Blimp-1 represses CD8 T cell expression of PD-1 using a feed-forward transcriptional circuit during acute viral infection

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Acute viral infection or immunization results in the generation of highly functional memory CD8 T cells that are poised to rapidly control secondary immune challenges to the cognate pathogen. During the early stages of CD8 T cell effector responses, the programmed cell death 1 (PD-1 or CD247) inhibitory receptor is transiently expressed (Wherry et al., 2007). As such, it is not surprising that PD-1 was found to play a role during acute infections. Depending on the infection system, genetic or immunological inhibition of the PD-1 pathway was shown to influence disease progression, pathology, and the generation of immunity (Lafon et al., 2008; Lázár-Molnár et al., 2008). The observed differences in PD-1’s role likely reflect the biology of the pathogen being tested, immunization regimens, and type of immune response required for clearance. Importantly, these systems demonstrate a role for PD-1 in acute infections.

In contrast, during persistent exposure to antigen or chronic infection, antigen-specific CD8 T cells do not acquire the heightened ability to recall effector function. These dysfunctional CD8 T cells were termed exhausted CD8 T cells (Zajac et al., 1998). The PD-1 signaling pathway is responsible for the generation of exhausted CD8 T cells in numerous settings involving persistent antigen, including those derived from chronic viral infections of HIV, HCV, HBV, lymphocytic choriomeningitis virus (LCMV), and SIV, as well as from cancer (Barber et al., 2006; Day et al., 2006; Urbani et al., 2006; Radziewicz et al., 2007; Velu et al., 2007; Peng et al., 2008; Fourcade et al., 2010). Antibody blockade of the PD-1 signaling pathway results in reinvigoration of exhausted CD8 T cell’s immune responses (Barber et al., 2006). Recently, antibody blockade of the PD-1 pathway was demonstrated to have a profound positive effect on variety of late stage cancers, including a few cases of complete cure (Brahmer et al., 2010, 2012; Topalian et al., 2012a,b). Together, these reports highlight the importance of the PD-1 pathway in the treatment of infectious disease and cancer.

In CD8 T cells, PD-1 expression is regulated at the level of transcription. Two upstream conserved regulatory regions termed conserved regions B and C (CR-B and CR-C) were found to be hypersensitive to DNase I and important for PD-1 expression during the early stages of CD8 T cell differentiation after acute infection with lymphocytic choriomeningitis virus (LCMV) strain Armstrong. Blimp-1 represses PD-1 expression through a feed-forward repressive circuit by regulating PD-1 directly and by repressing NFATc1 expression, an activator of PD-1 expression. Blimp-1 binding induces a repressive chromatin structure at the PD-1 locus, leading to the eviction of NFATc1 from its site. These data place Blimp-1 at an important phase of the CD8 T cell effector response and provide a molecular mechanism for its repression of PD-1.
to CR-C after translocation to the nucleus (Oestreich et al., 2008). The transient activation of PD-1 mentioned above suggests that there may be a mechanism that directly represses its expression after initial activation events. One candidate could have been T-bet, a transcriptional repressor which can modulate PD-1 expression through a region close to CR-B (Kao et al., 2011). However, T-bet’s repressive effect on PD-1 expression is not robust when overexpressed, suggesting that other factors may play a role in silencing PD-1 expression at late effector CD8 T cell stages.

The B lymphocyte–induced maturation protein 1 (Blimp-1) encoded by the pdim1 gene is a transcriptional repressor that is required for terminal differentiation of B cells into plasma cells (Turner et al., 1994; Shaffer et al., 2002; Shapiro-Shelef et al., 2003). Blimp-1 was found to be induced during the later stages of CD8 T cell activation and was shown to be required for the efficient terminal differentiation of effector CD8 T cells (Kallies et al., 2009; Rutishauser et al., 2009). Mice with Blimp-1–deficient CD8 T cells display reduced efficiency in clearing an acute infection due to reduction in some effector and homing functions (Kallies et al., 2009; Rutishauser et al., 2009). Moreover, these mice developed a greater quantity of memory CD8 T cells possessing enhanced abilities to recall effector cytokine expression than their WT littermates, suggesting that Blimp-1 controls the cell fate differentiation between terminal effector and memory CD8 T cells. In addition, Blimp-1 is a transcriptional antagonist of Bcl6, a factor which promotes differentiation and PD-1 expression in Tfh cells (Johnston et al., 2009). Together, these data suggest the hypothesis that Blimp-1 may directly regulate PD-1 expression during the acute phase of CD8 T cell activation. To test this hypothesis, we used three model systems that take advantage of the ability to conduct biochemical and molecular studies, as well as provide in vivo relevance. We found that Blimp-1 directly represses PD-1 gene expression in CD8 T cells using three distinct molecular mechanisms: regulation of the expression of PD-1’s activator NFATc1; alteration of the local chromatin structure; and eviction of the activator NFATc1 from its site that controls PD-1 expression. These mechanisms demonstrated that Blimp-1 at the center of the PD-1 pathway during the early stages of CD8 T cell responses as a direct modulator of the functional activity of these cells.

RESULTS
Blimp-1 expression inversely correlates with PD-1 expression during ex vivo CD8 T cell activation
During the initial phases of CD8 T cell activation, PD-1 is transiently expressed (Wherry et al., 2007). Given that the transcriptional repressor Blimp-1 has been shown to play important roles during CD8 T cell differentiation (Johnston et al., 2009; Kallies et al., 2009; Rutishauser et al., 2009) and that Blimp-1 expression is correlated with repression of PD-1 expression on Tfh and Treg cells during their differentiation (Johnston et al., 2009; Cretney et al., 2011), we proposed that Blimp-1 could also be the repressor of PD-1 expression during CD8 T cell activation. To test this, EL4 T cells, which constitutively express high levels of PD-1 (Oestreich et al., 2008), were transduced with an adenovirus expression vector that expresses Flag-tagged Blimp-1 and GFP or just GFP. PD-1 expression was reduced >10-fold in this system when the transduced virus expressed Blimp-1 (Fig. 1 A).

To further examine a role for Blimp-1, the expression of Blimp-1 and PD-1 was examined in murine splenic CD8 T cells after ex vivo activation using anti-CD3/CD28 beads. As observed in Fig. 1 B, this activation protocol induced PD-1 transiently over a 5-d (120 h) time course with the peak of expression appearing at 24 h. At the 120-h time point, PD-1 expression returned to baseline levels. In contrast, Blimp-1 gene expression was induced only a few-fold above base line at 24 h and peaked at 72 h. The expression of Blimp-1 was maintained at the peak levels through the 120-h time point. Thus, Blimp-1 and PD-1 expression were inversely correlated. Because loss of its activator NFATc1 (Oestreich et al.,...
Blimp-1 regulates PD-1 and NFATc1 gene expression

To determine if Blimp-1 was influencing PD-1 expression through its proximal upstream regulatory elements, luciferase gene reporter assays were conducted using a series of PD-1 promoter region constructs (Fig. 2 A). Both the PD-1B/C and PD-1B luciferase reporter constructs, which contain both CR-B and CR-C or just CR-B, respectively, were shown previously to possess moderate activity in EL4 cells, and treatment with PMA/ionomycin slightly enhances the activity of the PD-1B luciferase reporter and strongly enhances the activity of the PD-1B/C luciferase reporter (Oestreich et al., 2008). EL4 cells were co-transfected with the PD-1B/C or PD-1B reporter vectors and with a Blimp-1 cDNA expression vector or control vector. The reporter vector containing only CR-B was unaffected by Blimp-1 overexpression. In contrast, the reporter plasmid containing the entire PD-1 sequence between CR-B and CR-C was repressed by Blimp-1 to \( \leq 20\% \) of the levels of the control (Fig. 2 B). These data suggest that Blimp-1 acts on the sequence upstream of CR-B.

A bioinformatics search of this portion of the PD-1 sequence identified three potential Blimp-1 binding sites: site 1, 5'-TAGTTTCAGTC-3' (−366 to −355); site 2, 5'-AAGAGAAAGAT-3' (−786 to −775); and site 3, 5'-AAGAGAAAGAT-3' (−1,603 to −1,592; Fig. 2 A). These homologous potential Blimp-1 sites, which are equivalent in position to sites 1 and 2 in the mouse, also exist in the human PD-1 gene: −285 to −274 (5'-TATGGAAAGAT-3') and −670 to −659 (5'-GTTGGAAAGAT-3'). Nucleotide substitution mutations were introduced into each of these sites (Fig. 2 A) and their ability to resist Blimp-1–mediated repression was assessed as above in EL4 cells. Mutations at sites 1 and 3 did not affect Blimp-1 repression of the reporter, whereas the mutation at site 2 reverted expression to \( \leq 75\% \) of the control transfection (Fig. 2 C, left). Similarly, complete deletion of site 2 had a similar effect on restoring the expression of the luciferase.
reporter (Fig. 2 C, right). These data suggest that site 2 is responsible for Blimp-1-mediated repression of PD-1 expression.

It was expected that the deletion of the Blimp-1-response element (site 2) would have completely negated the repressive effect of Blimp-1 on the PD-1 reporter plasmid. One possibility explaining why this did not occur could be due to a loss of the activator, NFATc1. A previous report suggested that Blimp-1 antagonized NFATc1 expression (Martins et al., 2008), but a direct link was not provided. Therefore, to determine if NFATc1 expression was reduced upon Blimp-1 expression, EL4 cells were transduced with the Blimp-1 or control adenovirus expression vectors, and NFATc1 protein and mRNA were examined. The levels of NFATc1 protein and mRNA were reduced after ectopic Blimp-1 expression (Fig. 2 D and E). This repression is likely mediated directly at the level of transcription of the NFATc1 gene, as the expression of a luciferase reporter construct containing 5‘ NFATc1 DNA was also reduced in the presence of ectopic Blimp-1 expression (Fig. 2 F). Thus, these assays showed that Blimp-1 mediates repression of both PD-1 and NFATc1 through DNA sequences associated with their respective genes.

Blimp-1 binds directly to the PD-1 gene

Chromatin immunoprecipitation (ChIP) assays were conducted on EL4 cells and ex vivo activated CD8 T cells to determine if Blimp-1 was in fact bound to site 2 under conditions in which Blimp-1 was expressed. The adenovirus transduction expression system was again used to ectopically express Flag-Blimp-1 in EL4 cells, which constitutively express PD-1. The ChIP assays showed that the Flag-Blimp-1 protein was bound at site 2 and not at the other putative sites (Fig. 3, A and B). No binding was detected in the control samples or assays. Similarly, additional ChIP assays showed that endogenous Blimp-1 was bound to site 2 and not the other sites in splenic CD8 T cells that were stimulated ex vivo with anti-CD3/CD28 beads for 5 d (Fig. 3 C). In these assays, a polyclonal antisera to Blimp-1 (Rockland Immunocyticals, Inc.) was used. This antisera recognizes Blimp-1 in nuclear lysates prepared from LPS-induced plasma cells from WT mice but not from similarly treated B cells from a Blmp1fl/fl; TgCD19-Cre mouse (Shapiro-Shelef et al., 2003) in which Blimp1 is conditionally deleted in the B cell lineage (Fig. S1).

To determine if Blimp-1 binds to site 2 in antigen-specific CD8 T cells, naive Thy1.2+ Db-GP33+CD8 T cells were adoptively transferred into Thy1.2+ C57BL/6 mice and analyzed 8 d after infection with LCMV Armstrong. At day 8 after infection, a time point in which PD-1 expression has waned (Wherry et al., 2007), LCMV-specific P14 CD8 T cells were FACS-sorted using Db-GP33 tetramers (Murali-Krishna et al., 1998) and assessed for Blimp-1 binding by ChIP assays. As above, Blimp-1 was bound to site 2 but not the other sites (Fig. 3 D). These data therefore demonstrate that Blimp-1 directly binds site 2 of the PD-1 gene in vitro, ex vivo, and in vivo systems.

Blimp-1 binding leads to the eviction of NFATc1 from the PD-1 gene

Previously, we showed that NFATc1 bound to CR-C of the PD-1 gene in response to activation signals (Oestreich et al., 2008). To investigate the binding relationship between the factors, three model systems were used. ChIP assays after ectopic
expression of Blimp-1 were performed in EL4 cells. The results showed that NFATc1 was bound in GFP-transduced control cells but not in the Blimp-1–transduced cells (Fig. 4 A). In ex vivo activated splenic CD8 T cells, NFATc1 binding was readily detected at day 1 and significantly reduced at day 5 after stimulation. In contrast, Blimp-1 was bound at day 5 but not at day 1 in these cells (Fig. 4 B). Using the LCMV model as above, FACS-sorted, LCMV-specific CD8 T cells were examined for NFATc1 and Blimp-1 binding at days 6 and 8 after LCMV Armstrong infection. Although PD-1 expression is waning by day 6, this was the most practical time point to obtain sufficient antigen–specific cells for ChIP. Nonetheless, NFATc1 binding was detected at day 6 but not at day 8 (Fig. 4 C). Blimp-1 binding showed the reciprocal pattern in these cells. These results are consistent with the exchange of these factors and their positive and negative regulation of PD-1 gene expression.

The above data do not rule out the possibility that the loss of NFATc1 binding was due to Blimp-1 repressing its expression as implicated in Fig. 2. To determine if Blimp-1 influences the binding of NFATc1 directly, NFATc1 and Blimp-1 were expressed ectopically in a sequential manner using vectors that were not influenced by either factor. EL4 cells were first transduced with either an NFATc1-GFP chimeric protein or GFP-only adenovirus expression vector. At 24 h, a second transduction was performed with Blimp-1 or GFP adenovirus expression vectors, such that one culture received NFATc1 then Blimp-1. At the final 48-h point, cells were harvested and assessed for transcription factor binding, PD-1 mRNA expression, and expressed proteins. ChIP analyses demonstrated that NFATc1 was bound in the control (GFP) adenovirus expressing an NFATc1-GFP chimeric protein or GFP alone, stimulated with PMA/ionomycin for 24 h and then transduced again with adenovirus vectors expressing either Flag-tagged Blimp-1 or GFP. After a second 24-h period, the cultures were harvested and examined in D by ChIP for NFATc1/NFATc1-GFP (anti-NFATc1 or anti-GFP antibody) or Flag-Blimp-1 (anti-Flag antibody) binding as above (column numbers are for reference to the text); in E for expression of PD-1 mRNA by real-time RT-PCR; and in F by immunoblotting for expression of the transduced genes with anti-GFP and anti-Flag antibodies. Data are representative of at least three independent experiments (mean ± SD). A two-tailed Student’s t test was used to determine significance. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Blimp-1 binding results in the formation of a repressive chromatin structure at CR-C and CR-B

One way in which Blimp-1 could mediate repression of PD-1 could be through changes in accessibility to the PD-1 regulatory regions. To test this possibility, the sensitivity of the PD-1 promoter to DNase I and the histone modifications associated with CR-B and CR-C were determined. As above, EL4 cells were transduced with a GFP or Blimp-1 adenovirus expression vector and assessed. Control GFP-transduced EL4 cells showed a high level of sensitivity to DNase I at CR-C, whereas Blimp-1 adenovirus-transduced cells displayed near base line levels (Fig. 5 A). These data suggest that Blimp-1 binding to PD-1 leads to a closed or inaccessible chromatin domain at CR-C. Analyses of active and repressive histone modifications at CR-B and CR-C indicated that control adenovirus-transduced EL4 cells displayed only active modifications, including histone H3K9Ac, H3K27Ac, and H3Ac (K9Ac and K14Ac), as well as histone H3K4me2 and H3K4me3, with the latter being associated with active transcription (Fig. 5 B). In contrast, Blimp-1 adenovirus-transduced EL4 cells showed strong reductions in all of the active histone modifications. In agreement with a repressive role for Blimp-1, the Blimp-1 adenovirus-transduced cells displayed a marked increase in the three major repressive modifications H3K9me3, H3K27me3, and H4K20me3.
Blimp-1–deficient CD8 T cells do not acquire repressive histone modifications

Similar to other studies (Rutishauser et al., 2009), a conditional Blimp-1–targeted mouse (Shapiro-Shelef et al., 2003) was crossed to the Granzyme B Cre mouse (Jacob and Baltimore, 1999), and mice homozygous for the Blimp-1 alleles and a Granzyme B Cre allele were obtained. The mouse was termed Blimp-1 conditional KO (cKO) mouse. Granzyme B is expressed after T cell activation, resulting in a targeted deletion within the Blimp-1 gene after TCR stimulation. Ex vivo culture of splenic CD8 T cells from Blimp-1 cKO mice with anti-CD3/CD28 beads was sufficient to eliminate Blimp-1 mRNA accumulation (Fig. 6 A) and created a system to explore PD-1 regulation in the absence of Blimp-1. A time course of PD-1 expression was examined over 72 h and indicated that PD-1 levels were sustained in Blimp-1–deficient CD8 T cells compared with WT CD8 T cells. PD-1 mRNA was measured by real-time RT-PCR, and results were normalized to 18S rRNA and plotted relative to the expression in unstimulated WT CD8 T cells. Data were representative of three independent experiments (mean ± SD). A two-tailed Student’s t test was used to determine significance. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Control sequences and control antibody showed no modifications or significant precipitation in these assays (Fig. 5 B).

Using the ex vivo CD8 T cell activation system (Fig. 1 B), CD8 T cells at days 0, 1, and 5 were assessed for the same active and repressive marks described above. At day 1, all of the active modifications at CR-B were significantly increased compared with day 0, and the repressive modifications were similar to day 0 (Fig. 5 C). CR-C also showed increases for all the active marks, except H3K4me3 which was not increased. This was expected, as this modification is found on nucleosomes juxtaposed to transcription start sites and CR-C is ~1 kb upstream of that site. At day 5, all active modifications returned to the day 0 levels and the three repressive modifications were observed. This time point occurs after Blimp-1 is expressed and is bound to site 2. Together, these data strongly argue that Blimp-1 mediates the conversion of an active chromatin region to one that is repressive in its structure.
Thus, the absence of Blimp-1 leads to prolonged activation of PD-1 and a failure to remodel the chromatin of the PD-1 promoter region into a repressive state. To determine if the prolonged expression of PD-1 was due to the presence of NFAT activity, CD8 T cells were stimulated ex vivo for 24 h and then treated with cyclosporine A (CsA) to inhibit NFAT activation through the calcineurin pathway. Previously, we showed that inhibitors of this pathway could inhibit PD-1 expression (Oestreich et al., 2008; Araki et al., 2010). Indeed, CsA treatment of the Blimp-cKO CD8 T cells resulted in decreased PD-1 expression compared with untreated Blimp-cKO cells (Fig. 6 E). These data suggest that in the absence of Blimp-1, PD-1 expression is maintained by NFATc1.

Blimp-1–deficient LCMV-specific effector CD8 T cells have higher PD-1 expression after acute infection

To further assess the impact of Blimp-1 deletion on PD-1 expression in vivo, PD-1 expression in virus-specific CD8 T cells generated during acute viral infection was measured by flow cytometry. WT and Blimp-1 cKO mice were acutely infected with LCMV Armstrong, and effector CD8 T cells were harvested from the spleen 8 d after infection. Consistent with the above results (Fig. 6), Blimp-1 cKO antigen-specific effector CD8 T cells were also sustained at higher levels in the Blimp-1 cKO mice compared with the wild type (Fig. 6 A).

To examine an in vivo role for Blimp-1 in the regulation of PD-1, Blimp-1 cKO and WT mice were infected with LCMV Armstrong, and antigen-specific CD8 T cells were isolated for analysis at day 8 after infection. The day-8 time point is near the end of the acute infection where viral loads are reduced and Blimp-1 expression is high and PD-1 levels are low (Wherry et al., 2007; Rutishauser et al., 2009). Compared with CD8 T cells from WT mice, PD-1 and NFATc1 mRNA levels were increased four- and twofold, respectively, in the CD8 T cells from Blimp-1 cKO mice. Blimp-1 levels were substantially diminished in this system (Fig. 6 B). ChIP assays conducted on these cells showed that Blimp-1 was present at site 2 in the WT CD8 T cells but not in the Blimp-1–deficient CD8 T cells at day 8 after infection (Fig. 6 C). In contrast, NFATc1 was present at CR-C in the Blimp-1–deficient CD8 T cells but not in the WT CD8 T cells at day 8 after infection (Fig. 6 C), providing in vivo support for the in vitro analyses presented in Fig. 4. The Blimp-1–deficient antigen-specific CD8 T cells showed increased levels of the active histone modification H3K4me3 and decreased accumulation of the repressive modification H3K27me3 (Fig. 6 D).

Thus, the absence of Blimp-1 leads to prolonged activation of PD-1 and a failure to remodel the chromatin of the PD-1 promoter region into a repressive state. To determine if the prolonged expression of PD-1 was due to the presence of NFAT activity, CD8 T cells were stimulated ex vivo for 24 h and then treated with cyclosporine A (CsA) to inhibit NFAT activation through the calcineurin pathway. Previously, we showed that inhibitors of this pathway could inhibit PD-1 expression (Oestreich et al., 2008; Araki et al., 2010). Indeed, CsA treatment of the Blimp-cKO CD8 T cells resulted in decreased PD-1 expression compared with untreated Blimp-cKO cells (Fig. 6 E). These data suggest that in the absence of Blimp-1, PD-1 expression is maintained by NFATc1.

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effector CD8 T cells had a strikingly higher level of PD-1 expression relative to WT effector CD8 T cells, indicating that Blimp-1 serves as a repressor of PD-1 expression at the effector stage of the immune response (Fig. 7, A and B).

It has been previously shown that Blimp-1 cKO mice may have delayed clearance of LCMV infection (Rutishauser et al., 2009) and, thus, the higher level of PD-1 expression on CD8 T cells in the Blimp-1 cKO mice may have been skewed by the prolonged antigen presentation. To facilitate the control of the LCMV infection in the Blimp-1 cKO mice and to serve as an internal control for WT levels of PD-1 expression, 10^6 naive Thy1.1, LCMV GP33-specific TCR-transgenic CD8 T cells (P14), which are WT for Blimp-1, were adoptively transferred into WT and Blimp-1 cKO mice (Thy1.2+). After LCMV Armstrong infection, analysis of both the adoptively transferred and endogenous CD8 T cells was performed at day 8 after infection (Fig. 7, C–E). Using PD-1 expression from the transferred P14 CD8 T cells as a sensor for antigen, the relative level of antigen exposure to the endogenous CD8 T cells in the Blimp-1 cKO mice compared with that in WT mice could be assessed. PD-1 expression on the transferred P14 cells was the same regardless of the recipient host, indicating that the high level of transferred P14 CD8 T cells facilitated control of the viral infection in the Blimp-1 cKO mice (Fig. 7 C). In contrast, endogenous Blimp-1–deficient GP276 and NP396-specific CD8 T cells had a significantly higher level of PD-1 expression relative to the WT antigen-specific CD8 T cells (Fig. 7, C and D). These data indicate that the increase in PD-1 expression on the Blimp-1 cKO virus-specific CD8 T cells was a result of a deficiency in Blimp-1 repression of PD-1 expression. A pilot longitudinal analysis of PD-1 expression on the endogenous antigen-specific CD8 T cells revealed that Blimp-1 deficiency resulted in a delay in the down-regulation of PD-1 expression (Fig. 7 E).

To further differentiate between a cell-intrinsic contribution of Blimp-1 deficiency on the prolonged PD-1 expression in virus-specific CD8 T cells versus a cell extrinsic influence due to delayed viral clearance in Blimp-1 cKO mice, PD-1 expression was examined on WT and Blimp-1 cKO CD8 T cells derived from mixed bone marrow chimera mice. Chimeric mice were generated by adoptively transferring 2.5 × 10^6 congenically distinct WT and Blimp-1 cKO bone marrow chimera mice. Chimeric mice were housed in a sterile environment for 6 wk to allow for reconstitution. Chimeric mice were then acutely infected with LCMV Armstrong and the level of PD-1 expression was measured on antigen-specific WT and Blimp-1 cKO effector CD8 T cells at 8 d after infection (Fig. 8). Both WT and Blimp-1 cKO CD8 T cell populations generated a comparable number of antigen-specific CD8 T cells in response to the viral infection (Fig. 8 A). Similar to a previous study (Rutishauser et al., 2009), Blimp-1 cKO LCMV-specific CD8 T cells had a higher level of CD127 expression (not depicted). Importantly, Blimp-1 cKO effector CD8 T cells specific to multiple LCMV epitopes—GP33, GP276, and NP396—all had a significantly higher level of PD-1 expression relative to WT populations (Fig. 8, B and C). Collectively, these data suggest that Blimp-1 was responsible for the delay of PD-1 repression in vivo.

**DISCUSSION**

Evidence is increasing that PD-1 plays an important role in the development of T cell responses during the initial stages of immune responses through its immediate but transient expression (Wherry et al., 2007; Lafon et al., 2008; Lázár-Molnár et al., 2008; Brown et al., 2010). The down-regulation...
of PD-1 during acute infection suggested that there might be a direct transcriptional repressor whose identity was not previously determined. Here, we showed that Blimp-1 functions as that transcriptional repressor. We found that Blimp-1 uses multiple mechanisms to achieve its goal. First, Blimp-1 binds to a site between CR-B and CR-C of the PD-1 gene and directly represses PD-1 transcription. Second, Blimp-1 also inhibits the expression of NFATc1 and displaces it from CR-C, thereby removing the transcriptional activator that is induced after TCR stimulation. These direct and indirect processes form a coherent feed-forward repressive circuit that is similar to the one used by Blimp-1 to directly repress IL-2 expression and its activator c-Fos after T cell activation (Gong and Malek, 2007; Martins et al., 2008). By targeting both the activator of a gene and the gene itself, coherent feed-forward repressive circuits have greater potential to quickly and more effectively turn off target genes. Furthermore, the existence of potential Blimp-1-binding sites in the human PD-1 gene promoter suggests that this mechanism could be conserved in mammals. Blimp-1 is also important for the development of terminal effector CD8 T cells (Kallies et al., 2009; Rutishauser et al., 2009), suggesting that the silencing of PD-1 expression during acute infections may be critical for protective immunity.

The loss of NFATc1 binding to CR-C after Blimp-1 expression offered several mechanistic scenarios. The first was that Blimp-1 repression of NFATc1 transcription resulted in a passive loss in NFATc1 binding. A second part of the mechanism involved the direct removal of NFATc1 from CR-C. The ectopic expression of both NFATc1 and Blimp-1 showed that Blimp-1 binding and activity was dominant to NFATc1 and that subsequent Blimp-1 binding resulted in the eviction of NFATc1. Although possible, it is unlikely that Blimp-1 was simply displacing NFATc1 due to direct steric hindrance, as the two binding sites are ∼350 bp apart, a distance larger than in other genes in which such mechanisms are in play (Samoylenko et al., 2001; Wei et al., 2005). Instead, the most likely reason seems to be that Blimp-1 binding changed the local chromatin architecture around CR-C. As observed, increases in PD-1 transcription resulted in the accumulation of active histone modifications near the promoter and at the regulatory regions. In the presence of Blimp-1, these active histone marks were removed and replaced with repressive modifications. Associated with the repressive modifications was the conversion of CR-C from a region that was accessible to transcription factors such as NFATc1 to a region that was inaccessible. Coupled with a decrease in NFATc1 expression, the ability of NFATc1 to rebind and alter the local structure would be significantly reduced.

In addition to the site-specific transcription factors, PD-1 is also regulated by DNA methylation (Youngblood et al., 2011). Naive CD8 T cells display extensive DNA methylation at CR-B and CR-C. In response to LCMV infection, antigen-specific effector CD8 T cells lose DNA methylation across this region as PD-1 is expressed. At the later stages of the effector stage, the regulatory regions are remethylated in a manner that is coincident with the loss of PD-1 expression and Blimp-1 binding. Blimp-1 may facilitate this process. The histone modifications analyzed in this report were chosen to represent specific pairs. Histone H3K9 and H3K27 represent residues that function in active and repressive chromatin structures depending on whether the residue is acetylated or methylated. To be converted from an acetylated to a methylated mark, a histone deacetylase (HDAC), followed by a histone methyltransferase, is required. Although Blimp-1 can interact with HDACs (Yu et al., 2000), the specific HDAC used here is not known. In contrast, because the histone methyltransferases for H3K9 and H3K27 are known (Kouzarides, 2007), these data suggest a role for G9a and the polycomb group complex containing EZH2, respectively, in regulating PD-1 expression. A recent report shows Blimp-1 recruitment of G9a and HDAC2 to the IL2ra and CD27 genes during CD8 T cell differentiation (Shin et al., 2013). Thus, CD8 T cells use multiple epigenetic mechanisms to control PD-1 expression during activation and differentiation. As the action of the HDAC inhibitors would serve to potentially prolong PD-1 expression, our observations may suggest caution in the use of HDAC inhibitors for the treatment of diseases in which the immune system will be called upon to clear an infection or act.

Rutishauser et al. (2009) first reported a delay in control of an acute viral infection in the absence of Blimp-1 in CD8 T cells. Furthermore, they demonstrated that the reduced cytolytic capacity of the Blimp-1–deficient effector CD8 T cells, including reduced granzyme B expression, was likely the reason for inefficient viral clearance. Our data, demonstrating retained PD-1 expression and inhibitory signaling, provide a mechanism for the reduced viral clearance in a primary immune response. Several other findings are consistent with our observations that Blimp-1 represses PD-1 gene expression. In a similar manner, Blimp-1 deficiency has been shown to lead to higher/increased levels of PD-1 expression during Th1 and Tc1 cell differentiation (Johnston et al., 2009; Cretney et al., 2011). PD-1 expression is a hallmark of Th1 cells, which develop after the expression of the Blimp-1 transcriptional antagonist Bcl6 (Johnston et al., 2009).

In contrast to the acute infection scenario where antigen is ultimately cleared, antigen-specific CD8 T cells generated during a chronic LCMV infection where antigen is persistent displayed extremely high levels of both Blimp-1 and PD-1 (Shin et al., 2009). The high levels of Blimp-1 correlated with maintenance of the exhausted cell fate and appeared to control not only the expression of PD-1 but also of other inhibitory receptors, including LAG3, CD160, and 2B4 (Shin et al., 2009). Homozygous Granzyme B Cre x Blimp-1−/−/− mice (Blimp-1 cKO) in these chronic infection studies displayed ∼50% reduction of PD-1 expression on antigen-specific CD8 T cells compared with WT mice. However, it is important to point out that PD-1 levels were still higher than what is observed during acute infections. A mixed bone marrow chimera experiment examining the Blimp-1 cKO cells during chronic infection was performed but did not show a significant difference between wild type (Shin et al., 2009).
We interpret this finding to indicate that Blimp-1 cannot repress PD-1 under those conditions in which persistent antigen is present. Additionally, Agnellini et al. (2007) observed that NFATc1 translocation is impaired during chronic LCMV infection. Thus, coupled with the repression of NFATc1 transcription shown here and impaired NFATc1 translocation, it is unlikely that NFATc1 could play a major role in PD-1 expression during chronic LCMV infection. The combined data also suggest that a novel activator of PD-1 expression that would be induced during a chronic infection setting likely prolongs and maintains PD-1 expression. These results suggest that Blimp-1 activity may not be active at the PD-1 locus during chronic infection. Indeed, we previously showed that the repressive histone modifications for H3K9 and H3K27 were not present during chronic infection (Youngblood et al., 2011). Alternatively, Blimp-1 could act as an activator under conditions of persistent antigen that is associated with the exhausted CD8 T cell phenotype. Together these results imply that PD-1 regulatory mechanisms that function during CD8 T cell exhaustion are distinct from those that function during the initial phases of a CD8 T cell effector response where antigen is ultimately cleared.

In summary, the data presented here implicate Blimp-1 as a major silencer of PD-1 gene expression during the initial phases of CD8 T cell activation and formation of the effector phase. Because prolonged PD-1 expression and signaling would ultimately result in diminished T cell responses, the role that Blimp-1 plays is critical to the generation of an effector CD8 T response (Kallies et al., 2009; Rutishauser et al., 2009). Considering the contrasting roles that Blimp-1 and Bcl6 play in controlling T cell development, and the expression of PD-1 on the surface of these cells, it would not be surprising to find a similar molecular role for Blimp-1 in the expression of PD-1 in these cells. Understanding how Blimp-1 may function in different contexts could provide useful tools for manipulating T cell fate and responses to infection and immune settings.

**MATERIALS AND METHODS**

Luciferase reporters PD-1B/C and PD-1B were described previously (Oestreicht et al., 2008). pDNA3.1-Flag-Blimp-1 was a gift from P. Wade (National Institute of Environmental Health Sciences, Research Triangle Park, NC). The site-directed mutants of the PD-1B/C luciferase reporter were generated using QuickChange II Site-Directed Mutagenesis kit (Agilent Technologies) according to the manufacturer’s instructions. The 4 bp core sequences of the three potential Blimp-1–binding sites in the PD-1B/C luciferase reporter were mutated to random nucleotides (site 1, 5’-CTGAAACT to CTTGCGTCT-3’; site 2, 5’-GAGAAAGA to GAATCGGAA-3’; and site 3, 5’-GAGAAAGA to GAATCGGAA-3’). Deletion of the whole Blimp-1–binding site 2 in the PD-1 promoter was accomplished using overlap PCR techniques using an overlapping primer pair spanning the deletion sequence and two flanking primers, and the PCR fragment was cloned into pGL3-Basic at the Xhol–BamHI sites to make the Δsite2 mutant of the PD-1B/C luciferase reporter. Two sequential PCR fragments encompassing the 3.0 kb upstream region of NFATc1 gene were generated from mouse genomic DNA. The fragments of 1.1 and 1.9 kb in length were digested by BglII–PstI and PstI–HindIII, respectively, and ligated together into pGL3-Basic at the BglII–HindIII sites to construct the NFATc1 luciferase reporter. All primers used for cloning and mutagenesis are listed in Table S1.

**Mice.** C57BL/6 mice, Thy1.2+ C57BL/6 mice, Blimp-1 cKO mice, and P14 mice used in this study were maintained and manipulated in compliance with Emory University Institutional Animal Care and Use Committee-approved protocols. Breeding trios of C57BL/6 mice (The Jackson Laboratory) were bred inbred locally for up to 6 mos. *Pdmn* ß-6 x *TgCre-GNZB* mice, which delete Blimp-1 (*pdmn* gene) in the B cell lineage (Shapiro-Shelef et al., 2003), were provided by K. Calame (Columbia University, New York, NY). The human Granzyme B-Cre transgenic mouse *TgCre-GNZB* (Jacob and Baltimore, 1999) was provided by J. Jacob (Emory University, Atlanta, GA). This line was backcrossed six times with C57BL/6. Granzyme B promoter-driven Cre is expressed in activated CD8 T cells. A Blimp-1 ß-6 x *TgCre-GNZB* and a self-cross of the *TgCre-GNZB*-deficient F1 generated the Blimp-1 ß-6 x Granzyme B Cre mice. Mice carrying the Blimp-1 ß-6 and *TgCre-GNZB* alleles were used and termed Blimp-1 cKO in this study. The line was maintained by inbreeding afterward. PCR based genotyping was performed on each mouse to ensure that the correct alleles were being used. The number of backcrosses of the original *pdmn* ß-6 to C57BL/6 is not known. These were paired with WT C57BL/6 mice for the experiments shown.

**Cells and culture.** The murine T cell lymphoma EL4 cells were cultured in RPMI 1640 supplemented with 5% FBS (Sigma–Aldrich), 5% bovine calf serum (HyClone, Inc.), 4.5 g/liter glucose, 1.0 mM sodium pyruvate, 10 mM Hepes, and 100 U/ml penicillin/streptomycin. Primary murine CD8 T cells were isolated from spleens of C57BL/6 mice by negative selection using the CD8a+ T Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer’s protocol and cultured in the same media as that for EL4 cells. Anti-CD3/CD28 beads (Invitrogen) were directly added to the media to activate the cells. In some experiments, CsA was added to the cultures.

LCMV-specific P14 CD8 T cells for adoptive transfer were obtained from naive transgenic P14 mice that have an engineered TCR recognizing the Db-GP33–41 epitope of LCMV (Pircher et al., 1989). To generate LCMV-specific CD8 T cells, chimeric mice were generated by intravenous adoptive transfer of 105 congenically labeled Thy1.1+ naive P14 CD8 T cells into Thy1.1+ C57BL/6 recipients. 24 h after adoptive transfer, chimeric mice were infected with 2 ¥ 105 pfu of LCMV Armstrong. LCMV-specific effector P14 CD8 T cells were obtained from the spleen 6 or 8 d after infection by FACS using fluorescently labeled CD90.1 (Thy1.1) and CD8 antibodies as previously described (Murizli-Krstina et al., 1998; Blattman et al., 2002; Kersh et al., 2006). Naive P14 CD8 T cells were FACS purified and used as an antigen-specific naive control. In some experiments, 105 Thy1.1+ naive P14 CD8 T cells were transferred into WT C57BL/6 (Thy1.2+ ) mice or into Blimp-1 cKO mice as above. After LCMV Armstrong infection, both (endogenous and transferred) populations of cells were analyzed by flow cytometry for PD-1 expression on the indicated antigen-specific CD8 T cell subsets.

Adenovirus and transduction. The Flag-Blimp-1 adenovirus expression vector was provided by P. Wade. The NFATc1-GFP adenovirus was purchased from Applied Biological Materials Inc. Adenoviruses were prepared according to Qbiogene’s adenovirus purification protocol. To transduce EL4 cells with adenovirus, cells were seeded at the concentration of 2.5 ¥ 105 cells/ml, 1 d before transduction. At the time of transduction, cells were washed and resuspended in conditioned media composed of 50% old media and 50% fresh media. Cells were seeded at the concentration of 105 cells/ml. Adenovirus was directly added to the cells and mixed by swirling the plates. 6 h after transduction, cells were treated with 50 ng/ml Phorbol 12-myristate 13-acetate (PMA; Sigma–Aldrich) plus 2 µM ionomycin (Sigma–Aldrich) for 24 h to increase PD-1 expression (Oestreicht et al., 2008).

RNA isolation and real-time RT-PCR analysis. Total RNA was isolated with RNeasy mini prep kit (QIAGEN). Reverse transcription reactions contained 1 µg total RNA and were performed with SuperScript II reverse transcriptase (Invitrogen). SYBR Green incorporation-based real-time PCR was performed to quantify the mRNA levels of the specific genes. The mRNA levels were normalized to those of 18S rRNA. Each experiment was performed three times with technical triplicates. ANOVA was performed to determine the statistical significance of the data. The *p* values are indicated in the figure legends.

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Transfection and luciferase reporter assays. Transfection of EL4 cells was performed in 6-well plates by nucleofection. For each transfection, 5 µg firefly luciferase reporter plasmid and 0.2 µg pRL-TK that expresses Renilla luciferase together with 20 µg pcDNA3.1 or pcDNA3.1-Flag-Blimp-1 were co-transfected into 2 × 10⁶ cells. 16 h after transfection, cells were treated with 50 ng/ml PMA plus 2 µM ionomycin for 24 h to induce the expression of the reporters (Oestreich et al., 2008). Luciferase assays were conducted with the Dual-Luciferase Reporter Assay System (Promega, Inc.) according to the manufacturer’s instructions. The firefly luciferase activities were normalized to the Renilla luciferase activities. Each experiment was conducted from three different transfections.

DNase I hypersensitivity assays. DNase I hypersensitivity assays were performed as previously described (Oestreich et al., 2008) using 5 × 10⁵ cells, each treated with the indicated amounts of DNase I (Worthington Biochemical, Inc.). Real-time PCR was performed using amplicons spanning different regions of the PD-1 locus. The relative DNase I hypersensitivity was determined by quantifying the amount of DNA remaining after DNase I digestion. These values were normalized to those of a control amplicon that is insensitive to DNase I digestion. Each DNase I hypersensitivity assay was performed with nuclei from three independent experiments. The primers for real-time PCR of DNase I hypersensitivity assays are listed in Table S1.

ChIP assays. ChIP assays were conducted as previously described (Beresford and Boss, 2001; Choi and Boss, 2012). In this procedure, cross-linking in 1% formaldehyde was performed for 15 min. After sonication and isolation, 30 µg chromatin made from ~4 × 10⁶ cells was preclared with Protein A agarose beads and then subjected to immunoprecipitation with the indicated antibodies. The anti-Blimp-1 antibody was developed in collaboration with and obtained from Rockland Immunochemicals, Inc. The anti-Flag and anti-NFATc1 antibodies were purchased from Sigma-Aldrich, Inc. and Thermo Fisher Scientific, respectively. Anti-GFP and anti-H4K20me3 antibodies antibodies were purchased from Abcam. All the other histone antibodies used in this study were purchased from Millipore. Non-immune rabbit IgG was used as a nonspecific control for all rabbit antisera used. The mouse monoclonal antibody anti-HA (12CA5), which recognizes a short peptide from the influenza hemagglutinin protein, was produced from tissue culture supernatants and used as a nonspecific control for the mouse monoclonal antibodies used in this study, including anti-GFP, anti-Flag, and anti-NFATc1. After the immunoprecipitation and processing, the DNA in each immunoprecipitation was quantified by real-time PCR using a standard curve from sonicated murine genomic DNA. Each ChIP analysis was performed with chromatin purified from three independent experiments. The primers for real-time PCR of ChIP assays are listed in Table S1.

Online supplemental material. Fig. S1 shows validation of the Blimp–1 antibody. Table S1 shows PCR primers. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20130208/DC1.

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