Supporting Information

Bhadra et al. 10.1073/pnas.1015298108

SI Materials and Methods

Mice. Six- to 8-wk-old female C57BL/6 and B6-Ly5.2 (CD45.1) mice were purchased from the National Cancer Institute. Similarly aged female B6.PL-Thy1^a/CyJ (CD90.1) mice were purchased from the Jackson Laboratory. Animal studies were carried out in agreement with Institutional Animal Care and Use Committee approved guidelines at George Washington University Medical Center.

Parasites, Infection, and TLA Preparation. *T. gondii* cysts of ME49 strain were prepared from the brains of infected mice. For experimental infections, animals were infected (or rechallenged) with an average of 10 cysts per mouse via intragastric route. In some cases mice were infected i.p. with 5×10^5 parasites of RH strain (Type I). Infected animals were euthanized by CO_2 inhalation at different time points pi. TLA was extracted from RH strain of parasite and preparation was carried out as previously described (1).

Moribund Mice. Mice were scored daily on a scale of 1 (asymptomatic) to 5 (most severe) based on several aspects. Moribund mice were characterized by severe loss of mobility, hunched back, piloerection, ruffled fur, and weight loss.

In Vivo Antibody Treatment. For in vivo IFN-γ depletion, rat anti–IFN-γ (4 mg; clone XMG1.2; BioXCell) or rat IgG1 isotype-matched control antibody (4 mg; BioXCell) were injected intraperitoneally daily for 4 d starting at day 31 postinfection. Rat anti–PDL-1 (2) (200 μg; MIH5; prepared "in house") or rat IgG2a isotype-matched control antibody (200 μg; BD Biosciences) were administered intraperitoneally every third day for 2 wk beginning at week 5 postinfection unless otherwise mentioned in the figure legends. MIH5 is a nondepleting antibody (3). For CD8 depletion, anti–PDL-1 treated mice were administered rat anti-CD8 (500 μg; clone 2.43; BioXCell) intraperitoneally every third day beginning at day 126 postinfection until termination of the experiment. Control mice were administered rat IgG2b isotype-matched control antibody (200 μg; BD Biosciences).

Lymphocyte Isolation and Staining. A single-cell suspension was prepared from spleen, liver, blood, and brain using a standard protocol (4, 5). Briefly, for spleen, single-cell suspensions were made by mechanical disruption followed by red blood cell lysis. For hepatic lymphocyte enrichment, livers were perfused with 10 mL cold PBS, excised, minced, and passed through a meshed screen. The cells were washed in cold PBS, and one liver equivalent was suspended in 15 mL of 30% Percoll (Sigma) solution in the presence of 100 IU/mL heparin (Sigma). The suspension was centrifuged for 10 min at $1{,}000 \times g$ and the pellet was resuspended in cold PBS-2%FCS. For brain lymphocyte enrichment, brains were individually washed in HBSS-heparin (2 IU/mL) and then mashed through a 70-μm cell strainer, followed by gradient centrifuge ($1{,}000 \times g$ for 20 min) in 30% Percoll solution containing 100 IU/mL of heparin. Pellet was then resuspended in cold PBS-2% FCS. For peripheral blood, blood was initially collected in 2 mL of PBS containing 100 U/mL of heparin. Partial red blood cell lysis was then carried out. For immunophenotyping, 1×10^6 spleen or brain or liver cells were plated per well. For phenotyping peripheral blood cells, 3×10^6 cells per well was used.

The following antibodies were used in cell-surface staining and intracellular staining of lymphocytes: anti-CD8β (clone H35-17.2; eBioscience), PD-1 (clone J43; eBioscience), PDL-1 (clone MIH-5; eBioscience), PDL-2 (clone TY25; eBioscience), Eomes (clone 21Mags8; eBioscience), CD90.1 (clone OX-7; BioLegend), T-bet (clone 4B10; Santa Cruz Biotechnology), Granzyme B (clone GB11; Invitrogen), *K*i-67 (clone B56; BD Biosciences), active caspase-3 (clone C92-605; BD Biosciences), IFN-γ (clone XMG1.2; BD Biosciences), NK1.1(clone PK136; eBioscience), CD11b (clone M1/70; eBioscience), GR1 (clone RB6-8C5; eBioscience), F4/80 (clone BM8; BioLegend), CD3 (clone 145–2C11; eBioscience), and CD19 (clone 6D5; BioLegend). For sorting PD-1–expressing CD8$^+$ T cells, RMP1-30 (eBioscience) clone was used. This clone does not inhibit PD-1 interaction with its ligand (6).

Intracellular staining was performed after surface staining, using a Cytofix/Cytoperm Kit (BD Biosciences) as per the manufacturer's protocol. Cell fluorescence was measured with a BD FACS Calibur or BD FACS Aria or Cytek upgraded eight-color BD FACS Calibur cytometer, which accounts for differences in fluorescence scale. Data were analyzed using FlowJo (TreeStar) software. Cells were lymphocyte-gated based on forward-scatter and side-scatter. Gating for positive and negative populations was set up based on fluorescence-minus-one controls.

Intracellular Cytokine Detection. For cytokine detection, restimulation was carried out for 16 h with 30 μg/mL of *Toxoplasma* lysate antigen (TLA) in supplemented Iscove's Complete DMEM at 37 °C in 5% CO_2. Next, 0.65 μL/mL of monensin (BD Biosciences) and 0.65 μL/mL of brefeldin A (BD Biosciences) were added during the final 9 h of stimulation. For restimulation, 5×10^5 naive congenic splenocytes was mixed with an equal number of brain or splenic cells from infected mice and restimulation was similarly carried out.

Flow Cytometric Detection of *Toxoplasma gondii*-Infected Cells. Single-cell suspensions prepared as above were surface stained. Intracellular staining was performed as described above with some modifications. 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). Cells were analyzed on mononuclear leukocyte gate based on forward-scatter and side-scatter.

BrdU Injection and Staining. BrdU (BD Biosciences) was injected at 1 mg per mouse intraperitoneally on alternate days between week 4 and week 5 postinfection. Splenocytes and brain cells were analyzed for BrdU incorporation at week 5 and week 7 postinfection. Staining was performed using FITC BrdU Flow Kit (BD Biosciences) as per the manufacturer's protocol.

Cytotoxic T-Lymphocyte Assay. Cytotoxic T-lymphocyte assay was performed as previously described (7). Briefly, CD8$^+$ T cells stimulated with TLA were incubated with infected ^{51}Cr-labeled macrophages at various effector-target ratios in 96-well U-bottomed plates. After a 4-h incubation, the supernatants were measured for radioactive release and the percentage of cytotoxic response calculated.

Real-Time RT-PCR. Total RNA was harvested from flash-frozen infected mouse brains by homogenization in TRIzol reagent (Invitrogen), followed by extraction using phenol chloroform, digestion with DNase-1 (Roche), and then further purification with RNeasy spin columns (Qiagen). cDNAs were generated using M-MLV reverse-transcriptase (Invitrogen) and semiquantitative real-time PCR was performed using iQ SYBR Green Supermix (BioRad) with primers for *ENO-1*, *ENO-2*, *SAG-1*, *BAG-1*, and

Toxoplasma-specific actin (TgACT-1; Integrated DNA Technologies) on an iCycler iQ thermal cycler (BioRad). Samples used for RT-PCR were first normalized for constitutively expressed Tg-ACT1. PCR reactions were carried out using one cycle at 95 °C for 10 min followed by a two-step cycle, one at 95 °C for 15 s then another at 60 °C for 1 min repeated 40 times. PCR products were then analyzed for quality by melt curve analysis. Quantitation of transcripts for *ENO-1*, *ENO-2*, *SAG-1*, and *BAG-1* relative to day 10 postinfection (day 10 postinfection relative transcript level = 1.0) were calculated according to the Pfaffl method of quantitation (8). Primer sequences are as follows:

5′TgACT-1: TCCCGTCTATCGTCGGAAAG
3′TgACT-1: CCATTCCGACCATGATACCC
5′SAG-1: ATCGCCTGAGAAGCATCACTG
3′SAG-1: CGAAAATGGAAACGTGACTGG
5′BAG-1: GACGTGGAGTTCGACAGCAAA
3′BAG-1: ATGGCTCCGTTGTCGACTTCT
5′ENO-1: GGTATTGATATGCTTATGGTGGAG
3′ENO-1: GCGATGTATTTGTATAGTGGTAGG
5′ENO-2: CCGTGACAAGGACCAAAC
3′ENO-2: ACTCGTTCTTAGTTCCATCG

In Vitro Bradyzoite Induction and Imaging. As previously published, parasites (Type II) were cultured in human fibrolasts for 3 d at pH8 and 2 d at pH 7 for bradyzoite and tachzyzoite differentiation, respectively (9). Immunofluorescence assay was done as previously described (8). Briefly, samples were fixed for 30 min with 2% buffered formalin, washed three times with PBS, permeablized with 0.2% Triton ×100 for 20 min, washed three times with PBS, blocked with 1% BSA at 4 °C, washed three times with PBS, and then incubated with the primary antiserum (mAB 74.18: BAG-1 at 1:100 dilution and mAB DG52 SAG-1 at 1:500 dilution) for 1 h at 37 °C. Following incubation with the primary antiserum, the slides were then washed three times with PBS and then incubated with secondary antiserum (fluorescein or rhodamine anti-mouse IgG at 1:100) for 1 h at 37 °C, washed three times with PBS, and then examined under a Nikon Diaphot fluorescent microscope using the correct filters for the secondary antibody label (i.e., either rhodamine and fluorescein).

Drug Treatment. *Toxoplasma*-infected mice were administered sulfamethoxazole and trimethoprim oral suspension (Hi-Tech Pharmacal) via drinking water. A previous study has shown that this regimen has similar clinical efficacy to sulfadiazine-pyrimethamine treatment and produces fewer adverse effects (10). Five milliliters of the suspension containing 200 mg of sulfamethoxazole and 40 mg of trimethoprim were added to 245 mL of drinking water. Medicated water was replaced every third day.

Statistical Analysis. Differences in percentage, absolute number, MFI, and parasite gene expression levels of samples for each experiment were evaluated using Student's t test with $P < 0.05$ taken as statistically significant. Comparison of survival curves was performed using Log-rank (Mantel-Cox) Test. Error bars in graphs represent standard deviation of values of individual mice in the group from one experiment. All computations were performed using GraphPad Prism software.

1. Khan IA, Kasper LH (1996) IL-15 augments $CD8^+$ T cell-mediated immunity against *Toxoplasma gondii* infection in mice. *J Immunol* 157:2103–2108.
2. Tsushima F, et al. (2003) Preferential contribution of B7-H1 to programmed death-1-mediated regulation of hapten-specific allergic inflammatory responses. *Eur J Immunol* 33:2773–2782.
3. Dias P, et al. (2010) CD4 T-cell help programs a change in CD8 T-cell function enabling effective long-term control of murine gammaherpesvirus 68: Role of PD-1-PD-L1 interactions. *J Virol* 84:8241–8249.
4. Mueller SN, Ahmed R (2009) High antigen levels are the cause of T cell exhaustion during chronic viral infection. *Proc Natl Acad Sci USA* 106:8623–8628.
5. Shin H, Wherry EJ (2007) CD8 T cell dysfunction during chronic viral infection. *Curr Opin Immunol* 19:408–415.
6. Matsumoto K, et al. (2004) B7-DC regulates asthmatic response by an IFN-gamma-dependent mechanism. *J Immunol* 172:2530–2541.
7. Kasper LH, Matsuura T, Khan IA (1995) IL-7 stimulates protective immunity in mice against the intracellular pathogen, *Toxoplasma gondii*. *J Immunol* 155:4798–4804.
8. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45.
9. Weiss LM, et al. (1995) A cell culture system for study of the development of *Toxoplasma gondii* bradyzoites. *J Eukaryot Microbiol* 42:150–157.
10. Torre D, et al.; Italian Collaborative Study Group (1998) Randomized trial of trimethoprim-sulfamethoxazole versus pyrimethamine-sulfadiazine for therapy of toxoplasmic encephalitis in patients with AIDS. *Antimicrob Agents Chemother* 42: 1346–1349.

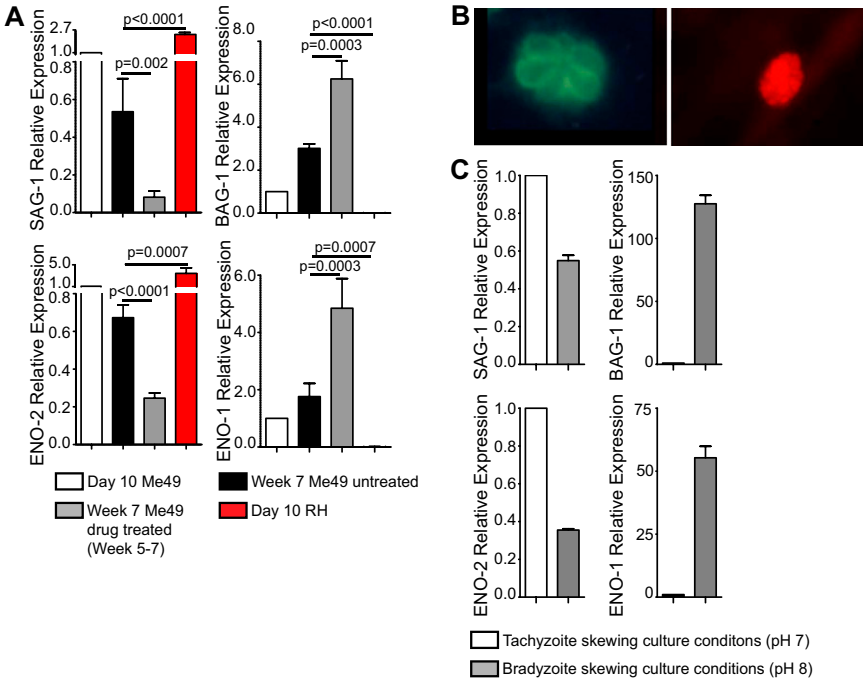

Fig. S1. Validation of in vivo parasite stage-specific gene transcription. (*A*) Relative expression of *SAG-1*, *BAG-1*, *ENO-2*, and *ENO-1* was computed in ME49- (type II strain) and RH- (type I strain) infected mice brains. Transcript levels at day 10 postinfection (ME49 infected) was taken as 1. The data represent two experiments with three to five mice per group. (*B*) (*Left*) SAG-1 staining of ME49 parasites cultured in vitro under tachyzoite-skewing conditions (pH 7). (*Right*) BAG-1 staining of ME49 parasite under bradyzoite skewing conditions (pH 8). (*C*) Relative expression of *SAG-1*, *BAG-1*, *ENO-2*, and *ENO-1* was calculated for in vitro cultured parasites. Transcript levels for parasites cultured in vitro under tachyzoite skewing conditions was taken as 1. (*B* and *C*) The data represent at least two experiments with three replicates per condition.

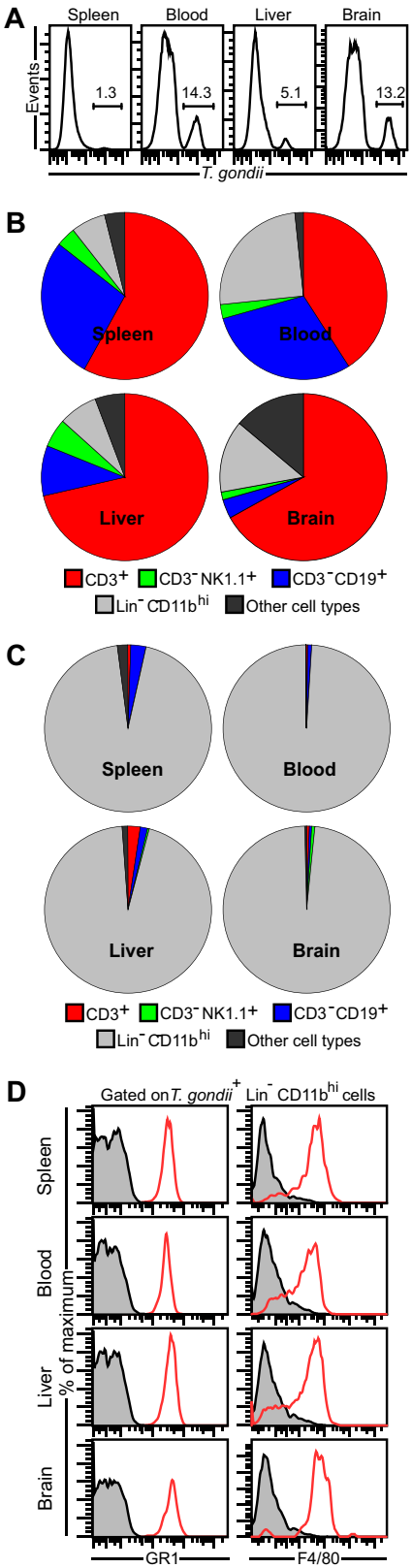

Fig. S2. Majority of *T. gondii*-infected leukocytes exhibit a myeloid lineage phenotype. (*A*) *T. gondii*-infected cells in spleen, blood, liver, and brain leukocyte gated samples (week 7 postinfection) were assayed by flow cytometry. (*B*) The mononuclear leukocyte composition of leukocytes in the above samples. (*C*) *T. gondii*-infected mononuclear cells were analyzed for various leukocyte subsets. (*D*) Expression of GR1 and F4/80 on *T. gondii*-infected $CD11b^{hi}$ cells. Lin denotes any of the following lineage markers: CD3 or CD19 or NK1.1. The data represent two experiments with four mice per group.

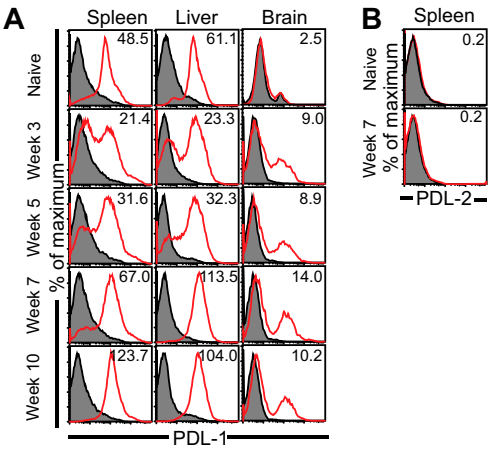

Fig. S3. PD-1 receptor, PDL-1 is up-regulated during late-chronic Toxoplasmosis. (*A* and *B*) PDL-1 and PDL-2 expression was evaluated on mononuclear cells in spleen, liver, and brain. Filled histogram represents staining with isotype-control antibody. The data represent three experiments with at least four mice per group.

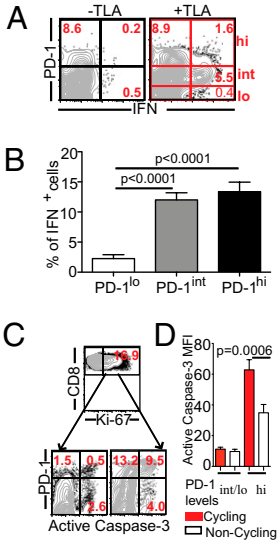

Fig. S4. High PD-1 expression correlates with apoptosis of CD8$^+$ T cells during late-chronic Toxoplasmosis. (*A* and *B*) Splenocytes from chronically infected mice were restimulated with TLA, and frequency of IFNg expressing CD8$^+$ T cells in PD-1^{lo}, PD-1^{int}, and PD-1^{hi} subsets was evaluated. (*C* and *D*) Splenocytes from chronically infected animals were incubated at 37 °C for 5 h, and active caspase 3, Ki-67, and PD-1 were detected on CD8$^+$ T cells by flow cytometry. The data represent three experiments with at least four mice per group.

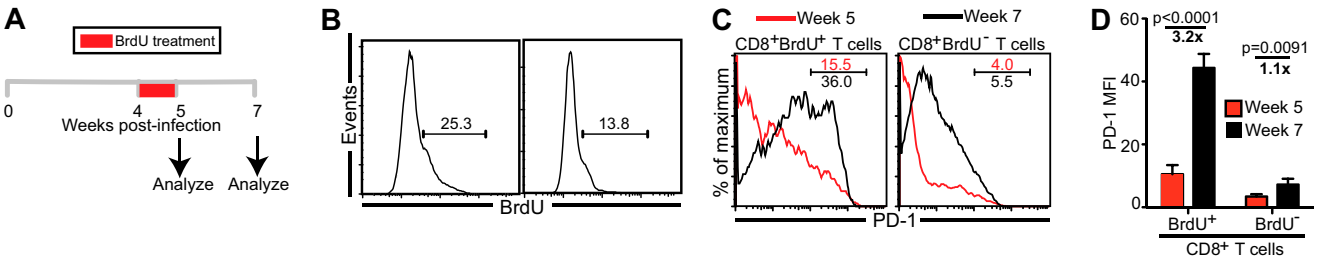

Fig. S5. High PD-1 up-regulation does not occur on newly generated CD8$^+$ T cells. (*A*) Using a pulse-chase approach, BrdU was injected into infected mice starting at week 5 postinfection. Treatment was performed on alternate days for 1 wk and animals were killed at weeks 5 and 7 postinfection. (*B*) Splenic CD8$^+$ T cells were evaluated for BrdU incorporation by flow cytometry. (*C*) Frequency of PD-1^{hi} cells was assessed on splenic BrdU$^+$ and BrdU$^-$ CD8$^+$ T cells. (*D*) PD-1 expression was also assessed in the above subsets. Fold-difference was computed as (Difference in mean PD-1 mean fluorescence intensity between the two time points)/(Mean PD-1 mean fluorescence intensity at week 5). Data represent two experiments with at least four mice per group.

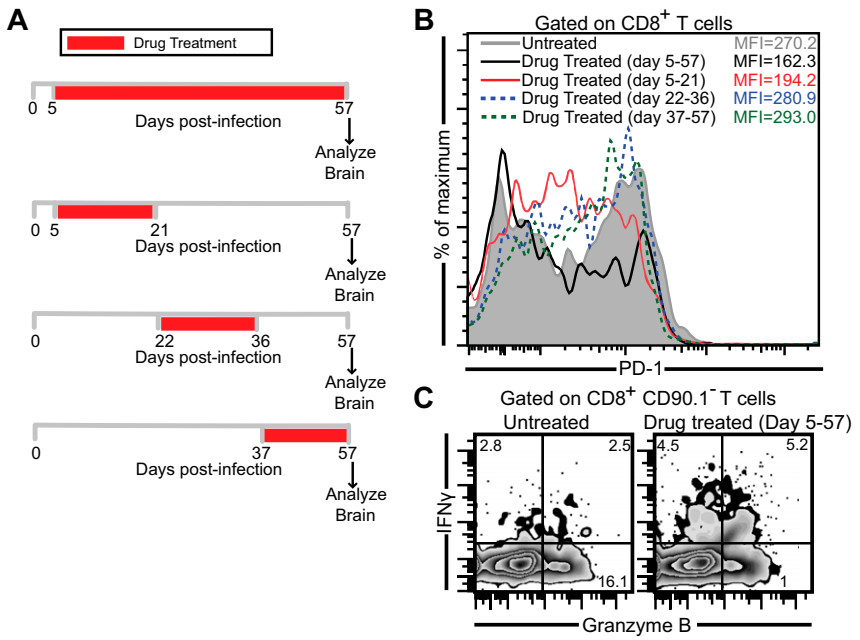

Fig. S6. Early initiation of drug treatment reduces CD8$^+$ T-cell exhaustion. (*A*) *Toxoplasma*-infected mice were treated with sulfamethoxazole and trimethoprim, as shown in the figure. Brains from untreated and various drug-treated animals were analyzed at day 57 postinfection. (*B*) PD-1 expression was assessed on CD8$^+$ T cells in brain by flow cytometry. (*C*) IFN-γ and Granzyme B production by CD8$^+$ T cells was evaluated in brain cells from infected mice in the presence of TLA. Data represent two experiments with at least four mice per group.

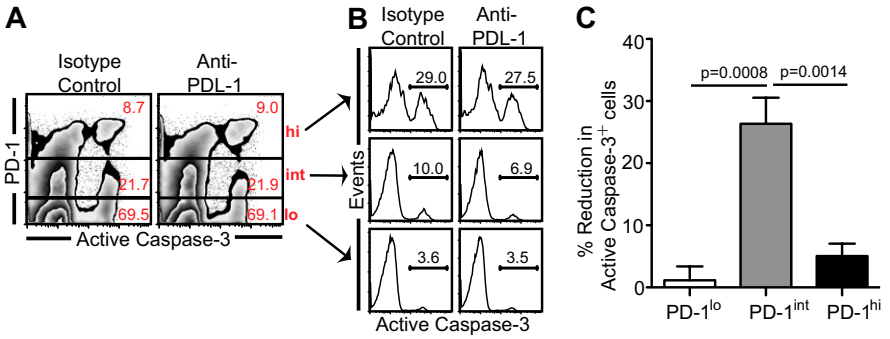

Fig. S7. Anti–PDL-1 treatment reduces apoptosis of PD1^{int}CD8$^+$ T cells. (*A* and *B*) Splenocytes from chronically infected mice (week 7 postinfection) were incubated in vitro for 5 h at 37 °C with isotype control or anti–PDL-1 antibody. This procedure was followed by evaluation of PD-1–expressing CD8$^+$ T-cell subsets for active caspase-3 expression. (*C*) Reduction in active caspase-3 expression in anti–PDL-1 treated splenocytes in PD-1^{hi}, PD-1^{int}, and PD-1^{lo} CD8$^+$ T cells. Data represent three experiments with at least four mice per group.

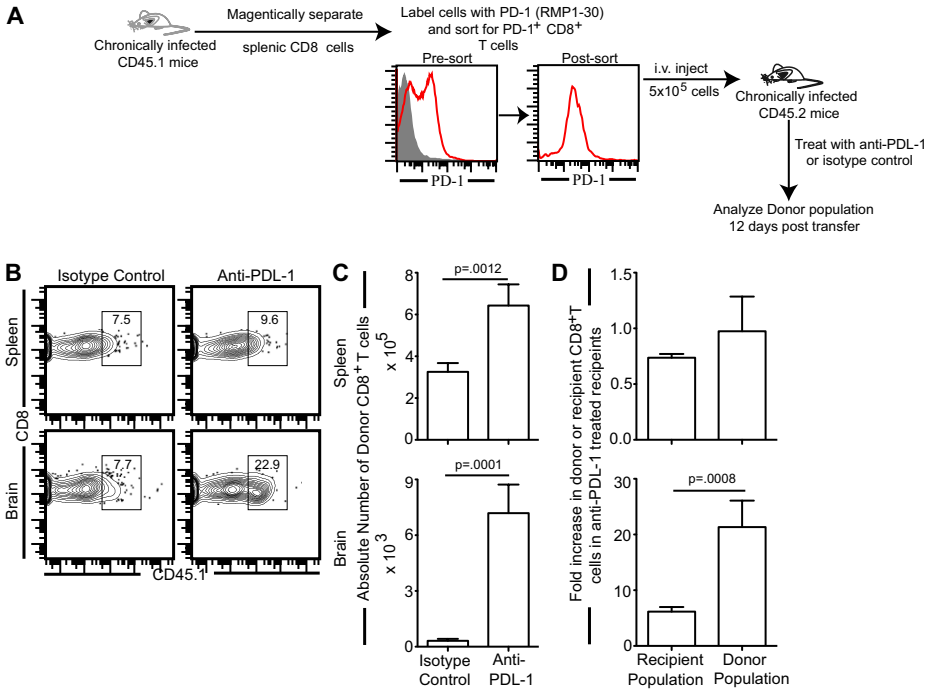

Fig. S8. Anti–PDL-1 treatment preferentially rescues PD-1–expressing CD8$^+$ T cells. (*A*) 5 × 10^5 PD-1–expressing splenic CD8$^+$ T cells isolated from chronically infected (week 5–6) CD45.1 mice were adoptively transferred to infected (weeks 5–6) CD45.2 mice. Recipients were then treated with isotype control or anti–PDL-1 antibody. Twelve days posttransfer, recipients were killed. Filled histogram represents staining with isotype-control antibody. (*B* and *C*) Frequency and absolute number of donor CD8$^+$ T cells was assessed in the spleen and brain of recipient mice. (*D*) Fold-increase in donor or recipient population on anti–PDL-1 treatment in recipient mice was computed as (Absolute number of donor or recipient CD8$^+$ T cells in anti–PDL-1 treated mice − Arithmetic mean of absolute number of donor or recipient CD8$^+$ T cells in isotype control antibody-treated mice)/(Arithmetic mean of absolute number of donor or recipient CD8$^+$ T cells in isotype control antibody-treated mice). Data represent two experiments with at least four mice per group.

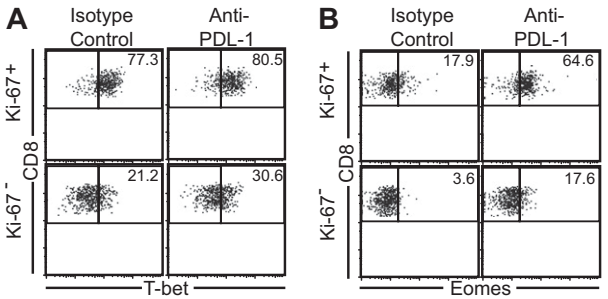

Fig. S9. PD-1–PDL-1 blockade increases Eomes but not T-bet expression on cycling CD8$^+$ T cells. (*A* and *B*) T-bet and Eomes expression was evaluated in cycling and noncycling splenic CD8$^+$ T cells from isotype or anti–PDL-1 antibody treated chronically infected mice (week 7 postinfection) by flow cytometry. The data represent two experiments with at least four mice per group.