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CD8⁺ T cell exhaustion during persistent viral infection is regulated independently of the virus-specific T cell receptor

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Abstract

During chronic viral infections, responses by virus-specific CD8⁺ T cells become marginalized by the acquisition of functional defects and reduced cell numbers in a process defined as T cell exhaustion. T cell tolerance to self-antigen is also characterized by impaired effector function and eventual deletion of self-reactive T cells. Induction of both tolerance and exhaustion involve many shared inhibitory mechanisms, thus similar therapeutic approaches have proven effective in these distinct environments. We previously demonstrated that tolerant self-reactive CD8⁺ T cells expressing dual-T cell receptors (i.e. dual-TCR) could be rescued by immunization through a second TCR specific for a foreign antigen. These data revealed that T cell tolerance was regulated at the level of the self-reactive TCR. Here, dual-TCR CD8⁺ T cells were used to examine if exhaustion during persistent viral infection could be rescued by an analogous strategy of immunization through a second TCR not involved in recognition of virus. In direct contrast to the rescue achievable in tolerant CD8⁺ T cells, exhausted T cells were equally impaired through both TCR. These findings suggest that exhaustion is maintained by defects downstream of the virus-specific TCR, and establish that exhaustion and tolerance are distinctly regulated states of T cell dysfunction.

Keywords

CD8⁺ T cells; Dual-T cell Receptor; Chronic Viral Infection; Exhaustion; Tolerance

INTRODUCTION

Maintenance of effective virus-specific CD8⁺ T cells is vital for the control and elimination of established viral infections. However, during many persistent or chronic infections, responding CD8⁺ cytotoxic T lymphocytes (CTL) become deleted or acquire functional defects in a process that has been defined as exhaustion. Exhausted T cells exhibit compromised proliferation and progressively lose the ability to produce effector molecules, like interleukin-2 (IL-2), tumor necrosis factor alpha (TNF- α), and interferon gamma (IFN-

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CONFLICT OF INTEREST

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γ) (1–4). T cell exhaustion has been described in patients chronically infected with human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV), and this loss of CTL immunity contributes to disease progression and limits the responses that may be achievable by therapeutic interventions (4–9).

Mice infected with lymphocytic choriomeningitis virus (LCMV) Clone 13 represent a well-characterized animal model of persistent viral infection that recapitulates many of the T cell defects seen in chronically infected humans (10–11). This model has been used to define the mechanisms of T cell exhaustion, identifying major roles for regulatory T cells (12), immunosuppressive cytokines (such as IL-10 and TGF- β) (13–14), and negative regulatory receptors such as PD-1 and LAG-3, among others (15). Therapeutic strategies aimed at overcoming these mechanisms, such as depletion of regulatory T cells, and blocking ligation of suppressive cytokines and negative regulatory proteins with their receptors have all proven effective strategies to improve anti-viral immune responses (6, 12–13, 15–20).

Several of the cellular defects associated with T cell tolerance have been attributed to regulation by similar mechanisms as those identified for exhaustion (21–24), and thus, similar therapeutic approaches have proven beneficial in these distinct environments (25–29). As achieved for exhausted T cells responding to persistent infection, inhibiting ligation of PD-1 and LAG-3 within a tolerizing environment also restores proliferation and effector cytokine defects characteristic of tolerant T cells (23, 27–28, 30–31). In an alternative strategy, we previously demonstrated that the function of tolerant self-reactive CD8⁺ T cells can be rescued by immunization through a second TCR (i.e. dual-TCR) specific for foreign-antigen (32). These latter studies redefined the mechanisms of T cell tolerance to self-antigen by demonstrating that dysfunction was controlled at the level of the self-reactive TCR, and further, elucidated a novel strategy for improving responses of tolerant T cells. Collectively, these observations suggest that bypassing the tolerizing self-reactive TCR (via a second TCR) is sufficient to overcome multiple suppressive influences, including negative regulatory receptor overexpression, associated with a tolerant phenotype.

A provocative question that arises in light of these results is whether responses by exhausted CD8⁺ T cells may also be enhanced via immunization through a TCR complex that is not continually engaged by persistent antigen. Here, we utilized CD8⁺ dual-TCR T cells to evaluate if T cell exhaustion during chronic virus infection is regulated at the TCR complex engaging persistent viral antigen, or rather by unique defects that distinguish exhausted and tolerant T cells. Our results demonstrated that stimulation via a secondary TCR was not able to rescue effector function or proliferation of exhausted T cells. Thus, unlike tolerance to self-antigen, which is governed locally at the self-reactive TCR, exhaustion during persistent viral infection represents a more global cellular defect. Our results identify an important difference in the way functional inactivation of CD8⁺ T cells is maintained under tolerizing versus exhausting conditions with implications for therapeutic interventions in these distinct immune modulatory environments.

RESULTS

CD8⁺ dual-TCR T cells express TCR reactive for LCMV and FMuLV epitopes

Peripheral CD8⁺ T cells from transgenic P14xTCR^{Gag} mice express two distinct TCR; one D^b-restricted P14 TCR specific for the KAVYNFATM epitope of lymphocytic choriomeningitis virus (LCMV) Gp33 protein, and a second D^b-restricted TCR specific for a Friend murine leukemia virus (FMuLV) Gag protein CCLCLTVFL epitope. Our previous studies using antibodies directed against the TCR $\alpha\beta$ chains suggested comparable cell surface expression of both receptors (32), which was confirmed here using a Gag H-2D^b tetramer in combination with a Gp33 H-2D^b tetramer (Fig. 1a). Dual-TCR expressing T cells

(P14xTCR^{Gag}) expressed less of each individual TCR when compared to single receptor-bearing T cells (P14 or TCR^{Gag}), likely the result of competition for surface expression among the two TCR (Fig. 1a). While about 45% of peripheral CD8⁺ T cells in P14xTCR^{Gag} mice co-expressed both receptors, a substantial proportion of CD8⁺ T cells from these same mice bound only the Gp33 H-2D^b tetramer (13.8%) or the Gag H-2D^b tetramer (26.8%) suggesting single TCR expression. To increase the frequency of dual-TCR expressing T cells, endogenous TCR $\alpha\beta$ gene rearrangement was prevented by crossing P14xTCR^{Gag} mice onto a *rag1*^{-/-} background. Although single TCR-bearing T cells were still present in these *rag1*^{-/-} P14xTCR^{Gag} mice, the frequency of dual-TCR T cells was elevated to more than 90% of peripheral CD8⁺ T cells (Fig. 1b). It was not practical to sort large numbers of T cells by tetramer staining, but antibodies against the V α chains from both TCR were used and routinely resulted in a 99% pure population of CD8⁺ T cells that co-expressed both V α 2 and V α 3 (Fig. 1c). These highly purified cells were used in all further studies described here, and henceforth are referred to as dual-TCR T cells.

CD8⁺ dual-TCR T cells respond similarly to immunization through either TCR

To examine *in vivo* responses by these cells following stimulation through each receptor independently, congenic CD90.1⁺ dual-TCR T cells were transferred into normal C57BL/6 (B6) recipients, which were subsequently infected with an ActA-deficient *Listeria monocytogenes* (Lm) control strain, or with an essentially identical strain engineered to express either Gp33 or Gag antigen. Four days after infection, splenocytes from these recipient mice were examined for the frequency of transferred dual-TCR T cells by flow cytometry. In the absence of antigen, a small but detectable population (0.07%) of undivided CD90.1⁺ T cells was observed in mice infected with Lm-control (Fig. 1d). In contrast, dual-TCR T cells proliferated and expanded more than 20-fold in response to immunization with either Lm-Gp33 or Lm-Gag, and these responses were profoundly similar regardless of the TCR engaged. It was not possible to assess tetramer staining on sorted dual-TCR T cells prior to transfer into mice due to interference by the TCR α chain antibodies used during sorting. However, such analysis was possible after 4 days *in vivo*, and demonstrated that a large percentage (>92%) of transferred CD8⁺ CD90.1⁺ T cells were indeed dual-TCR⁺ and maintained relatively high expression levels of both TCR (>86%) even after antigen stimulation (Fig. 1e).

While defects in effector cytokine production provide an early indication of impaired CD8⁺ T cell function during a persistent infection, such defects are not predicted during acute infection with an immunogenic and rapidly cleared pathogen like ActA-deficient *Listeria*. Here, effector cytokine production by dual-TCR T cells was examined on days 4 and 7 after transfer into mice acutely infected with attenuated Lm, as just described. Dual-TCR T cells immunized with either Lm-Gp33 or Lm-Gag were capable of mounting robust cytokine responses (defined as co-production of IFN- γ and TNF- α) following *ex vivo* restimulation with Gp33 or Gag peptide-pulsed antigen presenting cells (APC) (Fig. 1f-g and Supplemental Fig S1), but not control peptide (Ova). These data indicated that (i) dual-TCR T cells could be activated equivalently through either receptor *in vivo*, (ii) that both TCR were capable of inducing the differentiation of effector CD8⁺ T cells, and (iii) that both TCR were capable of responding to a secondary antigen encounter independent of the TCR engaged during primary antigen stimulation.

Activation through a secondary TCR is not sufficient to rescue exhausted CD8⁺ T cells

To directly compare T cell responses during acute and persistent viral infections, dual-TCR T cells were transferred into B6 mice infected with LCMV Armstrong (acute infection) or LCMV Clone 13 (persistent infection). One week after infection, transferred T cells had expanded to a similar frequency in response to either virus, and displayed an equivalent

activation phenotype with enhanced surface expression of CD44 and down-regulation of the lymphoid homing molecule CD62L (Fig. 2a). Even at this relatively early time point after infection, T cells responding either to Armstrong or Clone 13 displayed somewhat distinct surface expression of the negative regulatory receptors PD-1 and LAG-3. While expression of these markers was increased in response to both LCMV infections compared to naive cells, PD-1 and LAG-3 were higher on T cells from Clone 13 infected mice (Fig. 2b). This elevated expression corresponded with T cell dysfunction, as IFN- γ and TNF- α production were uniformly diminished in T cells from mice infected with Clone 13 compared with those from Armstrong infected mice (Fig. 2c and 2d). However, there was no obvious distinction between *ex vivo* restimulation with Gp33 or Gag peptide in either group, suggesting that immunization through the second TCR (Gag-specific) was not sufficient to induce more robust responses by dual-TCR T cells persistently encountering viral antigen through the Gp33-specific TCR. It should be noted that the failure of Gag-specific TCR stimulation to improve responses by exhausted dual-TCR T cells was not due to cross-reactivity (i.e. cross-exhaustion) with LCMV, as no LCMV-specific responses were observed in single-TCR⁺ Gag-specific T cells (Supplemental Fig S2).

To examine the function of exhausted T cells, dual-TCR T cells were analyzed 30 days after infection with LCMV. At this later time point, deletion of virus-specific dual-TCR T cells was evident in response to persistent Clone 13 infection, as the frequency of transferred CD90.1⁺ cells was routinely less than 10% of that observed in recipients acutely infected with LCMV Armstrong (Fig. 3a), despite similar frequencies at day 7. The majority (>78%) of transferred T cells remaining in persistently infected hosts were still dual-TCR positive as assessed by tetramer staining (Fig. 3a). Consistent with an exhausted phenotype, dual-TCR T cells in Clone 13 infected mice were distinctly positive for PD-1 and LAG-3 surface expression, whereas the same negative regulatory receptors were no longer observed on T cells from acutely infected mice at day 30 (Fig. 3b).

Our prior work demonstrated that CD8⁺ dual-TCR T cells rendered tolerant by encounter with self-antigen, while unresponsive to *ex vivo* restimulation by the same peptide antigen, could be rescued by activation through a virus-specific TCR that had not been engaged by tolerizing self-antigen (32). To determine if a similar strategy could be employed to rescue T cells from exhaustion, dual-TCR T cells were obtained from acutely and persistently infected recipients 30 days post-infection and restimulated directly *ex vivo*. T cells from acutely infected mice demonstrated vigorous production of IFN- γ (>68% positive cells) and co-production of IFN- γ and TNF- α (>38% positive cells), characteristic of healthy primed CD8⁺ effector T cells regardless of the TCR engaged during re-stimulation (Fig. 3c). In contrast, restimulation of T cells isolated from persistently infected mice with LCMV-derived Gp33 peptide induced a lower percentage of IFN- γ producing cells (45.1%), and even fewer that co-produced IFN- γ and TNF- α (5.1%) when compared to T cells from acutely infected mice. However, stimulation through the secondary TCR (here with Gag peptide) failed to rescue function of these exhausted T cells despite this receptor having no involvement in recognition of the persistent virus (Fig. 3c and 3d). Attempts to induce expansion of exhausted dual-TCR T cells *in vivo* with immunogenic Lm-Gp33 or -Gag vaccination or transfer of Gp33 or Gag peptide-pulsed APC also failed to generate disparate responses through the two TCR complexes (data not shown).

Blocking ligation of PD-1 and LAG-3 using antagonistic antibodies rescues exhausted CD8⁺ T cells *in vivo* (15), providing a window of time when otherwise dysfunctional T cells may respond to antigen immunization. To determine if such rescued T cells displayed any differential function through the Gp33 and Gag-specific TCR complexes, persistently infected recipients were administered blockade antibodies specific for PD-1 and LAG-3 starting on day 21 after T cell transfer and infection with Clone 13. Compared to control

antibody, blockade treatment resulted in a high frequency of polyfunctional T cells producing both IFN- γ and TNF- α after *ex vivo* restimulation (Fig. 4b and 4c). Again though, similar responses to antigen were observed following restimulation through either TCR, and this was not altered if T cells were exhausted or rescued. Thus, rescue of T cell exhaustion restores responsiveness at all expressed TCR, and collectively these data support a model wherein global cellular changes govern T cell exhaustion and functional rescue.

DISCUSSION

CD8⁺ T cell dysfunction following encounter with either tolerizing self-antigen (anergy) or chronic viral infection (exhaustion) is manifest by attenuated proliferative and effector responses to subsequent encounter with the same antigen (2–4, 32–35). In addition to similar functional impairments, many of the suppressive immune mechanisms restraining CD8⁺ T cell responses are conserved in both exhausted and tolerant T cells. Thus, approaches to enhance CD8⁺ T cell function in one context often prove to be of value in the other (13, 15–19, 24–25, 29, 36–37).

Our prior work demonstrated that expression of a second TCR specific for a foreign antigen on CD8⁺ T cells rendered anergic by encounter with tolerizing tumor/self-antigen could be exploited to immunize these cells, suggesting that tolerance was maintained at least in part by defects at the self-reactive TCR which left the other non-engaged TCR still functional (32). Subsequent work in our lab and others has further established that inhibition of overexpressed negative regulatory receptors like CTLA-4, PD-1 and LAG-3 can also restore function in tolerant T cells [our unpublished data and (23, 27–28, 30–31)]. Thus, tolerance is maintained by a constellation of changes which includes (but is not limited to) proximal defects at the self-reactive TCR continually engaged by antigen, and overexpression of negative regulatory receptors. Moreover, immunization through a second TCR is capable of overcoming these and other immune inhibitory mechanisms, rescuing tolerant T cell responses.

Overexpressed negative regulatory receptors are also known to influence exhausted T cell function. Blocking ligation of these molecules can improve T cell responses (6, 15, 17–18); however, a role for proximal TCR regulation has not yet been defined for exhausted T cells. Our current work compared the function of distinct TCR complexes expressed on exhausted dual-TCR T cells during persistent virus infection. Our data show that exhausted T cells are equally attenuated in response to stimulation through either TCR despite the fact that the secondary TCR remains unengaged by persistent viral antigen, and therefore suggest that dysfunction is not a consequence of defects at the virus-specific TCR. This is in agreement with a recent study demonstrating that PMA/Ionomycin stimulation (which bypasses TCR proximal signaling) likewise fails to rescue cytokine defects in exhausted LCMV-specific CD8⁺ T cells (38). Because stimulation through the second TCR was insufficient to overcome cytokine and proliferative defects characteristic of exhausted T cells in our work, it was presumed that viral control was also not being improved by vaccination through a second TCR; however, we do not directly exclude this possibility here. While vaccination through a second TCR proved insufficient to rescue exhausted CD8⁺ T cell function, blockade of the negative regulatory molecules, PD-1 and LAG-3, was able to improve cytokine responses to immunization at both receptors. These results are also consistent with a model wherein cellular changes downstream of the virus-specific TCR govern T cell exhaustion and functional rescue.

While not anticipated, the disparate responses achievable for tolerant and exhausted T cells are not entirely surprising either. Tolerance and exhaustion represent distinct T cell fates that are induced in unique environments. Whereas tolerance occurs when self-reactive T cells

fail to receive adequate co-stimulation upon recognition of cognate antigen, exhaustion occurs via progressive functional inactivation of fully differentiated cells persistently engaging viral antigen. It is conceivable that the unique microenvironments from whence exhausted and tolerant T cells emerge program molecular changes in these cells which poise them for differential responsiveness to select therapeutic strategies. Indeed, several recent studies have called attention to the discrete epigenetic and transcriptional regulatory events associated with these different CD8⁺ T cell fates (39–41). Moreover, a disparate response to therapy despite gross mechanistic similarity is not unprecedented in tolerant and exhausted T cells. For example, CTLA-4 is upregulated on both exhausted and tolerant T cells, yet CTLA-4 blockade as a therapeutic modality has shown promise for tolerant (42–43), but not exhausted T cells (17, 44). Thus, successful approaches to enhance CD8⁺ T cell immunity in the context of tolerance must be evaluated independently for merit in the setting of T cell exhaustion.

In contrast to the rescue achievable in tolerant CD8⁺ T cells, our work establishes that immunization through a second receptor is not a viable strategy for improving responses of exhausted CD8⁺ T cells. By demonstrating that the mechanisms of exhaustion render T cells defective in response to global TCR engagement, not just responses through the virus-specific TCR complex, our results expand upon our current understanding of the different mechanisms regulating dysfunction in exhausted and tolerant T cells, and reveal a novel and fundamental distinction between these two CD8⁺ T cell fates which holds important implications for future therapeutic approaches to overcome T cell exhaustion during chronic infections.

METHODS

Mice

C57BL/6 (B6) mice were purchased from Jackson Labs (Bar Harbor MA). P14 mice, TCR^{Gag} mice, and P14xTCR^{Gag} mice have been previously described (32–33). *RagI*^{-/-} P14xTCR^{Gag} mice were produced in house by crossing *ragI*^{-/-} P14 and *ragI*^{-/-} TCR^{Gag} mice. All mice were maintained in a specific-pathogen free environment and used in accordance with the guidelines of the Department of Comparative Medicine at Saint Louis University School of Medicine.

Adoptive transfers and infections

Cells from spleen and lymph node of *ragI*^{-/-} P14xTCR^{Gag} transgenic mice were stained for CD8, Vα2, and Vα3, and triple-positive cells purified by sorting on a FACSaria III (Bectin Dickinson). Sorted CD8⁺CD90.1⁺ T cells were adoptively transferred i.v. into 6–8 week-old CD90.2⁺ B6 recipients. For *Listeria* infections, 1×10⁵ sorted T cells were transferred, and recipients immunized i.v. with 2×10⁶ CFU recombinant Act-A deficient Lm expressing Gag, Gp33 or Ova on the day of cell transfer. For LCMV infections, 2×10³ sorted T cells were transferred and recipients infected one day later with either 2×10⁵ PFU LCMV Armstrong i.p. or 2×10⁶ PFU LCMV Clone 13 i.v., as previously described (39).

Ex vivo T cell stimulation and analysis

Splenocytes were harvested from recipient mice and simulated with peptide-pulsed APC for 12 hours in a 24-well plate in the presence of 1μg/ml Golgi Plug (BD Biosciences). To generate peptide-pulsed APC, B6 splenocytes were pulsed with 10μg/ml LCMV-derived Gp33–41 (KAVYNFATM), FMuLV-derived Gag (CCLCLTVFL), or chicken ovalbumin-derived (SIINFEKL) peptide (Pi proteomics) for 2 hours, and then excess peptide was washed away. After stimulation, responding T cells were stained for surface and intracellular proteins with antibodies purchased from eBiosciences (CD8, PD-1, LAG-3), or

BD Pharminogen (IFN- γ , TNF- α , CD44, CD62L) and using the BD Bioscience Cytotfix/CytoPerm protocol. After staining, cells were fixed in 4% formaldehyde and analyzed by flow cytometry on a FACS CantoII or LSRII (Bectin Dickenson). Tetramer staining was performed with Gp33- or Gag- peptide loaded MHC Class I tetramers (Fred Hutchison Cancer Research Center, Seattle WA).

Blockade Treatment

Anti-PD-1 (10F.9G2) was purchased from BioXCell and anti-LAG-3 was purified from supernatant of C9B7W hybridoma cells (provided by Dr. Dario Vignali, St. Jude Children's Research Hospital, Memphis TN). Twenty-one days after LCMV Clone 13 infection, blockade therapy (200 μ g α -PD-1 and 200 μ g α -LAG-3) was initiated and provided every third day for two weeks by i.p. injection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Dual-TCR CD8⁺ T cells respond similarly to immunization through either TCR. **a** Splenocytes from B6, P14, TCR^{Gag}, and P14xTCR^{Gag} transgenic mice were stained with Gag H-2Db and Gp33 H-2Db tetramers. Numbers indicate the percent of CD8⁺ tetramer-positive T cells within the inscribed regions. **b** Splenocytes from *rag1*^{-/-} P14xTCR^{Gag} transgenic mice were stained with Gag H-2Db and Gp33 H-2Db tetramers. Numbers indicate percent of CD8⁺ tetramer-positive T cells in each quadrant. **c** Vα2⁺ Vα3⁺ cells were FACS-purified from *rag1*^{-/-} P14xTCR^{Gag} donors for adoptive transfer. Numbers indicate the percent of gated CD8⁺CD44⁻ T cells within the inscribed region. **d** Sorted dual-TCR T cells (CD90.1⁺) stained with efluor 670 proliferation dye were adoptively transferred into B6 recipients (CD90.2⁺) immunized with Lm-control, Lm-Gp33, or Lm-Gag. Four days after immunization, the frequency of dual-TCR T cells in spleen and efluor 670 dilution were used to measure proliferation. **e** T cells were stained with H-2Db Gag and Gp33 tetramers to assess maintenance of dual-TCR expression post-immunization. Numbers indicate the percent of gated CD8⁺ CD90.1⁺ dual-TCR T cells (black dots) binding H-2Db Gag and Gp33 tetramers overlaid onto endogenous CD8⁺ T cells (grey dots) for comparison. **f** IFN-γ and TNF-α production by dual-TCR T cells from **d** after *ex vivo* restimulation with Gp33, Gag or Ova peptide-pulsed APC. Percent of gated CD8⁺CD90.1⁺ cells is depicted in each quadrant. **g** Graph shows pooled data from 5

separate experiments with a total of 10–15 mice per experimental group, and displays the percent of CD8+CD90.1+cells that co-produce IFN- γ and TNF- α following the indicated *in vivo* immunization and *ex vivo* restimulation. Error bars represent standard deviation.

Fig. 2.

Immunization through either receptor elicits similar cytokine responses by dual-TCR T cells from LCMV Clone 13 infected hosts seven days after infection. **a** CD8⁺CD90.1⁺ dual-TCR T cells were adoptively transferred into healthy B6 mice (CD90.2⁺), and infected one day later with LCMV Armstrong or Clone 13. One week after infection, expansion and activation status of CD90.1⁺CD8⁺ dual-TCR T cells was assessed by flow cytometry. Numbers represent the percent of total splenocytes in the gated region (upper panels) or percent of gated CD8⁺CD90.1⁺ dual-TCR T cells in each quadrant (lower panels). **b** Surface expression (mean fluorescence intensity, MFI) of PD-1 and LAG-3 on CD8⁺ dual-TCR T cells from LCMV Armstrong and Clone-13 infected mice seven days after infection. Surface expression of PD-1 and LAG-3 on naïve cells was determined by gating on endogenous CD90.1-negative CD8⁺CD44^{lo} T cells from Armstrong infected mice. **c** IFN- γ and TNF- α production by dual-TCR T cells in **a** after *ex vivo* restimulation with Gp33, Gag, or Ova peptide-pulsed APC. **d** Graph shows pooled data from 2 independent experiments, and displays the percent of CD8⁺CD90.1⁺ cells that co-produce IFN- γ and TNF- α following *ex vivo* restimulation. Error bars represent standard deviation.

Fig. 3.

Immunization through either receptor fails to rescue responsiveness of exhausted CD8+ dual-TCR T cells. **a** Dual-TCR T cells (CD90.1+) were transferred into B6 mice (CD90.2+) infected one day later with LCMV Armstrong or Clone 13. One month after infection, expansion of dual-TCR T cells and maintenance of dual-TCR expression were measured. Numbers represent the percent of total splenocytes in the gated region (upper panels) or percent of gated CD8+ CD90.1+ dual-TCR T cells (black dots) binding H-2Db Gag and Gp33 tetramers overlaid onto endogenous CD8+ T cells (grey dots) for comparison (lower panels). **b** Surface expression of PD-1 and LAG-3 (MFI) on dual-TCR T cells 30 days after infection. **c** IFN-γ and TNF-α production by dual-TCR T cells in **a** after *ex vivo* restimulation with Gp33, Gag, or Ova peptide-pulsed APC. Numbers represent the percent of total splenocytes within the inscribed square region or the percent of gated CD8+CD90.1+ cells in each quadrant. **d** Graph displays pooled data from 5 separate experiments showing the percent of transferred CD8+CD90.1+ T cells co-expressing both IFN-γ and TNF-α. Error bars represent standard deviation. Data represent 5 experiments with 6–9 mice per experiment.

Fig. 4.

Rescue of T cell exhaustion by blocking ligation of PD-1 and LAG-3 restores responses through either TCR. **a** Protocol for T cell transfer, infection and receptor blockade in **b-c**: Dual-TCR T cells (CD90.1⁺) were transferred into B6 mice (CD90.2⁺) infected one day later with LCMV Clone 13. Beginning 21 days after infection, anti-PD-1 and anti-LAG-3 were administered every 3 days for 2 weeks. On day 35 post-infection, splenocytes were restimulated *ex vivo* with Gp33, Gag, or Ova peptide-pulsed APC. **b** IFN- γ and TNF- α production by dual-TCR T cells after *ex vivo* restimulation. Numbers represent the percent of total splenocytes within the inscribed square region or the percent of gated CD8⁺CD90.1⁺ cells in each quadrant. **c** Graph displays pooled data from 3 separate experiments showing the percent of transferred CD90.1⁺ T cells co-expressing both IFN- γ and TNF- α . Error bars represent standard deviation. Data represent 3 experiments with 6–10 mice per experiment.