Blimp-1–mediated CD4 T cell exhaustion causes CD8 T cell dysfunction during chronic toxoplasmosis

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CD8, but not CD4, T cells are considered critical for control of chronic toxoplasmosis. Although CD8 exhaustion has been previously reported in *Toxoplasma* encephalitis (TE)–susceptible model, our current work demonstrates that CD4 not only become exhausted during chronic toxoplasmosis but this dysfunction is more pronounced than CD8 T cells. Exhausted CD4 population expressed elevated levels of multiple inhibitory receptors concomitant with the reduced functionality and up-regulation of Blimp-1, a transcription factor. Our data demonstrates for the first time that Blimp-1 is a critical regulator for CD4 T cell exhaustion especially in the CD4 central memory cell subset. Using a tamoxifen-dependent conditional Blimp-1 knockout mixed bone marrow chimera as well as an adoptive transfer approach, we show that CD4 T cell–intrinsic deletion of Blimp-1 reversed CD8 T cell dysfunction and resulted in improved pathogen control. To the best of our knowledge, this is a novel finding, which demonstrates the role of Blimp-1 as a critical regulator of CD4 dysfunction and links it to the CD8 T cell dysfunctionality observed in infected mice. The critical role of CD4-intrinsic Blimp-1 expression in mediating CD4 and CD8 T cell exhaustion may provide a rational basis for designing novel therapeutic approaches.

The Journal of Experimental Medicine

INTRODUCTION

Toxoplasma gondii is transmitted by food or water and infection occurs in many of the animals used for food in the United States (Scallan et al., 2011; Hill and Dubey, 2013). Human infection can result from the ingestion of undercooked or raw meat containing tissue cysts, or from the consumption of water or food contaminated by oocysts excreted in the feces of infected cats (Dubey, 1998; Torrey and Yolken, 2013). Because of the high prevalence of *T. gondii*, groups of individuals with compromised immune systems remain at risk of *Toxoplasma* encephalitis (TE). Indeed, in the early years of the HIV epidemic *T. gondii* was often observed in individuals with AIDS, sometimes leading to TE. Even in the era of combination antiretroviral therapy, fatal TE still occurs in HIV-infected individuals as a result of reactivation of latent infection, and remains a significant problem in AIDS patients who harbor this chronic parasitic infection (Grant et al., 1990; Zangerle et al., 1991). TE in HIV-infected individuals occurs coincident with the drop in CD4 T cell count (Luft and Remington, 1992), thus it is believed that reactivation of latent infection during AIDS is caused by reduced CD4 T cell help to CD8 T cells.

Although both CD4 and CD8 T cells have been reported to act synergistically to control *T. gondii* infection, CD8 T cells play a dominant role in host protection (Gazzinelli et al., 1991, 1992; Khan et al., 1994, 1999). Long-term immunity to *T. gondii* is believed to primarily depend on CD8 T cells (Parker et al., 1991), and depletion of this subset rather than CD4 T cells results in host mortality (Gazzinelli et al., 1992). The synergistic effect of CD4 T cells is most likely restricted to their helper role in the maintenance of a long-lived CD8 T cell response (Casciotti et al., 2002). Importantly, although CD4 T cell help most likely plays an important role during chronic *T. gondii* infection, the requirements for persistent CD4 T cell help in the control of chronic infections are not well defined. Studies conducted with viral pathogens like HBV, HCV, and lymphocytic choriomeningitis virus (LCMV) have observed CD4 T cell exhaustion in the infected host and it has been suggested that CD4 T cell dysfunction effects CD8 T cell functionality (Brooks et al., 2005; Yi et al., 2010; Crawford et al., 2014; Ye et al., 2015). The requirement for CD4 T cell help during chronic infections like toxoplasmosis is very crucial as CD8 T cells need to be maintained to keep the pathogen under control. Thus, studies focused on investigating CD4 T cell functionality during TE are needed. Previous studies from our laboratory reported several fold increases in the expression of the inhibitory receptor PD-1 on CD8 T cells from mice carrying chronic toxoplasmosis. This led to severe exhaustion and loss of functionality of these cells (Bhadra et al., 2011, 2012, 2013). In the current study, we demonstrate that similar to CD8 T cells, *T. gondii*–specific

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Abbreviations used: Blimp-1, B-lymphocyte-induced maturation protein 1; cKO mice, conditional KO mice; LCMV, lymphocytic choriomeningitis virus; MFI, mean fluorescence intensity; T_{CM}, central memory; TE, *Toxoplasma* encephalitis; T_{EM}, effector memory.

The Rockefeller University Press $30.00
J. Exp. Med. 2016 Vol. 213 No. 9 1799–1818
www.jem.org/cgi/doi/10.1084/jem.20151995

CD4 T cells have increased expression of inhibitory receptors like PD-1, 2B4 and LAG-3 during chronic infection. The parasite-specific CD4 T cells also exhibited decreased functionality, which was concomitant with reactivation of infection. Although some common features between exhausted CD4 and CD8 T cell exhaustion during chronic viral infection have been demonstrated (Shin and Wherry, 2007; Crawford et al., 2014), we observed that elevated expression of the transcription factor Blimp-1 in *T. gondii*–specific CD4 T cells was responsible for their dysfunction. Conditional deletion of Blimp-1 on CD4 T cells restored their functionality and reversed CD8 T cell exhaustion, leading to increased ability of the host to control infection. These studies link CD8 T cell dysfunctionality observed during chronic toxoplasmosis to CD4 T cell exhaustion.

RESULTS

T. gondii–specific CD4 T cells up-regulate inhibitory receptors and lose their functionality during chronic *T. gondii* infection

We have previously reported that chronic *T. gondii* infection results in progressive decline in CD8 T cell effector function concomitant with PD-1 up-regulation (Bhadra et al., 2011). Gazzinelli et al. (1992) demonstrated that depletion of CD8 T but not CD4 T cells in chronically infected C57BL6 (a susceptible mouse strain) results in TE. This led us to hypothesize that CD4 are more severely exhausted than CD8 T cells during chronic toxoplasmosis and, as a result, depletion of a highly exhausted subset (CD4 T cell) has minimal impact on pathogen control. Alternatively, it is possible that CD4 T cells may not be the relevant subset for *T. gondii* control during chronic infection. We addressed these possibilities by determining the functional status of *T. gondii*–specific CD4 T cells during chronic *T. gondii* infection. At first, we compared the expression of inhibitory receptor PD-1 on CD4 and CD8 T cells from *T. gondii*–infected animals. In agreement with our previous publication on CD8 T cells, PD-1 expression on CD4 T cells was up-regulated during early chronic phase (5–6 wk p.i.; Fig. 1 A). Significantly, PD-1^{hi} T cells were more enriched in CD4 T cell subset than CD8 T cell during early chronic phase. Studies related to T cell exhaustion, especially with CD8 T cells, have demonstrated that in addition to PD-1, up-regulation of other inhibitory receptors (2B4, CTLA4, etc.) can act cooperatively in exacerbating dysfunction on T cells (Blackburn et al., 2009; Jin et al., 2010; Butler et al., 2011). Significantly, up-regulation of inhibitory receptors concomitant with loss of T cell functionality are considered hallmarks of T cell exhaustion. Analysis of PD-1^{hi} CD4 and CD8 subsets revealed that both PD-1^{hi} CD4 and CD8 T cells expressed higher levels of 2B4 and CTLA4 than PD-1^{int} or PD-1^{lo} cells (Fig. 1 B). However, PD-1^{hi} CD4 T cells expressed significantly greater levels of 2B4 than PD-1^{hi} CD8 T cells (Fig. 1 C). To further characterize CD4 T cell dysfunction, we observed if down-regulation of co-stimulatory receptors on CD4 T cells correlated with increase in PD-1 expression on antigen-specific CD4 T cells. T cell subsets expressing different levels of PD-1 (PD-1^{hi}, PD-1^{int}, and PD-1^{lo}) were evaluated for the expression of co-stimulatory receptors (OX40, ICOS, and 41BB). We observed that during early chronic *T. gondii* infection (5-6 wk p.i.), CD4 PD-1^{hi} cells exhibited increased 2B4 (one of inhibitory receptors) and decreased ICOS and OX40 (co-stimulatory receptors) levels as compared with CD8 PD1^{hi} population (Fig. 1, D and E). The aforementioned data suggest that T cell dysfunction is more pronounced on CD4 T cells than CD8 T cells during early chronic phase.

Due to the limited number of class II epitopes defined for the *T. gondii* infection model (Grover et al., 2012), we used a surrogate marker strategy that has been successfully used by other groups to identify a broader representation of antigen-specific T cells (Butler et al., 2011; McDermott and Varga, 2011). This approach was validated by screening cells from animals infected with *T. gondii* for surrogate activation markers (CD11a and CD49d) and comparing this analysis to *T. gondii*–specific Class II tetramer (AS15) staining. At 3 and 7 wk p.i., >90% of tetramer^{+} cells from spleen and brain expressed CD11a^{hi} and CD49d^{hi} phenotype (Fig. 2 A). To identify the fraction of CD11a^{hi}CD49d^{hi} CD4 T cells that are tetramer (AS15) positive, CD11a^{hi}CD49d^{hi} cells were gated and analyzed. We observed that 1/5 of CD11a^{hi}CD49d^{hi} cells stained positive for tetramer (Fig. 2 B). These findings demonstrate that among the surrogate marker positive population, only 20% are specific for AS15 tetramer. To establish that surrogate marker positive CD4 T cells are antigen specific, the cells (CD11a^{hi}CD49d^{hi}) were separated into tetramer-positive and -negative population (Fig. 2 C). The two purified populations (>98% pure) were injected separately into CD4 KO animals and the next day the recipients were infected with *T. gondii*. The proliferation of donor CD4 T cells was measured by BrdU incorporation. As shown in Fig. 2 D (top column), both tetramer-positive and -negative CD4^{+}CD11a^{hi}CD49d^{hi} (surrogate marker positive) exhibited strong proliferation in response to *T. gondii* infection. Interestingly, tetramer-negative population showed significantly higher proliferation as compared with tetramer-positive cells (Fig. 2 D, top column). As expected, surrogate marker negative population exhibited minimal proliferation in response to *T. gondii* infection. These observations establish that the surrogate marker positive population is *T. gondii* specific, which responds strongly to recall antigen. This is further emphasized by the finding that a significantly higher percentage of donor CD4 population was observed in the spleens of animals treated with tetramer-negative cells from surrogate marker–positive population (Fig. 2 D bottom). *T. gondii* specificity of CD11a^{hi}CD49d^{hi} cell population was further established by the observation that, unlike the cells bearing CD11a^{lo}CD49d^{lo} phenotype, the former subset was able to produce cytokines like IFN-γ and TNF in response to stimulation with *T. gondii* antigen (Fig. 2 E). Furthermore, most IFN-γ^{+} cells expressing CD11a and CD49d phenotype were detected in spleen at 3 wk p.i.,

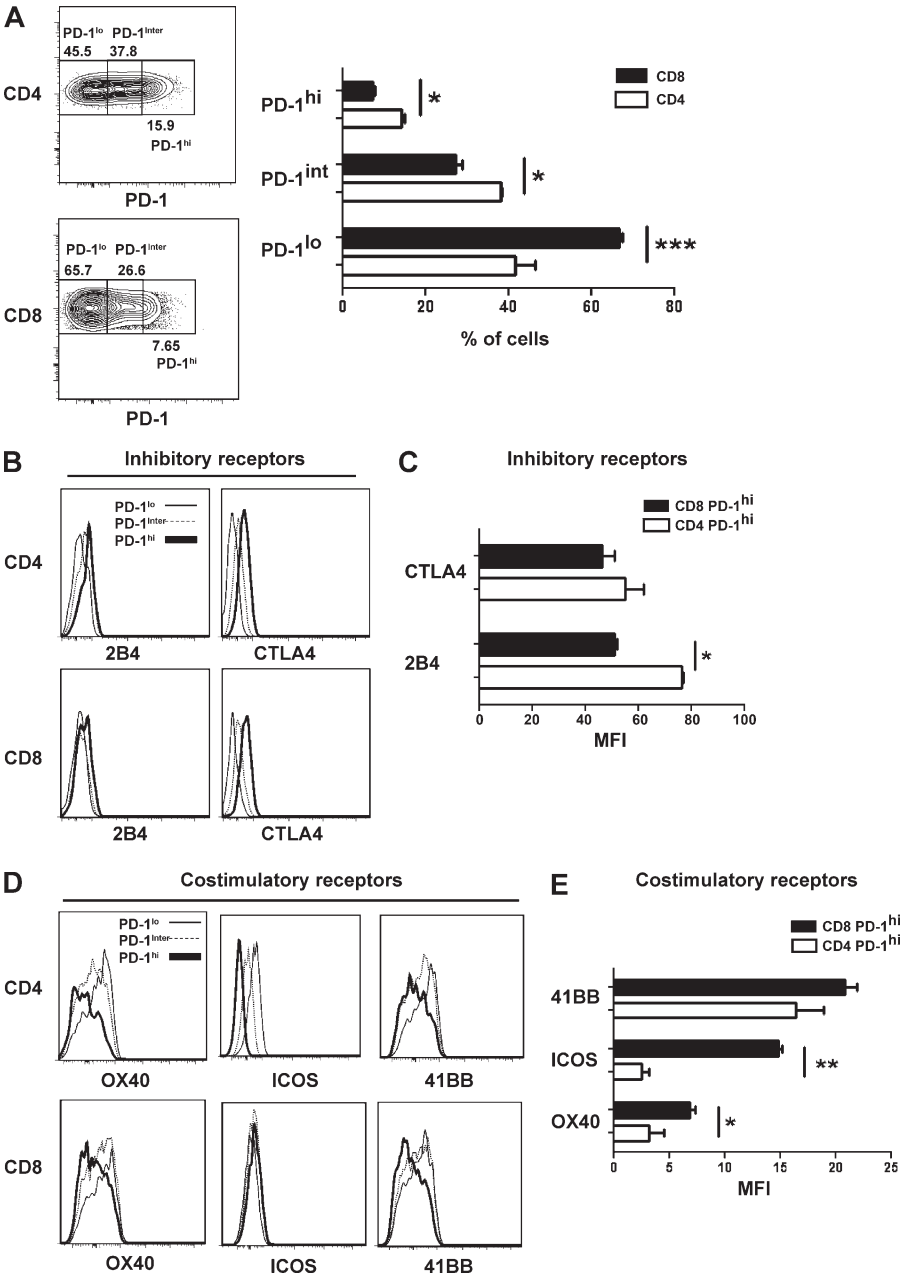

Figure 1. **CD4 T cells express increased levels of inhibitory receptors and decreased co-stimulatory receptors compared with CD8 T cells during early chronic infection.** C57BL/6 mice were perorally infected with 10 ME49 tissue cysts and splenocytes harvested during early chronic phase (5-6 wk p.i.). (A) PD-1^{lo}, PD-1^{Int}, and PD-1^{hi} gating was performed (left) on splenic CD4 cells (left, top) and CD8 (left, bottom). Percentage of PD-1^{hi}, PD-1^{int}, and PD-1^{lo} expression on CD4 or CD8 at 5-6 wk p.i. (right). (B) 2B4 and CTLA4 expressions on $CD4^+$ PD-1^{hi} and $CD8^+$ PD-1^{hi}. Black line, $CD4^+$ PD-1^{hi}; dot line, $CD4^+$ PD-1^{int}; bold line, $CD4^+$ PD-1^{lo} (top). Black line, $CD8^+$ PD-1^{hi}; dot line, $CD8^+$ PD-1^{int}; bold line, $CD8^+$ PD-1^{lo} (bottom). (C), MFI of CTLA4 and 2B4 on CD4 PD-1^{hi} and CD8 PD-1^{hi}. (D) Multiple co-stimulatory receptors expression on CD4 and CD8 cells in PD-1^{lo}, PD-1^{Int}, and PD-1^{hi} population at 5–6 wk p.i. (E) MFI of OX40, ICOS, and 41BB on splenic CD4 and CD8 cells. The data represents three experiments with at least three mice per group. P-values were obtained using the Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (mean ± SEM).

which decreased at 7 wk p.i. (Fig. 2 F). In addition, the trend of decreased cell numbers of $AS15^+$ ($tetramer^+$) CD4 T cells and $CD11a^{hi}CD49d^{hi}$ cells were comparable, although the $CD11a^{hi}CD49d^{hi}$ population was ∼10 times more than $AS15^+$ cells (Fig. 2, G and H). Thus, to more broadly evaluate the response of *T. gondii*–specific T cells to multiple antigens, our investigation focused on $CD11a^{hi}CD49d^{hi}$ *T. gondii*–specific CD4 T cells. We next proceeded to measure the kinetics of the $CD11a^{hi}CD49d^{hi}$ *T. gondii*–specific CD4 T cell response after *T. gondii* infection in spleen and brain. The total cell number of splenocytes increased at 3 wk (acute), but significantly decreased at 5 wk (early chronic) to 7 wk (late chronic) p.i. (Fig. 3 A, top). In contrast, the quantity of *T. gondii*–specific CD4 T cells were elevated at 5 wk in brain, but subsequently decreased at 7 wk p.i. (Fig. 3 A, bottom). Based on the decreased number of *T. gondii*–specific CD4 T cells, we sought to evaluate levels of $CD11a^{hi}CD49d^{hi}$ *T. gondii*–specific CD4 T cell exhaustion at 7 wk (late chronic phase). To determine if *T. gondii*–specific CD4 T cells had an exhausted phenotype similar to our prior report on CD8 T cells (Bhadra et al., 2011), we measured expression of the inhibitory receptor PD-1 at early and late stages of infection. As shown in Fig. 3 B, up-regulation of PD-1 expression was observed on the $CD11a^{hi}CD49^{hi}$ (*T. gondii*–specific) population of cells in spleen and brain at 7 wk p.i. (Fig. 3, B and C). The mean fluorescent intensity (MFI) of PD-1 expression in spleen was

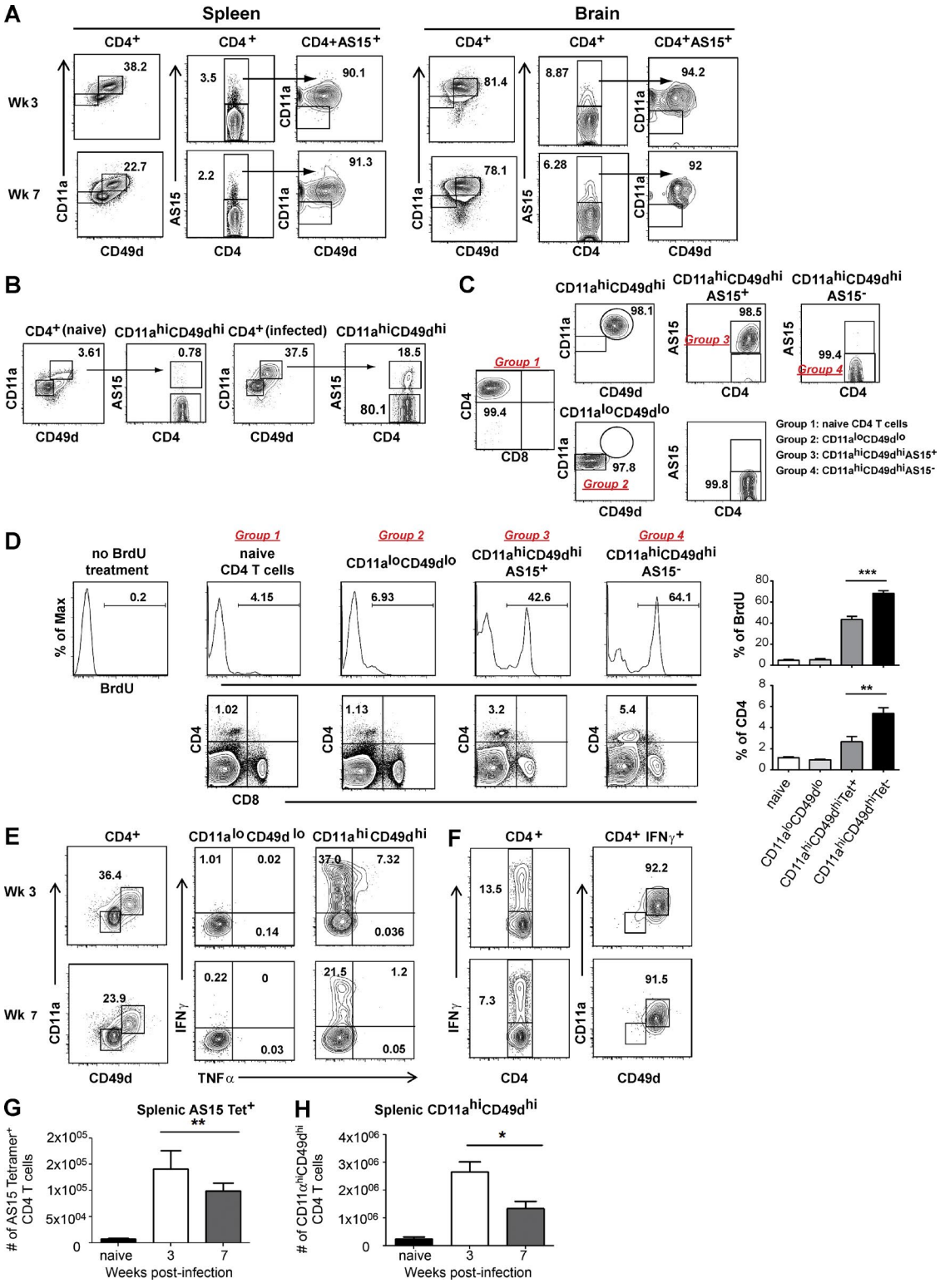

Figure 2. **Validation of surrogate markers on antigen-specific CD4 T cells during *T. gondii* infection.** (A) Splenocytes and brain cells were harvested at 3 and 7wk p.i., and subsequently stained with anti-CD4 and MHCII-restricted tetramer (AS15). Cells were gated on $CD4^+AS15^+$, analyzed by CD11a and CD49d surrogate markers (left, spleen; right, brain). (B) Splenocytes were stained for CD4 CD11a and CD49d at 3 wk p.i., and analyzed for the expression MHC II-restricted tetramer (AS15). (C) CD4CD11aCD49d population was sorted from the splenocytes of week 3 infected mice. The purified population (>98%) was stained with tetramer and separated into tet^+ and tet^- population. The cells were transferred separately into CD4KO mice (each animal received 50,000 cells).

significantly higher in the *T. gondii*–specific CD4 population at 7 wk p.i. and continued to increase on CD4 T cells in brain from 3–7 wk p.i (Fig. 3 C). As sustained expression of PD-1 is coupled to increased exhaustion and decreased functionality of *T. gondii*–specific CD4 T cells, we hypothesized that the PD-1^{hi} population would be associated with other inhibitory receptors. There was a significant increase in frequency of PD-1^{hi} expressing *T. gondii*–specific CD4 T cells at 7 wk p.i. (Fig. 3 D). Importantly, this population also had increased expression of LAG-3 and 2B4 (Fig. 3 E).

To determine if increases in the expression of inhibitory receptors correlated with the functionality, cells were assayed for the proinflammatory cytokines IFN-γ and TNF after stimulation with *T. gondii* antigen. Although the absolute number of splenic and brain cytokine producing *T. gondii*–specific CD4 T cells increased from 5 wk, there was a significant decrease in cytokine-producing cells at 7 wk p.i., coincident with their maximal expression of PD-1 (Fig. 4 A). Interestingly, the PD-1^{hi} subset of *T. gondii*–specific CD4 T cells in spleen and brain did not show a decrease in functionality (unpublished data) at 3 wk p.i. in spleen and at 5 wk p.i in brain, similar to our earlier study on CD8 exhaustion in which, at earlier time points, PD-1^{hi} population did not lose the ability to produce cytokines (Bhadra et al., 2011). However, at 7 wk p.i. there was a significant decrease in the functionality of PD-1^{hi} expressing *T. gondii*–specific CD4 T cells in both spleen and brain (Fig. 4, B–D). T reg (regulatory T) cells have been reported to express increased inhibitory receptors, such as CTLA4, among others. However, in the *T. gondii* model, no difference in T reg cell development was noted between the early chronic and late chronic infection (Fig. 4, E and F), arguing against the possibility of altered T reg cell development resulting in differential PD-1 expression or loss of IFN-γ during late chronic toxoplasmosis. Collectively, these findings suggest that the expression of inhibitory receptors on *T. gondii*–specific CD4 T cell during chronic toxoplasmosis contribute to the diminished ability of the cells to recall their effector functions.

Blimp-1 expression on *T. gondii*–specific CD4 T cell is correlated with CD4 T cell exhaustion during chronic toxoplasmosis

Recent studies have implicated changes in the expression pattern of the transcription factors, (T-bet, Eomes, Blimp-1, and Bcl6), being associated with the development of exhaustion in CD8 T cells (Shin et al., 2009; Kao et al., 2011; Paley et al., 2012; Buggert et al., 2014; Crawford et al., 2014). To better define the exhausted phenotype in parasite-specific CD4 T cells in a *T. gondii* infection model, we analyzed the expression pattern of those transcription factors at different time points after infection (Fig. 5, A and B). Similar to observations noted in CD8 T cells during chronic LCMV infection (Kao et al., 2011), we observed that in *T. gondii*–infected animals, concomitant with increased PD-1 levels, *T. gondii*–specific CD4 T cells showed decreased T-bet expression especially on the PD-1^{hi} subset (Fig. 5, A and B, panel 1). The PD-1^{hi} CD4 T cells also exhibited increased levels of Eomes (Fig. 5, A and B, panel 2), which was similar to the observations made with CD8 T cells from HIV infected individuals that exhibited T-bet^{dim}Eomes^{hi} expressional profile (Buggert et al., 2014; Gupta et al., 2015). Additionally, up-regulation of Blimp-1 an important transcriptional factor for CD8 T cell exhaustion (Shin et al., 2009), was observed in the CD4 T cell population at 7 wk p.i. (Fig. 5, A and B: panel 3). In contrast, a significant decrease in Bcl6 levels, a transcription factor considered to be a reciprocal antagonist to Blimp-1 (Johnston et al., 2009) and important for memory cell differentiation (Kaech and Cui, 2012), was noted at wk7 p.i. (Fig. 5, A and B, panel 4). To clarify the expression of transcription factors in multiple dimension, we performed the analysis with Blimp-1 versus different transcription factors (T-bet, Eomes, and Bcl6) at 7 wk p.i. Consistent with Fig. 5 B, we found that the Blimp-1^{hi} CD4 T cells exhibited decreased T-bet and Bcl6 expression, whereas increased expression of Eomes was observed on these cells (Fig. 5 C). Because the transcription factor Blimp-1 has been reported to be important for CD8 T cell exhaustion during chronic viral infection (Shin et al., 2009), we next assessed its role of regulating the function of *T. gondii*–specific CD4 T cells. We first measured the expression of PD-1 in Blimp-1^{lo}, Blimp-1^{int} and Blimp-1^{hi} CD4 T cell subsets (Fig. 5, D and E). As shown in Fig. 5 E, increased Blimp-1 expression correlated well with elevated PD-1 expression by these cells. Similarly, Blimp-1^{hi} CD4 T cell population also had increased levels of other inhibitory markers, including 2B4 and LAG-3 (Fig. 5, F and G). We next analyzed if the functionality of *T. gondii*–specific CD4 T cells correlated with their Blimp-1 expression. Compared with the Blimp-1^{int} population, Blimp-1^{hi}–expressing CD4 T cells exhibited decreased functionality in terms of their ability to produce both IFN-γ and TNF (Fig. 5, H and I). In total, these data suggest that elevated level of Blimp-1 in the PD-1^{hi} is coupled to exhaustion of the *T. gondii*–specific CD4 T cells.

Control animals received equal number of CD4 T cells from naive donors. The recipient animals were infected the next day and proliferation was measured using a pulse-chase approach. BrdU was injected into the recipient mice starting 1 d after transfer, and the treatment was repeated alternatively for a 5-d period. Animals were sacrificed 1 d after final treatment. (D) Donor CD4 T cells in spleens were evaluated for BrdU incorporation by flow cytometry (top) and CD4/CD8 profile (bottom). (E) CD11a^{hi}CD49d^{hi} cells and CD11a^{lo}CD49d^{lo} were gated then IFN-γ and TNF secretion was observed with TLA stimulation. (F) CD4^{+} IFN-γ^{+} cells were analyzed by CD11a and CD49d surrogate markers. (G) The graphs represent number of AS15 tetramer^{+} CD4 T cells and (H) number of CD11a^{hi}CD49d^{hi} CD4 T cells. The data represent three experiments with at least three mice per group (three experiments). P-values were obtained using the Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (mean ± SEM).

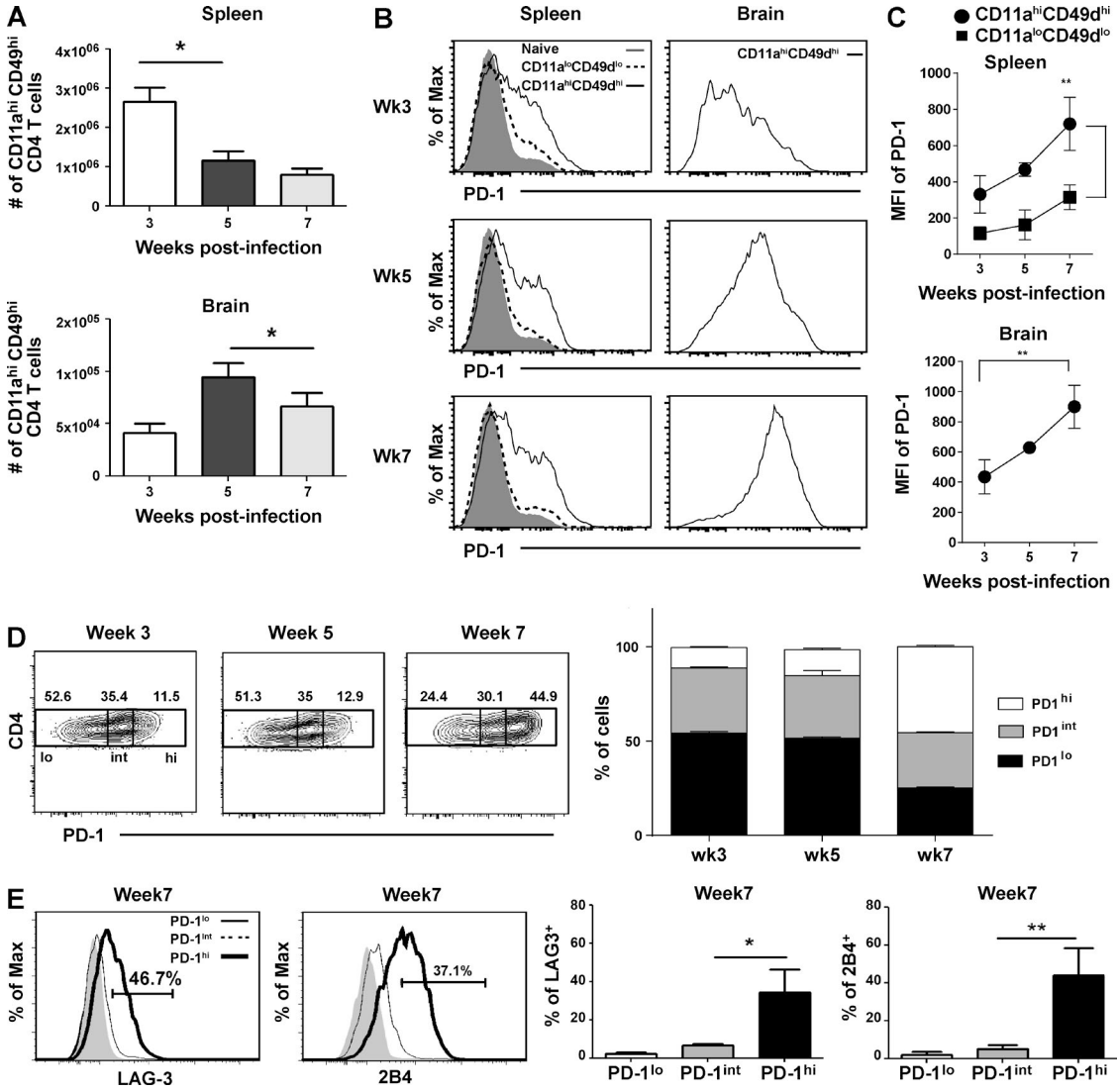

Figure 3. ***T. gondii*–specific CD4 T cells express increased levels of inhibitory receptors during chronic infection**. C57BL/6 mice were perorally infected with 10 ME49 tissue cysts. At 3, 5, and 7 wk p.i., absolute numbers of $CD4^+CD11a^{hi}CD49d^{hi}$cells in spleen (A, top) and brain (A, bottom) were evaluated. (B) PD-1 expression on $CD4^+CD11a^{hi}CD49d^{hi}$ cells in spleen and brain. Black line, $CD4^+CD11a^{hi}CD49d^{hi}$; dotted line, $CD4^+CD11a^{lo}CD49d^{lo}$; gray filled, naive $CD4^+$. (C) MFI of PD-1 expression at 3, 5, and 7 wk p.i. Circle, $CD4^+CD11a^{hi}CD49d^{hi}$; square, $CD4^+CD11a^{lo}CD49d^{lo}$. (D) PD-1 levels on the cells expressing surrogate markers was measured and ratio of $PD\text{-}1^{lo}$, $PD\text{-}1^{Int}$, and $PD\text{-}1^{hi}$ was performed. (E) Multiple inhibitory receptor expression on $CD4^+CD11a^{hi}CD49d^{hi}$ T cells in $PD\text{-}1^{lo}$, $PD\text{-}1^{Int}$, and $PD\text{-}1^{hi}$ population. LAG-3 and 2B4 expression on splenic *T. gondii*–specific CD4 T cells ($CD4^+CD11a^{hi}CD49d^{hi}$) at 7 wk p.i. Histogram overlays show the frequency of $PD\text{-}1^{hi}$ $CD4^+CD11a^{hi}CD49d^{hi}$ T cells that coexpress LAG-3 or 2B4 at 7 wk p.i. (left). The mean percentage of $PD\text{-}1^+LAG\text{-}3^+$ or $PD\text{-}1^+2B4^+$ cells (right). The data represent three experiments with at least three mice per group. P-values were obtained using the Student's *t* test. *, $P < 0.05$; **, $P < 0.01$ (mean ± SEM).

As earlier studies have shown that memory CD8 T cells are more prone to exhaustion (Bhadra et al., 2012), we next analyzed the pattern of expression of inhibitory receptor and transcription factor (PD-1 and Blimp-1) associated with T cell exhaustion in memory CD4 T cell subsets. CD4 effector memory (T_{EM}; $CD62L^{lo}CD44^{hi}$) or central memory (T_{CM}; $CD62L^{hi}CD44^{hi}$) cells were evaluated for expression of PD-1 and Blimp-1. As shown in Fig. 6, A and B, at the peak of exhaustion (7 wk p.i.), the T_{CM} subset exhibited higher levels of Blimp-1 and PD-1 expression as compared with the T_{EM} or cells bearing naive phenotype (Fig. 6, A and B). To further dissect the mechanism for the elevated expression of PD-1 on phenotypically defined memory T cells, we next examined the epigenetic program at the PD-1 promoter in naive and memory CD4 T cell subsets recovered from infected animals. This would determine if persistent *T. gondii* infection resulted in changes to the epigenetic program in *T. gondii*–specific CD4 T cells. Bisulfite sequencing methylation analysis of the PD-1

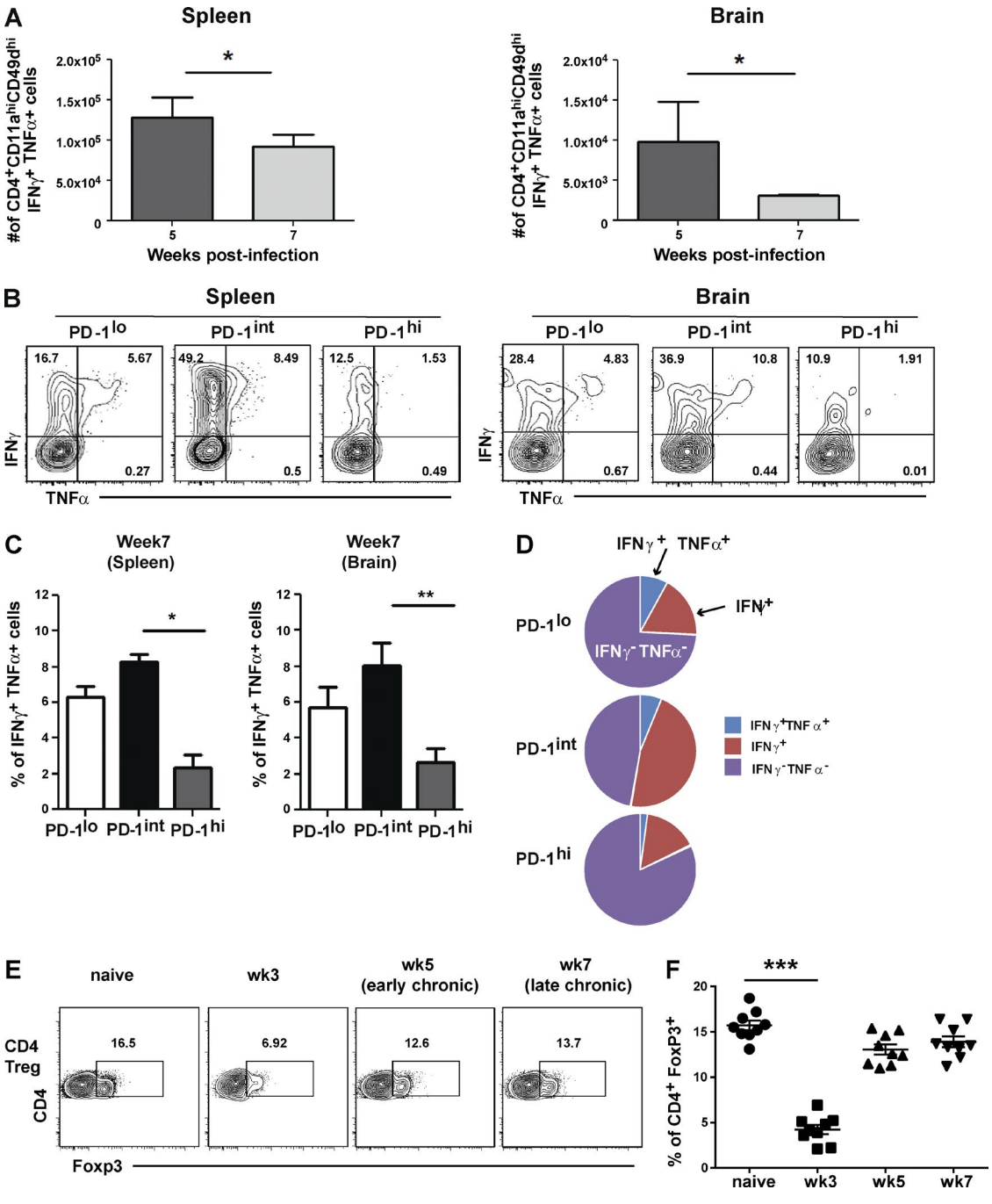

Figure 4. ***T. gondii*–specific CD4 T cells exhibit signs of functional exhaustion during chronic infection**. (A) Absolute number of functional (IFN-γ$^+$TNF$^+$) *T. gondii*–specific (CD11a^{hi}CD49d^{hi}) CD4 T cells at 5 and 7 wk p.i. in spleen and brain. (B) IFN-γ and TNF production of spleen and brain *T. gondii*–specific CD4 T cells was analyzed by gating on PD-1^{lo}, PD-1^{Int}, and PD-1^{hi} subsets from spleen (left) and brain (right) at 7 wk p.i. (C) Frequency of functional (IFN-γ$^+$TNF$^+$) T cells in PD-1^{lo}, PD-1^{Int}, and PD-1^{hi} subsets at 7 wk p.i. (D) Boolean gating analysis of the secretion of cytokines (IFN-γ$^+$TNF$^+$) by PD-1^{lo}, PD-1^{Int}, and PD-1^{hi} at 7 wk p.i. (E) CD4$^+$FoxP3$^+$ (T reg) cells were detected in splenocytes at 3, 5, and 7 wk p.i. (F) Frequency of T reg cells represented by dot graph. The data represent three experiments with at least three mice per group. P-values were obtained using the Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (mean ± SEM).

promoter was performed using genomic DNA isolated from purified naive and memory CD4 T cells from mice at 7 wk p.i. As expected, the PD-1 promoter in the T_{CM} and T_{EM} CD4 T cell subsets were dramatically demethylated compared with naive CD4 T cells from infected animals (Fig. 6, C and D). In addition, corresponding with the pattern of PD-1 protein ex-

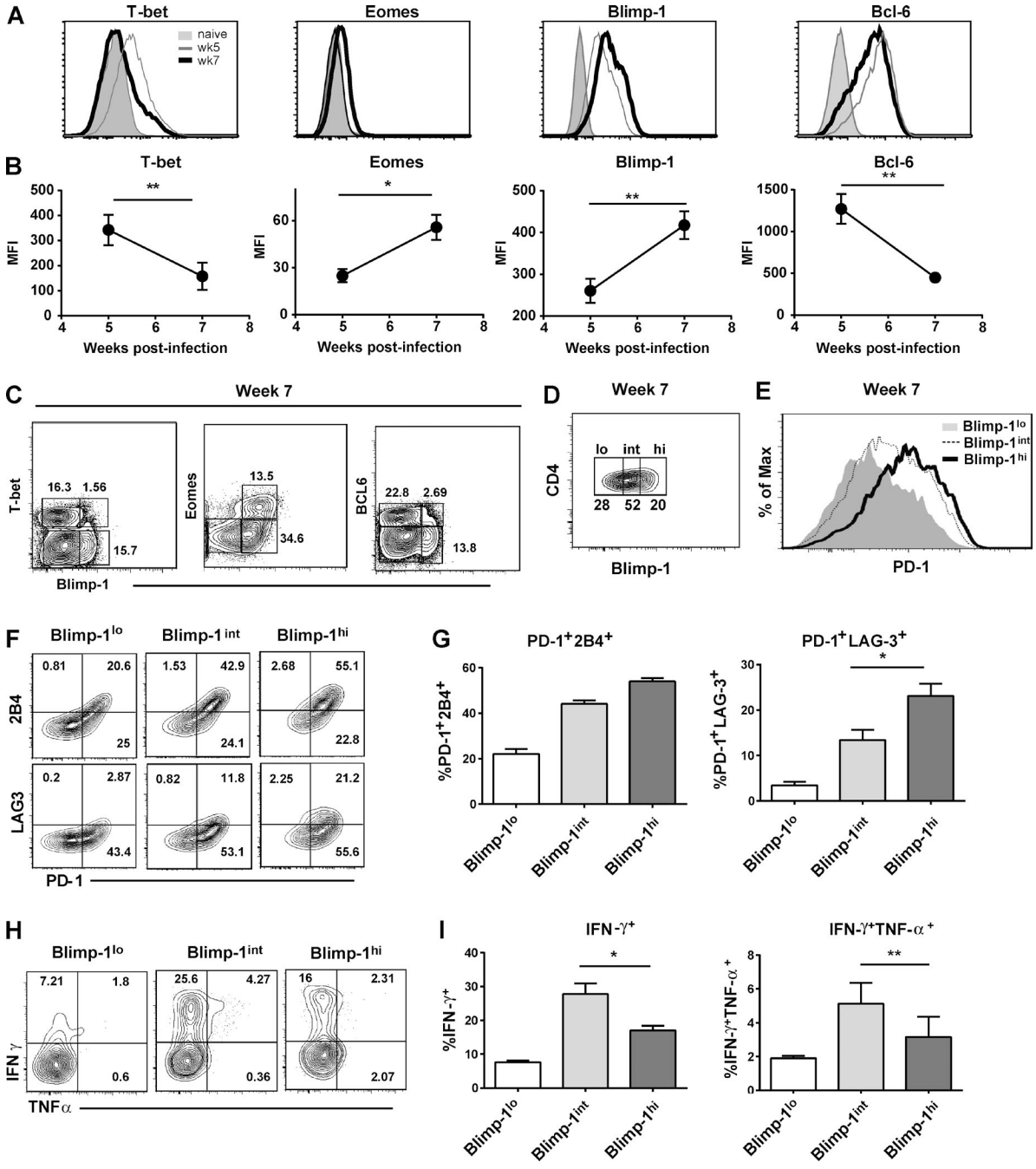

Figure 5. **Elevated Blimp-1 expression is directly correlated with increased inhibitory receptor expression and reduced functionality of *T. gondii*–specific CD4 T cells during chronic infection.** (A) Representative histograms of T-bet, Eomes, Blimp-1, and BCL6 expression in *T. gondii*–specific CD4 T cells at 5 and 7 wk p.i. Gray filled, naive; gray line, 5 wk pi; and black thick line, 7 pi. (B) MFI at 5 and 7 wk p.i. gated on PD-1^{hi} subset. (C) Analysis of T-bet, Eomes, and Bcl6 expression on Blimp-1^{hi} population. (D) Gating strategy for analyzing Blimp-1^{lo}, Blimp-1^{Int}, and Blimp-1^{hi} expressing *T. gondii*–specific CD4 T cells. (E) PD-1 expression on Blimp-1^{lo}, Blimp-1^{Int}, and Blimp-1^{hi} expressing cells (Black line, Blimp-1^{hi}; dot line, Blimp-1^{Int}; and gray filled, Blimp-1^{lo}). (F) Multiple inhibitory receptors expression (PD-1, 2B4, and LAG-3) on Blimp-1^{lo}, Blimp-1^{Int}, and Blimp-1^{hi} (G) Frequency of cells. (H) Functionality of Blimp-1^{lo}, Blimp-1^{Int}, and Blimp-1^{hi} on *T. gondii*–specific CD4 T cells. (I) Percentage of IFN-γ^{+} cells and IFN-γ^{+}TNF^{+}. The data represent three experiments with at least three mice per group. P-values were obtained using the Student's *t* test. *, $P < 0.05$; **, $P < 0.01$ (mean ± SEM).

pression, the methylation of PD-1 in the T_{EM} was significantly greater than in the T_{CM} (Fig. 6 E). These data indicate that the *T. gondii*–specific T_{CM} phenotype, CD4 T cells had acquired a transcriptionally permissive epigenetic program at the PD-1 promoter. Because the naive CD4 T cells from the infected animals had not acquired a demethylated PD-1 promoter, promoter demethylation in the *T. gondii*–specific CD4 T cell memory subsets is likely antigen mediated.

Prior studies have demonstrated that Blimp-1 expression enhances the terminal differentiation of CD8 T cells during LCMV infection (Rutishauser et al., 2009) and represses the acquisition of memory cell properties by effector cells. Therefore, to assess the role of Blimp-1 in CD4 T cell exhaustion, ERT cre *prdm1 fl/fl* (cKO mice; Reinert et al., 2012; Neumann et al., 2014; Tellier et al., 2016), mice were treated with tamoxifen at 6 wk after *T. gondii* infection, and the deletion of transcription factor was established by flow cytometry. After Blimp-1 deletion, the phenotype and function of *T. gondii*–specific CD4 T cells ($CD4^+CD11a^{hi}CD49d^{hi}$) were analyzed at 7 wk p.i. Interestingly, we observed a significant increase in the quantity of *T. gondii*–specific CD4 T cells ($CD4^+AS15^+$ and $CD11a^{hi}CD49d^{hi}$) after tamoxifen treatment (Fig. 6 F, left). We next measured the effect of Blimp-1 deletion on the T_{EM} and T_{CM} subsets. Consistent with the greater degree of functional exhaustion in the T_{CM} phenotyped cells, tamoxifen treatment resulted in a significant expansion in the percentage of T_{CM} ($CD44^{hi}CD62^{hi}$) phenotyped cells as compared with T_{EM} ($CD44^{hi}CD62^{lo}$) population (Fig. 6 F, right). Moreover, in the absence of Blimp-1, we also observed a significant reduction in PD-1 expression of T_{CM} population (Fig. 6, G and H). Collectively, these results demonstrate that the up-regulated expression of Blimp-1 results in the elevated expression of inhibitory receptors and is correlated with their reduced functionality during chronic *T. gondii* infection.

Recently, it has been reported that, compared with T_{EM}, T_{CM} have increased stem cell–like properties and can give rise to multiple effector and memory subsets upon antigen reencounter (Graef et al., 2014). Thus, increased expression of PD-1 by T_{CM}, and their exhaustion, may compromise the host's ability to maintain control of chronic *T. gondii* infection. In the intermediate host, *T. gondii* undergoes stage conversion into a rapidly replicating tachyzoite that is responsible for reactivation of toxoplasmosis (Lyons et al., 2002; Bhadra et al., 2011). Tamoxifen treatment led to significant decreases in the tachyzoite numbers in brain, blood, and spleen (Fig. 6, I and J). To determine this effect was not related to tamoxifen, the treatment of wild-type mice with the drug had no effect (Fig. 6 I). In spleen, analysis of the frequency of tachyzoite in the leukocyte subsets revealed that the parasite preferentially infected $CD11c^+$, $CD11b^+$, Macrophage ($F4/80^+$), and B cells, and tamoxifen treatment led to decreased cell infection in the cKO mice (Fig. 6 K). Collectively, these findings suggest that conditional deletion of Blimp-1 in the mice carrying chronic *T. gondii* infection results in the reduction of PD-1 expression on T_{CM} CD4 T cells, which leads to better parasite control by the infected host.

The role of Blimp-1 in the exhibition of *T. gondii*–specific CD4 T cell exhaustion is cell intrinsic

The aforementioned data bear the implication that absence of Blimp-1 affects both the quantity and quality of CD4 T cell response. However, it does not explain if CD4 T cell–intrinsic Blimp-1 expression in vivo can elicit a similar effect. Moreover, it does not rule out if Blimp-1 expression on other cell types via changes in microenvironment such as cytokine milieu or antigen burden affecting CD4 T cells. Next, to determine if Blimp-1 plays a cell-intrinsic role in CD4 T cell dysfunction, mixed bone marrow chimeras between WT and Blimp-1 conditional knock out (ERT cre Prdm1 *fl/fl*) at 1:1 ratio (WT/cKO mice) were generated (Fig. 7 A). The chimeras were infected and treated with tamoxifen for 1wk starting at 4 wk p.i. Flow cytometric assay was performed to confirm that treatment abrogates Blimp-1 expression in the CD4 T cells (Fig. 7 B). At 5 wk p.i., PD-1 and T-bet expression on *T. gondii*–specific CD4 T cells was measured. We observed that Blimp-1–deficient CD4 T cells had decreased levels of PD-1 and increased T-bet expression as compared with WT cells (Fig. 7 C). Furthermore, measurement of the cytokine profile ($IFN\text{-}\gamma^+TNF^+$) revealed that the *T. gondii*–specific CD4 T cells from the cKO had increased functionality as compared with WT cells (Fig. 7, D and E). These data demonstrate that Blimp-1 plays a cell-intrinsic role in mediating *T. gondii*–specific CD4 T cell dysfunction.

T gondii–specific CD4 T cells help to reduce CD8 T cell exhaustion in mice carrying chronic *T. gondii* infection

Because CD4 T cells play an important role in generation of functional CD8 T cell responses (Bevan, 2004), we next determined if the dysfunctional state of CD4 T cells during chronic toxoplasmosis influenced CD8 T cell response. To explore this hypothesis, *T. gondii*–specific and nonspecific CD4 T cell populations were isolated from mice at 2 wk p.i. and transferred into distinct congenically marked recipients that were infected 5 wk before transfer (Fig. 8 A, left). The donor population was recoverable at 2 wk after transfer as shown in Fig. 8 (right). Antigen specificity of CD8 T cells was measured by surrogate marker (CD11a and CD44) expression (Masopust et al., 2007), which was validated by using class I tetramer (Tg057; unpublished data). Similar to antigen–specific CD4 T cells, $CD8^+Tg057^+$ cells coexpressed CD11a and CD44 and the population secreted higher levels of IFN-γ than $CD11^{lo}CD44^{lo}$ (unpublished data). Furthermore, majority of splenic $IFN\text{-}\gamma^+GranzymeB^+$ CD8 population expressed $CD11a^{hi}CD44^{hi}$ in spleen (unpublished data). At 7 wk p.i., the CD8 T cells from the spleen and brain of recipient mice were evaluated for expression of various inhibitory receptors and transcription factors. Interestingly, transfer of nonexhausted *T.*

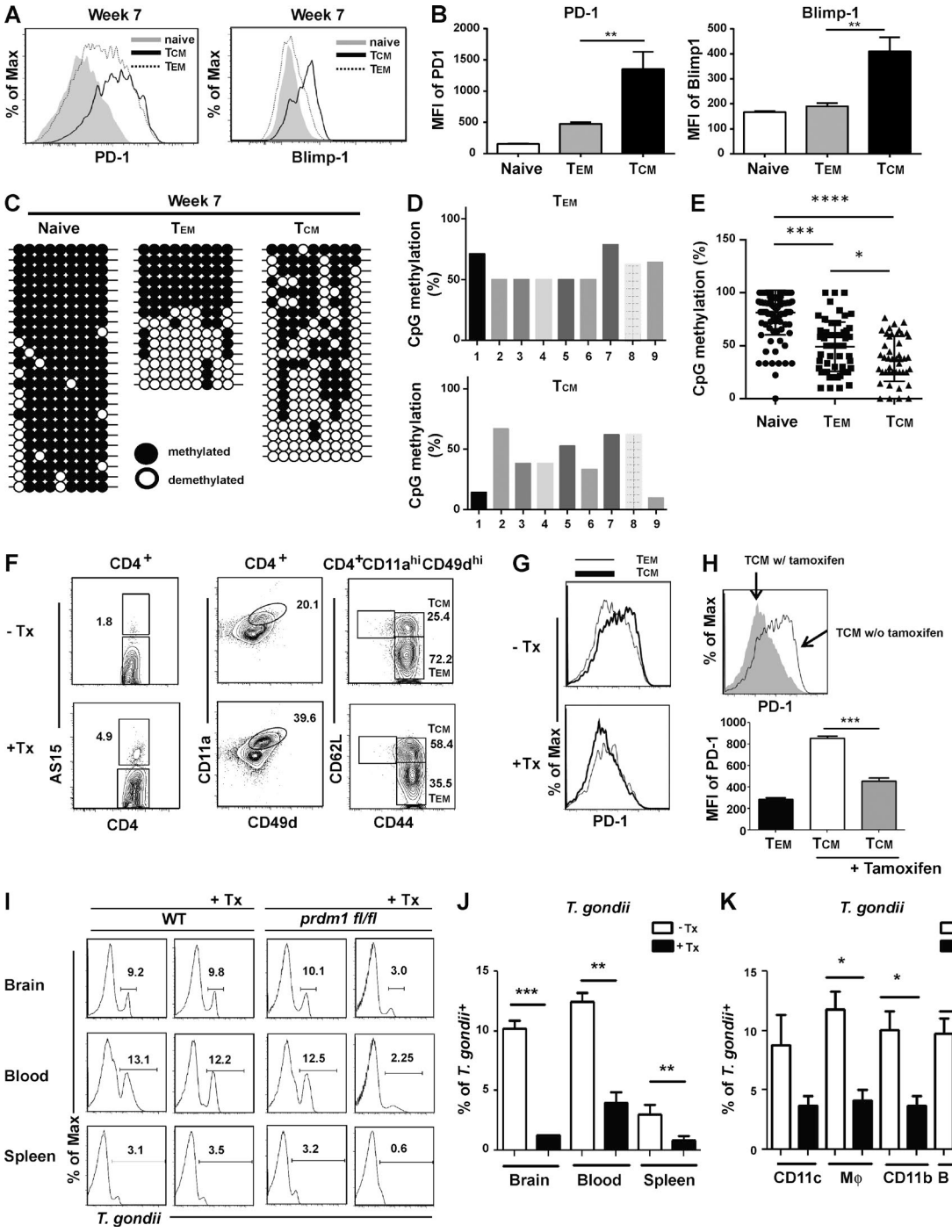

Figure 6. **Central memory-phenotype CD4 T cells are preferentially exhausted during chronic *T. gondii* infection.** (A) PD-1 and Blimp-1 expression in naive, T_{EM}, and T_{CM} CD4 T cells at 7 wk p.i. (B) MFI of PD-1 and Blimp-1. (C) Bisulfite sequencing methylation analysis of the PD-1 regulatory region in naive and *T. gondii*–specific CD4 T cells ($CD4^+CD11a^{hi}CD49d^{hi}$) at wk7 p.i. Genomic DNA was isolated from FACS-purified naive ($CD4^+CD11a^{lo}CD49d^{lo}CD44^{lo}CD62^{hi}$) and memory CD4 T cell subsets (T_{EM}, $CD4^+CD11a^{hi}CD49d^{hi}CD44^{hi}CD62L^{lo}$ vs. T_{CM}, $CD4^+CD11a^{hi}CD49d^{hi}CD44^{hi}CD62L^{hi}$). Genomic DNA was bisulfite treated and PCR amplified using PD-1 locus-specific primers. (D) Each horizontal line represents an individual sequenced clone. (E) CpG methylation rate of T_{EM} and T_{CM} in PD-1 regulatory region. Filled circles, methylated cytosine. Open circles, demethylated cytosine. (F) ERT cre *prdm1fl/fl* (cKO) mice were injected by tamoxifen at 6 wk p.i., mice were sacrificed at 7 wk p.i. Cells were stained with CD4 and MHCII-restricted tetramer (AS15) and gated on *T. gondii*–specific CD4 T cells ($CD4^+CD11a^{hi}CD49d^{hi}$, left). T_{EM} and T_{CM} were analyzed by CD62L and CD44. (G) PD-1 expression between T_{EM} and T_{CM} with tamoxifen treatment. (H) Overlay PD-1 expression on T_{CM} with tamoxifen to nontreated T_{CM}. (I) *T. gondii*–infected cells in brain, blood, and spleen. Cells were gated on leukocyte population and samples assayed by flow cytometry. (J) Bar

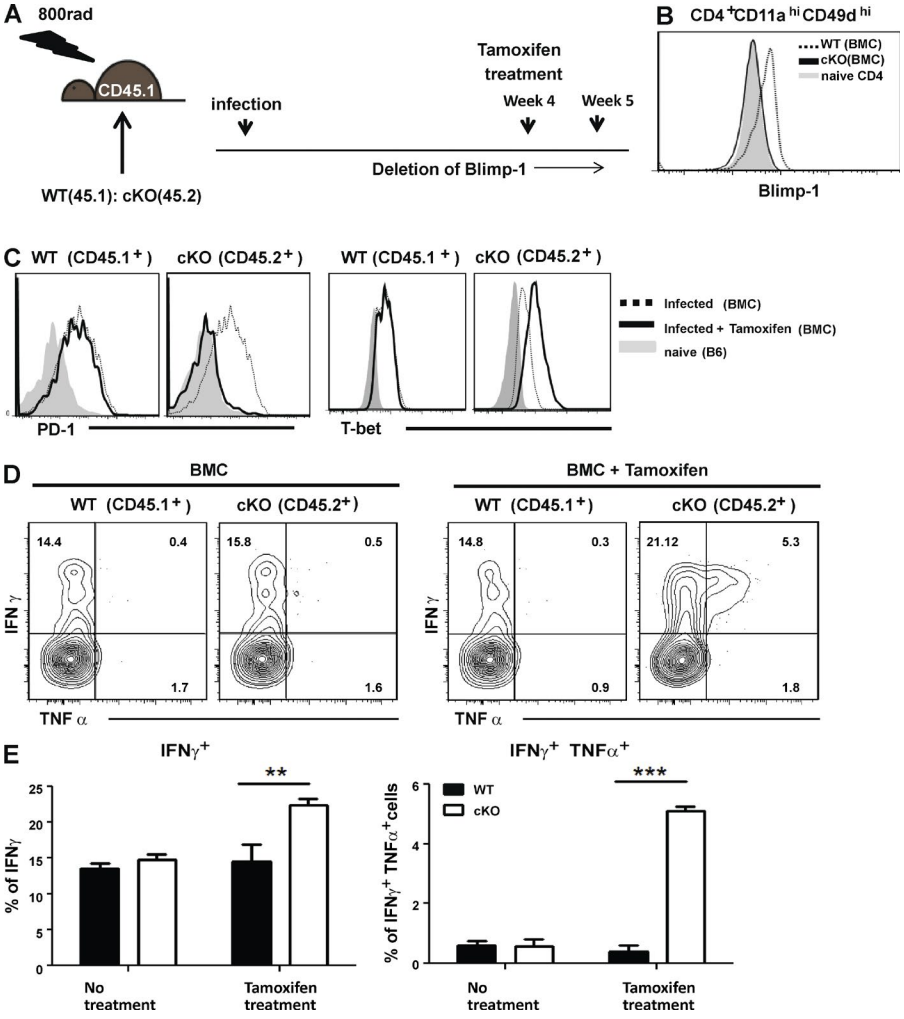

Figure 7. **The intrinsic role of Blimp-1 in *T. gondii*–specific CD4 T cells during chronic infection.** (A) Bone marrow cells purified from WT (CD45.1) and cKO (CD45.2) mice were transferred at 1:1 ratio to irradiated (800 rad) CD45.1 animals. 8 wk after transfer, the chimeric mice were infected perorally with 10 cysts. At 4 wk p.i., recipient mice were treated with tamoxifen for 5 d and 1 wk later, the animals were sacrificed. (B) Blimp-1 expression on *T. gondii*–specific CD4 T cells ($CD4^+CD11a^{hi}CD49d^{hi}$, dotted line) from WT animals, *T. gondii*–specific CD4 T cells ($CD4^+CD11a^{hi}CD49d^{hi}$, black line) from cKO mice and CD4 T cells from naive mice (gray filled) after tamoxifen treatment. (C) PD-1 and T-bet expression on *T. gondii*–specific CD4 T cells (dotted line, infected BMC; thick line, infected BMC with tamoxifen treatment; gray filled, naive B6 mice). (D and E) IFN-γ and TNF production of splenic *T. gondii*–specific CD4 T cells. The data represent three experiments with at least three mice per group. P-values were obtained using the Student's *t* test. **, $P < 0.01$; ***, $P < 0.001$ (mean ± SEM).

gondii–specific CD4 T cells significantly reduced PD-1 expression on *T. gondii*–specific CD8 T cells in both spleen and brain of recipient mice relative to animals receiving nonspecific CD4 T cells. (Fig. 8, B and C). *T. gondii*–specific CD8 T cells from the recipients treated with *T. gondii*–specific CD4 T cell exhibited increased T-bet (Fig. 8 B, panel 2), and decrease in Eomes and Blimp-1 expression (Fig. 8 B, panel 3 and 4). Additionally, treatment with *T. gondii*–specific CD4 T cells also enhanced the proliferation of *T. gondii*–specific CD8 T cells both in spleen and brain as measured by Ki67 expression (Fig. 8, B [panel 5] and C [panel 2]). Changes in the expression of PD-1, transcription factors, and proliferation of CD8 T cells from mice receiving *T. gondii*–specific CD4 T cells was also reflected by MFI (unpublished data). Transfer of *T. gondii*–specific CD4 T cells also increased the functionality of *T. gondii*–specific CD8 T cells in recipient mice, as defined by cytokine and Granzyme expression. Indeed, *T. gondii*–specific CD8 T cells from treated animals had increased IFN-γ and GranzymeB expression (Fig. 8 D). Surprisingly, transfer of nonexhausted *T. gondii*–specific CD4 T cells (CD45.2) did not significantly alter PD-1 or Blimp-1 expression or functionality (IFN-γ and TNF production) of recipient CD4 (CD45.1) T cells (Fig. 8, B–D). Thus, it is important to note that nonexhausted *T. gondii*–specific CD4 T cells help to rescue *T. gondii*–specific CD8 T cells, but not CD4 T cells, during advanced *T. gondii* infection.

Conditional deletion of Blimp-1 on CD4 T cells reduces CD8 T cell dysfunctionality and controls the reactivation of latent *T. gondii* infection

The aforementioned studies demonstrate that conditional deletion of Blimp-1 restores CD4 T cell helper function. We next

graph representing infected population in brain, blood, and spleen. (K) Percentage of infected mononuclear cells in spleen were evaluated. The data represent three experiments with at least three mice per group. P-values were obtained using the Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (mean ± SEM).

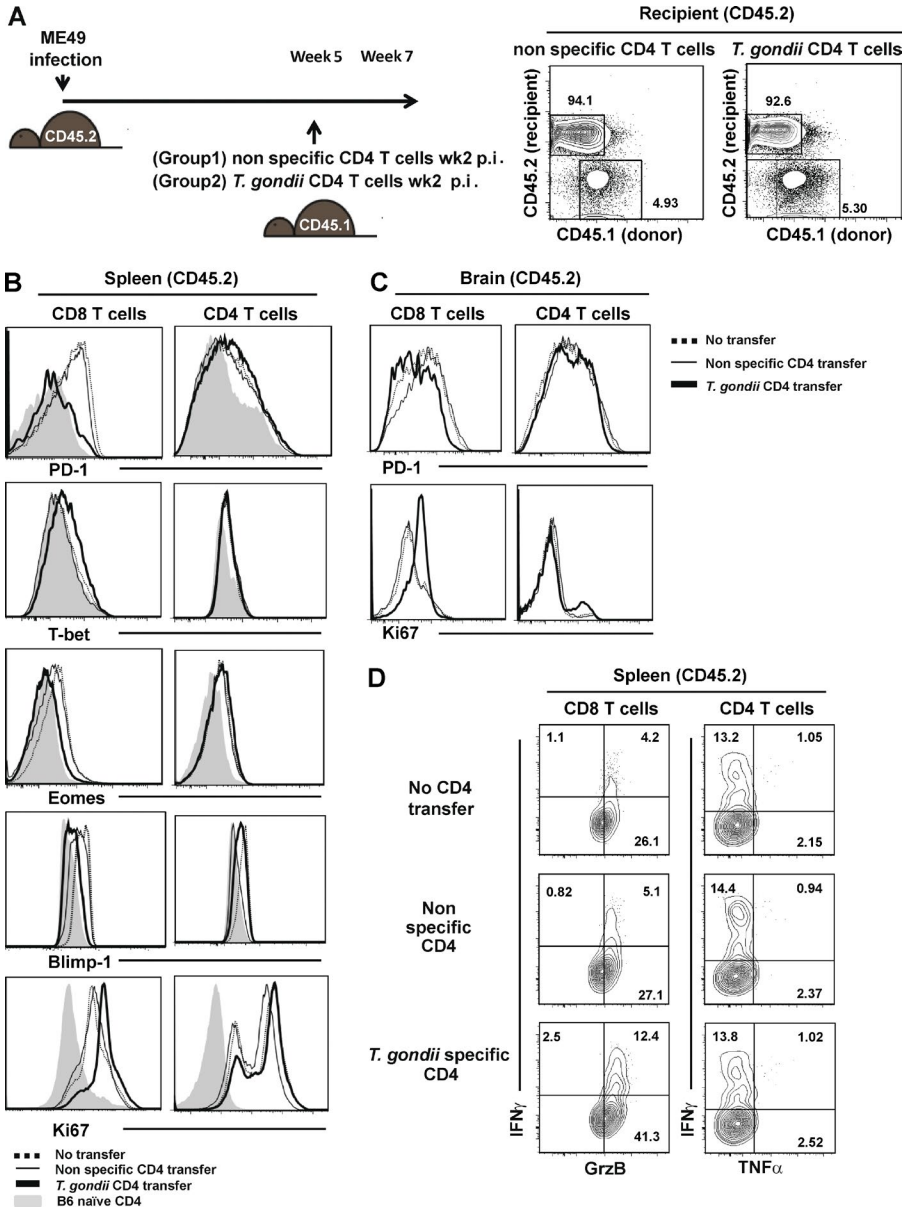

Figure 8. **Transfer of *T. gondii*–specific nonexhausted CD4 T cells reverses CD8 T cell response.** (A) Transfer strategy of *T. gondii*–specific nonexhausted CD4 T cells to mice carrying chronic infection (left). CD45.2 B6 animals (infected 5 wk before transfer) were treated with *T. gondii*–specific nonexhausted CD4 T cells ($CD4^+CD11a^{hi}CD49d^{hi}$) or nonspecific CD4 T cells ($CD4^+CD11a^{lo}CD49d^{lo}$) from CD45.1 donors. The cells from the donor mice were isolated at 2 wk p.i. and each recipient received 1×10^6 cells. Restoration of donor cell population in recipient mice (right). (B) *T. gondii*–specific splenic CD4 and CD8 population were analyzed for the expression PD-1, T-bet, Eomes, Blimp-1, and Ki67. (C) brain CD4 and CD8 T cells were assayed for PD-1 and Ki67 expression. (D) Polyfunctionality of *T. gondii*–specific CD8 T cells ($CD8^+CD11a^{hi}CD44^{hi}$) and CD4 T cells ($CD4^+CD11a^{hi}CD49d^{hi}$) in mice receiving nonexhausted *T. gondii*–specific CD4 T cells. The data represent three experiments with at least three mice per group.

determined if deletion of this transcription factor specifically reverses exhaustion of CD4 T cells and subsequently restores CD8 T cell functionality. To address a CD4 T cell–intrinsic mechanism for Blimp-1–mediated CD4 T cell exhaustion, adoptive transfer of cKO CD4 T cells into CD4 T cell–deficient mice was performed and reactivation of infection was monitored. CD4 T cells from cKO mice were purified and transferred into CD4 T cell–deficient mice (CD4 KO). The recipient animals were infected and treated with tamoxifen at 6 wk p.i. (Fig. 9 A). 1 wk later, animals were sacrificed and inhibitory receptors (Fig. 9, B and E), transcription factors (Fig. 9, C and F), and co-stimulatory receptors (Fig. 9, D and G) on recipient cerebral CD8 T cells were measured. As shown in Fig. 9 (B and E), deletion of Blimp-1 on CD4 T cells reduced the inhibitory receptors such as PD-1, CD160, and LAG-3 on *T. gondii*–specific CD8 T ($CD11a^{hi}CD44^{hi}$) cells, whereas co-stimulatory receptor (41BB) expression was up-regulated (Fig. 9, D and G). As expected, we observed down-regulation of inhibitory receptors and increased T-bet expression. Transcription factors (Blimp-1 and Eomes) were decreased on *T. gondii*–specific brain CD8 T cells as a result of tamoxifen treatment, indicating the reversal of exhaustion (Fig. 9, C and F). We next examined the ability of Blimp-1 conditional deletion to restore the functionality of recipient CD8 T cells. Indeed, Blimp-1 conditional deletion of CD4 T cells resulted in a significant increase in the population of polyfunctional CD8 T cells ($GranzymeB^+$, $IFN\text{-}\gamma^+$, and TNF^+; Fig. 9, H–J). Blimp-1 has been reported to repress inflammatory cytokines like IL-2 and IL-21 (Martins et al., 2008; Johnston et al., 2009) and induce IL-10 (Neumann et al.,

2014; Parish et al., 2014), a functionally suppressive cytokine. The evaluation of cytokine levels in the sera of the recipient mice treated with Blimp-1 deleted donor CD4 population was performed (Fig. 9, K–M). Interestingly, IL-2 and IL-21 were up-regulated and IL-10 was decreased in these animals. This suggested that these cytokines might be capable of helping *T. gondii*–specific CD8 T cell recover their proliferation and survival (IL-2) and maintain the memory function (IL-21) and escape from the suppressive cytokine (IL-10) released during chronic infection. As the reactivation of chronic toxoplasmosis leads to encephalitis, next the *T. gondii*–specific CD8 T cell response in the recipient animals treated with tamoxifen was assayed. As shown in Fig. 10 A, the donor CD4 T cell population was recovered in all tissues (blood, brain, and spleen) at 7 wk p.i. To determine if the increase in the *T. gondii*–specific CD8 ($CD8^+CD11a^{hi}CD49d^{hi}$) T cell poly-functional response translated into enhanced control of the parasite, the brains were assayed for the tachyzoites. As shown in Fig. 10 (B and C), significantly fewer parasites were detected in the tissues (blood, brain, and spleen) of recipients where donor CD4 T cells were deleted of Blimp-1. Tachyzoite–bradyzoite interconversion is believed to play a central role, not only in establishing the chronic infection, but also in disease recrudescence (Lyons et al., 2002; Bhadra et al., 2011). To further address if deletion of Blimp-1 results in control of infection, we performed confocal microscopic analysis using anti-SAG1 antibody (specific for tachyzoite antigen) to detect infected cells. Consistent with the flow cytometric data, the deletion of Blimp-1 on the donor CD4 T cells led to a decrease of tachyzoites in brain (Fig. 10 D). These findings suggest that the increase in inflammatory cytokines produced primarily by CD4 T cells may contribute in providing the help needed to maintain the functionality of an antigen-specific CD8 T cell population during chronic toxoplasmosis.

DISCUSSION

During chronic toxoplasmosis, CD4 T cells are believed to play an important secondary role by providing help to CD8 T cells, which are considered a primary effector population responsible for keeping the infection under control (Gazzinelli et al., 1992; Casciotti et al., 2002). Indeed, CD4 T cells have been described as an important source of IFN-γ during the acute phase of infection (Gazzinelli et al., 1991, 1992); however, their contribution in maintaining the chronicity of infection is not well defined. Earlier studies from our laboratory reported that a robust CD8 T cell immune response developed during *T. gondii* infection, but was unable to prevent the reactivation of latent infection in susceptible animals (Bhadra et al., 2011, 2012). This was attributed to increased expression of inhibitory receptors on CD8 T cells, which led to their dysfunctionality and subsequent reactivation of chronic infection in the host. However, the underlying causes for CD8 T cell dysfunction during chronic infection have not been explored. In this report, we demonstrate that the CD4 T cell subset during chronic toxoplasmosis gets exhausted due to increased Blimp-1 expression by these cells. We demonstrate that lack of adequate CD4 T cell help due to increased Blimp-1 expression is the primary cause of CD8 T cell exhaustion and that deletion of this transcription factor reverses CD8 T cell dysfunctionality and enhances host ability to contain chronic infection.

CD8 T cell exhaustion was first reported in mice infected with the chronic strain of LCMV (Zajac et al., 1998) and was subsequently extended to other persistent viral infections and cancer (Barber et al., 2006; Yi et al., 2010; Speiser et al., 2014; Pauken and Wherry, 2015). Recently, CD8 T cell exhaustion has also been reported during chronic parasitic infections, such as *T. gondii* and *Plasmodium*, as well as in mice infected with *Leishmania* (Hernández-Ruiz et al., 2010; Bhadra et al., 2011; Horne-Debets et al., 2013). In addition to CD8 exhaustion, CD4 T cell exhaustion has been reported during HIV, LCMV, and HCV infection (Fletcher et al., 2005; Day et al., 2006; Kasprowicz et al., 2008; Crawford et al., 2014; Morou et al., 2014). CD4 T cells have been reported to express inhibitory receptors during chronic infections similar to exhausted CD8 T cells (Butler et al., 2011; Mou et al., 2013), although there are differences in the degree of expression and some receptors like CTLA4 are biased toward CD4 T cells (Crawford et al., 2014).

However, the status of CD4 T cells and how they respond during chronic *T. gondii* infection has not been extensively evaluated. In this study, we observed that *T. gondii*–specific CD4 T cells during chronic infection exhibit increased expression of inhibitory receptors (PD-1, LAG-3, and 2B4) concomitant with decreased functionality, as shown by IFN-γ and TNF production. Remarkably, examination of inhibitory receptors and co-stimulatory receptors at early chronic infection revealed that CD4 T cell dysfunction is more pronounced than CD8 T cells. Similar to the studies performed with exhausted CD8 T cells during viral infection (Shin et al., 2009), Blimp-1 was highly up-regulated on *T. gondii*–specific $PD\text{-}1^{hi}$ CD4 T cells during chronic *T. gondii* infection. Interestingly, as compared with CD4 T_{EM}, Blimp-1 was increasingly expressed on CD4 T_{CM}, which was correlated with the high PD-1 expression by this subset. As long-term immunity against pathogens is attributed to T_{CM} population (Zaph et al., 2004; Pakpour et al., 2008), these findings identify a potentially serious flaw in the maintenance of immune-protection against the parasite in the animals carrying chronic infection due to defect in the T_{CM} population.

The critical role of Blimp-1 in CD4 T cell exhaustion/dysfunctionality was established by using conditional knockout mice for this transcription factor. Deletion of Blimp-1 in these animals reduced the expression of inhibitory factors on *T. gondii*–specific CD4 T cells, increased their functionality and ultimately led to better control of infection as demonstrated by the number of tachyzoites in various tissues.

Furthermore, our data demonstrates that CD8 T cell dysfunctionality is linked to CD4 T cell exhaustion. Adoptive transfer of nonexhausted parasite-specific CD4 T cells

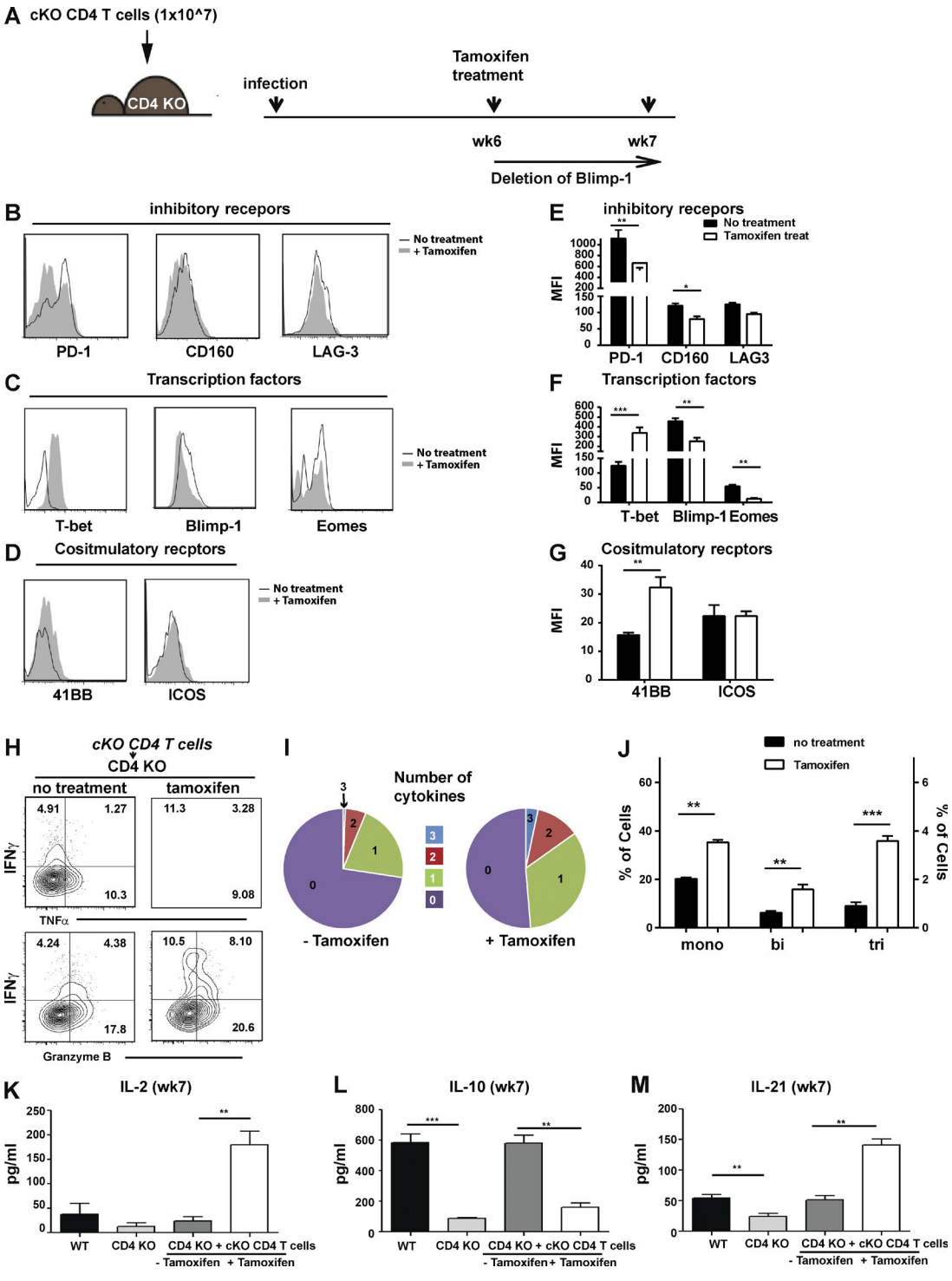

Figure 9. **Deletion of Blimp-1 on CD4 T cells reverses *T. gondii*–specific brain CD8 T cell exhaustion.** (A) Purified CD4 T cells (1×10^7) from cKO were transferred to CD4-deficient mice. The recipients were infected with 10 cysts of *T. gondii* and at 6 wk p.i. tamoxifen was administered to a group of recipients. (B) PD-1, CD160, and LAG-3 (inhibitory receptors). (C) T-bet, Blimp-1, and Eomes (transcription factors). (D) 41BB and ICOS (co-stimulatory receptors) expression on *T. gondii*–specific CD8 ($CD8^+CD11a^{hi}CD44^{hi}$) T cells in brain of recipient mice. (E) MFI of inhibitory receptors, (F) transcription factors, and (G) co-stimulatory receptors in *T. gondii*–specific CD8 T cells in brain. (H) Intracellular IFN-γ, TNF, and GranzymeB production of *T. gondii*–specific CD8 T cells in brain. (I) Boolean gating analysis with proportion of polyfunctional (IFN-γ, GranzymeB, and/or TNF) CD8 T cells in recipient mice. (J) Percentage of *T. gondii*–specific CD8 T cells exhibiting one, two, or three functions (IFN-γ, Granzyme B, and/or TNF), shown

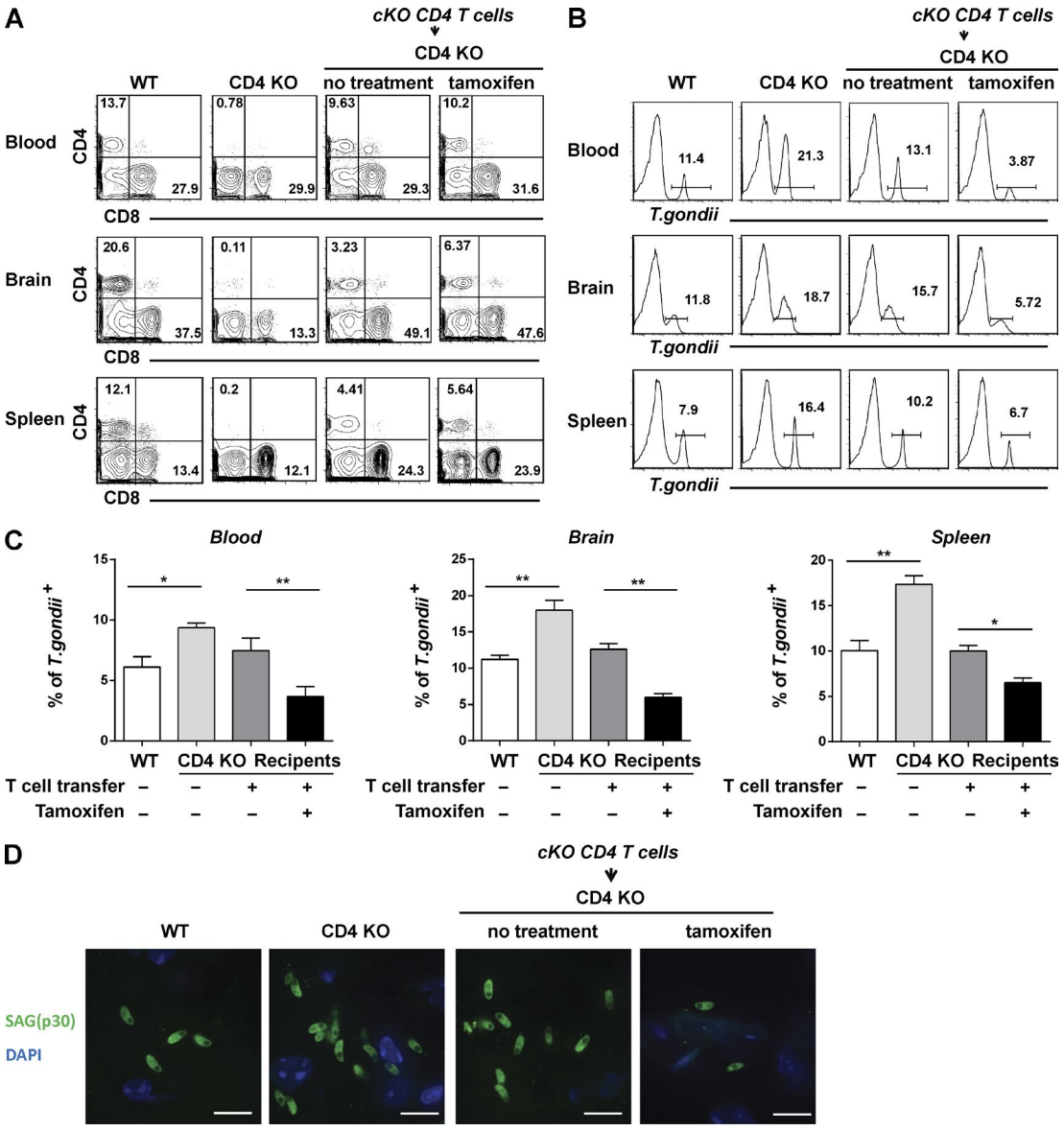

Figure 10. **Deletion of Blimp-1 on CD4 T cells inhibits parasites load during chronic stage of the infection.** (A) CD4/CD8 profile in CD4KO-recipient mice in blood, brain, and spleen. (B) Flow cytometric analysis of *T. gondii*–infected cells in blood, brain, and spleen on the samples gated on leukocytes. The assay was performed at 7 wk p.i. Bars, 5 µm. (C) Percentage of *T. gondii*–infected cells in each organ. (D) Tachyzoites were examined by anti-SAG (p30) with confocal microscopy. The data represent three experiments with at least three mice per group. P-values were obtained using the Student's *t* test. *, $P < 0.05$; **, $P < 0.01$ (mean ± SEM).

elevated T-bet levels and decreased PD-1 and Blimp-1 expression on *T. gondii*–specific CD8 T cells. More importantly, the transfer of nonexhausted CD4 T cells increased the poly-functionality of *T. gondii*–specific CD8 T cell population in the recipient mice and decreased parasite burden. Interestingly, in contrast to the effect on CD8 T cells, the transfer of nonexhausted CD4 T cells had minimal effect on the CD4 population from the recipient animals and no changes in terms of inhibitory receptor expression or functionality was noted. The precise mechanism by which CD4 T cells rescue CD8 T cell dysfunction is not well defined, but may be attributed to number of factors like CD40-40L pathway, CD4

as bar graphs (K–M). Serum was obtained by tail bleeding and analyzed for IL-2, IL-10, and IL-21 at wk7 p.i. by enzyme-linked immunosorbent assay (ELISA). Bar graph represents cytokine production (IL-2, IL-10, and IL-21), respectively at 7 wk p.i. The data represent three experiments with at least three mice per group. P-values were obtained using the Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (mean ± SEM).

and CD8 direct interaction, indirect DC help or cytokine effect (Bennett et al., 1997, 1998; Ozaki et al., 2002; Wilson and Livingstone, 2008; Wiesel and Oxenius, 2012). The roles of CD4 T cells in maintaining CD8 functionality may also be dependent on the nature of infection.

Another interesting finding is that exhausted CD4 T cells may have a defect in trafficking to the brain, which is an important site of *T. gondii* infection. In adoptive transfer studies, we observed that the reconstitution rate of Blimp-1 expressing exhausted CD4 T cell in brain was approximately twofold less than Blimp-1–deleted cells, whereas no differences in circulating CD4 T cell population in blood were observed (Fig. 10 A). In support of this data, decreased CD4 T cell recruitment in the brain was further confirmed with a confocal study that demonstrated that deletion of Blimp-1 on CD4 T cells led to their increased migration to the brain (Fig. 10 A). It is important to mention that decreased expression of CXCR3, a chemokine receptor, was observed on *T. gondii*–specific, antigen-specific CD8 T cells expressing high levels of PD-1 (unpublished data). A critical role of the chemokine receptor CXCR3 and its ligand CXCL-10 in the migration of CD8 T cells to the brain tissue during *T. gondii* infection has been reported (Khan et al., 2000; Harris et al., 2012). Thus, it is important to conduct studies that will address the issue of the ability of PD-1^{hi} and/or Blimp-1^{hi}–expressing T cell migration to functional sites of *T. gondii* infection.

In conclusion, our results reveal that during chronic infection, *T. gondii*–specific CD4 T cells express functional and phenotypic exhaustion, including reduced cytokine secretion and increased expression of multiple inhibitory receptors. Interestingly, this dysfunctionality is more pronounced on the T_{CM} subset of the CD4 T cell population. Remarkably, conditional deletion of Blimp-1 on CD4 T cells rescued CD8 T cell exhaustion and led to efficient control of parasite load in the infected host, linking CD8 T cell dysfunctionality to increased Blimp-1 expression on CD4 T cells. Our findings have important implications in HIV-infected individuals, in whom reduced CD4 T cell numbers are known to cause reactivation of latent infection (Sainz et al., 2013; Serrano-Villar et al., 2014). In addition to reduced CD4 T cell counts, dysfunctionality of these cells in these individuals may further compromise their ability to provide essential help to CD8 T cells that, as shown in (Fig. Figs 8–10), are primary effector cells against this opportunistic pathogen. Furthermore, Blimp-1 may be an important target for vaccination studies where the T_{CM} population needs to be maintained for long-term protection.

MATERIALS AND METHODS

Mice

6–8-wk-old female C57BL/6, B6.SJL(CD45.1), ERT cre, prdm1 *fl/fl*, CD4-deficient mice were purchased from Jackson laboratories. For generation of cKO (ERT cre Prdm1*fl/fl*) mice, ERT cre and prdm1 *fl/fl* cKO mice were bred. Animal studies were performed in agreement with Institutional Animal Care and Use Committee (IACUC)–approved guidelines at George Washington University Medical Center. All experiments were approved by the George Washington University Medical Center Animal Resource Facility IAC UC under protocol approval number A052 in accordance with the American Association for Accreditation of Lab Animal Care certified guidelines.

Parasites and infection

T. gondii cysts of ME49 strain were prepared from the brains of infected mice. Animals were infected with 10 cysts per mouse via intragastric route and sacrificed by CO_2 inhalation at 3, 5, or 7 wk after infection. *T. gondii* lysate antigen (TLA) was extracted from RH strain of parasite and preparation was performed as previously described (Khan and Kasper, 1996).

Tamoxifen preparation and administration

Tamoxifen (Sigma-Aldrich; T5648) was dissolved in corn oil to make solutions of 20 mg/ml, which were subsequently protected from light. The solutions were freshly prepared the day before each injection and dissolved overnight at room temperature. Recipient mice were administered the drug via peroral route at the dosage of 1 mg/animal every 24 h over a 5-d period.

Lymphocyte isolation and fluorescent antibody staining

Single-cell suspensions were prepared from spleen and brain of mice. Red blood cell lysis was performed to remove red blood cells from spleen cell suspensions. Brains were individually washed in PBS, and then passed through a 70-µm cell strainer, followed by gradient centrifuge (2,000 rpm for 20 min) in 30% Percoll solution containing 100 IU/ml of heparin. Pellet was then resuspended in cold PBS-2% FCS. The following antibodies were used in cell surface staining and intracellular staining of lymphocytes: CD4(GK1.5), CD8β (H35-17.2), PD-1 (RMP-1), LAG-3 (C9B7W), 2B4 (eBio244F4), CD45.1 (A20), CD45.2 (104), CD11a (M17/4), CD49d (R1-2), CD44 (IM7), CD62L(MEL-14), T-bet (eBio4B10), Eomes (Dan11mag), IFN-γ (XMG1.2), Ki-67 (SolA15), TNF(MP6-XT22), GranzymeB (NGZB), and Ki-67 (SolA15) were obtained from Affymetrix eBioscience. Blimp-1 (5E7) was purchased from BD. For intracellular staining for transcription factors, surface staining was performed, and then fix/perm buffer (eBioscience) were used. Next, T-bet, Eomes, Blimp-1, and BCL6 were stained after fixation.

Cell fluorescence was measured with a Cytek upgraded eight-color FACSCalibur cytometer (BD), which accounts for differences in fluorescence scale. Data were analyzed using FlowJo (Tree Star) software.

Intracellular cytokine detection

For cytokine detection, restimulation was performed for 20 h with 20 µg/ml of TLA in supplemented Iscove's Complete DMEM at 37°C in 5% CO_2. Monensin (BD) was added during the final 4 h of restimulation. After surface staining,

cells were then fixed using IC Fixation Buffer (Invitrogen) for 10 min. For detection of intracellular cytokines, cells were permeabilized by washing once with IC Permeabilization Buffer (Invitrogen). Next, cells were resuspended in IC Permeabilization Buffer and stained with anti-cytokine antibodies for 60 min at 4°C.

Cell purification and enrichment

Purified T cell populations were obtained from spleens using a magnetic bead/column system EasySep biotin selection kit (StemCell Technologies). CD4 T Cells were isolated by first labeling total splenocytes with anti-B220 biotin, anti-CD19 biotin, anti-CD11b biotin, anti-NK1.1 biotin, anti-Gr1 biotin, anti-Ter119 biotin, and anti Ly6G biotin. $CD4^+$ cells (unlabeled fraction) were isolated by magnetic columns after being washed and labeled with streptavidin microbeads. Purity was >90%.

Flow cytometric detection of *T. gondii*–infected cells

Single-cell suspensions were performed by surface staining. After permeabilization, staining with FITC-labeled polyclonal anti-Toxoplasma antibody (Abcam) was performed, followed by incubation with biotinylated anti-FITC antibody (FIT-22; BioLegend) and subsequent labeling with Streptavidin FITC (eBioscience).

Generation of mixed bone marrow chimeras

Bone marrow was extracted from cKO (CD45.2) and B6.SJL (CD45.1) mice, as previously described (Bhadra et al., 2014), and 5×10^6 total cells (at a 1:1 ratio for WT/cKO) were injected i.v. into lethally irradiated recipients (8 Gy/20 g body weight). Recipient animals received sulfamethoxazole- and trimethoprim-supplemented (Hi-Tech Pharmacal) water for 5 wk after transfer. Experiments were performed 8 wk after reconstitution.

PD-1 methylation analysis

DNA was isolated from FACS-purified T cell. Purified DNA was then bisulfite modified using the Zymo EZ DNA Methylation-Gold kit per the manufacturer's protocol. Bisulfite-modified DNA was then PCR amplified using Jumpstart Taq and published primers (Youngblood et al., 2011). The PCR amplicon was gel purified and cloned into the pGEM T-easy cloning vector. The vector was then transformed into XL-10 ultracompetent bacteria and colonies containing a vector with an insert were picked based on blue-white screening. The PCR insert from individual bacterial colonies was sequenced and the allelic frequency of cytosine preservation was determined by sequencing cloned vectors from many individual colonies. Cytosine preservation after bisulfite treatment (methylated) is represented as filled circles in the figure.

Immunohistochemistry and confocal imaging

T. gondii–infected frozen brain (5 µm) were fixed for 30 min with 4% buffered paraformalin, permeabilized with 0.2% Triton ×100 for 20 min and blocked with 1% BSA at 4°C. The samples were incubated with the primary antiserum (anti-SAG-1at 1:100 dilution) overnight at 4°C, and subsequently treated with secondary antiserum (fluorescein anti–mouse IgG at 1:100; L. Weiss, Albert Einstein College of Medicine, Bronx, NY) for 1 h at room temperature. For nuclear staining, the samples were treated with DAPI-NucBlue fixed cell stain Ready probes reagent (Molecular Probes). The analysis was performed with a spinning confocal system (Zeiss) with a scanning stage used to generate four-channel image sets controlled by Zen 2012 software. Images were captured with 150×/1.4 Plan Apochromat objective lens (Zeiss) at pixel size of 0.14 µm and pixel dwell time of 0.8 ms.

ELISA

Serum was taken from the tail bleed of *T. gondii*–infected mice at 7 wk p.i. The levels of cytokines (IL-2, -10, and -21) in the sera were determined by ELISA using commercially available kits (eBioscience).

Statistical analysis

Differences in percentage, absolute number, and MFI for each experiment were evaluated using Student's *t* test with $P < 0.05$ taken as statistically significant. Error bars in graphs represent standard deviation. All computations were performed using GraphPad Prism Software.

ACKNOWLEDGMENTS

We thank Dr. Luis Weiss at Albert Einstein College of Medicine for generously providing us with toxo-antibodies; Dr. David Leitenberg, Associate Professor Department of Microbiology, Immunology, and Tropical Medicine at George Washington University, for his critical reading and helpful insights; and Teresa Hawley of George Washington University Flow Cytometry Core Facility for technical assistance.

This work was supported by National Institutes of Health grant AI33325 awarded to I.A. Khan.

The authors declare no competing financial interests.

Submitted: 22 December 2015
Accepted: 3 June 2016

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