Control of *Toxoplasma* reactivation by rescue of dysfunctional CD8+ T-cell response via PD-1–PDL-1 blockade

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In this study, we document that *Toxoplasma gondii* differentiation and reactivation are mediated by systemic CD8 T-cell dysfunction during chronic infection. We demonstrate that CD8+ T-cell exhaustion occurs despite control of parasitemia during early-chronic toxoplasmosis. During later phases, these cells become exhausted, leading to parasite reactivation and mortality. Concomitant with increased CD8+ T-cell apoptosis and decreased effector response, this dysfunction is characterized by a graded elevation in expression of inhibitory receptor PD-1 on these cells in both lymphoid and nonlymphoid tissue. Blockade of the PD-1–PDL-1 pathway reinvigorates this suboptimal CD8+ T-cell response, resulting in control of parasite reactivation and prevention of mortality in chronically infected animals. To the best of our knowledge, this report is unique in showing that exposure to a persistent pathogen despite initial control of parasitemia can lead to CD8+ T-cell dysfunction and parasite reactivation.

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oxoplasmic encephalitis (TE) from reactivation of chronic *Toxoplasma* infection affects 24% to 47% of HIV-infected *Toxoplasma*-seropositive patients in the United States (1–3). Although the incidence of TE among AIDS patients declined threefold in the United States because of prophylactic treatment and highly active antiretroviral therapy, it still remains a significant problem in developing countries (4, 5). Hence, understanding the mechanisms responsible for parasite reactivation in chronically infected hosts is critical for evolving strategies for the development of immunotherapeutic agents against the parasite.

In the intermediate host, *Toxoplasma* undergoes stage conversion between the rapidly replicating tachyzoite that is thought to be responsible for acute toxoplasmosis and the slowly replicating, relatively quiescent, primarily encysted bradyzoite stage that can persist possibly for life (6). Effective CD8+ T-cell response, critical for control of both acute and chronic *Toxoplasma* in susceptible mice strains paradoxically do not ensure their long-term survival (7). Poor long-term survival of *Toxoplasma*-infected B10 mice is associated with reduced number of intracerebral T cells and TE development (8). Additionally, depletion of CD8+ T cells rather than CD4+ T cells had a greater effect on mortality of chronically infected animals (9). Taken together, these studies suggest that although critical for preventing parasite reactivation, CD8+ T-cell responses during late-chronic infection are incapable of preventing mortality in infected mice. However, the mechanism underlying this apparently ineffective CD8+ T-cell response during late-chronic infection has not been studied and it remains to be determined if this defect is responsible for loss of parasite control. We therefore tested whether CD8+ T cells during late-chronic toxoplasmosis become dysfunctional, as reported in some viral infections (10).

This article is unique in demonstrating that CD8+ T cells during later phases of chronic toxoplasmosis are unable to mount a potent anamnestic response, a hallmark of robust adaptive immunity. Moreover, CD8+ T cells from these mice exhibit a progressive increase in expression of inhibitory molecule PD-1 with concomitant parasite reactivation. In vivo blockade of PD-1 interaction with its receptor PDL-1 not only augmented polyfunctional CD8+ T-cell response, but it also inhibited parasite rerudescence and prevented the mortality of chronically infected mice in a CD8+ T-cell–dependent manner. To the best of our knowledge, this report of parasite reactivation caused by PD-1 mediated CD8+ T-cell dysfunction is unique.

**Results**

*Toxoplasma*-Infected Mice Show Increased Tachyzoite-Specific Gene Expression with Concomitant Decrease in CD8+ T-Cell Response During Chronic Toxoplasmosis. Humans are most commonly infected orally with Type II strains of *Toxoplasma* (11). To establish the loss of immune control during chronic infection, we first wanted to indentify the kinetics of long-term survival in B6 mice infected orally with type II strain (ME49) cysts. Most of these animals succumb to infection after 7 wk of challenge (Fig. 1D). Host innate and adaptive immunity are thought to cause differentiation of tachyzoites into chronic slow growing bradyzoites (12). Because encysted parasites can persist for life and are not believed to be associated with disease pathology, several studies have postulated that rather than an incidence of parasites, which remain primarily encysted in chronically infected brains, the degree of bradyzoite-tachyzoite interconversion is a better hallmark of disease reactivation (6, 13, 14). Therefore, we assayed parasite reactivation by changes in parasite stage-specific gene expression. We measured the transcription of the tachyzoite stage-specific Enolase-II (ENO-2) and the bradyzoite stage-specific Enolase-I (ENO-1), two genes that have been previously shown to change in very high levels (15–17). Concomitant with poor survival of *Toxoplasma gondii*-infected mice during the late-chronic phase, increased ENO-2 expression and decreased ENO-1 expression was noted at week 7 postinfection, suggesting parasite reactivation (Fig. 1B). To verify that ENO-2 and ENO-1 corresponded to the tachyzoite and bradyzoite stage of *T. gondii*, the gene expression of the parasite were examined again in vivo using established models of acute and chronic infection. Mice were infected with Type I RH strain (lethal acute infection) or Type II ME49 strain (chronic) (18, 19). Some of the ME49-infected animals were treated with drugs known to control reactivation of chronic infection (18, 19). As expected, RH-infected mice exhibited high tachyzoite-specific gene expression (SAG-1, ENO-2) and minimal bradyzoite-spe-


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In both the early-chronic and late-chronic stages, a significant increase in the absolute number of cycling CD8$^+$ T cells was noted, with a peak at weeks 3 to 5 postinfection followed by a gradual decline. This increase was accompanied by a decrease in the frequency of splenic IFN-γ-producing cells, as measured by flow cytometry. These data clearly demonstrate that a potent recall response occurs upon secondary challenge (23). To verify the potency of secondary response, Toxoplasma-infected animals were rechallenged during the early-chronic and late-chronic phases and assessed for cycling CD8$^+$ T cells (Fig. 1F). Mice rechallenged during the early-chronic phase demonstrated a significant increase in the absolute number of proliferating splenic CD8$^+$ T cells over unchallenged mice (Fig. 1F). In contrast, no significant change in cycling CD8$^+$ T cells was noted in rechallenged animals during the late-chronic phase. These data clearly demonstrate that a potent recall response, a hallmark of robust CD8$^+$ T-cell immunity, is compromised during later stages of chronic toxoplasmosis.

**CD8$^+$ T Cells Up-Regulate PD-1 During Chronic Toxoplasmosis.** The role of PD-1 in mediating dysfunction in chronic parasitic models has not been extensively addressed (24, 25). Although PD-1 expression during toxoplasmosis has been recently reported (26), its role in mediating CD8$^+$ T-cell dysfunction or pathogenesis as shown in chronic viral models like lymphoctic choriomeningitis virus (LCMV), hepatitis B virus (HBV), hepatitis C virus (HCV), and HIV (27), is currently unknown. Analysis of CD8$^+$ T cells revealed a progressive increase of PD-1 expression and frequency during the chronic stage of parasite infection (Fig. 2). Of the two PD-1 ligands, PDL-1 and PDL-2, the mean fluorescence intensity (MFI) of the former but not the latter increased during the course of chronic infection (Fig. S3).

Because increased PD-1 MFI correlated with decreased frequency of splenic IFN-γ$^+$ CD8$^+$ T cells, we hypothesized that PD-1$^+$CD8$^+$ T cells would have reduced capacity to release IFN-γ. To address this theory, we measured the proportion of IFN-γ-producing cells within PD-1$^+$CD8$^+$ and PD-1$^{−}$CD8$^+$ T-cell population. No significant difference in cytokine-positive cells between the two populations was noted (Fig. S4). Although
cells had a much lower MFI than T cells in vivo, we adoptively subset and only moderate de-T cells. T-Cell Response. cells was noted only in BrdU-labeled fraction. This Toxoplasma production in CD8 D T- and T cells during the T cells was noted in all of the tissues T cells, PD-1 CD8 CD8 T cells during the early-chronic and late-chronic cells (Fig. 3 o f6 T cells (Fig. 3). We then measured, if anti-PDL-1 treatment decreased the frequency of CD8 cells. Surprisingly, PDL-1 blockade caused a further increase in the frequency of PD-1-expressing CD8 cells (Fig. 3E). Because we demonstrated that high PD-1 expression was associated with apoptosis, we determined if rescue of CD8 T-cell response by PD-1–PDL-1 blockade was because of reduced apoptosis. To address this under steady-state conditions without confounding effects of proliferation and graded PD-1 increase, we evaluated apoptotic CD8 T cells in splenocytes from chronically infected mice incubated for 5 h with anti–PDL-1 or control antibody (Fig. S7). Anti-PDL-1 treatment mediated reduction in apoptosis occurred primarily in the PD-1hi subset and only moderate decrease in apoptosis was noted in the PD-1lo subset. To further address if anti–PDL-1 treatment was capable of rescuing pre-existing PD-1 expressing CD8 T cells in vivo, we adaptively transferred sorted PD-1hi CD8 T cells from chronically infected CD45.1 mice (week 5–6) to similarly infected CD45.2 mice and then treated recipients with anti-PDL-1 antibody. Anti-PDL-1 treatment increased the absolute number of donor cells in both spleen and brain (Fig. S8). However, although the recipient CD8 T-cell population (comprising of both PD-1hi and PD-1lo) expanded sixfold at the effector site (vis-à-vis isotype-treated), the donor population underwent over 20-fold expansion. This finding suggests that anti–PDL-1 treatment preferentially rescues pre-existing PD-1–expressing cells. Blockade of the PD-1–PDL-1 Pathway Controls Toxoplasma Recrudescence. Finally, we wanted to address if this augmented CD8 T-cell response was able to control recrudescence by examining the gene expression of Toxoplasma parasites in brains of anti–PDL-1 or control antibody treated mice. Anti–PDL-1 treated mice revealed more of a Bradyzoite-specific than tachyzoite-specific gene expression (Fig. 4/4). Moreover, anti–PDL-1 treated mice exhibited a much lower frequency of Toxoplasma-infected leukocytes in brain and blood than control group (Fig. 4B). As previously stated, because encysted parasites can persist for life in immunocompetent host brains without causing pathology, we examined parasite genomic DNA in blood (6). Detection of parasite DNA in blood by PCR has been used to diagnose cerebral toxoplasmosis in patents with AIDS (36). As shown in Fig. 4C, parasite genomic
increased survival of anti–PD-L1 treated mice was CD8-dependent, the surviving mice were treated with anti-CD8 antibody. As shown in Fig. 4E, CD8 depletion abrogated the survival of anti–PD-L1 treated animals. Taken together, the above data suggest that anti–PD-L1 treatment augments suboptimal CD8+ T-cell response in chronically infected mice, which prevents reactivation of infection leading to host survival.

**Anti–PD-L1 Treatment Up-Regulates Eomes in CD8+ T Cells from Chronically Infected Mice.** T-box factors, T-bet and Eomesodermin, play a critical role in development, survival, and function of CD8+ T cells (37, 38). Studies from our laboratory suggest that anti–PD-L1 treatment dramatically augments expression of Eomes but not T-bet (unaffected vis-à-vis acutely infected mice) in cycling CD8+ T cells (Fig. S9). Incidentally, the critical role of Eomes in mediating CD8+ T-cell responsiveness to IL-15 (38), a cytokine crucial for survival of memory as well as effector CD8+ T cells, and Granzyme B (39–41) expression in T cells has been recently elucidated. Significantly, our data show that CD8+ T-cell dysfunction affects Granzyme B expression more than IFN-γ production. Significance of Eomes expression in our current model will have to await further investigation.

**Discussion**

CD8+ T-cell exhaustion has been reported in several chronic viral infections, like LCMV, HBV, HCV, HIV, and Simian Immunodeficiency Virus, which are characterized by high levels of persisting viremia (42). This exhaustion is manifested by the gradual loss of CD8+ T-cell effector functions (cytokine production, cytotoxicity, proliferation, and recall responses) and, in extreme situations, CD8+ T cells can be physically deleted (43). In contrast, in infection models like murine cytomegalovirus, characterized by low levels of persistent viremia, antigen-specific T cells remain functional and respond vigorously to viral challenge (42, 44). In models of reactivation like HSV that induce latent infection punctuated by periods of reactivation, functional long-lived CD8+ T-cell response is generated (45). Based on these findings, it has been suggested that CD8+ T-cell exhaustion occurs only in the presence of uncontrolled persistent viremia (42, 46–48). Our current report documents that CD8+ T-cell dysfunction is not restricted to viral infections alone but can be extended to intracellular protozoan parasite, like T. gondii. Although CD8+ T-cell dysfunction observed in the present study is similar in certain facets to certain viral pathogens, in its dependence on PD-1–PD-L1 interaction, it discounts the conventional paradigm that CD8+ T-cell exhaustion occurs only in infectious disease models with uncontrolled persistent pathogen load. Moreover, notwithstanding the fact that the role of the PD-1–PD-L1 pathway has been addressed in chronic viral models of CD8+ T-cell exhaustion, its significance in bacterial or parasitic models of CD8+ T-cell dysfunction has not been extensively investigated.

Our studies demonstrate that despite a potent development of CD8+ T-cell immunity during the early stages of chronic infection, endogenous CD8+ T-cell response is unable to control parasite reactivation at the later time points. This finding is in agreement with a previous study by Gazzinelli et al. (49), which demonstrated that parasite reactivation in B6 mice during late-chronic phases is associated with increased expression of tachyzoite-specific genes in brain. Additionally, there is an increase in parasite levels in the periphery. A recent study has demonstrated the CD11b+ leukocytes can act as shuttles for parasite migration during the acute infection phase (22). Hence, it would be interesting to investigate in future studies whether during parasite reactivation, CD11b+ leukocytes, which are preferentially parasitized during the late-chronic phase, potentially serve a similar function and its role in mediating disease pathogenesis. Cumulatively, our results show that long-term exposure to a persistent pathogen despite initial control of parasitemia can lead to CD8+ T-cell dysfunction and parasite reactivation. This CD8 exhaustion with concomitant PD-1 up-regulation is characterized by reduced effector-cell numbers,

DNA was only detectable in control antibody-treated and not in drug or anti–PD-L1 treated blood. Next, we monitored long-term survival of anti–PD-L1 treated mice. Surprisingly, although 100% of control mice died by day 91 postinfection, 90% of anti–PD-L1 treated mice survived until the termination (day 125 postinfection) of the experiment (Fig. 4D). Furthermore, to demonstrate that
PDL-1 antibody was injected every third T-cell response but also prevented mortality in chronically infected mice. Our findings are further supported by a study in an LCMV model where antigen burden has been shown to be the key determinant for development of CD8 dysfunction (42). However, any control of infection or antigen burden can have such a profound effect during the later phases. Hence, future studies will be critical to discriminate the differential role of inflammatory milieu and antigen burden in mediating CD8 dysfunction.

Recent reports have identified role of IL-10 in mediating dysfunctional CD8+ T-cell response during LCMV infection (51). However, anti-IL10R treatment of mice carrying chronic *Toxoplasma* infection led to their increased mortality because of a strong inflammatory response (52). This finding suggests that any strategy for preventing *Toxoplasma* reactivation must involve an appropriate balance between enhanced proinflammatory mediators without excessive suppression of anti-inflammatory factors. Interestingly, anti–PDL-1 treatment not only augmented the CD8+ T-cell response but also prevented mortality in chronically infected mice by controlling parasite reactivation.

How blockade of the PD-1–PDL-1 pathway modulates CD8+ T-cell response at the molecular level is poorly understood in infectious disease models. Recent studies have shown that differential TCR signal strength can affect not only memory CD8 programming but also development of a potent polyfunctional CD8 response (53, 54). Considering that PD-1 ligation has been shown to inhibit TCR signaling via down-regulation of ZAP-70 and PKCδ in vitro, it is possible that anti–PDL-1 treatment restores robust CD8 response during chronic *Toxoplasma* infection via increased TCR sensitivity (55). Although the mechanistic basis of CD8 dysfunction and PD-1 up-regulation need to be investigated more thoroughly in future studies, the data presented in this article provide significant insights into the role of PD-1–PDL-1 pathway in mediating CD8+ T-cell dysfunction and concomitant parasite reactivation. These results are especially significant in view of a minimal role played by CTLA-4, a related inhibitory molecule in regulating T-cell response during TE (56). Our findings have important implications in the use of PD-1–PDL-1 blockade in combination with drug regimen for controlling TE in immunocompromised patients, as well as use of similar therapy in other dysfunction-induced reactivation-type diseases.

### Materials and Methods

**Mice, Parasites, and in Vivo Antibody Treatment.** Female C57BL/6, CD45.1, and CD90.1 mice were purchased from the National Cancer Institute or the Jackson Laboratory. Animal studies were carried out in agreement with Institutional Animal Care and Use Committee approved guidelines. Unless otherwise mentioned, *T. gondii* cysts of ME49 strain were used for i.g. infection throughout. Some mice were injected in vivo with the following antibodies i.p.: anti-PDL-1 (MiH5) or anti-CD8 (2.43) or isotype-matched control antibodies.

**Lymphocyte Isolation, Cell Surface Staining, and Intracellular Staining.** Single cell suspension was prepared from spleen, liver, blood, and brain using standard protocol. For cytokine detection, 5 × 10^5 naive congenic splenocytes was mixed with equal number of brain or splenic cells from infected mice, and restimulation was similarly carried out in the presence of TLA.

**Real-Time RT-PCR and PCR.** From total infected mouse brain RNA, real-time RT-PCR was performed with primers for ENO-1, ENO-2, SAG-1, and BAG-1 on *Toxoplasma* specific actin normalized samples. Transcript levels relative to day 10 post infection (day 10 post infection relative transcript level = 1.0) were calculated according to the Pfaffl method of quantitation (57).

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Fig. 4. Anti–PDL-1 treatment controls parasite reactivation and prevents mortality in chronically infected mice. (A) Relative expression of ENO-1 and ENO-2 was computed for each sample. Transcript levels at day 10 post-infection was taken as 1. (B) *T. gondii*-infected cells in the brain and blood leukocyte-gated samples (n = 5) were assayed by flow cytometry. (C) Parasite genomic DNA level was computed in whole blood before initiation of treatment (week 5 postinfection) or 2 wk after initiation of treatment (week 7 postinfection) (n = 4–5). (D) Anti–PDL-1 antibody was injected every third day, starting at week 5 postinfection (n = 10). To minimize the development of an allogeneic reaction, the antibody was administered with 2-wk interruptions until termination of the experiment. (E) Surviving anti–PDL-1 treated mice from the previous experiment were administered anti–PDL-1 along with anti CD8 (n = 4) or anti–PDL-1 and control antibody (n = 4) every third day until the conclusion of the experiment. Survival was monitored on a daily basis. Data represent two experiments.

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Additional details and techniques are provided in SI Materials and Methods.

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