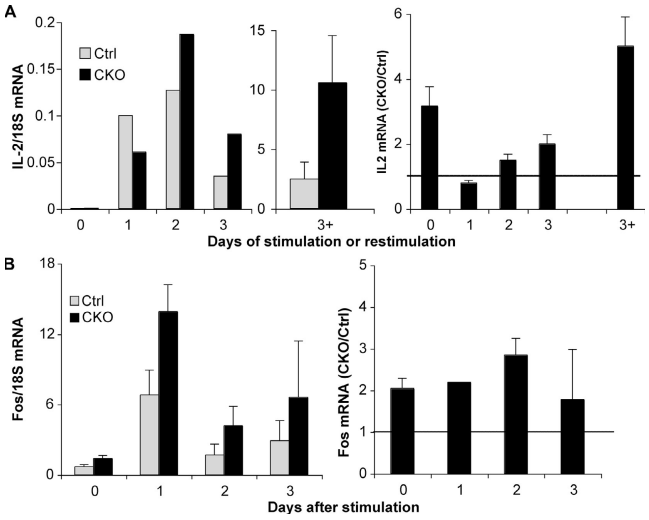

Figure 3. Blimp-1 deficiency results in increased levels of IL-2 and Fos steady-state mRNA. Steady-state IL-2 (A) and Fos (B) mRNA in naive (day 0) and stimulated CD4+ T cells from Control and CKO mice. In A, the middle graph shows steady-state IL-2 mRNA (normalized to 18 S) before and after restimulation. Ratios (CKO/Ctrl) from values in left graphs are shown in the right graphs. Results are representative of three to five experiments.

higher amounts of steady-state IL-2 mRNA than cells from Ctrl mice, before and at later time points after stimulation (days 2 and 3), but not earlier (Fig. 3 A). This suggests that low levels of Blimp-1 in naive cells (10) are sufficient to repress *Il2* before activation. Alternatively, or in addition, the repression of *Il2* by Blimp-1 in naive cells could be facilitated by the regulatory pathways operating before TCR stimulation, which differ considerably from the ones in place after stimulation (21). Upon activation of *Il2* by NFAT, members of AP-1 family and NF-κB (21), the relatively low amounts of Blimp-1 are apparently overcome, and Blimp-1 cannot repress *Il2*. However, as Blimp-1 levels rise, Blimp-1 is once again able to repress *Il2* transcription. If higher levels of Blimp-1 are, indeed, required to contain *Il2* transcription upon TCR stimulation, it would also explain why restimulation at day 3 results in more *Il2* mRNA in Blimp-1 CKO cells, as in these circumstances Blimp-1 expression in WT cells is further elevated (Fig. 1 C; unpublished data). Repression of *Il2* by Blimp-1 is also consistent with the recent observation that enforced expression of Blimp-1 represses IL-2 production upon TCR stimulation (12, 13).

Fos, a component of the AP-1 family of transcription factors, is one of the well-known transcriptional activators of the *Il2* gene in T cells (23, 24). Because our previous studies (25) showed that *Fos* is a direct target of Blimp-1–dependent repression in keratinocytes, we asked if Blimp-1 repressed *Fos* in T cells. Blimp-1–deficient CD4+ T cells stimulated (as described in Materials and methods) showed increased levels of Fos steady-state mRNA (Fig. 3 B). Thus, Blimp-1 normally down-regulates the steady-state mRNA of both *Il2* and its activator *Fos*.

Blimp-1 directly represses the *Il2* and *Fos* genes

Preliminary chromatin immunoprecipitation (ChIP) experiments showed increased specific binding of Pol II to the transcription initiation site of the *Il2* gene in activated CKO CD4 cells compared with Ctrl cells (unpublished data). Because a direct correlation between Pol II binding and transcription has been previously demonstrated for the *Il2* gene (26), this suggested that Blimp-1 directly represses *Il2* transcription. To test this hypothesis and identify Blimp-1 response elements, we searched the genomic sequence (plus 10 kb upstream and 10 kb downstream of the transcriptional start site [TSS]) of the mouse and human *Il2* genes, looking for Blimp-1 consensus binding sites (CBSs). We found 20 putative CBSs for Blimp-1 in the mouse *Il2* gene (Table S1, available at http://www.jem.org/cgi/content/full/jem.20080526/DC1), and 8 of these sites (−7,252, −5,547, −4,795, −4,765, −4,221, −4,207, −3,982, and −1,861) were contained in an 8.4-kb region upstream of the *Il2* TSS, which has been shown to be repressed by Blimp-1 in an in vitro reporter assay (12). We focused our efforts on these sites first; however, some sites were embedded in simple-sequence repeats and we were unable to design primers for efficient amplification of these regions. Thus, sites −3,982, −1,861, in the upstream region of the gene, and +10,215 and a region containing no Blimp-1 consensus sequence (+1,556 to +1,713 bp) relative to the TSS were investigated. We assayed binding of endogenous Blimp-1 by ChIP in WT CD4+ T cells after 6-d stimulation and restimulation in vitro, a condition where Blimp-1 expression is high (6). Out of the 3 Blimp-1 CBSs investigated, only site −1,861 was enriched for Blimp-1 binding (Fig. 4 A). Sites −3,982 and +10,215, as well as the irrelevant site, showed no significant enrichment (Fig. 4 A). Thus, Blimp-1 binds specifically at the site −1,861 of the *Il2* gene.

Previous studies showed that an 8.4-kb region of the *Il2* promoter, containing this site, conferred proper expression in

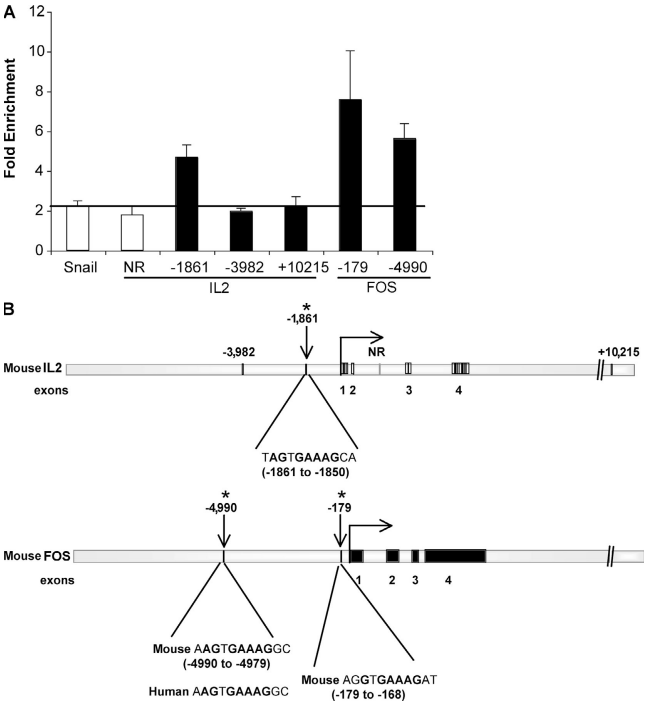

Figure 4. Blimp-1 binds to the mouse *Il2* and *Fos* genes. (A) ChIP of endogenous Blimp-1 bound in various regions of the *Il2* and *Fos* genes. WT purified CD4+ T cells were stimulated in vitro for 6 d, restimulated with PMA and ionomycin for 4 h, and cross-linked for ChIP. Data represent fold enrichment of αBlimp-1 antibody over control antibody. Snail3 is used as a negative control. NR is a region containing no Blimp-1 consensus sequence. Results shown are the mean and SEM from four to five different experiments. (B) Representation of the *Il2* and *Fos* mouse genes, with the sites shown in A marked. Sites enriched for Blimp-1 binding are marked with asterisks. The −4,990 site at the *Fos* gene is conserved between the human and mouse genes. No sites found at the *Il2* gene are conserved between mouse and human.

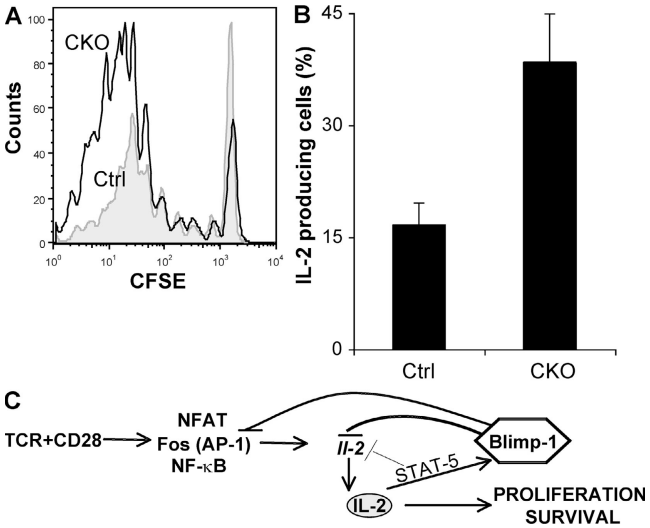

Figure 5. Lack of Blimp-1 results in increased proliferation and IL-2 production in vivo. CFSE-labeled naive Ctrl or CKO CD45.2 OT2 CD4+ T cells were injected i.v. into CD45.1 congenic WT mice. 2 d later, mice were immunized i.v. with antigen-pulsed APCs, and 5 d later, lymph nodes were collected and proliferation (A) and IL-2 production (B) were analyzed. Data in B are the mean (± SEM) of the percentage of IL-2–producing cells in the CD45-2+ gate in four different experiments. (C) Schematic representation of Blimp-1 participation in IL-2 autoregulation. Induction/activation of N-FAT, AP-1, and NF-κB upon TCR and CD28 stimulation leads to IL-2 transcription. IL-2 acting via STAT-5 leads to Blimp-1 expression. Blimp-1 then represses the *Il2* and *Fos* genes, inhibiting IL-2 production.

transgenic cells (27) and forced expression of Blimp-1 decreased expression of this transgene (12). Although another potential Blimp-1 CBS is present in the 8.4-kb region (site -3,982), this site does not seem to bind Blimp-1 in vivo (Fig. 5 A). Thus, the −1,862-bp site is likely to mediate repression of *Il2* by Blimp-1, although other currently untested sites in the 8.4-kb regulatory region (Table S1) may also contribute.

We also evaluated Blimp-1 binding to previously identified sites in *Fos* (25). Sites located at −179 and −4,990 bp from the TSS of mouse *Fos* are enriched for Blimp-1 binding in CD4+ T lymphocytes. These results, together with previous results (Figs. 1–3), demonstrate that Blimp-1 directly represses transcription of *Il2* and the *Il2* activator *Fos*.

We conclude that Blimp-1 attenuates IL-2 production both directly, by repression of the *Il2* gene, and indirectly, by repression of *Fos*. Nevertheless, the contribution of Fos to the overall increase in *Il2* transcription in the CKO cells is difficult to determine. Enforced expression of Fos in T cells caused elevated IL-2 production (28); however, deletion of Fos alone is insufficient to decrease IL-2 production (29), and the effects of Fos in regulating proliferation can only be observed when both FosB and Fos are missing (for review see [30]).

Lack of Blimp-1 leads to increased IL-2 production in vivo

Our data (Figs. 1–2 and 4), and that of others (12, 19), show that the transient nature of IL-2 production after T cell activation depends on induction of Blimp-1 by IL-2 and subsequent Blimp-1–dependent repression of IL-2 production via repression of *Il2* and *Fos*. To explore the role of Blimp-1–dependent repression of IL-2 in vivo, we transferred Ctrl and CKO CFSE-labeled naive CD4+ OT2 (CD45.2) into allotype congenic recipients (C57BL/6 SJ CD45.1), and then immunized the recipients with dendritic cells pulsed with OVA peptide. 5 d later, cells were recovered from lymph nodes of recipients and restimulated in vitro, and IL-2 production was evaluated in the CD45.2+ population by intracellular cytokine staining. At this time point, both proliferation and IL-2 production were more pronounced in the Blimp-1 CKO cells (Fig. 5, A and B). Approximately twofold more Blimp-1 CKO cells entered cell cycle compared with the Ctrl cells (Fig. 5 A). In addition, in mice injected with CKO cells, ∼40% of the CD45.2+ cells were producing IL-2, whereas 16% of the CD45.2+ cells were IL-2 producers in the mice injected with Ctrl cells (Fig. 5 B). Therefore, Blimp-1 attenuates CD4+ T cell proliferation and IL-2 production upon antigen-specific TCR stimulation in vivo (Fig. 5).

Thus, this study demonstrates that Blimp-1 represses IL-2 production after T cell activation and shows that the molecular mechanism responsible depends, at least in part, on Blimp-1–dependent repression of *Il2* and *Fos* transcription. Furthermore, we identify Blimp-1 response elements in these two genes. The conclusion that Blimp-1 represses IL-2 transcription is supported by several observations: (a) Blimp-1–expressing cells do not express IL-2 protein at detectable levels; (b) Blimp-1 mRNA induction correlates with IL-2 mRNA down-regulation; (c) IL-2 protein and steady-state mRNA are elevated in Blimp-1–deficient $CD4^+$ T cells; and (d) endogenous Blimp-1 specifically binds to a regulatory region in the *Il2* gene in activated primary $CD4^+$ T cells.

This establishes Blimp-1 as an important component in a recently described IL-2 autoregulatory loop (19) that operates in vivo to control the development and magnitude of T cell effector responses, and it confirms and extends the suggestion of Gong and Malek, that Blimp-1 plays an important role in IL-2 autoregulation (12).

Interestingly, after activation in vitro, IL-2 production in Blimp-1–deficient T cells eventually decreases, although more slowly than in Blimp-1–sufficient cells (Fig. 1 and Fig. 3 A). Thus, mechanisms in addition to Blimp-1 are apparently present for IL-2 down-regulation. Nonetheless, Blimp-1 is important physiologically for IL-2 repression because lack of Blimp-1 results in increased IL-2 production upon antigen immunization in vivo. Increased levels of IL-2 produced by CKO cells are associated with increased proliferation both in vitro and in vivo (Fig. S1 and Fig. 5), and with increased resistance to cytokine deprivation-induced cell death after activation in vitro (Fig. 2). Thus, deregulation of IL-2 production in the Blimp-1 CKO mice is likely to contribute to the aberrant T cell homeostasis and the inflammatory phenotype observed in these mice.

MATERIALS AND METHODS

Mice. C57BL/6 (B6) and B6 CD45.1 congenic mice were purchased from The Jackson Laboratory. *Prdm1*$^{flox/flox}$ (3) were backcrossed 10 times with B6 mice, and then crossed with B6CD4-Cre mice, which were purchased from Taconic, to generate B6 *Prdm1*$^{flox/flox}$CD4-Cre^+ (Blimp-1CKO) and *Prdm1*$^{+/+}$ CD4-Cre^+ (Control). For some in vitro experiments, mixed B6 × 129 *Prdm1*$^{flox/flox}$Lck-Cre *Prdm1*$^{+/+}$Lck-Cre (10) were also used. Mice bearing a BAC transgene-encoding membrane-target EGFP (mEGFP) under the control of Blimp-1 regulatory elements (Blimp-1 EGFP) (7) were a gift from M. Nussenzweig (The Rockefeller University, New York, NY) and were used as a reporter of Blimp-1 mRNA expression. In these mice, EGFP expression closely recapitulates Blimp-1 mRNA expression (7). For some experiments, spleen cells from Blimp-1 reporter mice were provided by S. Kaech's laboratory (Yale University, New Haven, CT). All mice were maintained in a specific pathogen–free animal facility at Columbia University and handled in accordance with the institutional guidelines. Animal experiments were approved by the Institutional Animal Care and Use Committee at Columbia University.

Cell isolation, stimulation, and IL-2 production. Naive $CD4^+$ ($CD44^{lo}$) cells were sorted using a FACSAria fluorescent cell sorter (BD Biosciences). Routinely, purity of all cell preparation was >90%. For Blimp-1 ChiP experiments, naive $CD4^+$ T cells were purified by negative selection using FITC-labeled antibodies and αFITC magnetic beads. The negatively isolated fraction contained >90% $CD4^+$ cells. Naive B6 OT2-TG *Prdm1*$^{F/F}$ CD4-Cre^+ or *Prdm1*$^{+/+}$ CD4-Cre^+ cells were stimulated in 48-well plates at a 1:1 ratio with APC (T cell–depleted and mytomycin-treated spleen cells) in a total of 5×10^5 cells per well with chicken OVA peptide (OVA 323–339; BP10-910 H2N-ISQAVHAAHAEINEAGR-OH; New England Peptide) for 3 d. IL-2 production was determined after incubation with 10 μg/ml Brefeldin A (BFA; Sigma-Aldrich) for the last 2 h of culture. When indicated, restimulation was done with 50 ng/ml PMA and 500 ng/ml ionomycin (both from Sigma-Aldrich) for 4 h, with BFA added in the last 2 h. IL-2 was detected by intracellular staining in combination with surface CD4 (both antibodies from BD Biosciences). Cells were analyzed in a LSRII flow cytometer (BD Biosciences). IL-2 staining was evaluated exclusively in the live, $CD4^+$ gated lymphocytes.

Simultaneous detection of Blimp-1 expression and IL-2 production. To evaluate IL-2 expression in Blimp-1-EGFP reporter cells, total spleen cells were stimulated with 1 μg/ml soluble αCD3 and 0.5 μg/ml αCD28 (both from BioExpress) with or without rHuIL-2 (QIAGEN). GFP expression was detected in permeabilized cells using an αGFP antibody (rabbit αGFP; Rockland Immunochemicals, Inc.) added simultaneously with the αIL-2 antibody. The αGFP antibody specifically stained GFP produced under the stimulation of the Blimp-1 transgenic promoter, as no staining was detected in nontransgenic cells stimulated and stained in the same way (Fig. 1 C).

Quantitative real-time PCR. For detection of Blimp-1, IL-2, and Fos mRNAs, naive $CD4^+$ T cells were stimulated with 5 μg/ml plate-bound αCD3, 2.5 μg/ml μg/ml αCD28, and 25 U/ml IL-2 for various time points. Total mRNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Reverse transcription was performed on equal amounts of RNA for each sample using SuperScript III (Invitrogen). SYBR Green incorporation quantitative real-time PCR was performed using a FastStart SYBR Green mix (Roche) in the ABI7400 Sequence Detection System (Applied Biosystems). Primers used were as follows: IL-2 forward, 5′-AGCAGCTGTTGATGGACCTA-3′; IL-2 reverse, 5′-CGCAGAGGTCCAAGTTCAT-3′ (designed using Primer 3 software). Primers for Blimp-1 and Fos were previously described (10, 25).

ChIP. ChIP assays were performed as previously described (25), with few modifications. Cells were fixed with 1.1% paraformaldehyde for 10 min at room temperature. Sonicated chromatin from $4–5 \times 10^7$ cells was immunoprecipitated with 25 μl of either rabbit αBlimp-1 polyclonal antibody serum (clone 267) or preimmune serum as a control. SYBR Green incorporation quantitative real-time PCR was performed in DNA recovered from immunoprecipitation and input samples (primers sequences in Table S2, available at http://www.jem.org/cgi/content/full/jem.20080526/DC1). Fold enrichment was calculated dividing the percentage of input values obtained with αBlimp by the one obtained with Ctrl antibody. Analysis of sequence homology and identification of putative Blimp-1 consensus sites were performed using the ECR browser (http://ecrbrowser.dcode.org) and rVista 2.0 software. Genomic sequences were obtained from Ensembl.

In vivo immunization. CFSE-labeled naive B6 OT2-TG *Prdm1*$^{F/F}$ CD4-Cre^+ or *Prdm1*$^{+/+}$ CD4-Cre^+ cells ($CD45\text{-}2^+$) (1×10^6) were transferred (i.v.) to allotype-marked congenic recipients (C57Bl/6 SJ CD45.1). 2 d after cell transfer, mice were immunized i.v. with GMCSF-bone marrow–derived dendritic cells matured with LPS and IL-4 and pulsed with the cognate peptide (1 μg/ml OVA 334–339). 5 d later, lymph nodes were recovered and CFSE dilution was evaluated in the $CD45.2^+$ cells. Alternatively, freshly harvested total lymph node cell suspensions were restimulated in vitro with PMA and ionomycin (with BFA added in the last 2 h), and IL-2 production was evaluated at the single-cell level in the $CD45\text{-}2^+$ population by intracellular cytokine staining.

Cytokine deprivation cell death assay. Naive $CD4^+$ T cells were stimulated (as described in Quantitative real-time PCR) for RNA isolation and

cultured for 5 d. On day 5, cells were washed extensively in complete RPMI medium and replated at the same initial density in the presence (50 U/ml) or absence of rHuIL-2. The percentage of live cells (7-AAD$^-$/Annexin V$^-$) was assessed 1, 2, or 3 d later.

Online supplemental material. Fig. S1 shows that lack of Blimp-1 results in increased proliferation upon antigen-specific stimulation in vitro. Table S1 shows the putative Blimp-1 binding sites at the *Il2* gene. Table S2 lists the primers used for Blimp-1 ChIP at the *Il2* gene. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20080526/DC1.

We thank Dr. H. Gu for advice and for critically reading the paper; the Calame laboratory for advice; Dr. Boris Reizis and his laboratory for advice (especially Jose Galan-Caridad and Michele Caton for advice on ChIP and i.v. injections). We thank Kristie Gordon and the staff at Columbia FACS core facility for sorting. We are grateful to Dr. Michel Nussenzweig for the Blimp-1 reporter mice and to Rachel Rutshauser and Susan Kaech for advice and reagents for Blimp-1 reporter mice experiments. We thank QIAGEN for the gift of rHuIL-2.

This work was supported by RO1-AI50659 and RO1-AI43576 to K. Calame.

The authors have no conflicting financial interests.

Submitted: 12 March 2008
Accepted: 17 July 2008

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