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Interplay between regulatory T cells and PD-1 in modulating T cell exhaustion and viral control during chronic LCMV infection

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Regulatory T (T reg) cells are critical for preventing autoimmunity mediated by self–reactive T cells, but their role in modulating immune responses during chronic viral infection is not well defined. To address this question and to investigate a role for T reg cells in exhaustion of virus–specific CD8 T cells, we depleted T reg cells in mice chronically infected with lymphocytic choriomeningitis virus (LCMV). T reg cell ablation resulted in 10–100–fold expansion of functional LCMV–specific CD8 T cells. Rescue of exhausted CD8 T cells was dependent on cognate antigen, B7 costimulation, and conventional CD4 T cells. Despite the striking recovery of LCMV–specific CD8 T cell responses, T reg cell depletion failed to diminish viral load. Interestingly, T reg cell ablation triggered up–regulation of the molecule programmed cell death ligand–1 (PD–L1), which upon binding PD–1 on T cells delivers inhibitory signals. Increased PD–L1 expression was observed especially on LCMV–infected cells, and combining T reg cell depletion with PD–L1 blockade resulted in a significant reduction in viral titers, which was more pronounced than that upon PD–L1 blockade alone. These results suggest that T reg cells effectively maintain CD8 T cell exhaustion, but block–ade of the PD–1 inhibitory pathway is critical for elimination of infected cells.

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Abbreviations used: Arm, Arm-strong; cl-13, clone 13; DT, diphtheria toxin; LCMV, lymphocytic choriomeningitis virus; MFI, mean fluorescence intensity; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand-1.

Regulatory T cells expressing transcription factor Foxp3 are indispensable for preventing immune responses to self, and their absence results in multi-organ autoreactivity and death (Kim et al., 2007; Sakaguchi et al., 2008). In addition to their major role in maintaining peripheral tolerance, T reg cells also control immune responses to infections. During acute infection, T reg cells can promote migration of effector immune cells to infection sites by modulating chemokine production (Lund et al., 2008), and prevent the activation of low avidity CD8 T cells (Pace et al., 2012). However, in cancer and persistent infections, T reg cells may expand and facilitate disease progression due to inhibition of T cell responses (Zou, 2006; Li et al., 2008; Belkaid and Tarbell, 2009; Dietze et al., 2011; Punkosdy et al., 2011).

In cancer and persistent infections, chronic antigenic stimulation causes deterioration of T cell responses. T cell exhaustion is manifested by progressive loss of proliferative potential, cytokine production, and for CD8 T cells, killing capability (Zajac et al., 1998; Wherry, 2003, 2011). This progressive T cell dysfunction is associated with expression of programmed cell death-1

Pablo Penaloza-MacMaster and Alice O. Kamphorst contributed equally to this paper.

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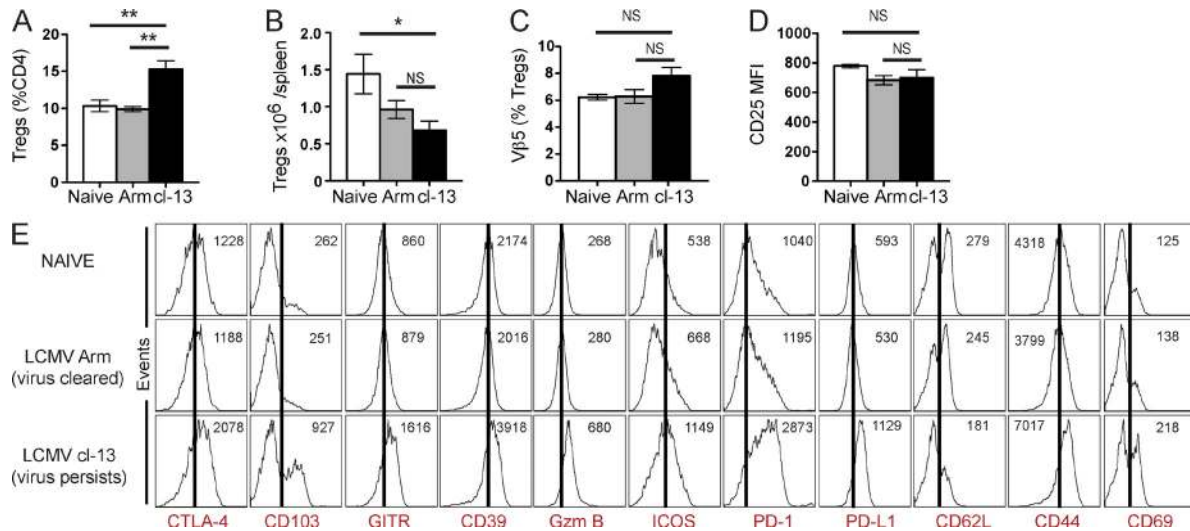
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Figure 1. **T reg cells have an activated phenotype during chronic viral infection.** Analysis was done on splenocytes from naive uninfected mice, mice infected with LCMV Arm, and analyzed at least 100 d after acute infection and LCMV cl-13 chronically infected mice (mice transiently depleted of CD4 T cells and infected with LCMV cl-13, with analysis done at least 40 d after infection). (A) Percentage of T reg cells (Foxp3^{+}) among CD4 T cells. (B) Absolute number of T reg cells in spleen. (C) Frequency of Vβ5^{+} cells among T reg cells. Mice infected with LCMV cl-13 and analyzed 21 d later were included as positive controls (22–40% Vβ5^{+} cells). (D) Graph shows MFI of CD25 expression on T reg cells. (E) Histograms show representative expression of different markers by T reg cells and numbers represent MFI. Data are a compilation of 4 experiments (A and B) or show representative results from 3 experiments (C–E; n = 3–4 mice per group). Error bars indicate SEM. Non-parametric Mann Whitney test, where *, $P < 0.05$; **, $P < 0.01$; NS = not significant.

(PD-1) and other inhibitory receptors such as Tim-3 and LAG-3 (Barber et al., 2006; Blackburn et al., 2009; Jin et al., 2010). Importantly, proliferation and function of exhausted T cells can be rescued by blockade of inhibitory pathways, which can result in restoration of effective immune responses that control infections and tumors (Barber et al., 2006; Fourcade et al., 2010; Sakuishi et al., 2010; Butler et al., 2012; Topalian et al., 2012).

Multiple pathways contribute to T cell dysfunction. Besides expression of inhibitory receptors by T cells, extrinsic factors such as cytokines also play a fundamental role in T cell exhaustion (Wherry, 2011). In addition, lack of CD4 help exacerbates CD8 T cell exhaustion (Matloubian et al., 1994; Zajac et al., 1998; Lichterfeld et al., 2004), and its restoration via adoptive transfer of CD4 T cells can reinvigorate virus-specific responses in mice chronically infected with lympho-cytic choriomeningitis virus (LCMV; Aubert et al., 2011). IL-21 produced by CD4 T cells plays an important role in sustaining CD8 T cells during chronic infection (Elsaesser et al., 2009; Fröhlich et al., 2009; Yi et al., 2009), and it was recently reported that IL-21 may also aid CD8 T cells by restricting T reg cell expansion (Schmitz et al., 2013). Thus, conventional CD4 T cells have a positive impact on modulating CD8 T cell function during persistent antigenic stimulation. In contrast, it has been described that T reg cells are detrimental to virus-specific T cell responses during persistent infection in mice (Dittmer et al., 2004; Dietze et al., 2011; Schmitz et al., 2013); nevertheless, the role of T reg cells in maintaining T cell exhaustion has not been well characterized or fully explored as a therapeutic approach.

To analyze the effects of T reg cells on exhausted virus-specific CD8 T cells, we used LCMV clone 13 (cl-13) infected

Foxp3[DTR] knock-in mice in which Foxp3^{+} T reg cells express the human diphtheria toxin (DT) receptor under control of the endogenous *Foxp3* locus and can be efficiently and specifically deleted by administration of DT (Kim et al., 2007). Using this approach, we found that T reg cell ablation in chronically infected mice leads to a striking rescue of exhausted viral-specific CD8 T cells. Restoration of antiviral CD8 T cell responses was dependent on cognate antigen, B7 costimulation, and conventional CD4 T cells. Interestingly, viral control was not achieved unless T reg cell depletion was combined to blockade of the PD-1 pathway. Thus, we propose that even though T reg cells maintain CD8 T cells in an exhausted state during persistent infections, the PD-1 inhibitory pathway further operates by inhibiting the cytotoxic activity of rescued CD8 T cells toward target cells expressing high levels of programmed cell death ligand-1 (PD-L1).

RESULTS
Regulatory T cells modulate exhausted CD8 T cells during chronic viral infection

To establish a lifelong viral infection of multiple organs accompanied by exhaustion of virus-specific CD8 T cells, we infected mice with LCMV cl-13 after antibody-mediated transient depletion of CD4 T cells (Matloubian et al., 1994). By day 45 after infection, CD4 T cells bounced back to normal numbers in infected mice. When compared with naive or LCMV Arm-strong (Arm)–infected mice that had cleared virus, LCMV chronically infected mice had increased frequency of T reg cells (Fig. 1 A). However, due to decreased splenic cellularity, the absolute numbers of T reg cells were actually reduced in chronically infected mice (Fig. 1 B). Punkosdy et al. (2011)
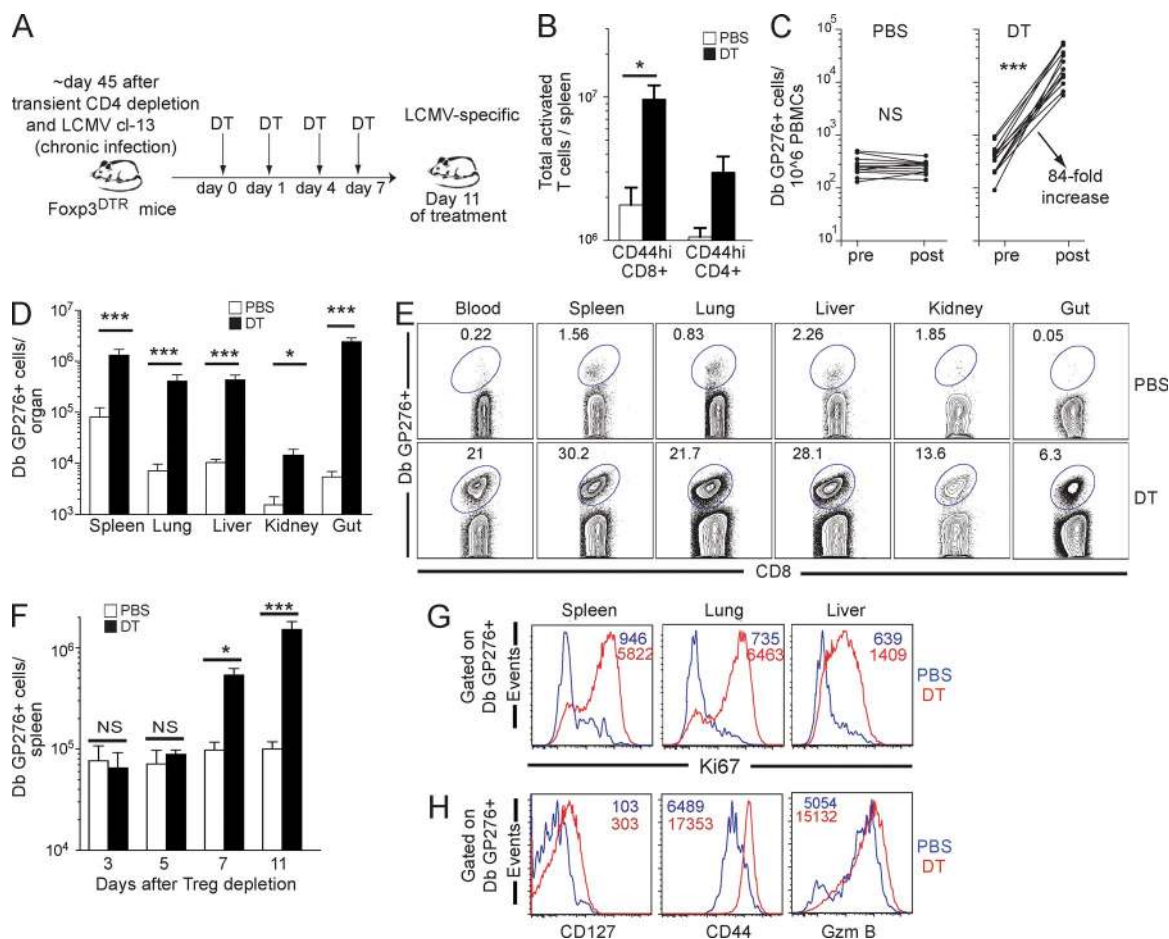
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Figure 2. Exhausted CD8 T cells expand and undergo phenotypic changes upon T reg cell ablation. (A) Experimental outline. (B) Total numbers of activated T cells in the spleen of PBS-treated or T reg cell–depleted (DT) mice chronically infected with LCMV. (C) Number of LCMV-specific (Db GP276) CD8 T cells among PBMCs before and after DT treatment. (D and E) Absolute numbers (D) and frequency (E) of LCMV-specific (Db GP276) CD8 T cells within total CD8 T cell population. (F) Absolute numbers of LCMV DbGP276-specific CD8 T cells in the spleen at different days after T reg cell ablation. (G) Proliferative activity of LCMV-specific (Db-GP276) CD8 T cells. Numbers show MFI of Ki67 expression. (H) Phenotype of LCMV-specific (Db GP276) CD8 T cells in spleen. Numbers show MFI of different CD8 T cell markers. Data are a compilation of 5 independent experiments with 3–5 mice per group. Error bars indicate SEM. Non-parametric Mann Whitney test, where *, P < 0.05; ***, P < 0.001; NS = not significant.

have reported a transient expansion of Vβ5+ T reg cells recognizing Mtv-9 endogenous retrovirus that peaks at day 17 of chronic LCMV infection in B6 mice. Hence, the frequency of Vβ5+ T reg cells was not significantly increased in LCMV chronically infected mice analyzed at 40 or more days after infection (Fig. 1 C).

To further characterize the T reg cell population in LCMV chronically infected mice, we assessed the expression of several cell surface molecules. CD25 expression was not significantly different between T reg cells of chronically infected mice, naive mice, or LCMV Arm–infected mice that had cleared virus (Fig. 1 D). However, based on the expression levels of several other markers, such as CD69, CD44, CD62L, ICOS, GITR, and CTLA-4, T reg cells from chronically infected mice appeared more activated (Fig. 1 E).

To examine the role of T reg cells during chronic infection, we infected Foxp3DTR knock-in mice with LCMV cl-13 after antibody-mediated transient depletion of CD4 T cells,

and at least 45 d after infection, T reg cells were depleted by DT administration (Fig. 2 A). T reg cell ablation in LCMV chronically infected mice led to a marked increase in absolute numbers of activated T cells (Fig. 2 B) and LCMV-specific CD8 T cells (Fig. 2, C–E). There was a >50-fold increase in Db GP276 LCMV-specific CD8 T cells in blood (Fig. 2 C), 10-fold increase in the spleen, and up to 100-fold increase in multiple nonlymphoid tissues (Fig. 2, D and E). This striking reversal of CD8 T cell exhaustion occurred between day 5 and 7 after T reg cell depletion (Fig. 2 F) and was accompanied by restoration of proliferative capacity assessed by the expression of the cell cycle progression marker Ki67 (Fig. 2 G). These results show that during chronic infection, T reg cell depletion enables expansion of previously exhausted T cells.

In addition to expansion, the surface phenotype of exhausted LCMV-specific CD8 T cells was also altered by T reg cell ablation manifested by up-regulation of IL-7 receptor (CD127), and increased CD44 and granzyme B expression
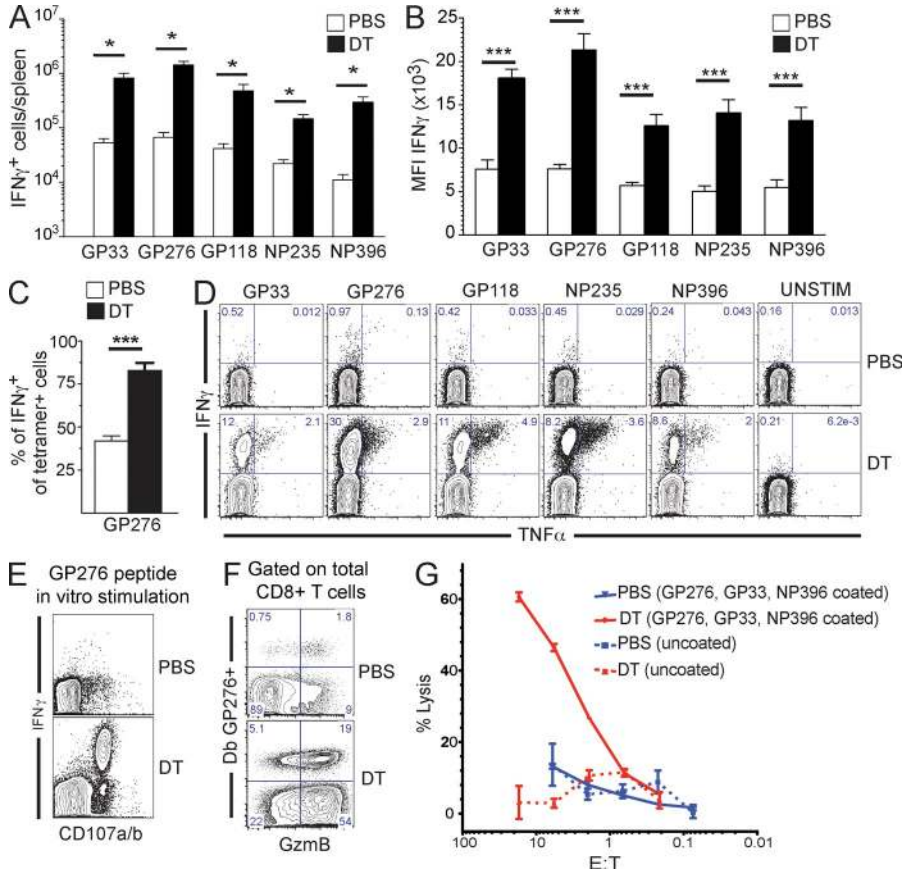
Figure 3. LCMV-specific CD8 T cells regain effector function upon T reg cell ablation in chronically infected mice. LCMV chronically infected *Foxp3^{DTR}* knock-in mice were depleted of T reg cells for 10 d by DT administration as in Fig. 2. (A) Absolute numbers of CD8 T cells in spleen producing IFN-γ. (B) MFI of IFN-γ production by CD8 T cells after in vitro restimulation with various LCMV peptides. (C) Percentage among DbGP276-specific cells that express IFN-γ in spleen. (D) Frequency of CD8 T cells producing both IFN-γ and TNF after in vitro restimulation with LCMV peptides. (E) Degranulation and surface expression of CD107 a/b in CD8 T cells after in vitro restimulation with GP276 LCMV peptide. (F) Granzyme B expression on LCMV DbG276-specific CD8 T cells in spleen. (G) Ex vivo cytotoxic activity of splenic CD8 T cells measured by ^{51}Cr release from MC57 target cells unpulsed (control) or pulsed with a mix of LCMV peptides (GP33, GP276, and NP396). Data are a compilation of 5 independent experiments with 3–5 mice per group. Error bars indicate SEM. Non-parametric Mann Whitney test, where *, P < 0.05; ***, P < 0.001.

(Fig. 2 H). CD127 is expressed on naive and memory cells to promote their long-term survival but is down-regulated on exhausted T cells (Kaech et al., 2003; Wherry and Ahmed, 2004). Interestingly, a recent report described that IL-2 therapy induces CD127 expression on exhausted CD8 T cells during chronic LCMV infection (West et al., 2013). Hence, it is possible that an increase in IL-2 availability upon T reg cell ablation triggers CD127 up-regulation on exhausted cells.
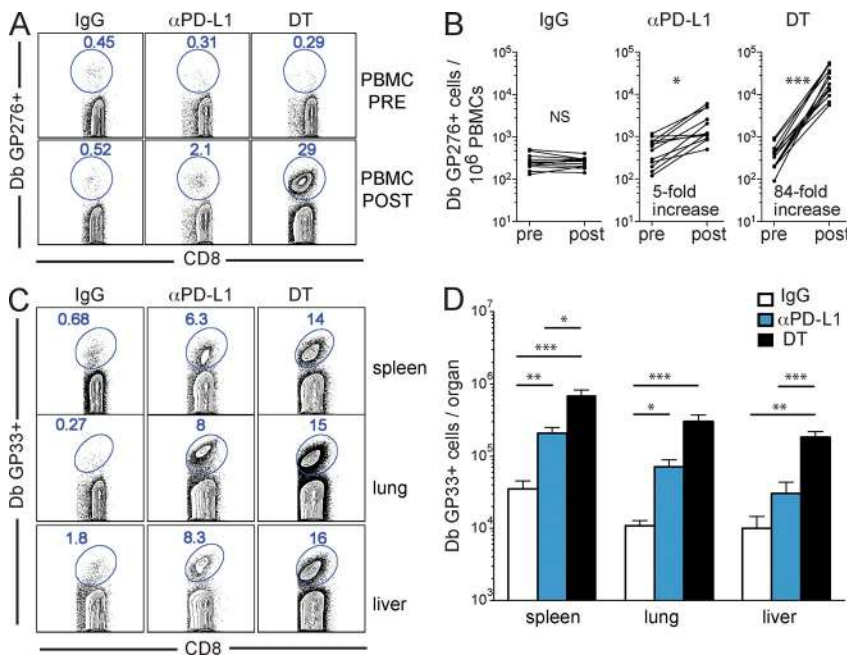

Figure 4. Rescue of LCMV-specific CD8 T cell responses by T reg cell ablation is greater than by blockade of the PD-1 pathway. LCMV chronically infected *Foxp3^{DTR}* knock-in mice were depleted of T reg cells for 10 d by DT administration as in Fig. 2 or mice received 3 doses of PD-L1 blocking antibody, every 3 d. (A and B) Dot plots (A) show frequency in the same mouse and graphs (B) show number of LCMV-specific (Db GP276) CD8 T cells among PBMCs before and after treatment. (C and D) Dot plots show frequency (C) and graphs show number (D) of LCMV-specific (Db GP33) CD8 T cells in different organs 11 d after treatment. Data are a compilation of 3 independent experiments with 3–5 mice per group. Error bars indicate SEM. Non-parametric Mann Whitney test, where *, P < 0.05; **, P < 0.01; ***, P < 0.001; NS = not significant.
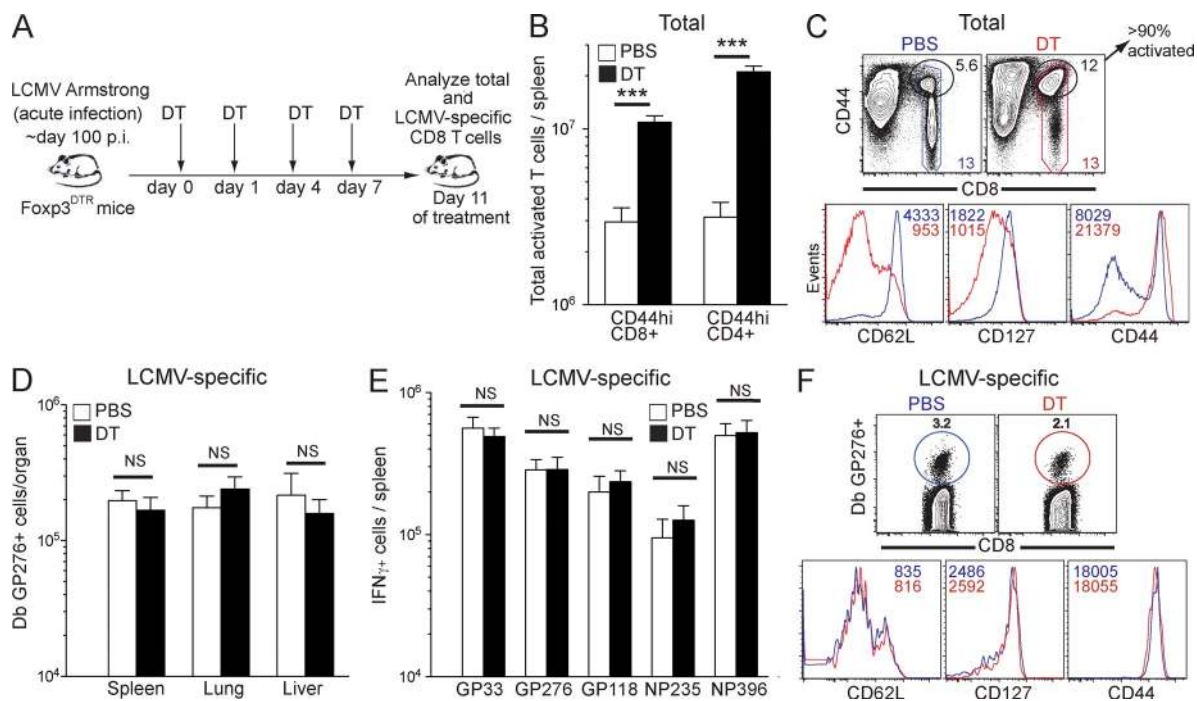
Figure 5. Cognate antigen is necessary for activation of antiviral T cells after T reg cell depletion. (A) Experimental outline. (B) Total numbers of activated T cells in the spleen of PBS-treated or T reg cell–depleted (DT) mice 100 d after infection with LCMV Arm. (C) Phenotype of total CD8 T cells in spleen. (D) Absolute numbers of LCMV-specific (Db GP276) CD8 T cells. (E) Absolute numbers of cells in the spleen that produce IFN-γ after in vitro restimulation with various LCMV peptides. (F) Phenotype of splenic LCMV-specific (Db GP276) CD8 T cells. A representative of 3 independent experiments is shown, with 5–6 mice per group. Error bars indicate SEM. Non-parametric Mann Whitney test, where ***, P < 0.001; NS = not significant.

Next, we wanted to ascertain whether T reg cell ablation in chronically infected mice restored functionality of exhausted T cells. We observed a marked increase in frequency and absolute numbers of virus-specific CD8 T cells that could produce IFN-γ upon stimulation with LCMV peptides (Fig. 3 A). T reg cell depletion not only affected the number of LCMV-specific functional T cells but also substantially increased the amount of IFN-γ produced per cell as indicated by the mean fluorescence intensity (MFI) of intracellular IFN-γ staining (Fig. 3 B). After T reg cell depletion, >75% of Db GP276 LCMV-specific T cells became functionally competent and acquired ability to produce IFN-γ (Fig. 3 C). We also observed increased TNF production to all LCMV epitopes tested and even severely exhausted CD8 T cells specific for the NP396 LCMV epitope were rescued upon T reg cell ablation (Fig. 3 D).

Furthermore, after T reg cell depletion, LCMV-specific CD8 T cells showed heightened ability to degranulate upon cognate peptide stimulation as measured by surface expression of the lysosomal marker CD107a/b (Fig. 3 E). As much as 73% of all CD8 T cells expressed granzyme B (Fig. 3 F), and rescued LCMV-specific T cells were capable of in vitro killing of antigen-bearing target cells (Fig. 3 G). Hence, T reg cell ablation in LCMV chronically infected mice rescued virus-specific CD8 T cells to proliferate and recover function.

The impressive increase in LCMV-specific responses after T reg cell depletion was of a much higher magnitude than the one caused by other means of rescue of exhausted CD8 T cells.

For example, blockade of the PD-1 inhibitory pathway increased the frequency of LCMV-specific CD8 T cells in blood by an average of fivefold, whereas T reg depletion led to an increase of almost 100-fold (Fig. 4, A and B). Similarly, the frequency and total number of LCMV-specific CD8 T cells in spleen, lung, and liver achieved by T reg cell depletion was higher than by blockade of the PD-1 inhibitory pathway in LCMV chronically infected mice (Fig. 4, C and D). Thus, T reg cells play a prominent role in the maintenance of CD8 T cell exhaustion during chronic viral infection.

Cognate antigen is necessary for activation of antiviral T cells after T reg cell depletion

Our results so far revealed that T reg cell ablation leads to a striking rescue of virus-specific CD8 T cells during chronic LCMV infection. To explore if cognate antigen was necessary for T cell activation that ensues after T reg cell depletion, we examined antiviral CD8 T cells after acute infection when antigen has been cleared. We infected *Foxp3*[DTR] knock-in mice with LCMV Arm, and T reg cells were depleted by DT administration at least 100 d after infection (Fig. 5 A). LCMV Arm causes an acute infection that is cleared within 8–10 d, and generates virus-specific long-lived memory CD8 T cells that persist in the absence of cognate antigen (Lau et al., 1994; Murali-Krishna et al., 1999; Wherry and Ahmed, 2004). Similar to mice chronically infected with LCMV, T reg cell ablation triggered expansion of total activated CD4 and CD8
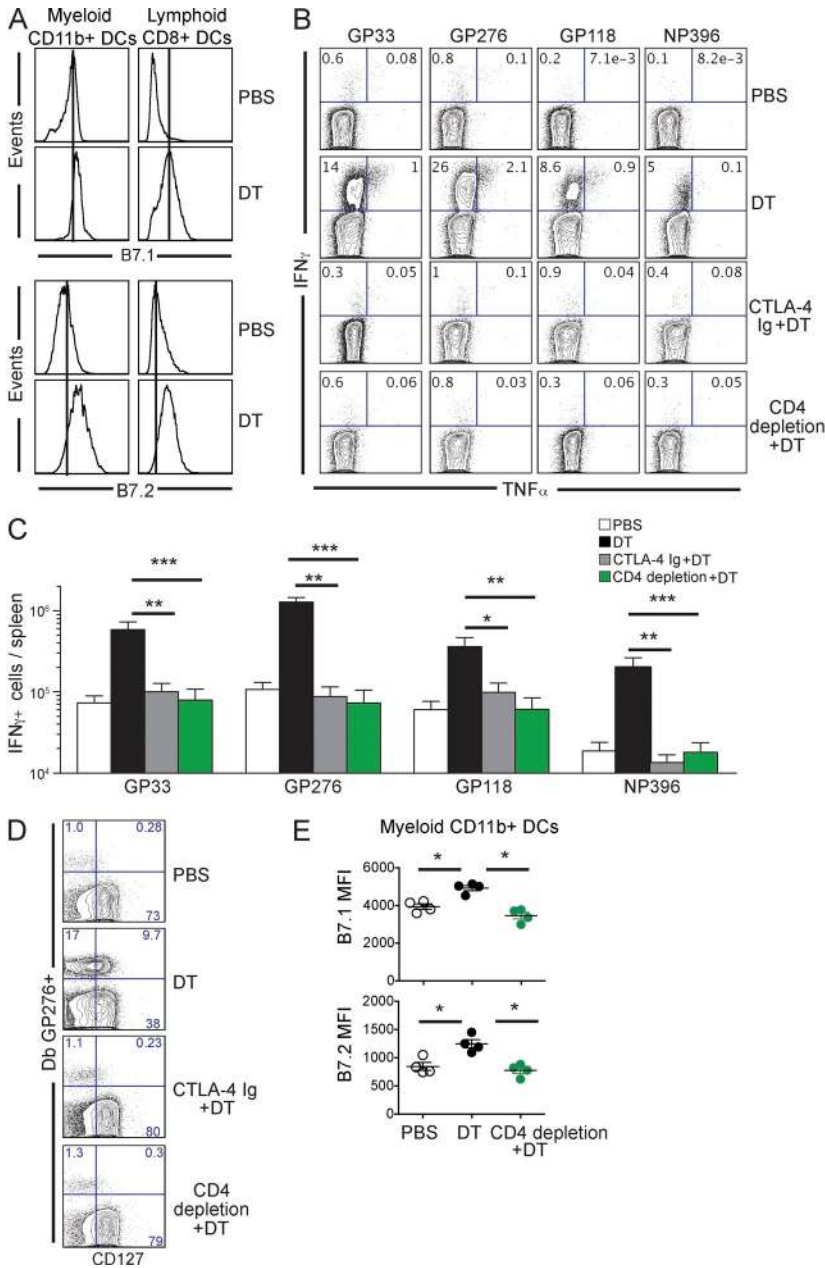
Figure 6. Co-stimulation and CD4 T cells are required for the rescue of exhausted CD8 T cells upon T reg cell depletion. *Foxp3^{DTR}* knock-in mice chronically infected with LCMV cl-13 received DT for 10 d (as in Fig. 2). CTLA-4 Ig was administered every other day during T reg cell depletion starting at day −2, and CD4 T cells were depleted upon administration of CD4 antibody on days −2 and −1 of T reg cell ablation. (A) Representative dot plots show expression of B7.1 and B7.2 on splenic DCs after 8 d of T reg cell ablation. (B and C) Frequency of splenic CD8 T cells that co-express IFN-γ and TNF (B) and absolute numbers of CD8 T cells that produce IFN-γ (C) after in vitro restimulation with LCMV peptides. (D) Frequency of LCMV-specific (Db-GP276) and CD127-expressing splenic CD8 T cells. (E) Graphs show B7.1 and B7.2 MFI on CD11b^+ splenic DCs after 8 d of T reg cell ablation preceded or not by conventional CD4 T cell depletion. The data are representative of 3 independent experiments with 3–5 mice per group. Error bars indicate SEM. Non-parametric Mann Whitney test, where *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

T cells (Fig. 5 B). After T reg cell depletion, CD8 T cells showed extensive phenotypic changes associated with activation such as down-regulation of CD62L and CD127 (Fig. 5 C). However, despite massive activation of the bulk CD4 and CD8 T cell subsets upon T reg cell ablation, the absolute numbers of LCMV-specific CD8 T cells remained unchanged in the spleen and nonlymphoid organs in mice that had cleared LCMV (Fig. 5 D). Analysis of T cell responses to multiple LCMV epitopes also showed no alterations in the functionality of LCMV-specific memory CD8 T cells after T reg cell depletion (Fig. 5 E). In addition, the phenotype associated with long-lived memory CD8 T cells was unaltered by depletion of T reg cells (Fig. 5 F). Thus, T reg cells do not play a significant role in regulating the homeostasis of memory T cells in the absence of cognate

antigen. Our results show that bystander effects upon T reg cell ablation do not impact memory T cells once the antigen has been cleared and indicate that T reg cells mostly suppress antigen-driven proliferation of T cells.

Rescue of exhausted CD8 T cells by T reg ablation is dependent on B7 costimulation and conventional CD4 T cells

To further explore potential mechanisms of T reg cell–dependent enforcement of an exhausted state of T cells, we looked at DC activation status. Under steady-state conditions, T reg cells control expression of costimulatory molecules on DCs (Kim et al., 2007; Wing et al., 2008; Schildknecht et al., 2010; Qureshi et al., 2011). Consistent with previous reports, we noticed increased expression of costimulatory molecules
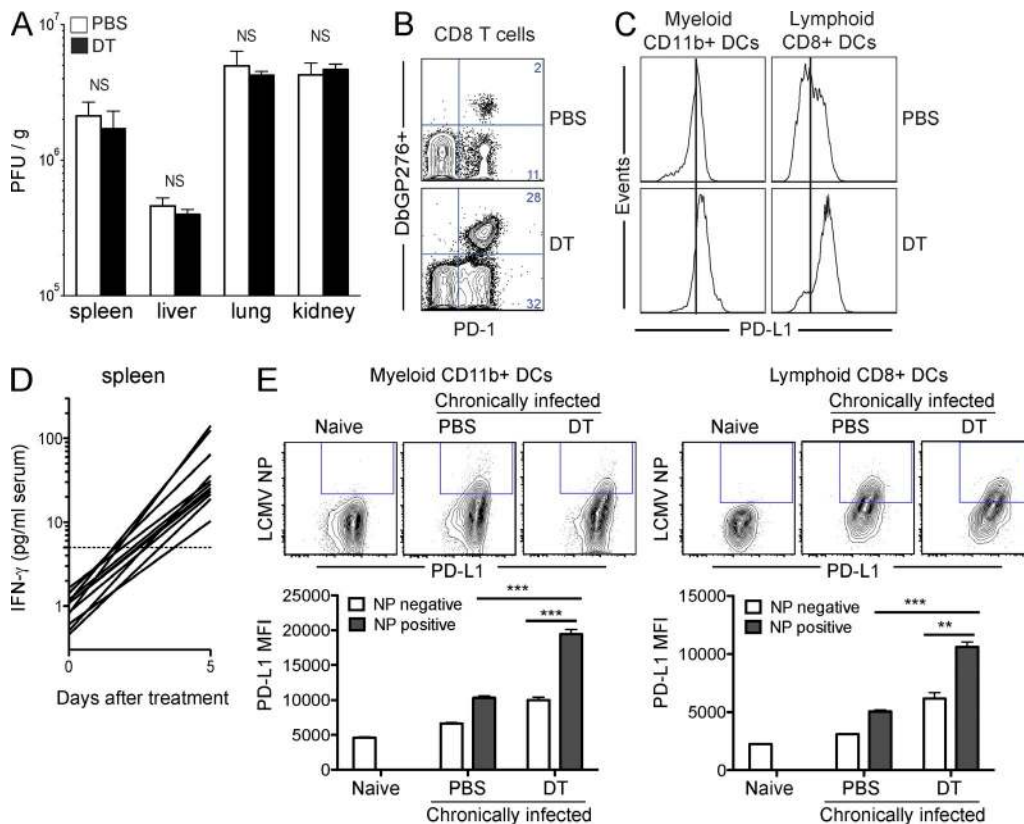
Figure 7. Inability to clear virus coincides with PD–L1 up–regulation. *Foxp3^DTR* knock-in mice chronically infected with LCMV received DT for 10 d (as in Fig. 2). (A) Viral load after 10 d of T reg cell depletion. (B) Dot plots show PD-1 expression on DbGP276-specific CD8 T cells. (C) PD-L1 expression on splenic DCs after 7 d of T reg cell ablation. (D) Graph shows IFN-γ in serum before and at day 5 after initiation of DT treatment. Dashed line indicates the limit of detection of this assay. (E) Representative dot plots show intracellular staining of LCMV nucleoprotein (NP) and PD-L1 expression on splenic DCs. Staining of DCs in uninfected mice is shown as a control for specificity of NP staining and for basal PD-L1 expression. PD-L1 expression is shown as MFI on infected (NP positive) or noninfected (NP negative) DCs. Data are a compilation of 3 experiments (A) or show representative results of 2–3 experiments (B–E) with 3–6 mice per group. Error bars indicate SEM. (A) Non-parametric Mann Whitney test, where NS = not significant. (D) Wilcoxon matched pairs test, where *, $P < 0.05$. (E) Student's *t* test, where **, $P < 0.01$; ***, $P < 0.001$.

B7-1 (CD80) and B7-2 (CD86) on CD11b⁺ and CD8α⁺ DC subsets after T reg cell depletion (Fig. 6 A). To explore whether increased costimulation was necessary to rescue virus-specific CD8⁺ T cells, we treated T reg cell ablated mice with CTLA-4 Ig, a fusion protein which binds B7-1 and B7-2 and thus blocks the B7/CD28 costimulatory pathway. In mice subjected to T reg cell depletion in combination with CTLA4-Ig–mediated B7 blockade, the number of functionally competent LCMV-specific CD8 T cells (Fig. 6, B and C) and their phenotype (Fig. 6 D) remained similar to that of untreated mice. Thus, B7 costimulation blockade prevented the increase in the response of virus-specific CD8 T cells that ensues upon T reg cell ablation in chronically infected mice. These results imply a critical role for costimulation in the rescue of exhausted T cell responses after T reg cell depletion and suggest an important role of DC activation in this process.

It was previously proposed that conventional CD4 T cells mediate the DC activation that ensues after T reg cell depletion (Kim et al., 2007). To address the role of conventional CD4 T cells in our model, we depleted total CD4 cells in concert with T reg cell depletion. We observed that CD4 T cell depletion reduced DC activation, especially of the CD11b⁺ DCs (Fig. 6 E), a DC subset which efficiently presents antigens to CD4 T cells (Dudziak et al., 2007). Importantly, CD4 T cell depletion completely abrogated the rescue of LCMV-specific CD8 T cells observed upon T reg cell ablation in chronically infected mice (Fig. 6, B–D). These findings suggest that in chronically infected mice, T reg cells modulate DC activity and conventional CD4 T cells to maintain an exhausted state of virus-specific CD8 T cells. In the chronic viral infection model used in our studies, CD4 T cells were transiently depleted before infection with LCMV cl-13 and even after restoration of CD4 T cell numbers, LCMV-specific CD4 T cells were not detected (unpublished data), as it was expected due to the infection of the thymus and negative selection (Matloubian et al., 1994). Therefore, it is highly unlikely that cognate CD4 T cell help is involved in the rescue of CD8 T cells upon T reg cell depletion, at least in this model of chronic LCMV infection.
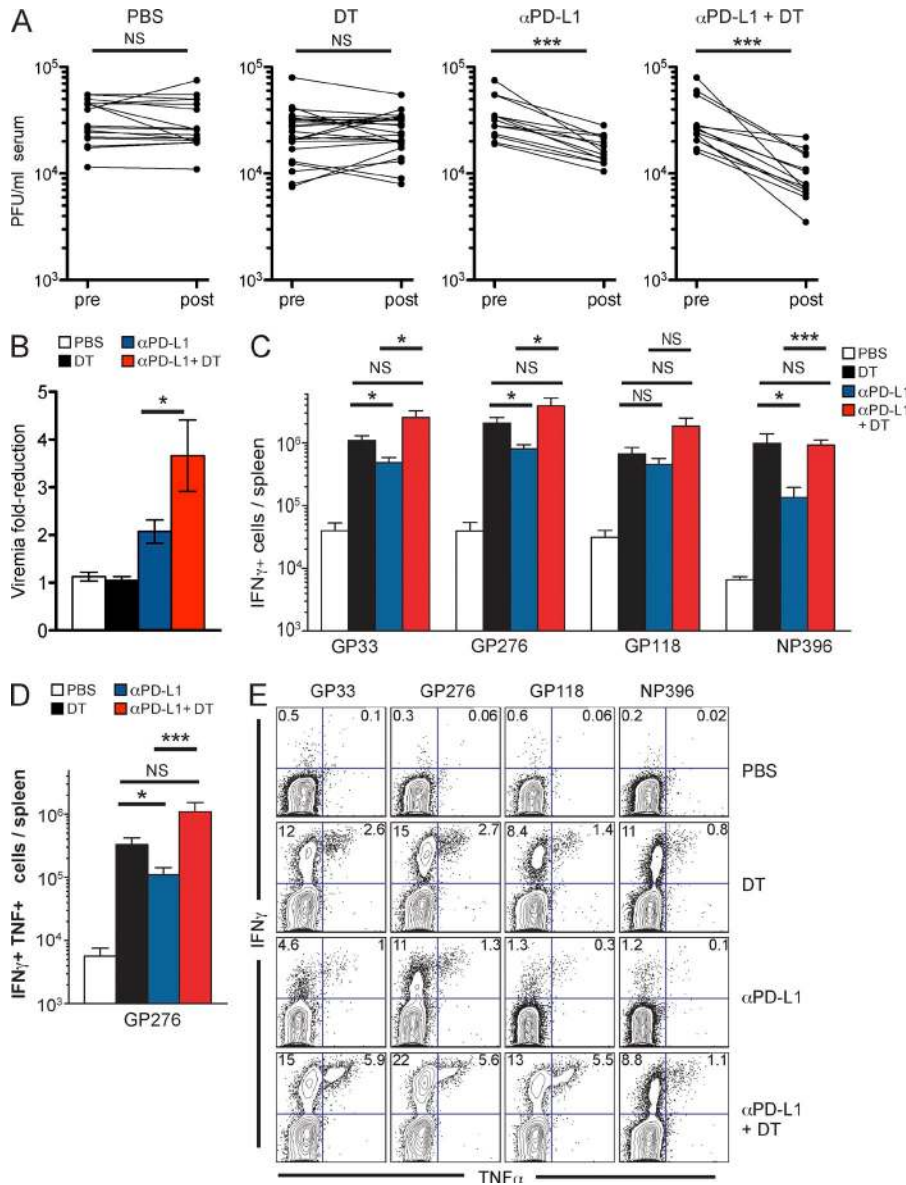
Figure 8. Essential role for PD–L1 blockade in viral control after T reg cell depletion. *Foxp3^{DTR}* knock-in mice chronically infected with LCMV received DT for 10 d (as in Fig. 2) in combination with PD-L1 blocking antibody. PD-L1 antibody was administered on days 1, 4, and 7 of DT treatment. (A) Viral titers in serum before and after treatment. (B) Fold reduction in serum viral titer after treatment. (C) Absolute numbers of splenic CD8 T cells that produce IFN-γ after in vitro restimulation with LCMV peptides. (D) Absolute numbers of CD8 T cells co-expressing IFN-γ and TNF after in vitro restimulation with GP276 LCMV peptide. (E) Representative dot plots showing frequency of CD8 T cells producing cytokines as in D. Data are a compilation (A and B) or show representative results of 3 independent experiments, with 4–6 mice per group. Error bars indicate SEM. Non-parametric Mann Whitney test, where *, P < 0.05; ***, P < 0.001; NS = not significant.

Failure to control virus by T reg cell ablation, despite striking rescue of LCMV–specific CD8 T cell responses, may be due to PD–L1 up-regulation on infected cells

We then tested whether the functional rescue of LCMV-specific CD8 T cells was associated with viral control. Unexpectedly, we did not observe a significant reduction in viral load in chronically infected mice (Fig. 7 A) despite the impressive rescue of LCMV-specific CD8 T cell responses induced by T reg cell depletion. Antiviral T cells are known to control virus by direct killing of infected cells and by production of cytokines (Kaech et al., 2002). Because T reg cell depletion resulted in a substantial increase in the number of LCMV-specific functional CD8 T cells (Fig. 3), we reasoned that the lack of viral control might be due to an inhibitory process that limits killing of infected cells in vivo.

Although LCMV-specific T cells were rescued by T reg cell ablation and regained function, they still remained PD-1^+

(Fig. 7 B). PD-1 is triggered by TCR signaling; thus, as long as virus persists, LCMV-specific CD8 T cells maintain PD-1 expression (Blattman et al., 2009). Furthermore, T reg cell ablation also led to increased expression of PD-L1 in both CD11b^+ and CD8α^+ DCs (Fig. 7 C). To gain an insight into potential reasons for PD-L1 up-regulation, we assessed inflammatory cytokine levels in the serum of LCMV chronically infected mice 5 d after T reg cell depletion. Most notably, we found increased levels of serum IFN-γ (Fig. 7 D), which has been described to modulate PD-L1 expression (Dong et al., 2002). Interestingly, we found higher PD-L1 expression on infected, LCMV nucleoprotein–positive DCs in comparison with their uninfected counterparts (Fig. 7 E). In addition to DCs, LCMV cl-13 replicates extensively in nonhematopoietic cells, such as fibroblastic reticular cells, endothelial cells, and diverse parenchymal cell types, and PD-L1 expression in these cells is known to restrict viral control (Mueller
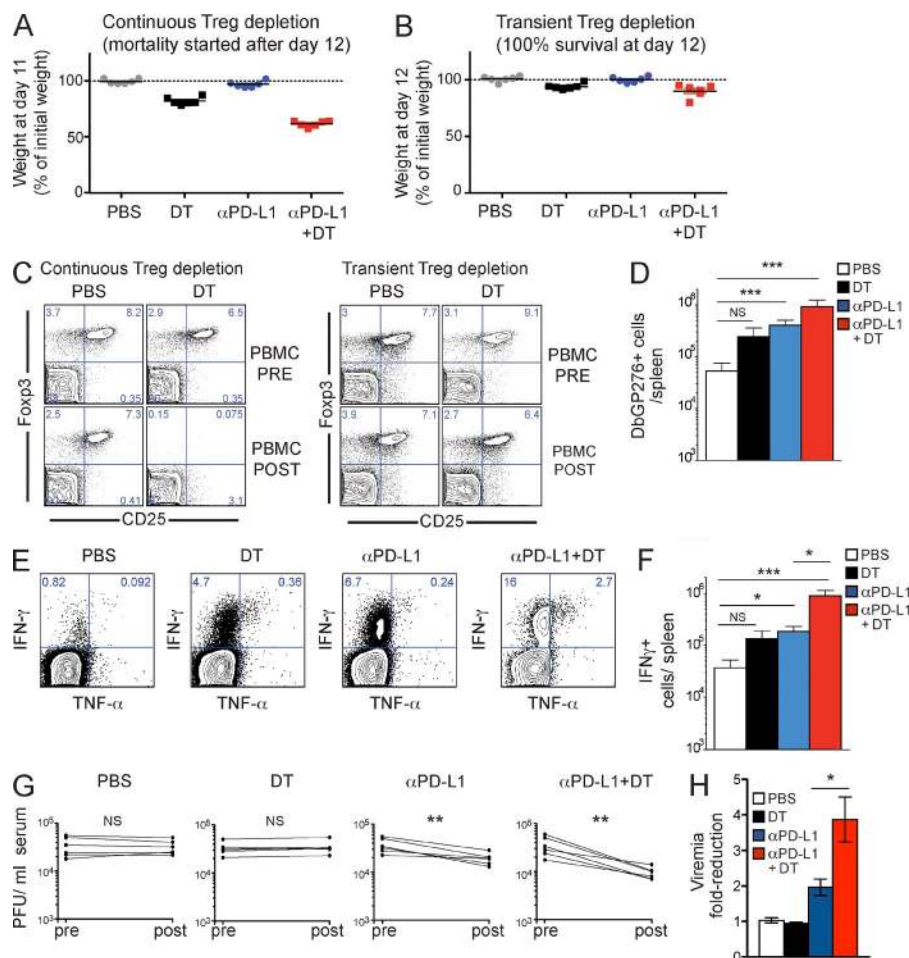
Figure 9. Transient T reg cell depletion improves CD8 T cell rescue and viral control when combined with PD-L1 blockade without causing overt disease in LCMV chronically infected mice. (A) *Foxp3^DTR* knock-in mice chronically infected with LCMV received continuous DT (on days 0, 1, 4, and 7) and/or PD-L1 antibody (on days 1, 4, and 7), as in Fig. 8. Mice weight after 11 d of treatment, relative to initial weight before treatment. (B) As in A, but mice received transient DT treatment (on days 0, 1, and 4). Mice weight after 12 d of treatment. (C) Representative dot plots show frequency of T reg cells in PBMC before and after continuous or transient DT treatment. (D) Absolute numbers of LCMV-specific (Db GP276) CD8 T cells within the total splenic CD8 T cell population. (E and F) Frequency of CD8 T cells producing both IFN-γ and TNF (E) and absolute numbers of CD8 T cells in the spleen that produce IFN-γ after in vitro restimulation with LCMV peptide GP276 (F). (G) Viral titers in serum before and after treatment. (H) Fold reduction in serum viral titer after treatment. A representative of 3 independent experiments is shown, with 6 mice per group. Error bars indicate SEM. Non-parametric Mann Whitney test, where *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS = not significant.

et al., 2007, 2010; Frebel et al., 2012). Thus, we reasoned that PD-1 interactions with the increased levels of PD-L1 expressed by infected cells might oppose viral clearance by functionally competent CD8 T cells in T reg cell–depleted mice.

Combining T reg cell depletion to blockade of the PD-1 pathway results in viral control

To test the idea that PD-L1 expression could be protecting LCMV-infected cells from cytotoxic T cells, we administered PD-L1 blocking antibodies to LCMV chronically infected *Foxp3^DTR* knock-in mice in conjunction with T reg cell depletion. Combining blockade of the PD-1 pathway with T reg cell ablation resulted in a significant reduction of viral load (Fig. 8 A), which was more pronounced than that upon blockade of the PD-1 pathway alone (Fig. 8 B). These results demonstrated an essential role for the PD-1–PD-L1 pathway in limiting viral control after CD8 T cell rescue. Importantly, the overall magnitude of multiple LCMV-specific CD8 T cell responses observed after T reg cell depletion alone or in combination with the PD-1 pathway blockade was similar (Fig. 8, C–E), with the exception of an additive increase in the frequency of IFN-γ and TNF double–producing cells (Fig. 8 E). Thus, these results demonstrated an essential role for the PD-1–PD-L1 pathway in limiting viral control after CD8 T cell

rescue. Because T reg cell depletion resulted in a greater number of functional virus-specific T cells when compared with PD-1 blockade alone (Fig. 8, C–E), and the combined therapy further improved viral control (Fig. 8, A and B), our data support the use of combination therapies based on modulation of T reg cell function and the PD-1 pathway to rescue exhausted T cell responses. Moreover, our data indicate that the magnitude of virus-specific CD8 T cell responses and decrease in viral load are not always directly correlated. These results show that target cell elimination is affected both by intrinsic cytotoxic potential of antigen-specific CD8 T cells and by inhibitory ligands such as PD-L1 displayed by target cells that may limit cytotoxicity.

Transient T reg cell depletion improves antiviral T cell responses when combined to blockade the PD-1 pathway

In adult healthy mice, chronic T reg cell ablation triggers autoimmunity as early as 10 d after the start of DT administration, and all mice succumb to overt disease by 3 wk of sustained absence of T reg cells (Kim et al., 2007). Not unexpectedly, sustained T reg cell ablation in LCMV chronically infected mice also induced immune-mediated wasting disease, which was further exacerbated by blockade of the PD-1 pathway (Fig. 9 A). To establish the therapeutic utility of our approach,

we subjected chronically infected mice to a transient T reg cell depletion regimen where mice received three DT doses instead of four doses. This regimen of DT administration did not cause an overt disease since these mice showed only minimal weight loss (Fig. 9 B). Although continuous DT treatment resulted in nearly complete absence of T reg cells in *Foxp3^DTR* knock-in mice, upon transient depletion the T reg cell subset had mostly recovered by the time of analysis (day 12; Fig. 9 C). To ensure that wasting disease and mortality were associated with the chronic T reg cell depletion rather than nonspecific DT toxicity, we treated LCMV chronically infected B6 mice with DT as a control and did not observe significant weight loss or mortality.

Transient T reg cell ablation in LCMV chronically infected mice did not significantly improve antiviral responses as a single therapy, but improved T cell rescue and viral control when combined to blockade of the PD-1 pathway (Fig. 9, D–H). Most importantly, combination treatment was more effective for viremia control than anti–PD-L1 treatment alone (Fig. 9, G and H). Additionally, even partial (fivefold lower DT dose) and transient T reg cell depletion was able to improve LCMV-specific T cell responses obtained by PD-L1 blockade, and mice remained healthy (unpublished data). Thus, careful optimization of the dose and timing of immunotherapy based on a combination of T reg cell and PD-1 signaling pathway can maximize the desired T cell responses while minimizing adverse events.

DISCUSSION

In this study, we show that T reg cells play a major role in T cell exhaustion during chronic viral infection. T reg cell ablation in LCMV chronically infected mice led to a striking rescue of exhausted CD8 T cells. Virus-specific CD8 T cells expanded 10–100-fold after depletion of T reg cells, and rescued CD8 T cells were able to degranulate, produce IFN-γ, TNF, and granzyme B, and also kill LCMV peptide-pulsed cells. Notably, the anti-LCMV CD8 T cell response obtained upon T reg cell ablation was of much higher magnitude than the rescue obtained in LCMV chronically infected mice by PD-1–PD-L1 blockade (Barber et al., 2006) or even combination of PD-1 pathway blockade with IL-2 therapy (West et al., 2013). In spite of this, the antiviral CD8 T cell responses that ensued after T reg cell depletion were ineffective to reduce viral burden. When we tried to understand this unexpected lack of viral control, we detected up-regulation of the inhibitory molecule PD-L1 after T reg cell ablation, especially in infected cells. Blockade of the PD-1 pathway in combination with T reg cell depletion resulted in significant control of viral load, despite minimal improvement of CD8 T cell rescue when compared with T reg cell ablation alone. We propose that the increased PD-L1 on target cells binds PD-1 on the rescued exhausted CD8 T cells and inhibits cytotoxic activity. These results support the hypothesis that T reg cells modulate T cell exhaustion, but the PD-1–PD-L1 pathway operates to prevent destruction of target cells. Our data show that rescue of effective T cell responses can be improved by combining blockade of the PD-1 pathway to T reg cell depletion.

In this study, we uncovered a major role of T reg cells in T cell exhaustion. Interestingly, T reg cells from LCMV chronically infected mice were phenotypically different from mice that had cleared LCMV infection or naive mice. During chronic infection, T reg cells displayed an activated phenotype, as previously reported (Punkosdy et al., 2011), with lower CD62L and higher CD44 and CD69 expression, as well as an increase in the inhibitory molecule PD-1. T reg cells from persistently infected animals also had increased expression of CD103, an important molecule for lymphocyte retention in skin and other epithelial rich sites (Suffia et al., 2005). Furthermore, we observed increased expression of molecules that have been associated with T reg suppressive activity, such as ICOS (Chen et al., 2012), granzyme B (Gondek et al., 2005; Cao et al., 2007), and CD39, an ectoenzyme which catabolizes proinflammatory extracellular ATP (Borsellino et al., 2007).

Most notably, T reg cells in chronically infected mice showed a twofold increase in the levels of CTLA-4, an inhibitory protein implicated in T reg cell–mediated suppressive function (Wing et al., 2008). It has been shown that CTLA-4 interacts with and internalizes B7 molecules by trans-endocytosis from the surface of the interacting cells, which results in APCs with lower stimulatory capacity for effector T cells (Qureshi et al., 2011). In our model, T reg cell depletion leads to increased B7 expression on DCs and we demonstrate that B7 stimulation is essential for rescue of exhausted LCMV-specific CD8 T cells. T reg cells may affect DC activation status directly (possibly through CTLA-4 interactions with B7 molecules) and also indirectly (possibly through conventional CD4 T cells). It is important to point out that during chronic LCMV infection CTLA-4 blockade does not rescue exhausted CD8 T cells (Barber et al., 2006), suggesting that CTLA-4 does not constitute a major nonredundant suppressive mechanism in this model.

We found that T reg cell control of CD8 T cell exhaustion operates by restraining activation of DCs and conventional CD4 T cells. After depletion of T reg cells we observed generalized activation of CD4 T cells but no LCMV-specific CD4 T cells that could provide cognate help to virus-specific CD8 T cells. Most likely, when autoreactive CD4 T cells are released from T reg cell inhibition, they become activated and then trigger activation of DCs presenting autoantigens, as it has been previously suggested (Kim et al., 2007). Consistent with the concept that CD4 T cells activate DCs, we found that depletion of conventional CD4 T cells abrogated activation of splenic CD11b⁺ DCs triggered by T reg cell ablation in LCMV chronically infected mice. Nonetheless, T reg cells might also affect DC activation status by other mechanisms as we observed that depletion of conventional CD4 T cells could not completely abrogate activation of splenic CD8α⁺ DCs after T reg cell ablation. There might be important differences between DC subsets with regard to interaction with T reg cells and how distinct DC subsets respond to the lack of T reg cells.

Even though our results suggest that DC activation plays an important role in the rescue of exhausted CD8 T cells, it is important to take into account that the presence of conventional

CD4 T cells was required for the rescue of exhausted CD8 T cells after T reg cell depletion. CD4 T cells can directly provide factors for the rescue of exhausted CD8 T cells, such as IL-2 (Blattman et al., 2003; West et al., 2013) and IL-21 (Elsaesser et al., 2009; Fröhlich et al., 2009; Yi et al., 2009). Activation of CD4 T cells and DCs constitute a very intricate relationship and interfering with one will affect the other, thus, in our study we could not determine exactly the factors necessary for the rescue of exhausted CD8 T cells. Our data highlight that manipulation of costimulatory pathways and identification of activating signals from CD4$^+$ T cells would provide pivotal knowledge for treating chronic infections and cancer.

During chronic LCMV infection, antigen is abundant, yet virus-specific CD8 T cells are suppressed and maintained by minimal proliferation. Our data indicate that in chronic viral infection T reg cell ablation promotes T cell activation but does not elicit de novo T cell responses. Consistent with this notion, T reg cell depletion did not generate LCMV-specific CD8 T cell responses in carrier mice infected with LCMV at birth in which there are no LCMV-specific T cells in the periphery due to negative selection in the thymus (Pircher et al., 1989; King et al., 1992). After 11 d of sustained T reg cell depletion in LCMV carrier $Foxp3^{DTR}$ knock-in mice (>10,000 PFU/ml in serum), despite prominent T cell activation (85.73%, SD = ±2.55 CD44^{hi} CD8 T cells in DT treated vs. 34.4%, SD = ±2.81 in untreated mice), we were unable to detect any LCMV-specific CD8 T cells (DbGP33$^+$ or DbGP276$^+$). This important observation implies that antigen-specific T cells must be present to elicit T cell responses by T reg cell manipulation.

In addition, our data suggest that the T cell activation that occurs upon T reg cell ablation is antigen-driven. In contrast to the impressive rescue of virus-specific CD8 T cells in LCMV chronically infected mice, T reg cell depletion in mice that had cleared the infection did not affect the numbers and the activation state of virus-specific CD8 T cells. This observation reveals that T reg cells do not control maintenance of memory cells and suggests that T reg cell manipulation would not affect homeostasis of memory cells generated by vaccination or acute infection.

In this study, we compared the effect of T reg cell depletion and blockade of the PD-1 pathway on the antiviral response in chronically infected mice. T reg cell depletion in LCMV chronically infected mice resulted in an unprecedented rescue of antiviral CD8 T cells. Distinctly, after T reg cell ablation, LCMV-specific CD8 T cells re-expressed CD127, which may be indicative of increased IL-2 signals (West et al., 2013). T cells integrate signals received from APCs and the environment to determine their differentiation program. Besides increased systemic IFN-γ, we also observed an increase in TNF (10–140 pg/ml), MCP-1 (45–360 pg/ml), and IL-6 (6–55 pg/ml), as it has been previously reported after T reg cell depletion in naive mice (Chinen et al., 2010). Interestingly, we could not detect any significant amount of inflammatory cytokines and chemokines (IFN-γ, TNF, MCP-1, and IL-6) in the serum of LCMV chronically infected mice treated with anti–PD-L1 blocking antibodies for 5 or 8 d.

Thus, the presence of inflammatory cytokines in the serum is most likely the result of autoreactivity unleashed by T reg cell depletion and not a measure of antiviral T cell responses. Thus, it is likely that LCMV-specific CD8 T cells rescued by T reg cell ablation might be quite different from CD8 T cells rescued by blockade of the PD-1 pathway, and better understanding of these differences might help shed light into potential molecular mechanisms involved in rescue of T cell exhaustion.

Importantly, our study shows that even a 10–100-fold increase in the number of functional viral-specific CD8$^+$ T cells does not necessarily correlate with a decline in viral load. We show that the PD-1 pathway plays a fundamental role in viral clearance and we propose that PD-L1 expression in virus-infected cells is a potent inhibitor of target cell elimination. We observed an increase in B7 and PD-L1 expression on DCs after T reg cell depletion. We propose that LCMV-specific CD8 T cells are rescued because there is a net positive balance of signals from APCs presenting viral antigens to exhausted CD8 T cells. However, the bulk viral burden in chronically infected mice is largely due to LCMV infection of nonhematopoietic cells that express PD-L1 but no B7 molecules (Mueller et al., 2007, 2010). Thus, even though LCMV-specific CD8 T cells regain function after T reg cell depletion, there is no improvement in viral control because rescued CD8 T cells maintain PD-1 expression and their cytotoxic activity is inhibited by PD-L1–expressing infected cells (Mueller et al., 2010; Frebel et al., 2012). Notably, PD-L1 also protects tumor cells from CD8 T cell–mediated killing (Iwai et al., 2002). When T reg cell depletion was combined with PD-L1 blocking antibodies, rescue of exhausted T cells was only marginally improved as reflected by higher frequency of LCMV-specific CD8 T cells coproducing IFN-γ and TNF. However, T reg cell depletion in combination with blockade of the PD-1 pathway resulted in marked improvement of viral control. In summary, our data show that the magnitude of T cell responses and effective control of infected cells (and possibly tumors) are not always coupled together.

PD-L1 is ubiquitously expressed and its expression is further increased by several cytokines, including IFN-γ (Dong et al., 2002; Keir et al., 2008). We showed that T reg cell depletion in LCMV chronically infected mice causes a systemic increase in IFN-γ as early as day 5 after treatment, which may modulate PD-L1 expression. In addition, functional rescue of CD8 T cells might directly induce PD-L1 up-regulation, especially on infected cells presenting LCMV antigens due to cognate interactions with T cells and local IFN-γ production. It is important to point out that LCMV cl-13 and other viruses that cause chronic infections are relatively resistant to IFNs (Moskophidis et al., 1994) and IFNs may even have a detrimental effect on the course of the infection (Teijaro et al., 2013; Wilson et al., 2013). Thus, therapies that promote cytotoxic T cell responses still need to ensure that further inhibitory mechanisms will not hamper destruction of target cells.

Reversal of T cell exhaustion is the ultimate goal of immunotherapies aiming to treat chronically infected patients as well as cancer patients. There have been many recent advances

in this field, and although single therapies such as blockade of the PD-1 pathway and anti–CTLA-4 antibody treatment have achieved success in clinical trials with advanced cancer patients, most studies suggest that a combination of therapies may achieve better results. Rational combination of therapies that operate through different mechanisms will probably improve synergy to achieve better response rates as well as longevity of the immune response. Our study uncovers a potential mechanism for synergistic effects of T reg cell depletion and blockade of the PD-1 pathway.

Recent evidence suggests that anti–CTLA-4 may function by depletion of T reg cells at the tumor site (Bulliard et al., 2013; Selby et al., 2013; Simpson et al., 2013). Importantly, it was recently shown that combining anti–CTLA-4 to PD-1 blockade resulted in >40% objective responses in advanced melanoma patients (Wolchok et al., 2013). In this study, among responders, most patients had >80% tumor regression, which exceeds the rate of responses reported previously in monotherapy trials. Thus, our results provide important concepts that may have direct relevance to cancer treatment. Nevertheless, additional studies need to be done to determine which chronic infections and cancers would be responsive to this combinatorial immunotherapy involving T reg cell depletion and blockade of the PD-1 pathway because there may be heterogeneity in the response to such therapies (Bos et al., 2013).

In LCMV chronically infected mice, virus is widespread and there is a delicate balance between an effective immune response that controls viral load and overt immunopathology. Using $Foxp3^{DTR}$ knock-in mice, we were able to achieve very efficient depletion of T reg cells. Although the complete absence of T reg cells may increase T cell rescue, serious adverse effects may limit its application in a clinical setting. We show that transient T reg cell depletion, as a single therapy, failed to rescue antiviral responses, but when combined to PD-1 pathway blockade, transient T reg cell depletion improved both CD8 T cell responses and viral control. Thus, milder T reg cell depletion regimens, which could be achieved in patients with antibody-based depletion strategies, might improve immune responses induced by other therapies without overt adverse effects. Alternative approaches that would preferentially affect a subset of T reg cells, such as $CCR4^+$ T reg cells which are predominant at the tumor site (Sugiyama et al., 2013) or $Nrp1^+$ T reg cells which are recruited to VEGF-producing tumors (Hansen et al., 2012), have also been proposed as potential strategies that would evoke immune responses while minimizing autoimmunity.

In summary, our studies demonstrate that T reg cells control chronically stimulated exhausted CD8 T cells during persistent viral infection. We show that this T reg cell–mediated control of exhausted CD8 T cells involves restraint of B7 costimulation and of help from conventional CD4 T cells. The findings presented here also underscore a critical role of PD-L1 in determining viral control after T cell rescue from an exhausted state.

MATERIALS AND METHODS

Mice and infections. 4–8-wk-old homozygous $Foxp3^{DTR}$ knock-in mice on a C57BL/6 background (Kim et al., 2007) or C57BL/6 (The Jackson Laboratory) were infected with LCMV Arm or LCMV cl-13. Memory T cell responses were generated by i.p. injection with 2×10^5 PFU of LCMV Arm, which results in an acute infection that is cleared within 8 d. Chronic infections with exhausted T cell responses and lifelong viremia were induced by antibody-mediated transient depletion of CD4 T cells with 2 doses of 500 µg GK1.5 (Bio X Cell) i.p., followed by i.v. injection with 2×10^6 PFU LCMV cl-13 as described previously (Matloubian et al., 1994). To analyze memory T cell responses, we waited at least 100 d after LCMV Arm infection, and for exhausted T cell responses, we waited at least 45 d after LCMV cl-13 infection. Titration of virus was performed on Vero cell monolayers as described previously (Ahmed et al., 1984). All mouse experiments were performed according to institutional guidelines and were approved by the Emory University Institutional Animal Care and Use Committee.

Antibody treatments and T reg cell depletion. 500 µg CTLA-4 Ig (gift from the Ford and Larsen laboratory, Emory University, Atlanta, GA) was injected i.p., on days −2, 0, 2, 4, 6, 8, and 10 of T reg cell ablation. $CD4^+$ T cell depletion was achieved by i.p. injection of 500 µg GK1.5 on days −2 and −1 of T reg cell ablation. 200 µg PD-L1 blocking antibody (10F.9G2) was administered i.p. on days 1, 4, and 7 after T reg cell ablation. Each mouse received ∼1 µg DT (Sigma-Aldrich) i.p. (50 µg/kg).

Cytotoxicity activity by 51 chromium–release assay. MC57 mouse fibroblast cell targets were coated with a mix of GP33, GP276, and NP396 peptides or no peptide, and labeled with 350 µCi 51-Cr. Target cells were incubated for 6 h with different amounts of effector splenic $CD8^+$ T cells enriched using mouse CD8 beads (Miltenyi Biotec). Absolute numbers of viral-specific $CD8^+$ T cells was retroactively calculated and plotted. E:T = effector to target ratio. MC57 cells with 1% Triton X-100 were used as positive control (total release), and MC57 cells without effectors were used to calculate spontaneous release.

Antibodies and flow cytometry. Single cell suspensions were obtained from blood, spleen, lungs, liver, kidney, and gut as previously described (Masopust et al., 2001). For analysis of dendritic cells, spleens were digested with 0.4 U/ml collagenase D (Roche) for 30 min at 37°C. Single cell suspensions were stained with anti-CD8α (53–6.7), -CD4 (RM4-5), -CD25 (PC61), CD40 (3/23), CD80 (16-10A1), CD86 (GL1), -CD107a (1D4B), -CD107b (ABL-93), Vβ 5.1 5.2 (MR9-4), and Ki67 (B56; from BD); -ICOS (7E.17G9), −PD-L1 (MIH5), -CD11c (N418), -CD11b (M1/70), -CD127 (A7R34), -MHCI (AF6-88.5.5.3), and -Foxp3 (FJK-16s; from eBioscience); -CD44 (IM7) and −PD-1 (RMP1-30; from BioLegend); and −granzyme B (MHGB04; Invitrogen). Anti-LCMV NP antibody was a gift from the Buchmeier Laboratory (University of California, Irvine, Irvine, CA). Dead cells were excluded by gating out cells positive for Live/Dead fixable dead cell stain (Invitrogen). LCMV MHC class I tetramers were prepared and used as previously described (Wherry et al., 2003). The LCMV MHC class II tetramer (I-Ab GP61-80) was obtained from the National Institutes of Health tetramer facility and stains were performed at 37°C for 3 h, gently mixing cells every 30 min. LCMV-specific responses were assessed by restimulating splenocytes with 0.1 µg/ml GP33, NP396, GP276, NP118, or NP235 LCMV peptides in the presence of brefeldin and monensin for 5 h at 37°C. Intracellular staining for IFN-γ, TNF, Ki67, and granzyme B were performed with the Cytofix/Cytoperm kit (BD). Intracellular staining of Foxp3 was performed according to manufacturer's instructions (eBioscience). Serum cytokines were measured by cytometric bead array according to manufacturer's instructions (BD). Samples were acquired with a FACSCanto or LSRII (BD) and analyzed using FlowJo (Tree Star).

Statistical analysis. Statistical analysis was performed on Prism software (GraphPad Software).

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