Antigen-specific CD4 T-cell help rescues exhausted CD8 T cells during chronic viral infection

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Edited by Peter K. Vogt, The Scripps Research Institute, La Jolla, CA, and approved November 18, 2011 (received for review April 12, 2011)

CD4 T cells play a critical role in regulating CD8 T-cell responses during chronic viral infection. Several studies in animal models and humans have shown that the absence of CD4 T-cell help results in severe dysfunction of virus-specific CD8 T cells. However, whether function can be restored in already exhausted CD8 T cells by providing CD4 T-cell help at a later time remains unexplored. In this study, we used a mouse model of chronic lymphocytic choriomeningitis virus (LCMV) infection to address this question. Adoptive transfer of LCMV-specific CD4 T cells into chronically infected mice restored proliferation and cytokine production by exhausted virus-specific CD8 T cells and reduced viral burden. Although the transferred CD4 T cells were able to enhance function in exhausted CD8 T cells, these CD4 T cells expressed high levels of the programmed cell death (PD)-1 inhibitory receptor. Blockade of the PD-1 pathway increased the ability of transferred LCMV-specific CD4 T cells to produce effector cytokines, improved rescue of exhausted CD8 T cells, and resulted in a striking reduction in viral load. These results suggest that CD4 T-cell immunotherapy alone or in conjunction with blockade of inhibitory receptors may be a promising approach for treating CD8 T-cell dysfunction in chronic infections and cancer.

CD8 T cells activated during acute viral infections develop into highly functional effector CD8 T cells capable of killing infected cells and secreting antiviral cytokines. After resolution of the primary infection, memory CD8 T cells persist long term via homeostatic turnover and remain poised for rapid effector function and proliferation in response to secondary challenges (1). In contrast, CD8 T cells generated during many chronic viral infections have impaired ability to proliferate, kill virally infected targets, and produce effector cytokines. CD8 T-cell dysfunction has been well documented in several chronic viral infection models and in chronic human infections with hepatitis C virus, hepatitis B virus, and HIV (2). In addition, CD8 T-cell dysfunction occurs in other situations of prolonged antigen persistence, such as cancers (3, 4). Increased understanding of the events that drive and maintain this exhausted state in CD8 T cells remains critical for the development of clinical therapies to treat patients with chronic infections.

Early studies using lymphocytic choriomeningitis virus (LCMV) showed that CD4 T-cell help is critical for maintaining CD8 T-cell function during chronic infection. Mice transiently depleted of CD4 T cells before infection with chronic strains of LCMV exhibit profound CD8 T-cell exhaustion and higher viral burden compared with mice with an intact CD4 T-cell compartment (5–7). Elimination of CD4 T-cell help also results in impaired long-term viral control during murine gammaherpesvirus infection (8). Likewise, loss of CD4 T-cell help has been implicated in CD8 T-cell dysfunction and disease progression in human chronic infections with HIV and hepatitis C virus (9–11).

Immunotherapy in humans with transfer of antigen-specific CD8 T-cell clones has prevented viral infection during bone marrow transplantation (12), and CD8 T-cell immunotherapy also has proven effective in treating some cancers (13, 14). Interestingly, CD4 T-cell help has been suggested to be important for the maintenance and survival of transferred virus-specific CD8 T cells after bone marrow transplantation (15–17). In addition, cultured autologous CD4 T cells have shown some promising results against metastatic melanoma (18, 19), and CD4 T cells isolated during acute HIV infection have been shown to restore proliferation in cocultured exhausted CD8 T cells from patients with progressive HIV infection (20). These results suggest that CD4 T-cell therapy may be useful in treating patients with chronic infections and cancer.

In this study, we examined whether the restoration of CD4 T-cell help can revert established CD8 T-cell exhaustion. Transfer of LCMV-specific CD4 T cells to mice with complete lack of viral control and pronounced levels of CD8 T-cell dysfunction (5) resulted in enhanced virus-specific CD8 T-cell proliferation and function, along with reduced viral burden. Previous studies have shown that inhibitory receptors on exhausted CD8 T cells play a pivotal role in T-cell dysfunction during chronic infections (21, 22), and that blockade of the programmed cell death (PD)-1 pathway enhances proliferation and function of CD8 T cells during chronic LCMV (23). In this study, we found that PD-1 blockade after CD4 T-cell transfer into chronically infected mice improved the functionality of CD4 T cells. Furthermore, the combined immunotherapeutic CD4 T-cell transfer with blockade of PD-1 increased the rescue of virus-specific CD8 T-cell function and greatly improved viral control during chronic LCMV infection.

Results

LCMV-Specific CD4 T Cells Undergo Rapid Antigen-Driven Activation and Proliferation and Persist Long Term After Transfer into Chronically Infected Mice. We initially characterized the fate of naïve LCMV-specific CD4 T cells when transferred into chronically LCMV-infected mice. CD4 T cells from SMARTA transgenic mice, which have CD4 T cells specific for the gp67-77 epitope of LCMV (24, 25), were labeled with carboxyfluorescein succinimidyl ester (CFSE) and transferred into either uninfected (naïve) or chronically infected recipients. Recipient mice were infected with LCMV for ~2–3 mo and had high levels of persistent virus in multiple tissues and serum. SMARTA CD4 T cells transferred into infected recipients up-regulated activation markers such as CD44, underwent rapid antigen-driven proliferation (between four and seven divisions by 2.5 d posttransfer), and were detected in spleen and nonlymphoid (e.g., liver, lung) tissues. In contrast, SMARTA

Author contributions: R.D.A., A.O.K., S.S., V.V., S.-J.H., D.L.B., and R.A. designed research; R.D.A., A.O.K., S.S., S.-J.H., and L.Y. performed research; A.H.S. and G.J.F. contributed new reagents/analytic tools; R.D.A., A.O.K., S.S., and R.A. analyzed data; and R.D.A., A.O.K., and R.A. wrote the paper.

Conflict of interest statement: R.A., A.H.S., and G.J.F. hold patents for the PD-1 inhibitory pathway.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1118450109/-/DCSupplemental.

CD4 T cells transferred into uninfected mice remained undivided and retained the naïve CD44^{low} phenotype (Fig. 1*A*). Accumulation of LCMV-specific CD4 T cells was seen in the spleen, lung, and liver of infected recipients (Fig. S1*A*).

We next examined the long-term fate of SMARTA CD4 T cells by monitoring their persistence in the blood of chronically infected recipients. The peak expansion of SMARTA CD4 T cells occurred at approximately 1 wk posttransfer. The percentage of SMARTA CD4 T cells remained fairly high, demonstrating a slow contraction but long-term term persistence in chronically infected mice (Fig. 1*B* and Fig. S1*B*). Transferred LCMV-specific CD4 T cells were detectable in the spleen, liver, and bone marrow at 4 mo posttransfer (Fig. S1*C*). Given previous reports of loss of CD4 T-cell function during chronic LCMV infection (26, 27), we also examined SMARTA CD4 T-cell cytokine production. The percentage of SMARTA CD4 T cells producing IFN-γ was ~50% (n = 5; range, 39–64%), with a smaller fraction of the SMARTA CD4 T cells producing TNF-α (average, 32%; range, 26–42%) or IL-2 (average, 18%; range 10–27%) on ex vivo restimulation (Fig. 1*C*). This percent was reduced compared with acute infection, where usually more than 50–70% of memory SMARTA CD4 T cells coproduce both IFN-γ and TNF-α, and with 50% of SMARTA CD4 T cells also making IL-2 (Fig. S1*D*).

Antigen-Specific CD4 T-Cell Help Enhances LCMV-Specific CD8 T-Cell Responses and Reduces Viral Load in Chronically Infected Mice. We then monitored LCMV-specific CD8 T-cell responses after SMARTA CD4 T-cell transfer. Mice receiving a single transfer of naïve LCMV-specific CD4 T cells had significantly more LCMV-specific CD8 T cells in the blood by 2 wk posttransfer. This elevated response was long lasting and could be detected at 2 mo posttransfer, whereas untreated animals continued to maintain very low to undetectable frequencies of LCMV-specific CD8 T cells (Fig. 2*A*). Mice receiving LCMV-specific CD4 T cells also had elevated frequencies of virus-specific CD8 T cells in the spleen, liver, lung, and bone marrow (Fig. 2*B*). The average number of LCMV-specific CD8 T cells increased by approximately fourfold for the GP33 epitope and sixfold for the GP276 epitope in the spleen. Substantial increases in LCMV-specific CD8 T-cell numbers were also seen in the liver and lungs (Fig. S2).

Exhausted CD8 T cells make very low levels of IFN-γ in response to restimulation with cognate peptide (7, 28). At 1 wk after LCMV-specific CD4 T-cell transfer, there was an elevated number of CD8 T cells in the spleen producing IFN-γ after stimulation with LCMV-specific peptides (Fig. 2*C*). Therefore, transfer of LCMV-specific CD4 T cells enhanced both the number and function of exhausted CD8 T cells in chronically infected recipients. Most importantly, this rescue of LCMV-specific CD8 T-cell responses resulted in an approximately fourfold decrease in viral titers in the serum within 1 mo (Fig. 2*D*).

We also tested whether the recovery of the LCMV-specific CD8 T cells relies on cognate CD4 T-cell interactions by transferring either SMARTA or OT-II (specific for MHC class II epitope 323–339 of ovalbumin) transgenic CD4 T cells into chronically infected recipients. Mice that received no cells or OT-II cells had a low frequency of LCMV-specific CD8 T cells

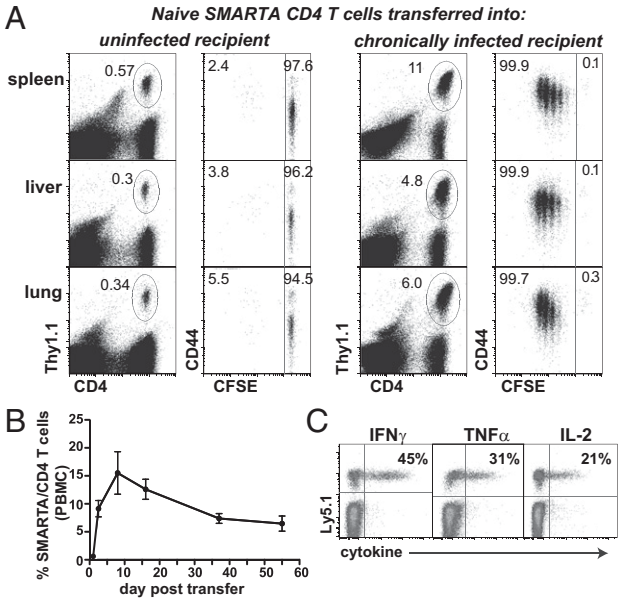

Fig. 1. LCMV-specific transgenic CD4 T cells proliferate and persist long term after transfer into chronically infected hosts. LCMV-specific CD4 T cells were isolated from naïve Thy1.1 or Ly5.1 SMARTA transgenic mice, labeled with CFSE, and transferred into congenic B6 recipients. (*A*) Frequency of SMARTA cells at day 2.5 posttransfer. CFSE dilution and CD44 expression for transferred cells (gated on Thy1.1). (*B*) SMARTA CD4 T-cell expansion in the blood, summarized from several experiments (n = 10–15 mice per time point). (*C*) LCMV-specific cytokine production at 4 mo posttransfer. Shown is the percentage of SMARTA CD4 T cells producing cytokine after ex vivo stimulation with the GP61-80 peptide in one representative mouse (n = 5).

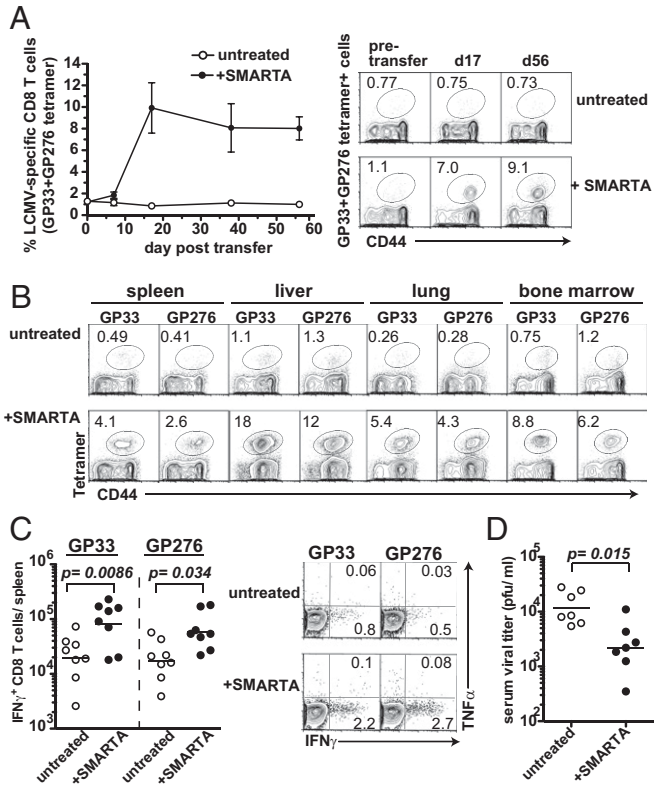

Fig. 2. Transfer of CD4 T-cell help enhances LCMV-specific CD8 T-cell responses. (*A*) LCMV-specific CD8 T cells in the blood after SMARTA T-cell transfer (n = 4 untreated mice and 6–8 treated mice). Representative LCMV-specific tetramer staining (gated on CD8 T cells) is shown. (*B*) LCMV-specific CD8 T-cell responses in tissues at day 35 posttransfer. (*C*) Summarized LCMV-specific IFN-γ production by CD8 T cells in the spleen at day 7 posttransfer (n = 8 mice per group, combined from two independent experiments) and representative IFN-γ and TNF-α staining for CD8 T cells. (*D*) Serum viral titers as determined by plaque assay at 1 mo posttransfer. Similar results were found in several independent experiments. P values were determined by the Student t test.

producing IFN-γ, whereas mice receiving LCMV-specific SMARTA CD4 T cells had an increased frequency of IFN-γ^+ CD8 T cells (Fig. S3*A*). Importantly, we also observed that this increased function in LCMV-specific CD8 T cells occurred across a wide range of LCMV-specific CD8 T-cell epitopes (Fig. S3*B*).

LCMV-Specific CD4 T Cells Also Enhance B-Cell Responses in Chronically Infected Recipients. LCMV-specific B-cell responses are not critical for the initial control of acute LCMV infection, which is mediated primarily by the CD8 T-cell response (29). However, the absence of LCMV-specific antibodies results in incomplete clearance and relapsing low levels of virus (30, 31). In the model of chronic LCMV infection used in the present study, CD4 T cells were transiently depleted during the early phase of LCMV infection, resulting in the inability to develop virus-specific CD4 T-cell responses, even after the recovery of total CD4 T-cell numbers (5). Consequently, we were interested in determining whether transfer of SMARTA CD4 T cells could initiate B-cell responses. At 1 mo after transfer of SMARTA cells, chronically infected mice developed germinal center reactions, as identified by PNA$^+$FAS$^+$ B cells in the spleen compared with untreated controls (Fig. 3*A*). Furthermore, mice receiving SMARTA CD4 T cells had significantly increased levels of LCMV-specific antibodies compared with untreated controls, albeit at lower titers than seen after acute LCMV Armstrong infection (Fig. 3*B*). Therefore, in addition to improving CD8 T-cell responses, transferred LCMV-specific CD4 T cells were able to initiate antigen-specific antibody responses in chronically infected recipients.

PD-1 Blockade Improves CD4 T-Cell Function in Chronically Infected Recipients. Previous studies have shown that blocking the PD1/PD-L1 pathway enhances function in exhausted LCMV-specific CD8 T cells during chronic viral infection (23). Whether the PD-1/PD-L1 pathway regulates CD4 T-cell responses during chronic viral infection has not been fully addressed. However, SMARTA CD4 T cells transferred into chronically infected mice expressed high levels of PD-1 by 2 wk after transfer (Fig. 4*A*). Thus, we wanted to determine whether blockade of the PD-1 pathway would influence the proliferation and function of the transferred CD4 T cells, and whether PD-1 blockade in combination with CD4 T-cell transfer could further improve the rescue of exhausted CD8 T cells and reduction of viral load in LCMV chronically infected mice.

Chronically infected mice were given naïve SMARTA CD4 T cells and then treated with the blocking αPD-L1 antibody for 2 wk starting at day 1 posttransfer. The total number of SMARTA

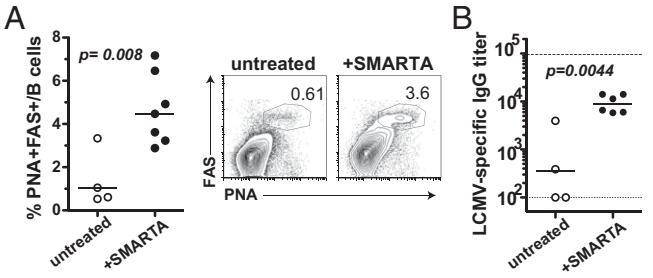

Fig. 3. Increased B-cell responses after SMARTA CD4 T-cell transfer. (*A*) Germinal center activity in chronically infected recipients at 1 mo after SMARTA CD4 T-cell transfer. Shown is a summary graph of the percentage of PNA$^+$FAS$^+$ B220$^+$ cells in the spleen, with one representative flow plot per group. (*B*) At day 55 posttransfer, LCMV-specific antibody levels were measured by serum ELISA. The bottom dotted line indicates the lower limit of detection, and the top line indicates the endpoint titer in LCMV Armstrong immune mice at >60 d postinfection.

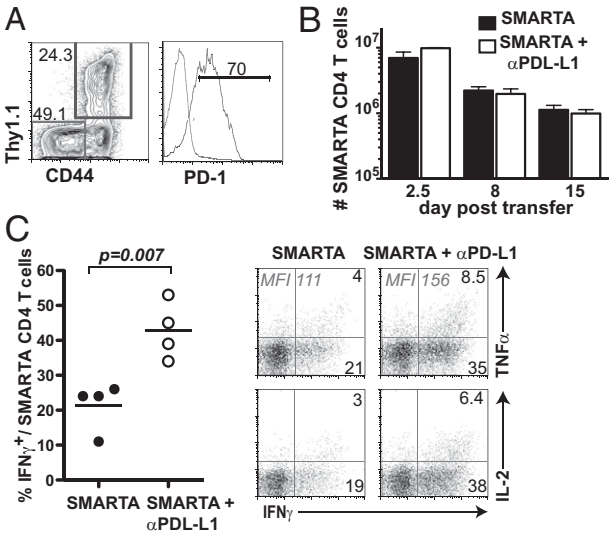

Fig. 4. PD-1 blockade improves function but not proliferation of LCMV-specific CD4 T cells. (*A*) PD-1 expression on SMARTA CD4 T cells in chronically infected hosts at day 15 posttransfer. Histogram shows percent of Thy1.1 CD4 T cells expressing PD-1 compared to naive CD44low (left histogram) CD4 T cells. (*B*) Number of SMARTA cells in spleen (n = 3–5 mice/group). (*C*) Percentage of SMARTA CD4 T cells producing IFN-γ at day 15 posttransfer (n = 4 mice per group). Polyfunctionality of the SMARTA cells in chronically infected recipients after αPD-L1 blockade gated on SMARTA (Thy1.1), frequency of transferred cells producing cytokine, and mean fluorescence intensity of the IFN-γ staining.

CD4 T cells recovered at days 2.5, 8, and 15 posttransfer was similar in mice treated with αPD-L1 blockade and those not treated (Fig. 4*B*); however, αPD-L1 therapy augmented the functionality of the transferred CD4 T cells, with a greater percentage of SMARTA cells producing IFN-γ. A comparison of the mean fluorescence intensity of the IFN-γ^+ cells also suggested increased cytokine production on a per-cell basis after PD-1 blockade. Most critically, there was an increased frequency of polyfunctional CD4 T cells capable of coproducing both IFN-γ and TNF-α or both IFN-γ and IL-2 (Fig. 4*C*).

PD-1 Blockade Complements CD4 T-Cell Therapy by Enhancing CD8 T-Cell Function and Further Reducing Viral Load. We next examined whether blockade of the PD-1/PD-L1 pathway could complement CD4 T-cell help to enhance rescue of exhausted CD8 T cells when performed for 2 wk after SMARTA CD4 T-cell transfer. The rescue of CD8 T-cell function was greater in mice receiving the combination of CD4 T-cell help with PD-1 blockade and was observed for both dominant and subdominant CD8 T-cell epitopes (Fig. S4 *A* and *B*). Mice receiving SMARTA CD4 T cells and transient PD-1 blockade had a significantly greater number of LCMV-specific CD8 T cells capable of producing both IFN-γ and TNF-α compared with the mice that received either treatment alone (Fig. 5*A* and Fig. S4*A*). Most remarkably, mice receiving SMARTA CD4 T cells and transient PD-1 blockade also had better viral control at 1 mo posttransfer. Chronically infected mice receiving the combination therapy had an ∼10-fold reduction in viral titer compared with untreated mice, with some of the treated mice suppressing serum virus to levels below the limit of detection by plaque assay (Fig. 5*B*). Thus, short-term blockade of inhibitory signals such as the PD-1/PD-L1 pathway complemented the transfer of CD4 T cells to improve CD8 T-cell responses and significantly enhanced viral control during established chronic LCMV infection. Such striking viral reduction has not been reported previously in this very stringent model of chronic LCMV infection lacking CD4 T-cell

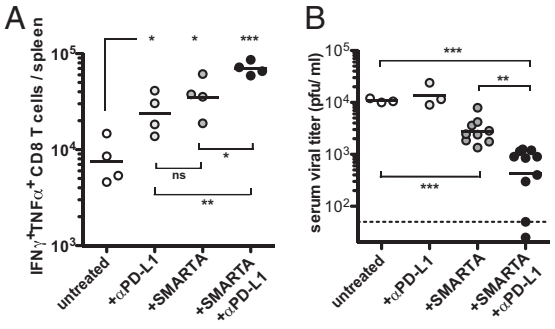

Fig. 5. PD-1 blockade complements CD4 T-cell therapy to enhance function in exhausted CD8 T cells and further reduce viral loads. (*A*) Number of LCMV-specific CD8 T cells coproducing IFN-γ and TNF-α at 2 wk posttreatment, summed from individual staining for six LCMV epitopes (GP33, GP276, NP205, NP235, GP118, and GP92). (*B*) Viral titers in the serum at 1 mo after CD4 T-cell transfer (LOD 50 pfu/mL). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, Student *t* test.

help. These results further support the use of combination therapies to achieve resolution of chronic infections.

Effector SMARTA CD4 T Cells Also Enhance LCMV-Specific CD8 T-Cell and B-Cell Responses in Chronically Infected Mice. So far, we have shown that transfer of naïve SMARTA CD4 T cells can rescue CD8 T cell exhaustion. However, current human T-cell therapies require the expansion of rare antigen-specific T-cell populations before transfer into patients (32). To better model this type of immunotherapy, we ascertained whether pre-expanded effector SMARTA CD4 T cells also could provide help and rescue exhausted CD8 T cells. Effector SMARTA CD4 T cells were isolated from mice at 7 d after acute LCMV infection. Transfer of SMARTA CD4 effector T cells induced a significant increase in LCMV-specific CD8 T cells in the blood (Fig. 6*A*), as well as in lymphoid and nonlymphoid tissues of chronically infected recipients (Fig. 6*B*). In addition, there was also an increase in the numbers of splenic CD8 T cells that produced IFN-γ after stimulation with either GP33 or GP276 LCMV-specific peptides (Fig. 6*C*). Effector SMARTA CD4 T cells also provided B-cell help, as demonstrated by increases in germinal center B cells (Fig. 6*D*) and virus-specific antibody responses. In summary, these data show that transfer of preactivated CD4 T cells into chronically infected mice also can rescue exhausted CD8 T cells and provide B-cell help and further support the feasibility of similar therapies in clinical settings.

Discussion

We have previously shown that exhausted CD8 T cells express a unique pattern of genes, and that the exhausted phenotype develops longitudinally over the course of chronic LCMV infection (33). CD4 T cells play a critical role in this process, and even a transient depletion of CD4 T cells during the initial CD8 T-cell priming results in the establishment of severely exhausted CD8 T cells and high viral loads in chronically infected mice (5). In this study, we examined whether the restoration of antigen-specific CD4 T-cell help could rescue CD8 T-cell function after the establishment of this exhausted phenotype. We found that the transfer of antigen-specific CD4 T cells during chronic LCMV infection enhanced CD8 T-cell number and function, resulting in decreased viral burden. We also found that PD-1 played an important role in controlling CD4 T-cell function and blockade of the PD-1 pathway after CD4 T-cell transfer enhanced cytokine production in both LCMV-specific CD4 and CD8 T cells. Finally, the combined CD4 T-cell immunotherapy and PD-1 blockade further enhanced the rescue of exhausted CD8 T cells, resulting

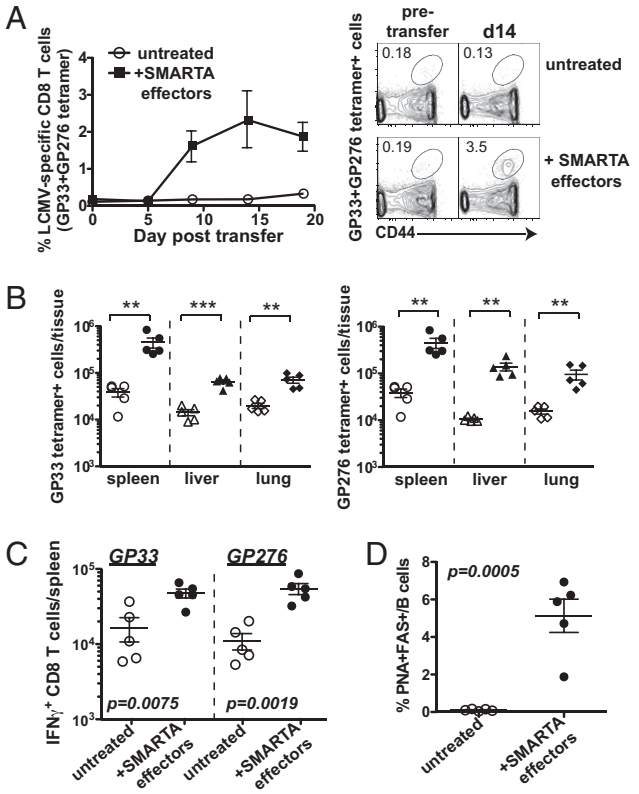

Fig. 6. Transfer of effector SMARTA CD4 T cells enhances LCMV-specific CD8 T-cell and B-cell responses in chronically infected mice. Here, 10×10^6 effector SMARTA CD4 T cells (isolated from day 7 LCMV Armstrong mice) were transferred into LCMV chronically infected recipients. (*A*) LCMV-specific CD8 T cells in blood (n = 5/group) and representative LCMV-specific tetramer staining (gated on CD8 T cells). (*B*) Total numbers of LCMV-specific CD8 T cells in tissues on day 19 posttransfer. Open symbols indicate untreated mice; closed symbols, SMARTA effector recipients. (*C*) Number of LCMV-specific CD8 T cells in the spleen producing IFN-γ at day 19 posttransfer. (*D*) Graph of average percentage and range of germinal center B cells (gated on $B220^+$ $CD19^+$ B cells) detected in the spleen at day 19 posttransfer. Shown is a representative experiment of two independent experiments with similar results. $**P < 0.01$; $***P < 0.001$, Student *t* test. In the chronically infected mice receiving SMARTA effectors, all LCMV-specific CD8 T cells were Ly5.2, and thus were not contamination from the donor LCMV Armstrong-infected mice (Ly5.1).

in polyfunctional CD8 T-cell responses, and a significant reduction of viral load during chronic LCMV infection.

The transferred antigen-specific CD4 T cells persisted long term despite the presence of high levels of antigen and mediated a long-lasting increase in LCMV-specific CD8 T cells. In addition, this enhanced CD8 T-cell function was seen for a wide range of virus-specific CD8 T-cell epitopes, which may be critical for treating infections in which viral escape to dominant CD8 T-cell epitopes has been demonstrated (9, 34, 35). The mechanism by which antigen-specific CD4 T cells drive the increased CD8 T-cell proliferation and function in the presence of high levels of virus remains an important question. One possibility is that soluble factors produced by activated CD4 T cells act directly on exhausted CD8 T cells. Recent studies have shown that IL-21 produced by CD4 T cells plays a critical role in maintaining CD8 T-cell function during chronic LCMV infection (36–38). IL-21 has also been shown to enhance HIV-specific CD8 T cell function in vitro (39). Ongoing studies are addressing whether IL-21 production by the transferred SMARTA cells is critical for rescuing exhausted LCMV-specific CD8 T cells. In addition, exogenous IL-2 has been shown to enhance CD8 T-cell function

during chronic LCMV (40) and HIV (20) infection, and thus could be important in our CD4 T-cell therapy model.

Besides providing soluble factors to CD8 T cells directly, CD4 T cells can provide signals to antigen-presenting cells that can then alter the activation of exhausted CD8 T cells. The transferred antigen-specific CD4 T cells may act on these antigen-presenting cells by direct contact (e.g., CD40L/CD40) or by production of cytokines and chemokines that can alter activation or survival of the antigen-presenting cells (41). It would be of interest to examine dendritic cell populations after CD4 T-cell transfer in these chronically infected recipients to determine whether changes in the number or status of these cells occurs and is required for CD8 T-cell rescue.

The significant lag between the CD4 T-cell transfer and the reduction in viral titers suggests that reductions in viremia are not due to the direct effects of CD4 T cells, but require an LCMV-specific CD8 T-cell response. B cells also might play an important role in the control of viral load, as suggested by the significant levels of LCMV-specific antibody found in all mice receiving LCMV-specific CD4 T cells at 2 mo posttransfer. Previous studies have implicated a role for the B-cell response in resolving chronic LCMV infection (31, 42). Thus, development of an LCMV-specific antibody response after SMARTA CD4 T-cell transfer likely would be beneficial to enhance viral control. Interestingly, mice receiving a combination of CD4 T-cell help and PD-1 blockade exhibited significantly larger reductions in viral loads, but no increase in LCMV-specific antibodies compared with mice receiving CD4 T cells alone. Thus, although antibodies may play a role in viral control, LCMV-specific antibody titer alone did not correlate with decreased viral loads in chronically infected mice after CD4 T-cell transfer.

Blockade of inhibitory receptors such as PD-1 has been shown to enhance both exhausted CD8 T-cell proliferation and function during chronic LCMV infection (23). Although PD-1 blockade did not increase expansion, it enhanced effector function in LCMV-specific CD4 T cells. A slight increase in CD4 T-cell function was already detectable during the initial expansion (day 2.5), suggesting that the improved CD4 T-cell function by PD1 blockade was not solely a result of decreased viral titers or improved CD8 T-cell function. Future studies with PD-1 deficient CD4 T cells should provide more information on the role of PD-1 signaling and CD4 T-cell function during chronic LCMV infection. PD-1 signaling also may play an important role in CD4 T-cell differentiation, since T follicular help cells (Tfh) which produce IL-21 and provide B cell help, express high levels of PD-1 (43). It may be interesting to examine other inhibitory receptors expressed by CD4 T cells, such as CTLA-4 (44), which could modulate the function of the transferred virus-specific CD4 T cells.

Combined CD4 T-cell transfer and PD-1 blockade increased the polyfunctionality of the rescued CD8 T cells. Although both CD4 T-cell immunotherapy and αPD-L1 blockade enhanced IFN-γ production, the combined therapy resulted in a significantly higher frequency of CD8 T cells coproducing IFN-γ and TNF-α. This increased functionality may be related in part to the reduced viral load at 1 wk after PD-1 blockade (23); however, enhanced CD8 T-cell rescue was also seen during the first week posttransfer, and thus we favor the model in which PD-1 blockade has direct effects on CD8 and CD4 T cells. Importantly, mice receiving both CD4 T cells and short-term αPD-L1 therapy exhibited ~10-fold decreases in viral burden in the serum, significantly better than mice receiving either CD4 T cells or αPD-L1 treatment alone. Most notably, complete suppression of serum virus in this stringent exhaustion model of LCMV infection (without CD4 T-cell help) had not been achieved previously.

To further support the application of our findings to human therapies, we have shown that transfer of preactivated effector LCMV-specific CD4 T cells can rescue exhausted CD8 T cells as well. Current immunotherapy approaches in humans rely on the isolation of rare antigen-specific cells, which then require several rounds of in vitro expansion before being transferred into patients. Preactivated CD4 T cells have reduced expansion potential compared with naïve CD4 T cells; thus, strategies that use multiple transfers and/or further manipulation (with cytokines or by genetic engineering) may be required to achieve long-term effects (13, 45, 46).

In conclusion, we have shown that both naïve and effector antigen-specific CD4 T-cell help can rescue already exhausted CD8 T cells in vivo. Moreover, short-term blockade of the PD-1 pathway increased this rescue, thereby supporting the development of T-cell therapies combined with blockade of inhibitory pathways as a novel approach to treating patients with chronic diseases.

Materials and Methods

Mice and Infection. The 4- to 6-wk-old female C57BL/6 mice were purchased from Jackson Laboratory. SMARTA transgenic mice, which have CD4 T cells specific for the gp67-77 (KGVYQFKSV) epitope of LCMV (25), were maintained at Emory University. LCMV clone 13 was propagated and titered as described previously (47). Mice were infected with 2×10^6 pfu of LCMV clone 13 intravenously after CD4 T-cell depletion as described previously (5). A 2-wk αPD-L1 (10F.9G2) blockade was administered as described previously starting at 1 d after SMARTA CD4 T-cell transfer (23). All mice were handled in accordance with National Institutes of Health and Emory University's Institutional Animal Care and Use Committee guidelines.

CD4 T-Cell Isolation and in Vivo Antibody Blockade. CD4 T cells from SMARTA transgenic mice were enriched by depletion of non-CD4 T cells (Miltenyi). Then 4×10^6 CD4 T cells were injected intravenously into uninfected (naïve) B6 mice or mice infected with LCMV clone 13 for 2–3 mo before transfer. CFSE labeling was done as described previously (48). SMARTA effectors were obtained at day 7 after LCMV Armstrong infection from mice receiving 1×10^5 SMARTA CD4 T cells 1 d before infection. SMARTA (Thy1.1) effectors were isolated by depletion of non-CD4 and Th1.2 T cells (Miltenyi).

Antibodies, Flow Cytometry, and ELISA. All antibodies were obtained from BD Biosciences except PD-1 (Biolegend) and PNA (Vector Laboratories). All surface stains and intracellular cytokine stimulations were done as described previously (48). All samples were read using the BD FACSCalibur or LSRII and analyzed using Flowjo software (Tree Star). LCMV-specific IgG ELISAs were performed as described previously (47). LCMV-specific antibody titers were determined by an endpoint titer 0.1 OD over background (naïve serum).

Statistical Analysis. Statistical analyses were performed with the two-tailed unpaired Student *t* test or the Mann–Whitney test (where noted) using Prism software (GraphPad Software).

ACKNOWLEDGMENTS. We thank B. T. Konieczny and H. Wu for technical assistance and members of R.A.'s laboratory for helpful discussions. This work was supported by National Institutes of Health Grants R01 AI030048 and P01 AI080192 (to R.A.), P01 AI056299 (G.J.F., R.A., A.H.S.), and by the Cancer Research Institute's Irvington Institute Fellowship Program (A.O.K.).

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