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Research Article **Immunology**

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PD-L1 blockade synergizes with IL-2 therapy in reinvigorating exhausted T cells

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The inhibitory receptor programmed cell death 1 (PD-1) plays a major role in functional exhaustion of T cells during chronic infections and cancer, and recent clinical data suggest that blockade of the PD-1 pathway is an effective immunotherapy in treating certain cancers. Thus, it is important to define combinatorial approaches that increase the efficacy of PD-1 blockade. To address this issue, we examined the effect of IL-2 and PD-1 ligand 1 (PD-L1) blockade in the mouse model of chronic lymphocytic choriomeningitis virus (LCMV) infection. We found that low-dose IL-2 administration alone enhanced $CD8^+$ T cell responses in chronically infected mice. IL-2 treatment also decreased inhibitory receptor levels on virus-specific $CD8^+$ T cells and increased expression of CD127 and CD44, resulting in a phenotype resembling that of memory T cells. Surprisingly, IL-2 therapy had only a minimal effect on reducing viral load. However, combining IL-2 treatment with blockade of the PD-1 inhibitory pathway had striking synergistic effects in enhancing virus-specific $CD8^+$ T cell responses and decreasing viral load. Interestingly, this reduction in viral load occurred despite increased numbers of Tregs. These results suggest that combined IL-2 therapy and PD-L1 blockade merits consideration as a regimen for treating human chronic infections and cancer.

Introduction

CD8 T cells play a key role in eliminating and intracellular infections and tumors. However, in the setting of chronic antigen stimulation, such as that seen in chronic infections and tumors, CD8 T cells undergo exhaustion, causing them to become dysfunctional. This exhaustion is characterized by decreased proliferative capacity, loss of cytokine secretion, reduced cytotoxic killing abilities, and phenotypic changes, including low expression of canonical memory markers, such as the IL-7 receptor α chain (CD127), and also an increase in inhibitory receptors (1–3).

While multiple mechanisms contribute to the process of exhaustion, the inhibitory receptor programmed cell death 1 (PD-1) has emerged as a major player in this process. PD-1 is the most well-characterized inhibitory molecule upregulated during chronic antigen stimulation and is associated with disease progression and immune dysfunction (2). Importantly, recent data from 2 clinical trials have highlighted the role of PD-1 inhibition in human cancers and have shown that PD-1 blockade, by in vivo administration of humanized anti–PD-1 or anti–PD-1 ligand 1 (anti–PD-L1) antibodies, is an effective immunotherapeutic for increasing tumor clearance. Notably, in vivo PD-1 blockade resulted in durable tumor reduction or clearance in multiple cancers, including lung cancer, which is highly refractory to any treatment (4–6). These data correspond well with previous in vitro and in vivo animal model data showing that PD-1 plays a central role in T cell dysfunction during chronic infections and cancer and that PD-1 blockade can restore T cell function (2, 3, 7–16). Overall, these data indicate that PD-1 may be an important immunotherapeutic for cancers and chronic infections and signify that it is vital to find ways to increase the efficacy of PD-1 blockade. Multiple inhibitory mechanisms regulate CD8 T cell exhaustion, and, thus, combining PD-1 blockade along with other therapies, such as simultaneous blockade of multiple inhibitory receptors or therapeutic vaccination, results in enhanced reduction of viral loads and increased CD8 T cell responses in animal models of chronic infection. However, it is important to note that the mechanisms underlying the synergy of combined treatments has not been well explored (17–19). Overall, this suggests that combining strategies or treatments to combat chronic infections and cancer may be a valid strategy to increase efficacy.

IL-2 is a cytokine that has a pleiotropic effect on multiple immune cell types and has been used as a therapy for several human diseases/conditions. IL-2 has been used to augment T cell responses against virus or tumor antigens in HIV and patients with metastatic cancer. While high-dose intermittent IL-2 therapy has increased long-term survival for some patients with metastatic renal cell carcinoma (20) and IL-2 therapy alone or in combination with a peptide vaccine has resulted in clinical improvement for patients with metastatic melanoma (21, 22), it has shown very limited success when given during chronic human viral infections, such as when it is combined with antiretroviral drugs during HIV (23–28). Greater improvement was seen in one trial, with IL-2 administration combined with antiretroviral drugs and therapeutic vaccination during HIV infection (29), although other small studies suggest that a long-term effect is not seen after antiviral therapy is discontinued (30–32). However, continuous IL-2 administration, along with therapeutic vaccination and antiretroviral treatment, in macaques infected with chronic SIV increases SIV-specific CD8 T cell responses and results in decreased viral burden (33, 34).

Overall, a major limitation of high-dose intermittent IL-2 therapy is that it can result in severe toxicity issues, such as vascular leakage. By comparison, daily, much lower doses of IL-2 can ame-

Conflict of interest: Rafi Ahmed, Sang-Jun Ha, and Gordan J. Freeman have patents and receive patent royalties related to the PD-1 pathway. Gordon J. Freeman is a scientific founder and scientific advisory board member of CoStim Pharmaceuticals.

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liorate these toxicity issues (35). Recently promising human data indicate that daily low-dose IL-2 therapy may be useful for increasing Treg numbers and reducing autoimmune complications in patients with graft-versus-host disease as a result of undergoing an allogeneic hematopoietic stem cell transplantation (36) and also in patients with hepatitis C–induced vasculitis (37). Importantly, these recent studies indicate that daily low-dose IL-2 therapy is well tolerated by patients (36, 37). While daily low-dose IL-2 therapy increases Tregs in the context of autoimmune complications, in contrast, our laboratory has previously shown that daily low-dose IL-2 treatment during chronic mouse lymphocytic choriomeningitis virus (LCMV) infection results in enhanced virus-specific CD8 T cell numbers and function and slightly reduces viral burden (38). These data indicate that daily low-dose IL-2 therapy may be beneficial during persistent infections, but a better understanding of the action of low-dose IL-2 therapy on exhausted T cells and its role on Treg numbers during chronic infection is needed.

Additionally, while the clinical data on high-dose intermittent IL-2 therapy during chronic viral infections has not been very promising, combining IL-2 therapy with other immunomodulatory regimens may allow for the positive effects of IL-2 to be enhanced, while diminishing the negative toxicity issues associated with IL-2, through the use of a daily low-dose treatment of IL-2. Therefore, in this study, we explored the idea of combining the positive signal of IL-2 along with the blockade of an inhibitory pathway, PD-1, as a therapy for chronic infection. Using the mouse LCMV model of chronic infection, we determined the effect of daily low-dose IL-2 treatment given alone, or as a combined immunotherapy in conjunction with PD-1 blockade, on exhausted virus-specific CD8 T cells, Tregs, and viral control. Herein, we show that when used as a combination therapy, IL-2 administration strikingly enhances the effectiveness of PD-1 blockade and this combined therapy may be an important clinical therapeutic for fighting human cancer and chronic infections.

Results

IL-2 therapy synergizes with PD-L1 blockade to enhance virus-specific CD8 T cell control of chronic LCMV infection. To determine the effects of IL-2 therapy, PD-L1 blockade, and combined IL-2 therapy and PD-L1 blockade on CD8 T cells and viral control during chronic infection, we used the chronic LCMV mouse model. Mice were infected with the clone-13 (cl-13) strain of LCMV, which results in a protracted viral infection with >2 months of viremia. After chronic infection was well established, beginning at day 23 to 27 after infection, mice were treated with anti–PD-L1 blocking antibody once every 3 days for a total of 5 treatments, and during the last 8 days of anti–PD-L1 treatment 15,000 IU (1 μg) of recombinant human IL-2 was administered once daily. This regimen was decided upon after consideration of previously published data in this model (38) and after some titration of the dose of IL-2, as similar LCMV-specific CD8 T cell responses and viral reduction were seen when 15,000 IU IL-2 was injected twice daily and when it was injected once daily (Supplemental Figure 1, A–C; supplemental material available online with this article; doi:10.1172/JCI67008DS1). Administration of IL-2 or anti–PD-L1 alone increased LCMV-specific CD8 T cells in both the blood and tissues (Figure 1, A–C) that had an enhanced ability to produce both IFN-γ and TNF-α after ex vivo restimulation with LCMV-specific peptides covering multiple epitopes (Figure 1D and Supplemental Figure 2, A and B). This increase in functional LCMV-specific CD8 T cells after treatment with IL-2 or PD-L1 blockade alone correlated with some reduction in viral loads, with a trend toward PD-L1 blockade resulting in a greater reduction of viral burden than that seen with IL-2 therapy alone (Figure 1E). Strikingly, mice given combined IL-2 therapy and PD-L1 blockade had a much larger expansion of LCMV-specific CD8 T cells in the blood and tissues (~4-fold expansion for the D^bGP33-41 epitope) (Figure 1, A–C). In addition, after combined IL-2 therapy and PD-L1 blockade, production of IFN-γ and coproduction of IFN-γ and TNF-α by CD8 T cells after ex vivo stimulation with LCMV-specific peptides were greatly increased in response to multiple epitopes, including the previously undetectable NP396 epitope (Figure 1D). These data indicate the expansion of CD8 T cells with a broader response, which may help reduce the selection of viral escape mutants. This effect was distinct compared with that seen with either treatment given alone, as IL-2 or PD-L1 blockade alone did not enhance the NP396 response (Figure 1D). Last, combined IL-2 therapy and PD-L1 blockade resulted in undetectable viral loads in the majority of mice, indicating faster viral control than that seen with either treatment given alone (Figure 1E). These data indicate that IL-2 therapy synergizes with PD-L1 blockade, resulting in a greatly expanded and functional LCMV-specific CD8 T cell response to multiple epitopes and increased viral clearance during chronic LCMV cl-13 infection.

IL-2 therapy and PD-L1 blockade have distinct effects on virus-specific CD8 T cell expansion and viral loads during chronic LCMV infection. Transient depletion of CD4 T cells in mice before infection with LCMV cl-13, leads to a deeper exhaustion of LCMV-specific CD8 T cells and high levels of viremia for the life of the mouse. We next tested the ability of combined IL-2 therapy and PD-L1 blockade to rescue LCMV-specific CD8 T cells in the absence of CD4 T cell help during chronic LCMV infection ("unhelped" chronic infection model). To address this, we transiently depleted mice of CD4 T cells, using an anti-CD4 depleting antibody, prior to infection with chronic LCMV cl-13. Following 60 days after infection, when CD4 T cell numbers returned to normal but no LCMV-specific CD4 T cells existed, we began treating the mice with PBS/isotype control, IL-2 alone, PD-L1 blockade alone, or combined IL-2 therapy and PD-L1 blockade. The appropriate groups were given PD-L1 blocking antibody once every 3 days for 5 total treatments, and 15,000 IU IL-2 was given every 12 hours i.p. continuously during the 12 days of PD-L1 blockade (Figure 2A). This regimen of treatment was decided upon after determining that IL-2 administration given continuously during PD-L1 blockade resulted in increased LCMV-specific CD8 T cells compared with IL-2 given early after, in the middle of, or late after the start of PD-L1 blockade (Supplemental Figure 3, A–D). Furthermore, administration of IL-2 every 24 hours had less effect on the LCMV-specific CD8 T cells than giving IL-2 every 12 hours in this model of more severe CD8 T cell exhaustion (data not shown).

Both IL-2 treatment alone and PD-L1 blockade alone resulted in an increase in the GP33- and GP276-specific cells in the blood and tissues (Figure 2, B–E). However, combined IL-2 therapy and PD-L1 blockade led to a huge expansion of LCMV-specific CD8 T cells, even as early as day 8 after the beginning of treatment, resulting in up to approximately 30% of the CD8 T cells in the blood of some mice being specific for one LCMV epitope 2 days after the last treatment (Figure 2B) and an overall increase in both GP33- and GP276-specific CD8 T cells in the blood (Figure 2C). In addition, combined IL-2 therapy and PD-L1 blockade resulted in a large increase in the frequency and numbers of LCMV-specific

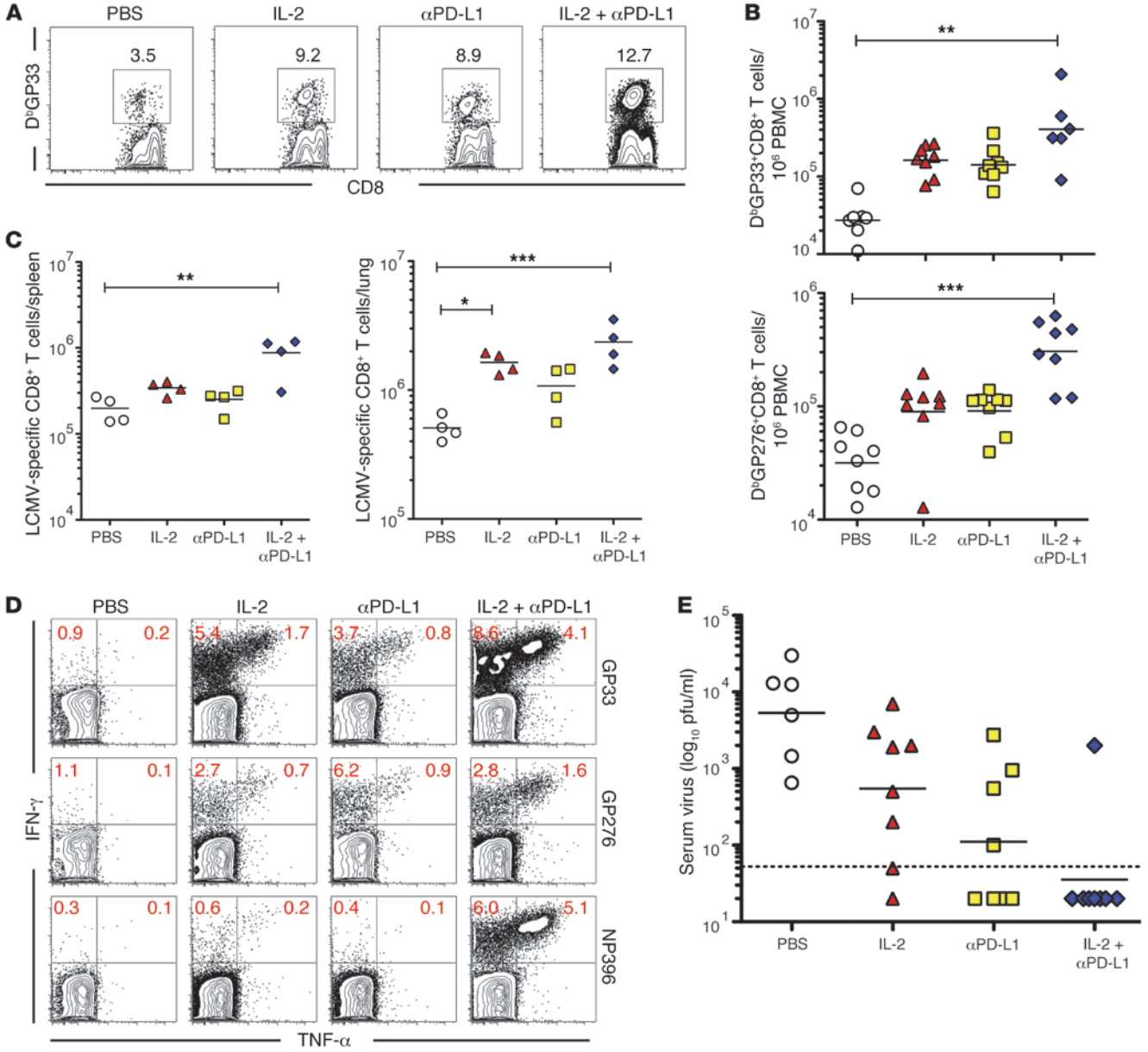

Figure 1

IL-2 therapy combined with PD-L1 blockade enhances antiviral CD8 T cell responses during chronic LCMV infection. C57BL/6 mice were infected with LCMV cl-13, and beginning on day 23 to 27 after infection, the mice were treated with 200 μg anti–PD-L1 antibody every 3 days for 12 days (5 total treatments). All IL-2–treated groups were given 1.5×10^4 IU IL-2 (i.p.) once a day for the last 8 days of anti–PD-L1 treatment. (**A**) Frequency of H-2D^b GP33-specific CD8 T cells in the blood 1 day after last treatment (gated on CD8 cells). (**B**) Number of H-2D^b GP33- and GP276-specific CD8 T cells in the blood 1 day after last treatment. (**C**) Number of LCMV-specific CD8 T cells (GP33 and GP276 combined) in the spleen and lung 1 day after last treatment. (**D**) Representative dot plots of IFN-γ– and TNF-α–producing CD8 T cells in the spleen after ex vivo restimulation with the indicated peptides. (**E**) Viral titer in the serum 1 day after last treatment, as quantified by plaque assays using Vero E6 cells. Individual symbols represent individual mice, and horizontal bars represent the mean. The dashed line represents the limit of detection for the assay. Results are representative of 3 separate experiments, with at least 4 mice per group per experiment. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

CD8 T cells in both lymphoid and nonlymphoid tissues at 2 days after the last treatment ($P < 0.01$, $P < 0.001$, $P < 0.5$ for number of GP33$^+$ CD8 T cells in the combined treatment group compared with PBS/isotype controls in the spleen, lung, and liver, respectively; $P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.05$ for number of GP276$^+$ CD8 T cells in the spleen, lung, liver, and bone marrow, respectively) (Figure 2, D and E).

We next determined whether these CD8 T cells that had expanded after combined IL-2 therapy and PD-L1 blockade had also regained function. After ex vivo restimulation with broad range of LCMV-specific peptides, we assessed the ability of the CD8 T cells to produce cytokines after treatment in order to address this question. While both IL-2 or PD-L1 blockade alone increased the number of CD8 T cells producing IFN-γ or copro-

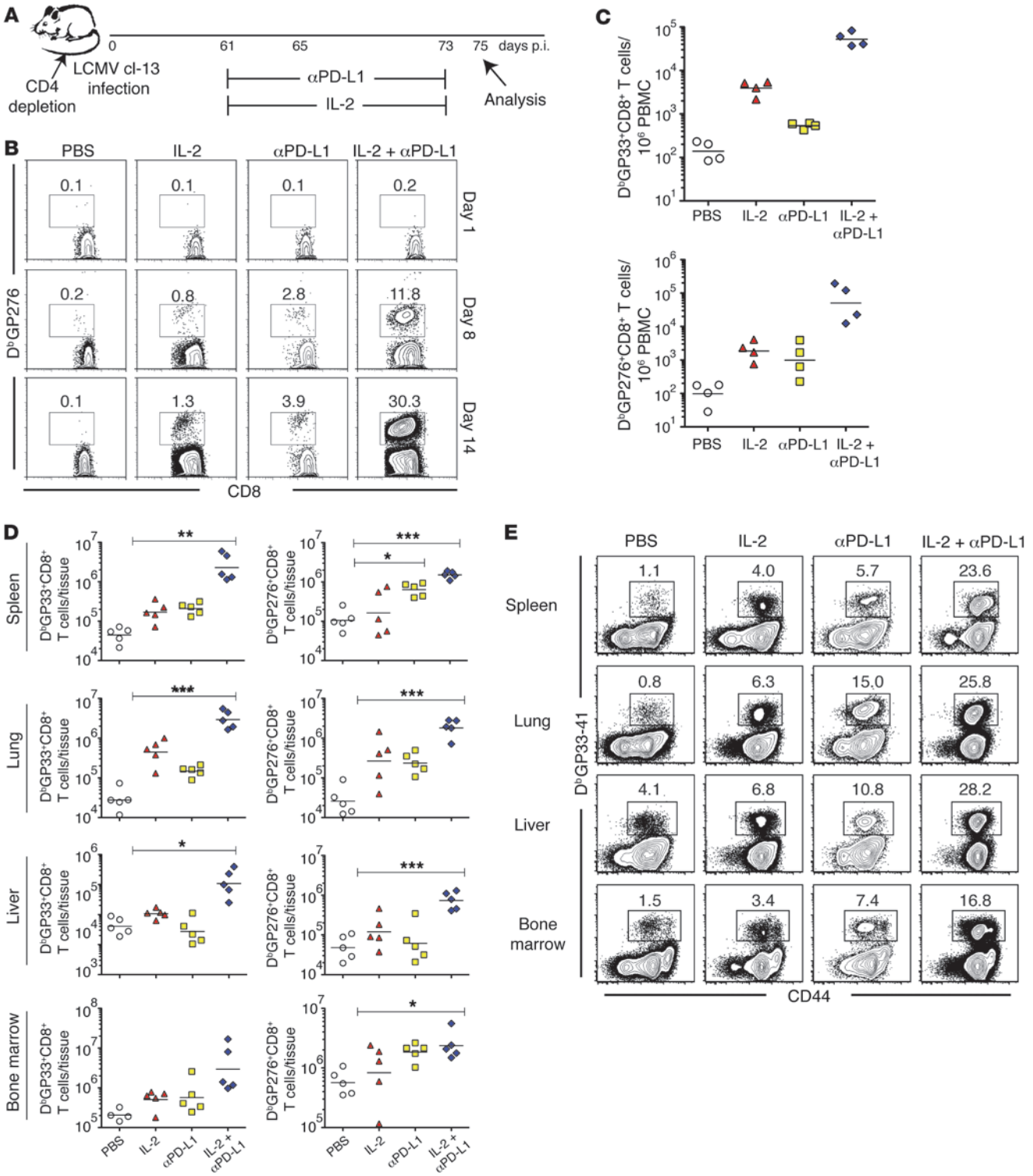

Figure 2

Combined IL-2 therapy and PD-L1 blockade enhance antiviral CD8 T cell responses during "unhelped" chronic LCMV infection. C57BL/6 mice were depleted of CD4 T cells and infected with LCMV cl-13. Following day 60 after infection, appropriate groups of mice were treated with PBS/isotype antibody, 200 μg anti–PD-L1 antibody every 3 days for 12 days (5 total treatments), and/or IL-2 (i.p.). IL-2–treated groups were given 1.5×10^4 IU IL-2 (i.p.) twice a day for the duration of the anti–PD-L1 treatment. (**A**) Experimental set up. p.i., after infection. (**B**) Frequency of H-2D^b GP276-specific CD8 T cells in the blood before (day –1), during (day 8), and after (day 14) treatment (gated on CD8 cells). (**C**) Number of H-2D^b GP33- and GP276-specific CD8 T cells in the blood 1 day after last treatment. (**D**) Number and (**E**) frequency of GP33 and GP276 CD8 T cells in tissues 2 days after last treatment. Results are representative of 3 separate experiments, with at least 4 mice per group per experiment. Individual symbols represent individual mice, and horizontal bars represent the mean. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

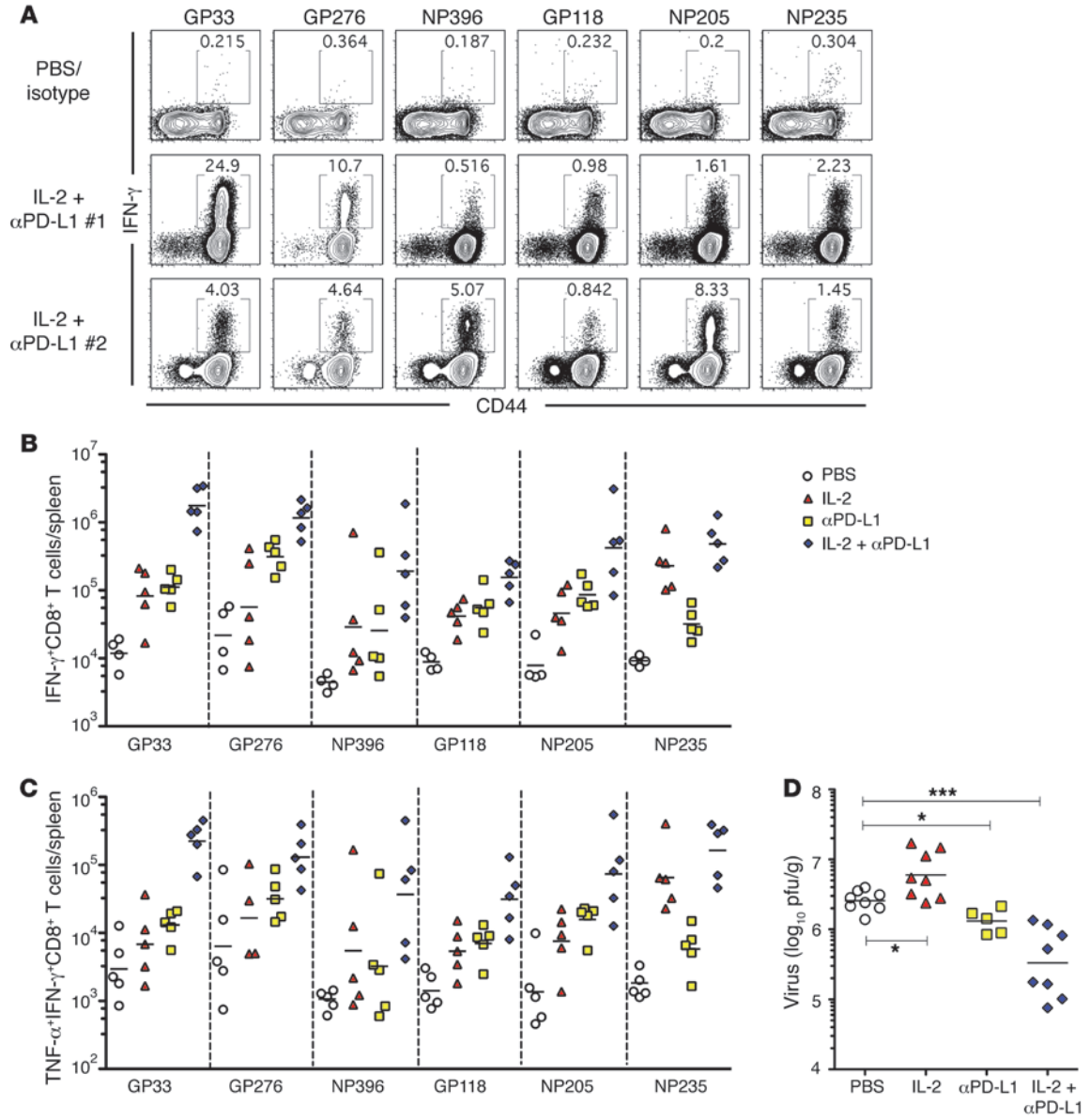

Figure 3

Distinct effects of IL-2 therapy and PD-L1 blockade on antiviral CD8 T cell responses and viral load during chronic LCMV infection. C57BL/6 mice were depleted of CD4 T cells and infected with LCMV cl-13. Following day 60 after infection, appropriate groups of mice were treated with PBS/isotype antibody, 200 μg anti–PD-L1 antibody every 3 days for 12 days (5 total treatments), and/or IL-2 (i.p.). IL-2–treated groups were given 1.5×10^4 IU IL-2 (i.p.) twice a day for the duration of the anti–PD-L1 treatment. (**A**) Frequency and (**B**) number of IFN-γ–producing CD8 T cells in the spleen after ex vivo restimulation with the indicated peptides. (**C**) Number of simultaneous IFN-γ– and TNF-α–producing CD8 T cells in the spleen. (**D**) Viral titer in the spleen 2 days after last treatment, as quantified by plaque assays. Results are representative of 3 separate experiments, with at least 4 mice per group per experiment. Individual symbols represent individual mice, and horizontal bars represent the mean. $^*P < 0.05$; $^{***}P < 0.001$.

ducing IFN-γ and TNF-α in response to a broad range of LCMV epitopes, combined IL-2 therapy and PD-L1 blockade resulted in massive increases (up to ~25%) in the frequency of CD8 T cells producing IFN-γ in response to a broad range of LCMV epitopes (Figure 3A). Notably, combined therapy resulted in striking responses to both dominant (GP33 and GP276) and subdominant epitopes (GP118, NP205, and NP235), including the previously undetectable epitope, NP396 (Figure 3A). Interestingly, the distribution of IFN-γ–producing cells to different LCMV peptides was altered in individual mice, with some mice making more of a response to the dominant epitopes, (IL-2 + αPD-L1 no. 1 in Figure 3A) and other mice making a larger response to subdominant epitopes (IL-2 + αPD-L1 no. 2 in Figure 3A). This difference in the breadth of the responses in individual mice may be a reflection of the differential degree of exhaustion of T cell subsets and/or differences in the precursor frequency in individual mice. Furthermore, combined treatment resulted in immense increases in the number of CD8 T cells producing IFN-γ, even up to an approximately 100-fold increase in cells responding to the GP33 epitope, and multifunctional cells coproducing IFN-γ and

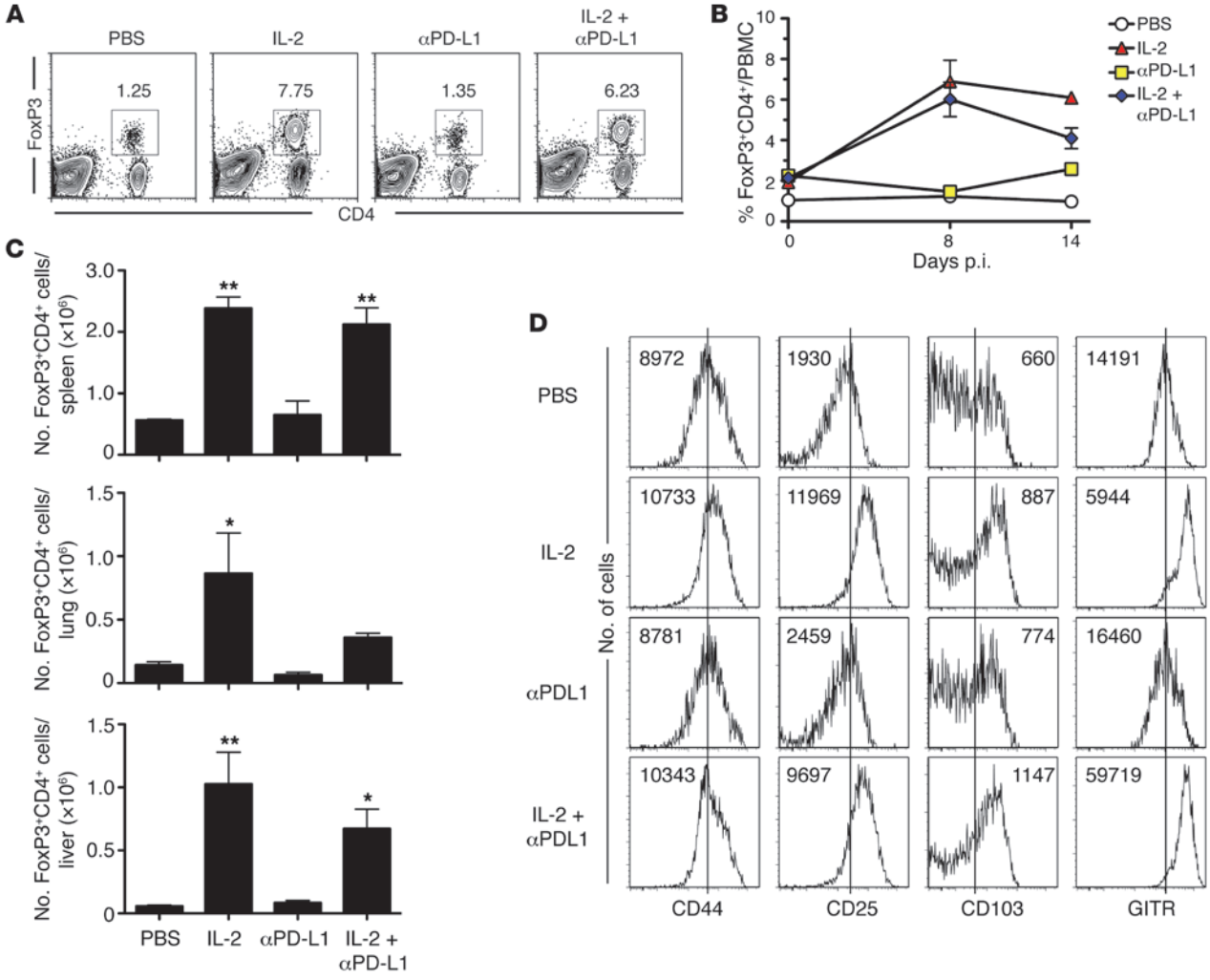

Figure 4

IL-2 therapy affects Tregs during chronic LCMV infection. C57BL/6 mice were depleted of CD4 T cells and infected with LCMV cl-13. Following day 60 after infection, appropriate groups of mice were treated with PBS/isotype antibody, 200 μg anti–PD-L1 antibody every 3 days for 12 days (5 total treatments), and/or IL-2 (i.p.). IL-2–treated groups were given 1.5×10^4 IU IL-2 (i.p.) twice a day for the duration of the anti–PD-L1 treatment. (**A**) Representative flow plots showing the frequency of FoxP3+ CD4 T cells in the blood in the middle of treatment (day 8 after start of treatment). (**B**) Frequency of FoxP3+ CD4 T cells in the blood before (day 0), during (day 8), and after (day 14) treatment. (**C**) Number of FoxP3+ CD4 T cells in the tissues 2 days after final treatment. (**D**) Representative histograms showing the expression of CD44, CD25, CD103, and GITR on FoxP3+ CD4 T cells in the spleen after treatment. Numbers represent mean fluorescence intensity. Results are representative of 2 separate experiments, with at least 4 mice per group per experiment. $^*P < 0.05$; $^{**}P < 0.01$. Error bars indicate the standard deviation of the mean.

TNF-α in response to a broad range of dominant and subdominant LCMV epitopes (Figure 3, B and C).

Next, we addressed whether the increased numbers of functional LCMV-specific CD8 T cells seen after therapy correlated with viral reduction. First, while IL-2 therapy alone resulted in a large expansion of LCMV-specific CD8 T cells, there was no reduction in viral loads after treatment, but, surprisingly, a slight increase in the levels of virus was observed (Figure 3D). In contrast, PD-L1 blockade alone, which results in less of an expansion of LCMV-specific CD8 T cells compared with IL-2 therapy alone, reduced viral loads (Figure 3D). Importantly, combined IL-2 and PD-L1 therapy resulted in a more significant decrease in viral load than that provided by PD-L1 blockade alone (Figure 3D). Therefore, IL-2 therapy synergizes with PD-L1 blockade in this model of extreme exhaustion, resulting in a massive expansion of functional LCMV-specific CD8 T cells and a greater reduction of viral load than that seen with PD-L1 blockade alone.

Effectiveness of combined IL-2 therapy and PD-L1 blockade compared with that of other combination therapies. Multiple combinational strategies have been tested in the LCMV chronic infection model (17–19, 39). In the past, in our laboratory, we tested 3 other combined modalities: therapeutic vaccination combined with PD-L1 blockade (19), coblockade of IL-10R and PD-L1 (39), and combined Tim-3 and PD-L1 blockade (17). Comparison of our new IL-2 and anti–PD-L1 combined therapy data with these other previously published data indicate that IL-2 and anti–PD-L1 therapy has a more impressive effect on increasing the functional LCMV-specific CD8 T cell response and decreasing viral titers than these other treatments during chronic viral infection.

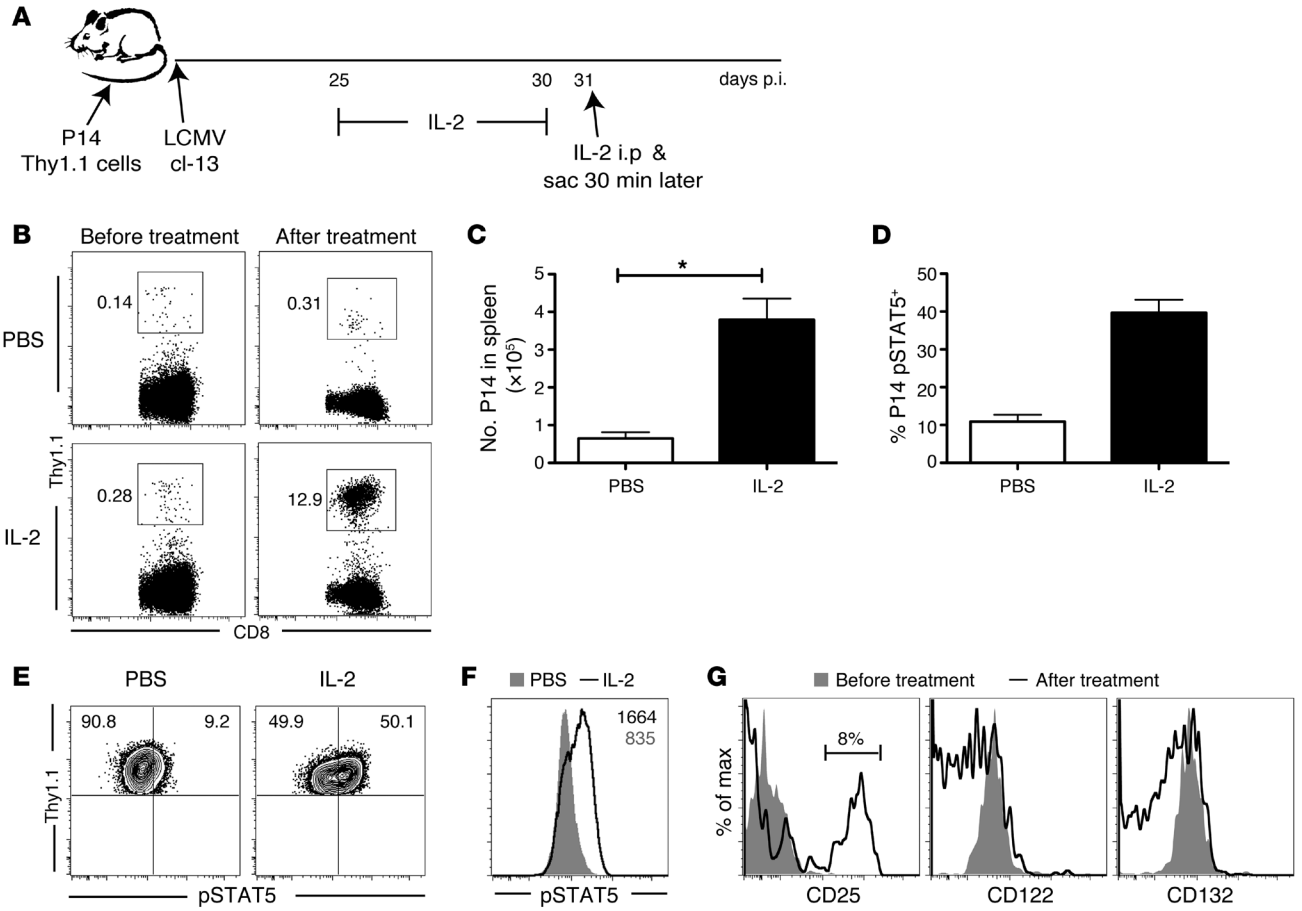

Figure 5

IL-2 therapy modulates exhausted antiviral CD8 T cells. 2 × 10^3 Thy1.1$^+$ D^bGP33 LCMV-specific CD8 T cells (P14 transgenic) were transferred i.v. into C57/BL6 mice (Thy1.2$^+$) that were subsequently infected with LCMV cl-13, and beginning on day 23 to 27 after infection, the mice were treated with 1.5 × 10^4 IU IL-2 (i.p.) every 24 hours for 6 days. On the seventh day, the mice were either treated with PBS or 1.5 × 10^4 IU IL-2 and mice were sacrificed 30 minutes after IL-2 treatment. Splenocytes were removed and stained with Thy1.1, CD8, and phospho-STAT-5 antibodies. (**A**) Experimental set up. (**B**) Frequency of P14 T cells in the blood (gated on CD8) and (**C**) number in the spleen before and after 6 days of IL-2 treatment. (**D**) Percentage of P14 T cells in the spleen that are phospho-STAT-5 positive. (**E**) Representative FACS plots showing phospho-STAT-5 staining of P14 T cells. (**F**) Representative histogram showing phospho-STAT-5 staining of P14 T cells. Numbers on graph indicate MFI. (**G**) Representative histograms of CD25, CD122, and CD132 expression on P14 T cells in the blood before and after IL-2 treatment. Results are representative of 2 independent experiments, with at least 4 mice per group. *P < 0.05. Error bars indicate the standard deviation of the mean.

In addition, to control for the variable of the treatments (mentioned above) not being directly compared within the same experiment, we performed a new experiment directly comparing IL-2 therapy and anti–PD-L1 blockade side by side with the previously published combined blockade of Lag-3 and PD-L1 (18). We accomplished this by transiently treating CD4-depleted cl-13–infected mice (after day 100 after infection) with these two combination therapies. IL-2 plus anti–PD-L1 treatment resulted in approximately 10- to 20-fold greater increase in virus-specific CD8 T cells in the tissues, spleens, livers, and lungs of mice infected with chronic LCMV compared with that in the group receiving combined Lag-3 and PD-L1 blockade (Supplemental Figure 4A). Furthermore, these virus-specific CD8 T cells were highly functional in the IL-2 plus anti–PD-L1 group, evidenced by a significant (P < 0.05) increase in the number of IFN-γ–producing cells in the IL-2 plus anti–PD-L1 group and increased double-producing IFN-γ$^+$TNF-α$^+$ cells compared with the anti–Lag-3 plus anti–PD-L1 group (Supplemental Figure 4, B and C). Last, combined IL-2 therapy and PD-L1 blockade resulted in an approximately 10-fold greater reduction of viral burden than anti–Lag-3 plus anti–PD-L1 treatment (Supplemental Figure 4D). Taken together, these data suggest that combined IL-2 therapy and anti–PD-L1 blockade may be a more effective therapy than previously published combined therapies during chronic antigen persistence.

IL-2 and combined therapy influences Treg numbers and activation markers. Thus far, we have focused on the LCMV-specific CD8 T cell responses after treatment; however, since Tregs express constitutively high levels of the high-affinity IL-2Rα chain and IL-2 is known to expand their numbers and increase their suppressive function (40, 41) and low-dose IL-2 treatment in humans has been shown to increase Treg numbers (36, 37), we assessed the Treg compartment (FoxP3$^+$ CD4 T cells) after administration of IL-2 or PD-L1 blockade alone and after combined IL-2 therapy and PD-L1 blockade during chronic LCMV infection. IL-2 adminis-

tration alone resulted in an increase in the frequency of FoxP3$^+$ CD4 T cells in the blood during ongoing treatment (day 8 after the start of treatment) and also after the completion of treatment (day 14 after the start of treatment) (Figure 4, A and B). Furthermore, upon the completion of treatment, there were increased FoxP3$^+$ CD4 T cells in the tissues ($P < 0.01$ for spleen and liver; $P < 0.05$ for lung compared with PBS/isotype control group) (Figure 4C). In contrast, PD-L1 blockade alone did not significantly affect the frequency of FoxP3$^+$ CD4 T cells in the blood or their numbers in the tissues (Figure 4, A–C). Combined IL-2 therapy and PD-L1 blockade resulted in an increase in the frequency of FoxP3$^+$ CD4 T cells in the blood (Figure 4, A and B) and increased numbers in the tissues ($P < 0.01$ for spleen and $P < 0.05$ in liver compared with PBS/isotype control); however, this increase was very similar to that seen after administration of IL-2 therapy alone (Figure 4C). This indicates that IL-2 expands the FoxP3$^+$ CD4 T cell population, but PD-1 blockade does not. To determine whether these FoxP3$^+$ CD4 T cells had a more activated phenotype after treatment, we assessed CD44, CD25, CD103, and GITR expression on these cells after treatment by measuring the MFI of these markers using flow cytometry. CD44, CD25, CD103, and GITR expression was increased on the FoxP3$^+$ CD4 T cells after IL-2 or combined IL-2 plus PD-L1 blockade, as assessed by MFI, indicating that these FoxP3$^+$ CD4 T cells had a more activated phenotype (Figure 4D). In contrast, PD-L1 blockade alone only very slightly affected the expression of these markers (Figure 4D). These data indicate that IL-2 therapy increases the number of "activated" Foxp3$^+$ CD4 T cells during chronic infection, unlike PD-1 blockade. However, in spite of the increase in activated Tregs after combined treatment, virus-specific CD8 T cell numbers and function were augmented and virus levels were reduced after treatment (Figures 1–3).

IL-2 modulates existing exhausted virus-specific CD8 T cells. Since exhausted CD8 T cells have low surface expression of the high-affinity IL-2Rα chain (42), it is unclear how IL-2 is modulating these CD8 T cells. To better understand whether IL-2 may modulate existing exhausted virus-specific CD8 T cells, we transferred Thy1.1$^+$ LCMV-specific D^{b}GP33-41 transgenic TCR (P14) T cells into Thy1.2$^+$ B6 mice, infected them with LCMV cl-13, and then assessed the P14 T cell numbers and IL-2 signal transduction in the Thy1.1$^+$ P14 cells after establishment of chronic infection (day 23–27) by measuring the level of STAT-5 phosphorylation (phospho-STAT-5) after in vivo treatment with IL-2 (Figure 5A). Following 6 days of daily IL-2 administration, the frequency of P14 T cells in the blood was increased from approximately 0.3% of CD8 T cells before treatment to approximately 13% after treatment, indicating a large expansion of the P14 T cells in the blood after IL-2 therapy (Figure 5B). In addition, there was an approximately 4-fold increase in the number of P14 T cells in the spleens of mice treated with IL-2 for 6 days compared with those treated with PBS (Figure 5C). This indicates that IL-2 causes the expansion of preexisting exhausted CD8 T cells and does not act just on newly primed CD8 T cells coming from the thymus during the ongoing infection. Second, to address whether IL-2 may act directly on exhausted LCMV-specific CD8 T cells, we assessed phospho-STAT-5 staining after IL-2 administration. Notably, STAT-5 remained unphosphorylated 60 minutes after 1 injection of IL-2 (data not shown). However, when 15,000 IU IL-2 was administered i.p. every 24 hours for 6 days and then on the seventh day either PBS or IL-2 was given to the mice followed by analysis of the P14 T cells 30 minutes after IL-2 injection, striking increases of phospho-STAT-5 expression were seen in the P14 T cells of mice given IL-2 30 minutes prior (~40% are phospho-STAT-5$^+$ after 30 minutes) (Figure 5, D–F). These data are consistent with IL-2 signaling directly on the LCMV-specific CD8 T cells; however, they do not exclude the effect that IL-2 therapy may also have on other cell types during chronic infection or a possible additional nondirect effect of therapy on the LCMV-specific CD8 T cells. Notably, the IL-2 receptor components, IL-2Rβ (CD122) and IL-2Rγ (CD132) were expressed by LCMV-specific T cells (Figure 5G); however, these cells did not express IL-2Rα, except for a small population (8% or less) in some mice treated with IL-2 therapy (Figure 5G), consistent with the idea that IL-2 may be able to act directly on LCMV-specific CD8 T cells mostly via the lower-affinity IL-2 receptor (IL-2Rβ plus IL-2Rγ). Taken together, these data indicate that IL-2 can modulate existing exhausted virus-specific CD8 T cells.

IL-2 decreases inhibitory receptors and increases CD127 expression on exhausted virus-specific CD8 T cells. We have shown that IL-2 can modulate existing exhausted CD8 T cells, so we next determined whether IL-2 changes the expression of markers known to be important in the exhaustion process. Multiple inhibitory receptors are expressed on CD8 T cells during chronic infection and have been shown to play a central role in inhibiting their function (2, 43); so to begin, we first assessed the expression of the inhibitory receptors PD-1, 2B4, Tim-3, and Lag-3 on exhausted CD8 T cells after IL-2 therapy. LCMV-specific P14 TCR transgenic cells congenically marked with Thy1.1 were transferred into mice that were subsequently infected with LCMV cl-13. After chronic infection was established, at 27 days after infection, the mice were treated with either PBS or 15,000 IU IL-2 every 24 hours for 8 days, and the following day, the expression of an activation marker, CD44, and the inhibitory receptors on the P14 T cells was assessed by flow cytometry. The P14 transgenic system was used to show how IL-2 acts on preexisting exhausted cells (eliminating the contribution of any newly primed CD8 T cells); however, similar results were also seen with endogenous LCMV-specific CD8 T cells (data not shown). Furthermore, IL-2 treatment was given alone, not in combination with anti–PD-L1, so that virus loads would still be high, thereby helping to eliminate the impact of viral load on inhibitory receptor expression. The activation molecule CD44 was substantially increased on the LCMV-specific CD8 T cells after IL-2 treatment (~2-fold increase in MFI) (Figure 6A). Interestingly, there was a large decrease in the inhibitory receptors PD-1, 2B4, and Tim-3, as measured by MFI, after IL-2 treatment. In contrast, Lag-3 was not altered by IL-2 treatment (Figure 6A). In addition to this decrease in expression of the inhibitory receptors on P14 T cells after IL-2 therapy, we found that these cells expressed higher levels of the transcription factor T-bet (Figure 6B). A recent paper by Kao et al. showed that LCMV-specific CD8 T cells that expressed high levels of the transcription factor T-bet had decreased expression of inhibitory receptors and increased functional capabilities during chronic infection (44). These data indicate that IL-2 can influence inhibitory receptor expression on exhausted CD8 T cells during chronic LCMV infection, and, thus, this may help lead to decreased inhibition of these cells.

Second, we assessed the expression levels of 2 other markers, Bcl-2 and granzyme B, which have been shown to be increased in CD8 T cells after in vivo or in vitro IL-2 administration (45–49). Bcl-2 is a known negative regulator of apoptosis and is important for T cell survival, and while IL-2 has been shown to increase Bcl-2 expression in CD8 T cells (47–49), we found that Bcl-2 expression

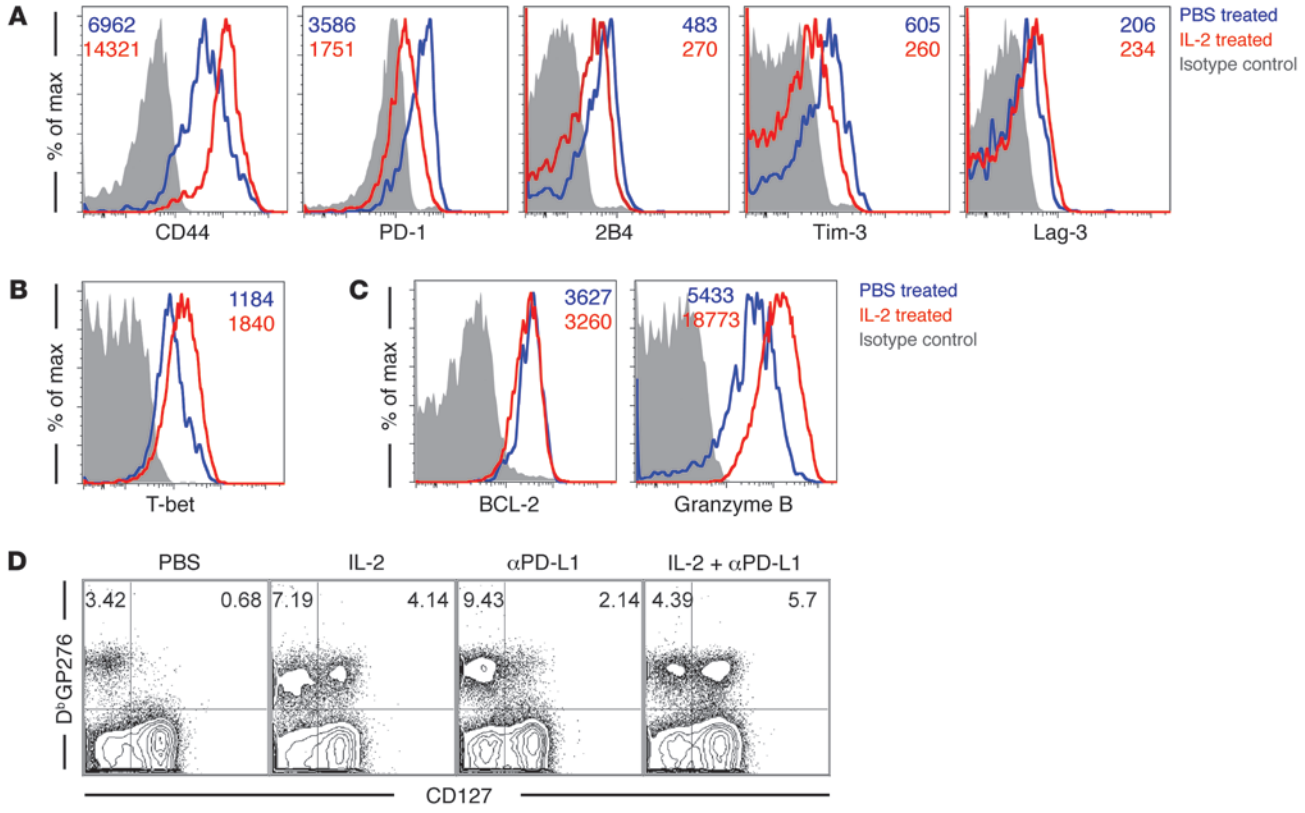

Figure 6

IL-2 therapy affects inhibitory receptor expression and the phenotype of antiviral CD8 T cells during chronic LCMV infection. 2×10^3 Thy1.1$^+$ D^bGP33 LCMV-specific CD8 T cells (P14 transgenic) were transferred i.v. into C57/BL6 mice (Thy1.2$^+$) that were subsequently infected with LCMV cl-13, and beginning on day 23 to 27 after infection, the mice were treated with either PBS or 1.5×10^3 IU IL-2 (i.p.) every 24 hours for 8 days. After the end of treatment, the splenocytes were removed and stained with Thy1.1, CD8, CD44, and the indicated antibodies. The MFI of the markers is indicated by numbers in the histograms. Representative histograms showing the expression of (**A**) CD44 and the inhibitory receptors, (**B**) intracellular T-bet expression, and (**C**) intracellular Bcl-2 and granzyme B expression 1 day after the last PBS or IL-2 treatment. (**D**) C57/BL6 mice were infected with LCMV cl-13. 23–27 days after infection, the appropriate mice were treated with either isotype control antibody or anti–PD-L1 (every 3 days for 5 total treatments), and 1.5×10^3 IU IL-2 was given to the appropriate groups every 24 hours for the last 8 days of anti–PD-L1 treatment. Representative dot plots showing CD127 expression on D^bGP276 tetramer CD8 T cells at 3 weeks after end of anti–PD-L1 and IL-2 treatment. (**A**–**C**) Results are representative of 2 independent experiments, with at least 5 mice per group, or (**D**) 3 independent experiments, with at least 4 mice per group.

was similar on LCMV-specific CD8 T cells after IL-2 and PBS treatment during chronic infection (Figure 6C). In contrast, granzyme B expression was increased in the LCMV-specific CD8 T cells after IL-2 therapy during chronic infection (Figure 6C), indicating that these cells may have increased cytolytic potential.

Last, we looked at the expression of the IL-7 receptor α chain (CD127) on exhausted CD8 T cells after treatment with IL-2 or PD-L1 blockade or combined IL-2 therapy and PD-L1 blockade. CD127 is a CD8 T cell marker that helps define functional memory cells in acute infection, as the responding CD8 T cells that reexpress CD127 are preferentially destined to become memory CD8 T cells (50). However, in contrast, exhausted CD8 T cells do not reexpress this memory marker during chronic infection (51), and as of yet there is not a reported treatment that increases CD127 expression on CD8 T cells during chronic infection. Interestingly, we found that CD127 was upregulated on the LCMV-specific CD8 T cells after IL-2 therapy alone or after combined IL-2 therapy and PD-L1 blockade treatment during chronic LCMV infection (Figure 6D). This increase in CD127 expression was not due to viral clearance, as mice treated with only IL-2 alone (which does not result in viral clearance) also had increased expression of CD127 (Figure 1E and Figure 6D). In addition, in the "unhelped" model of chronic LCMV infection, in which viremia is maintained at high levels for the life of the animal, only combined IL-2 therapy and PD-L1 blockade resulted in increased CD127 expression (data not shown). This increased expression of CD127 may indicate a reprogramming away from the exhausted state and may lead to increased cell survival.

Discussion

Herein we show that combined daily low-dose IL-2 therapy and PD-L1 blockade enhance CD8 T cell responses and function during chronic LCMV infection and result in decreased viral burden. The effects of the combined therapy were greater than those of either treatment given alone. To better understand the way in which IL-2 affected the T cell responses, we treated mice solely with daily low-dose IL-2 therapy. IL-2 administration alone was shown to modulate CD8 T cells, causing the phosphorylation of STAT-5.

Furthermore, daily low-dose IL-2 therapy resulted in a decrease in the expression of the inhibitory receptors Tim-3, PD-1, and 2B4 (CD244). This reduction in inhibitory receptor expression was seen, even though IL-2 treatment alone does not clear virus, indicating that IL-2 therapy can reduce their expression, even in the presence of high levels of virus. In addition to a reduced expression of inhibitory receptors, we found that the activation molecule CD44 was upregulated after IL-2 therapy. CD8 T cells that have increased CD44 expression and intermediate PD-1 expression have been shown to have greater proliferative potential, increased ability to control infections, and are more responsive to PD-1 blockade than their counterparts with intermediate CD44 and high PD-1 high expression (52). This suggests that IL-2 administration may result in the generation of a pool of CD8 T cells that are more responsive to PD-L1 blockade. Furthermore, a recent report showed that antigen-specific CD8 T cells expressing high levels of T-bet were more functional during chronic LCMV infection and had reduced inhibitory receptor expression (44), and our data show that IL-2 treatment increases T-bet expression. Last, IL-2 increased granzyme B expression, consistent with enhanced cytolytic potential.

Importantly, IL-2 treatment alone increased IL-7 receptor α expression (CD127), which is a marker of long-lived highly functional memory CD8 T cells that has not previously been shown to be upregulated on CD8 T cells during chronic infection. This increase in CD127 expression was seen even in cases in which antigen persisted, such as in the mice treated only with IL-2 alone, therefore it is reexpressed even in the presence of high viral loads. Additionally, during normal chronic LCMV infection, CD127 was induced by IL-2 alone or by combined IL-2 therapy and PD-L1 blockade; however, in the more severe "unhelped" chronic LCMV infection only the combined IL-2 therapy and PD-L1 blockade was able to induce CD127 expression. This interesting difference between the 2 models may be due to the fact that the CD8 T cells are exhausted more extremely during "unhelped" LCMV infection and therefore need more immunomodulatory stimulus to "revive" them and allow for reexpression of CD127. Expression of CD127 may indicate that these cells are more functional and have increased survival capacity compared with the exhausted CD8 T cells found during chronic infection in untreated mice.

Our result of IL-2 increasing CD127 expression on exhausted CD8 T cells during chronic infection is the opposite result seen when early effector CD8 T cells are given IL-2 either in vitro or in vivo during an acute infection, in which IL-2 sways the cells toward a CD127-negative and more terminally differentiated state (53–55). This distinct effect of IL-2 on CD127 expression in cases of limited antigen stimulation and persistent stimulation during chronic infection may be due to the different programming/state of exhausted cells compared with that of early effector CD8 T cells in acute infection. It has been well documented that exhausted cells are molecularly distinct from naive, effector, or memory CD8 T cells (2, 42). Moreover, IL-2 has been shown to have differential effects on CD8 T cells when administered in the presence of inflammatory signals (53). Therefore, IL-2 may act differentially on these exhausted CD8 T cells during a chronic infection that has a distinct inflammatory milieu compared with that seen by CD8 T cells during a quickly resolved acute infection. Overall, IL-2 appears to play a unique role on exhausted CD8 T cells, compared with CD8 T cells during acute infection.

While IL-2 therapy, PD-L1 blockade, or IL-2 therapy combined with PD-L1 blockade greatly increased antigen-specific CD8 T cell numbers and increased their function, there was not a similar effect on total or LCMV-specific CD4 T cell numbers or LCMV-antibody production (data not shown). In contrast, IL-2 therapy alone or combined therapy resulted in an increase of FoxP3$^+$ CD4 T cells (Tregs). These data are consistent with previous work, which has shown that IL-2 is important for Treg development and function and IL-2 can increase FoxP3 expression (56, 57). Notably, combined treatment did not enhance Treg numbers above those seen with IL-2 therapy alone and PD-1 blockade alone did not increase Treg numbers, thus indicating that IL-2 itself is responsible for increasing Tregs in this system. These data highlight the fact that low-dose IL-2 therapy increases Tregs during chronic infection in mice, similar to what has been shown recently with low-dose IL-2 treatment in humans for chronic graft-versus-host disease and hepatitis C virus–induced vasculitis (36, 37). However, even in mice in which Tregs were increased, LCMV-specific CD8 T cells were greatly expanded, regained function, and were able to reduce viral loads, showing that the CD8 T cells are still functional, even in the presence of increased Treg numbers. This emphasizes the distinct roles that IL-2 therapy can have on multiple cell types in differing environments, in which, during an autoimmune manifestation, low-dose IL-2 therapy can increase Tregs, resulting in clinical improvement (36, 37); in the mouse, therapy increases Tregs during chronic infection, but virus-specific CD8 T cells are expanded to a greater extent and reacquire functional capabilities.

IL-2 treatment alone resulted in a reduction of viral loads during chronic cl-13 infection (in which mice are viremic for ~2 months after infection), as previously reported (38). However, surprisingly, even though IL-2 treatment alone resulted in a large expansion of LCMV-specific CD8 T cells, it did not result in significant viral reduction in the more stringent "unhelped" chronic LCMV infection (in which mice are highly viremic for life). One possible explanation for the inability of these cells to reduce viral loads in these highly viremic mice is that while IL-2 therapy alone expands LCMV-specific CD8 T cells that are capable of cytokine secretion, these cells still express PD-1, albeit at lower levels than CD8 T cells from untreated mice. Therefore, while IL-2 therapy effectively increases the number of functional CD8 T cells, these cells still may be unable to kill their target cells due to inhibition by PD-1 binding to it's widely expressed ligand, PD-L1, on target cells. Accordingly, nonhematopoietic cells are a major source of viral burden, and PD-L1 expression on nonhematopoietic cells has been shown to impair the ability of CD8 T cells to clear virus during chronic LCMV infection (58). This indicates that PD-1 blockade therapy works at the level of the cells to allow for effective CTL killing of infected target cells. Importantly, when IL-2 therapy and PD-L1 blockade are combined, LCMV-specific CD8 T cells are expanded, thereby increasing the effector-to–infected target ratio, and blockade of the inhibitory signal PD-1 then results in increased ability of the cells to effectively reduce viral burden. This implies a distinct and synergistic effect of IL-2 therapy and PD-L1 blockade on CD8 T cell responses and viral control.

Overall these data indicate that combined IL-2 and PD-1 blockade therapy may be a promising therapy for increasing CD8 T cell function and reducing viral loads during chronic infections and possibly cancers. In addition, since both IL-2 therapy alone and PD-1 blockade alone have been used clinically, combined therapy is something that could be implemented for in vivo use in human clinical studies. Moreover, combining these two therapies may allow for titration of the IL-2 dose, allowing for the posi-

tive effects of IL-2 to be gained while using a low enough dose to minimize toxicity issues. Additional studies will need to be performed to determine whether IL-2 or combined IL-2 therapy helps "reprogram" exhausted CD8 T cells in humans, making them more like the functional memory CD8 T cells that express high levels of CD127 found after acute infections. Moreover, since IL-2 therapy or combined IL-2 therapy and PD-L1 blockade resulted in increased expression of the IL-7 receptor α chain (CD127), it may render these cells more responsive to IL-7 therapy. While IL-7 therapy has recently been shown to enhance CD8 T cell responses and decrease viral loads when given for long periods of time in the less stringent model of chronic LCMV infection (59, 60), in our hands, we found little effect of IL-7 therapy alone in the more stringent model of chronic LCMV infection (in which CD4 T cell help is absent) (data not shown). However, since IL-2 therapy increases expression of the IL-7 receptor α chain on CD8 T cells during chronic infection, combining IL-2 therapy (plus or minus PD-L1 blockade) along with IL-7 therapy may have enhanced therapeutic benefit during chronic infection, especially in cases of very high viral loads and extreme exhaustion.

Last, a major concern for the use of in vivo IL-2 therapy as a treatment for cancers and infections is that IL-2 can increase Tregs, which may dampen the effector T cell response (36, 37, 40, 41). However, herein, we have shown that although low-dose IL-2 therapy does increase Treg numbers, it still results in a highly augmented and functional antiviral CD8 T cell response and decreased viral burden.

In conclusion, combining daily low-dose IL-2 therapy with blockade of the inhibitory receptor PD-1 may be a useful clinical strategy for reversing CD8 T cell exhaustion during chronic infections, leading to an enhanced reduction in viral burden or viral control. Further, since PD-1 also plays an important regulatory role in some cancers, combined IL-2 therapy and PD-1 blockade might be an important clinical tool for treating cancer. Overall, this work may help us to design rational strategies for developing immunotherapies for chronic infections and cancer.

Methods

Mice, infections, and cell transfers. Six-week-old female C57BL/6 mice were purchased from The Jackson Laboratory. P14 TCR transgenic mice were bred in house. Mice were infected with 2×10^6 PFU of LCMV cl-13 i.v. by tail vein. Viral titers were determined by plaque assay on Vero E6 cells as described previously (1). For chronic infection in an "unhelped" environment, mice were given 500 μg of the CD4-depleting antibody GK1.5 i.p. (BioXcell) 1 day prior to infection and again on the day of infection. For experiments using P14 T cells, 2×10^3 Thy1.1$^+$ P14 T cells were transferred into mice i.v. 1 day prior to infection.

Lymphocyte isolation and flow cytometry. Lymphocytes were isolated from the blood, spleen, liver, lungs, and bone marrow as previous described (3, 61). All antibodies were purchased from BD except CD44, Thy1.1, and Thy1.2 (Biolegend); anti–PD-1 and anti-FoxP3 (ebioscience); and anti–Tim-3 (R&D Systems). MHC class I tetramers were prepared and used as previously described (1). Intracellular cytokine staining was performed as previously described (1). Phospho-STAT-5 staining was done following the manufacturer's protocol (BD Biosciences). Cells were analyzed on a LSR II or Canto flow cytometer (BD Immunocytometry Systems). Dead cells were excluded by gating on Live/Dead NEAR IR (Invitrogen).

In vivo blockade and IL-2 therapy. For blockade of the PD-1 pathway, 200 μg rat anti-mouse PD-L1 antibody (10F.9G2 prepared in house) or rat IgG2b isotype control was administered i.p. every 3 days for 5 total treatments beginning on the day after infection, as indicated in the appropriate figure or figure legend. For blockade of Lag-3, 200 μg rat anti-mouse Lag-3 antibody (C9B7W, Biolegend) was administered i.p. every 3 days for 5 total treatments beginning on the day after infection, as indicated in the appropriate figure or figure legend. For IL-2 therapy, 15,000 IU (1 μg) of recombinant human IL-2 (Amgen) diluted in PBS with 0.1% normal mouse serum was given i.p. to the mice either every 12 or 24 hours for 8 to 12 consecutive days (as indicated in the appropriate figure legend) beginning after chronic infection was established (at the time point indicated in the appropriate figure legend).

Statistics. Statistical analysis was performed using 2-tailed unpaired Student's *t* tests (when comparing 2 groups) or 1-way ANOVA (when comparing >2 groups) using Prism 5.0 (GraphPad) software. *P* values of less than 0.05 were considered statistically significant.

Study approval. Mice were maintained and used according to institutional and NIH guidelines in a specific pathogen–free facility. All animal studies were approved by the IACUC of Emory University.

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1. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol*. 2003;77(8):4911–4927.
2. Wherry EJ. T cell exhaustion. *Nat Immunol*. 2011; 12(6):492–499.
3. Barber DL, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*. 2006;439(7077):682–687.
4. Brahmer JR, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med*. 2012;366(26):2455–2465.
5. Topalian SL, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl*

J Med. 2012;366(26):2443–2454.
6. Brahmer JR, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol*. 2010;28(19):3167–3175.
7. Velu V, et al. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature*. 2009;458(7235):206–210.
8. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A*. 2002;99(19):12293–12297.
9. Strome SE, et al. B7-H1 blockade augments adoptive T-cell immunotherapy for squamous cell carcinoma. *Cancer Res*. 2003;63(19):6501–6505.
10. Blank C, Gajewski TF, Mackensen A. Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy. *Cancer Immunol Immunother*. 2005;54(4):307–314.
11. Dong H, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med*. 2002;8(8):793–800.
12. Wang S, Chen L. Co-signaling molecules of the B7-CD28 family in positive and negative regulation of T lymphocyte responses. *Microbes Infect*. 2004; 6(8):759–766.
13. Trautmann L, et al. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med*. 2006; 12(10):1198–1202.
14. Petrovas C, et al. PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *J Exp Med*. 2006;203(10):2281–2292.
15. Day CL, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature*. 2006;443(7109):350–354.
16. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. 2008;26:677–704.
17. Jin HT, et al. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc Natl Acad Sci U S A*. 2010;107(33):14733–14738.
18. Blackburn SD, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol*. 2009; 10(1):29–37.
19. Ha SJ, et al. Enhancing therapeutic vaccination by blocking PD-1-mediated inhibitory signals during chronic infection. *J Exp Med*. 2008;205(3):543–555.
20. Klapper JA, et al. High-dose interleukin-2 for the treatment of metastatic renal cell carcinoma: a retrospective analysis of response and survival in patients treated in the surgery branch at the National Cancer Institute between 1986 and 2006. *Cancer*. 2008;113(2):293–301.
21. Smith FO, et al. Treatment of metastatic melanoma using interleukin-2 alone or in conjunction with vaccines. *Clin Cancer Res*. 2008;14(17):5610–5618.
22. Schwartzentruber DJ, et al. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. *N Engl J Med*. 2011;364(22):2119–2127.
23. Pandolfi F, et al. Low-dose IL-2 reduces lymphocyte apoptosis and increases naive CD4 cells in HIV-1 patients treated with HAART. *Clin Immunol*. 2000;94(3):153–159.
24. Durier C, et al. Long-term effects of intermittent interleukin-2 therapy in chronic HIV-infected patients (ANRS 048-079 Trials). *AIDS*. 2007; 21(14):1887–1897.
25. Martinez-Marino B, Shiboski S, Hecht FM, Kahn JO, Levy JA. Interleukin-2 therapy restores CD8 cell non-cytotoxic anti-HIV responses in primary infection subjects receiving HAART. *AIDS*. 2004; 18(15):1991–1999.
26. Levy Y, et al. Comparison of subcutaneous and intravenous interleukin-2 in asymptomatic HIV-1 infection: a randomised controlled trial. *ANRS*. 1999; 353(9168):1923–1929.
27. Kovacs JA, et al. Controlled trial of interleukin-2 infusions in patients infected with the human immunodeficiency virus. *N Engl J Med*. 1996;335(18):1350–1356.
28. Abrams D, et al. Interleukin-2 therapy in patients with HIV infection. *N Engl J Med*. 2009;361(16):1548–1559.
29. Levy Y, et al. Immunological and virological efficacy of a therapeutic immunization combined with interleukin-2 in chronically HIV-1 infected patients. *AIDS*. 2005;19(3):279–286.
30. Hardy GA, et al. A phase I, randomized study of combined IL-2 and therapeutic immunisation with antiretroviral therapy. *J Immune Based Ther Vaccines*. 2007;5:6.
31. Kilby JM, et al. A randomized, partially blinded phase 2 trial of antiretroviral therapy, HIV-specific immunizations, and interleukin-2 cycles to promote efficient control of viral replication (ACTG A5024). *J Infect Dis*. 2006;194(12):1672–1676.
32. Smith KA, et al. Immunotherapy with canarypox vaccine and interleukin-2 for HIV-1 infection: termination of a randomized trial. *PLoS Clin Trials*. 2007;2(1):e5.
33. Tryniszewska E, et al. Vaccination of macaques with long-standing SIVmac251 infection lowers the viral set point after cessation of antiretroviral therapy. *J Immunol*. 2002;169(9):5347–5357.
34. Nacsa J, et al. Contrasting effects of low-dose IL-2 on vaccine-boosted simian immunodeficiency virus (SIV)-specific CD4+ and CD8+ T cells in macaques chronically infected with SIVmac251. *J Immunol*. 2005;174(4):1913–1921.
35. Jacobson EL, Pilaro F, Smith KA. Rational interleukin 2 therapy for HIV positive individuals: daily low doses enhance immune function without toxicity. *Proc Natl Acad Sci U S A*. 1996;93(19):10405–10410.
36. Koreth J, et al. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med*. 2011; 365(22):2055–2066.
37. Saadoun D, et al. Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. *N Engl J Med*. 2011;365(22):2067–2077.
38. Blattman JN, Grayson JM, Wherry EJ, Kaech SM, Smith KA, Ahmed R. Therapeutic use of IL-2 to enhance antiviral T-cell responses in vivo. *Nat Med*. 2003;9(5):540–547.
39. Brooks DG, Ha SJ, Elsaesser H, Sharpe AH, Freeman GJ, Oldstone MB. IL-10 and PD-L1 operate through distinct pathways to suppress T-cell activity during persistent viral infection. *Proc Natl Acad Sci U S A*. 2008;105(51):20428–20433.
40. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol*. 2005;6(4):331–337.
41. Malek TR, Bayer AL. Tolerance, not immunity, crucially depends on IL-2. *Nat Rev Immunol*. 2004; 4(9):665–674.
42. Wherry EJ, et al. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity*. 2007;27(4):670–684.
43. Virgin HW, Wherry EJ, Ahmed R. Redefining chronic viral infection. *Cell*. 2009;138(1):30–50.
44. Kao C, et al. Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection. *Nat Immunol*. 2011;12(7):663–671.
45. Liu CC, Rafii S, Granelli-Piperno A, Trapani JA, Young JD. Perforin and serine esterase gene expression in stimulated human T cells. Kinetics, mitogen requirements, and effects of cyclosporin A. *J Exp Med*. 1989;170(6):2105–2118.
46. Janas ML, Groves P, Kienzle N, Kelso A. IL-2 regulates perforin and granzyme gene expression in CD8+ T cells independently of its effects on survival and proliferation. *J Immunol*. 2005;175(12):8003–8010.
47. Akbar AN, et al. Interleukin-2 receptor common gamma-chain signaling cytokines regulate activated T cell apoptosis in response to growth factor withdrawal: selective induction of anti-apoptotic (bcl-2, bcl-xL) but not pro-apoptotic (bax, bcl-xS) gene expression. *Eur J Immunol*. 1996;26(2):294–299.
48. Deng G, Podack ER. Suppression of apoptosis in a cytotoxic T-cell line by interleukin 2-mediated gene transcription and deregulated expression of the protooncogene bcl-2. *Proc Natl Acad Sci U S A*. 1993;90(6):2189–2193.
49. Miyazaki T, et al. Three distinct IL-2 signaling pathways mediated by bcl-2, c-myc, and lck cooperate in hematopoietic cell proliferation. *Cell*. 1995;81(2):223–231.
50. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol*. 2003;4(12):1191–1198.
51. Wherry EJ, Barber DL, Kaech SM, Blattman JN, Ahmed R. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc Natl Acad Sci U S A*. 2004;101(45):16004–16009.
52. Blackburn SD, Shin H, Freeman GJ, Wherry EJ. Selective expansion of a subset of exhausted CD8 T cells by alphaPD-L1 blockade. *Proc Natl Acad Sci U S A*. 2008;105(39):15016–15021.
53. Pipkin ME, Sacks JA, Cruz-Guilloty F, Lichtenheld MG, Bevan MJ, Rao A. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity*. 2010;32(1):79–90.
54. Xue HH, et al. IL-2 negatively regulates IL-7 receptor alpha chain expression in activated T lymphocytes. *Proc Natl Acad Sci U S A*. 2002;99(21):13759–13764.
55. Kalia V, Sarkar S, Subramaniam S, Haining WN, Smith KA, Ahmed R. Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo. *Immunity*. 2010;32(1):91–103.
56. Smith KA, Popmihajlov Z. The quantal theory of immunity and the interleukin-2-dependent negative feedback regulation of the immune response. *Immunol Rev*. 2008;224:124–140.
57. Popmihajlov Z, Smith KA. Negative feedback regulation of T cells via interleukin-2 and FOXP3 reciprocity. *PLoS One*. 2008;3(2):e1581.
58. Mueller SN, et al. PD-L1 has distinct functions in hematopoietic and nonhematopoietic cells in regulating T cell responses during chronic infection in mice. *J Clin Invest*. 2010;120(7):2508–2515.
59. Pellegrini M, et al. IL-7 engages multiple mechanisms to overcome chronic viral infection and limit organ pathology. *Cell*. 2011;144(4):601–613.
60. Nanjappa SG, Kim EH, Suresh M. Immunotherapeutic effects of IL-7 during a chronic viral infection in mice. *Blood*. 2011;117(19):5123–5132.
61. Masopust D, Vezys V, Marzo AL, Lefrancois L. Preferential localization of effector memory cells in nonlymphoid tissue. *Science*. 2001;291(5512):2413–2417.