Exhausted CD8^+ T cells exhibit low and strongly inhibited TCR signaling during chronic LCMV infection

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Chronic viral infections are often associated with impaired CD8^+ T cell function, referred to as exhaustion. Although the molecular and cellular circuits involved in CD8^+ T cell exhaustion are well defined, with sustained presence of antigen being one important parameter, how much T cell receptor (TCR) signaling is actually ongoing in vivo during established chronic infection is unclear. Here, we characterize the in vivo TCR signaling of virus-specific exhausted CD8^+ T cells in a mouse model, leveraging TCR signaling reporter mice in combination with transcriptomics. In vivo signaling in exhausted cells is low, in contrast to their in vitro signaling potential, and despite antigen being abundantly present. Both checkpoint blockade and adoptive transfer of naive target cells increase TCR signaling, demonstrating that engagement of co-inhibitory receptors curtails CD8^+ T cell signaling and function in vivo.
CMV (lymphocytic choriomeningitis virus) infection in mice is a well-established model for chronic infection, leading to T-cell exhaustion following chronic infection in mice. Compared to effector CD8 T cells generated following an acute infection, exhausted CD8 T cells have impaired effector functions and are transcriptionally different. An important feature of exhausted CD8 T cells is the co-expression of multiple co-inhibitory receptors (such as PD-1, CTLA-4, LAG-3, TIM-3, CD39, TIM-3), which dampen T-cell activation by various mechanisms. These include limiting co-stimulation by receptor competition (CTLA-4, TIGIT), direct inhibition of signal transduction downstream of T-cell receptor (TCR) engagement by limiting the phosphorylation of signaling molecules, such as CD3, ZAP70, and PCK (PD-1, TIM-3), restraining metabolic changes, changes in expression of key co-inhibitory receptors, are very efficient in improving CD8 T-cell numbers and effector function of exhausted CD8 T cells in both cancer and chronic infections. The phenotypic and functional landscape of exhausted cells is very diverse, translating into differential responsiveness to checkpoint inhibition. A specific subset of non-terminally exhausted cells, termed memory-like and characterized by expression of TCF1 and SLAMF6, was shown to replenish the pool of terminally exhausted cells and to respond to checkpoint blockade by proliferation and differentiation into more effector-like, and eventually terminally exhausted cells. Despite the compelling evidence that CD8 T-cell function is impaired in chronic LCMV infection and that continued exposure to antigen significantly contributes to exhaustion, there is little insight into how much TCR signaling is actually ongoing in exhausted CD8 T cells in vivo during established chronic infection.

Here, we characterize in vivo TCR signaling in virus-specific CD8 T cells in the setting of chronic LCMV infection. We use virus-specific TCR transgenic CD8 T cells expressing the Nr4a1-GFP reporter as proxy for TCR signaling in adoptive transfer experiments. Despite abundant availability of antigen in the form of peptide-MHC class I complexes, there is very limited TCR signaling ongoing in exhausted CD8 T cells during chronic infection, evidenced by low expression of the GFP reporter and by RNAseq analysis of TCR signaling-associated genes. We observe enhanced TCR signaling after in vivo blocking of PD-1/PD-L1 interaction or in vivo exposure of exhausted CD8 T cells to antigen on naive target cells, which have not been exposed to the inflammatory milieu of chronic infection. This observation suggests that the engagement of co-inhibitory receptors, such as PD-1, exerts a pronounced inhibition of TCR signaling in vivo.

Results

RNAseq indicates low TCR signaling in chronic infection. Previous studies showed that, compared to classical effector CD8 T cells formed in acute infection, exhausted virus-specific CD8 T cells are transcriptionally distinct and fail to exert several effector functions, such as production of the inflammatory cytokines IFN-γ and TNF-α. To test whether this defect is due to intrinsically impaired TCR signaling, we set out to investigate whether ex vivo restimulation of these cells in absence of the chronic environment leads to upregulation of new transcriptional modules. CD45.1+ TCR-transgenic P14 CD8 T cells recognizing the LCMV-derived gp33-41 peptide were adoptively transferred into CD45.2+ hosts one day prior infection with a low (200 flu) or high (2 x 10^6 flu) dose of LCMV clone 13 to induce an acute or chronic infection. Two weeks later, P14 cells were isolated from the spleens and restimulated in vitro with plate-bound anti-CD3 and anti-CD28 antibodies. As described previously, cells isolated from chronic infection have a distinct transcriptional profile compared to the ones isolated from an acute infection (Fig. 1a). Upon in vitro restimulation in presence anti-CD3 and anti-CD28 antibodies, there were significant changes in CD8 T cells isolated from both infection conditions, especially regarding inhibitory receptor genes, such as Pdcd1 (encoding PD-1) and Cd160, and cytokine production-related transcripts like Ifng, Trif, Il10, and Xcl1, demonstrating that exhausted CD8 T cells are not inherently unable to transmit TCR signals into a transcriptional output. In fact, 429 genes showed similar changes in expression in both CD8 T cells isolated from acute infection and from chronic infection. Surprisingly, only few genes (35) were differentially expressed in exhausted cells when compared to effector/memory cells after stimulation (Fig. 1b), most of which include inhibitory receptor genes that were already found at higher levels before stimulation and were, therefore, not increased as strongly as in effector/memory cells. Moreover, genes involved in TCR signaling, such as Nr4a1 and TCR-induced genes Ifng and Trif had a low expression in chronic infection ex vivo, which increased after antibody stimulation, suggesting that the cells were either not properly activated and/or strongly inhibited in vivo.

NUR77 is downregulated rapidly in P14 cells in vivo. In order to investigate the TCR signaling in an in vivo setting, we took advantage of a transgenic mouse model, in which GFP is expressed under the control of the Nr4a1 promoter. NUR77, encoded by Nr4a1, is triggered by antigen stimulation in T cells and has been used as a proxy for TCR signaling. In vitro stimulation of transgenic CD8 T cells labeled with a cell proliferation dye (CPD) with plate-bound anti-CD3 and anti-CD28 antibodies for 20 h led to fast upregulation of GFP. After the stimulus was removed, the signal was rapidly lost: after approximately 50 h, the GFP levels were similar to the ones observed in naive cells (Supplementary Fig. 1a). Moreover, GFP was down-regulated before cells started dividing (29 h after stimulation), suggesting that the initial drop in the observed signal was due to transcriptional regulation and protein degradation, but not proliferation-related dilution. We further sought to characterize the TCR signaling in vivo in chronic LCMV infection. Nr4a1-GFP mice were crossed to the P14 strain to generate P14-Nr4a1-GFP mice. CD45.1+ P14-Nr4a1-GFP CD8 T cells were adoptively transferred into wild-type CD45.2+ C57BL/6j mice 1 day prior infection with a high dose of 2 x 10^6 flu LCMV clone 13. Mice were sacrificed at different time points after infection to investigate TCR signaling ex vivo by measuring GFP in P14 cells. Only 1 day after chronic infection, P14-Nr4a1-GFP cells isolated from the spleen were highly activated (Fig. 2a). Consistent with this observation, the activation marker and inhibitory receptor PD-1 was also upregulated within 24 h after infection (Fig. 2a). However, the GFP levels were rapidly downregulated in vivo already 3 days post infection and kept decreasing with time, despite the maintenance of high PD-1 levels (Fig. 2a–c). Since LCMV has a broad tropism and induces a systemic infection, P14-Nr4a1-GFP cells isolated from various infected tissues (lymph nodes, bone marrow, spleen, blood, liver, lung, and kidney) were also analyzed 3 weeks post LCMV infection. Nr4a1-GFP levels were extremely low in all screened tissues 3 weeks into chronic infection compared to in vivo primed cells 1 day after infection (Fig. 2d and Supplementary Fig. 1b–d).

In vitro stimulation of P14 cells increases TCR signaling. There is extensive work showing that exhausted CD8 T cells have a
dysfunctional transcriptional network compared to functional cells developed upon an acute infection. To test whether TCR signaling can be increased in exhausted cells, P14-Nr4a1-GFP cells were isolated 3 weeks post chronic or acute LCMV infection, sorted, and stimulated in vitro with anti-CD3 and anti-CD28 antibodies for 6 h. There was a strong upregulation of Nr4a1-GFP in cells that responded to stimulation (CD107a⁺) compared to the ones that did not (CD107a⁻) for both P14 cells isolated from acute or chronic infection (Fig. 3). However, the extent to which the Nr4a1-GFP signal could be induced in cells isolated from

Fig. 1 Transcriptional profiling of functional or exhausted P14 cells with or without restimulation. P14 cells were adoptively transferred into mice 1 day prior high or low-dose LCMV clone 13 infection. Animals were sacrificed after 14 days. CD8⁺ P14 cells were stimulated with anti-CD3 and anti-CD28 for 4 h. RNA was extracted and sequenced. a Heatmap of the 200 most variable gene profiles was generated using hierarchical clustering (k = 4). Selected genes are annotated. Heatmap colors indicate normalized gene expression ranging from high (red) to low (blue). Gene color highlights differentially expressed genes between groups indicated in b. b Number of differentially expressed genes between the conditions are indicated. The nested effect model disentangles the effect of chronic infection and stimulation and contains an interaction term for stimulation in chronic infection. Color code of gene names indicates the group of the differential expression analysis (see Methods for details). Data from one experiment are shown. Source data are provided as a Source Data File.
chronically infected mice, which also expressed high levels PD-1, was significantly lower compared to cells isolated from acutely infected mice. Similar observations were made in virus-specific cells isolated from various tissues such as LN, blood, BM, lung, liver, and kidney (Supplementary Fig. 2). Given the fact that chronic LCMV infection is characterized by high viral titers, the difference between in vivo and in vitro stimulated virus-specific exhausted CD8 T cells regarding Nr4a1-GFP signal was surprising.

Antigen is present 3 weeks into chronic LCMV infection. The in vitro data suggested that the TCR signaling was still functional to some extent, documented by increased GFP expression after restimulation of exhausted P14-Nr4a1-GFP cells. Several hypotheses could explain this difference: (i) the GFP signal is diluted in vivo due to proliferation, (ii) there is little antigen available in vivo despite abundance of infectious viral particles 3 weeks post chronic infection, and (iii) there is strong inhibition of TCR signaling in vivo. Since the proliferation rate of exhausted cells is low in vivo, we decided to focus on the last two hypotheses. To test whether antigen sensed by P14 CD8 T cells is present 3 weeks post chronic LCMV infection, naive P14-Nr4a1-GFP cells were transferred into 3 weeks chronically infected or naive mice and the GFP signal was assessed 12 h later (Fig. 4a). Nr4a1-GFP was highly upregulated in the transferred P14 cells isolated from all screened tissues, suggesting that antigen was abundantly present in mice with established chronic infection, leading to potent activation of naive gp33-41-specific CD8 T cells (Fig. 4b, c).

P14 cells are PD-1⁺, while most infected cells are PD-L1⁺. There are numerous reports documenting the role of multiple co-inhibitory receptors on exhausted CD8 T cells, such as CTLA-4, PD-1, LAG3, CD39, TIM-3, 2B4,9. PD-1 is perhaps the most important inhibitory receptor because its absence from the onset of chronic LCMV infection leads to death caused by T-cell-related immunopathology, while its blocking at later stages reinvigorates exhausted CD8 T cells. One of its ligands, PD-L1, is expressed on most infected cells (denoted by intracellular staining for the viral nucleoprotein using VL4 antibody) which are found in all screened tissues (Supplementary Fig. 3a-c). Expression of several inhibitory receptors (PD-1, CD39, TIM-3) on P14 cells was high (~50%) during chronic infection (Supplementary Fig. 3c, d); notably, PD-1 is expressed in over 90% of P14 cells isolated from various tissues. Moreover, the fraction of infected hematopoietic cells expressing PD-L1 was quite similar across tissues (Fig. 4a, c), whereas the fraction of PD-L1⁺ infected non-hematopoietic cells was higher in peripheral tissues compared to hematopoietic organs (Fig. 5).

PD-L1 blockade leads to increased TCR signaling in vivo. In order to assess the effect of PD-1/PD-L1 interaction on TCR signaling in vivo, we performed PD-L1 blockade in mice that had been chronically infected for 3 weeks. Three hours after administration of the blocking antibody, various tissues were harvested and P14 cells were analyzed (Fig. 6a). Blockade efficiency was confirmed by failure of in vitro counterstaining with
In vivo killing of naive targets enhances TCR signaling. Previous work showed that, despite being less functional than virus-specific cells generated upon an acute infection, exhausted CD8 T cells are capable of efficient in vivo killing of target cells isolated from a naive host. To corroborate that gp33-41-pulsed target cells originating from naive mice and transferred into recipients with established chronic LCMV infection leads to their killing, and test whether such recognition and killing would be associated with in vivo TCR signaling in exhausted CD8 T cells, peptide-pulsed or unpulsed naive splenocytes were transferred into chronically infected hosts 21 days post infection (Fig. 7a). Gp33-41-pulsed targets were killed very efficiently only 3 h after transfer (Fig. 7b, c and Supplementary Fig. 6a). Moreover, TCR signaling was increased in cells isolated from the recipients that received the pulsed targets compared to the control group, which received unpulsed splenocytes (Fig. 7d–f and Supplementary Fig. 6b, c). Given the fact that the virus is not cleared in vivo, the killing efficiency of target cells originating from naive mice might seem surprising. However, there are several factors, which might explain these observations. First of all, the splenocyte suspension treated mice compared to the control mice (Fig. 6e and Supplementary Fig. 4b, c). Moreover, PD-1 levels were also higher in P14 cells isolated from anti-PD-L1-treated mice compared to the control mice (Fig. 6f and Supplementary Fig. 4b, c). Since PD-1 is also an activation marker, this increase is likely a result of increased stimulation. Additional short-term co-blockade of other inhibitory receptors (LAG-3, TIM-3, CTLA-4, 2B4, TIGIT) did not significantly enhance TCR signaling compared to PD-L1 blockade (Supplementary Fig. 5), suggesting that PD-1/PD-L1 interaction is the main inhibitory signal.

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PD-1/PD-L1 axis with respect to cytotoxicity, irrespective of the exhausted subset (Supplementary Fig. 7).

Discussion

We have characterized TCR signaling in virus-specific exhausted CD8 T cells on the transcriptional level and in an in vivo setting by using theNr4a1-GFP reporter mouse in the context of chronic LCMV infection. The transcriptomic data shows that there are significant differences in virus-specific CD8 T cells from either acutely or chronically infected mice ex vivo without stimulation. These include many co-inhibitory receptors and the transcription factorTox, which has been recently shown to be specifically expressed in exhausted cells33–35. However, there are many genes upregulated after in vitro restimulation in both conditions with hardly any difference between the chronic and acute infection, indicating that even CD8 T cells from chronic infection are capable of transcriptionally responding to stimulation.

The low-TCR signaling observed on the transcriptional level was confirmed in vivo, by measuring the activity of theNr4a1promoter used as a proxy for TCR signaling24. There was a strong signal induced after initial priming, which was rapidly down-regulated in vivo. The fast decrease of the signal could be attributed, at least at this stage (1–5 days) post infection, to signal dilution due to proliferation and/or downregulation ofNr4a1-GFP. Nevertheless, in vivo data showed that the signal is rapidly downregulated before first cell division, suggesting a tight regulation ofNr4a1, consistent with its short half-life25.

Surprisingly, despite abundant antigen being present 3 weeks post infection, TCR signaling is very low in vivo. At this later stage in chronic infection, the low signal is unlikely to be due to proliferation-related dilution because exhausted CD8 T cells do not divide at a fast rate36. TCR signaling could be increased by in vitro stimulation of exhausted cells, but not to the same extent as observed in functional CD8 T cells (generated upon an acute infection). Altogether with the RNAseq data and previous studies3, this suggests that TCR signaling is strongly regulated in exhausted cells, as maximum signaling levels are not reached in vivo, despite the antigen being abundantly present. Additionally, recent work showedNr4a1transcription is not induced by NFAT alone37 and there is evidence for ERK signaling mediated AP-1 induction being involved inNr4a1transcription38.

In chronic LCMV infection, the formation of NFAT/AP-1 dimers is impaired39, implying thatNr4a1does not report the full extent of TCR signaling in this setting.

IFN-γ secretion and degranulation were also significantly lower in exhausted cells compared to functional cells (generated upon acute LCMV infection), as previously shown28,40 (Fig. 3 and Supplementary Fig. 3). Not surprisingly, exhausted virus-specific CD8 T cells co-expressed a multitude of inhibitory receptors, which dampen TCR signaling1. Indeed, both short-term PD-L1 blockade and adoptive transfer of pulsed target cells isolated from naive mice led to increasedNr4a1-GFP and PD-1 expression in vivo, linked to efficient killing of these target cells as shown previously41. The highest increase in TCR signaling was observed in P14-Nr4a1-GFP cells isolated from spleen and lungs after adoptive transfer of pulsed target cells isolated from naive mice, probably due to the nature and delivery of targets. The pulsed cells were splenocytes, mainly composed of naive lymphocytes, which are primarily in circulation and home to secondary lymphoid tissues. Additionally, due to the intravenous delivery, most targets would initially reach the lungs where there are many P14 cells30 that could kill the pulsed targets specifically, resulting in fewer pulsed targets reaching other peripheral organs. Importantly, the adoptively transferred target cells from naive mice expressed lower levels of PD-L1 compared to VL4+ LCMV-infected cells in chronically infected hosts, thus, lowering negative regulation of TCR signaling in exhausted CD8 T cells. This difference might explain why naive targets are recognized and eliminated, while most endogenous infected targets are not42. Altogether, these results suggest that TCR signaling is strongly inhibited in vivo.

Compared to PD-L1 blockade alone, short-term co-blockade of several inhibitory receptors (PD-1, LAG-3, CTLA-4, TIM-3,
TIGIT) did not show a significant increase of Nr4a1-GFP expression in exhausted LCMV-specific CD8 T cells in vivo (Supplementary Fig. 5), suggesting that the PD-1/PD-L1 axis exerts the strongest inhibition on TCR signaling. This observation is consistent with previous work, which showed that PD-1/PD-L1 interaction protects mice from CD8-mediated lethal immunopathology\(^3^0\). Several studies proved that co-blockade of PD-L1 and another inhibitory receptor had synergistic effects, showing benefits over PD-L1 blockade alone\(^9\). Moreover, in some cases, blocking an inhibitory receptor is only efficient when combined with PD-L1 blockade. For example, blocking LAG-3 in a chronic LCMV infection has no effect on the function of exhausted virus-specific CD8 T cells\(^6\), while co-blockade of PD-L1 and LAG-3 leads to their reinvigoration\(^4\). This suggests that blocking LAG-3 is only efficient when PD-1/PD-L1 interaction is inhibited. The fact that there was no increase of TCR signaling compared to single PD-L1 short-term blockade suggests that the direct inhibition of TCR signaling transduction is mainly due to the PD-1/PD-L1 axis. Nevertheless, other inhibitory receptors are able to interfere with signaling cascade downstream the TCR as well. For example, CTLA-4 can inhibit AKT\(^1^1\), while TIGIT ligation can lead to downregulation of TCR components\(^4^3\). However, unlike PD-1 whose ligand is widely expressed\(^4^4\), the ligand expression of CTLA-4 and TIGIT is restricted to APCs\(^1^0,^1^1\). It is possible that the short-term blockade did not allow for a substantial fraction of exhausted cells to interact with target cells expressing ligands of these inhibitory receptors. Our results suggest that, out of the screened blocking antibodies (PD-L1, LAG-3, CTLA-4, TIM-3, TIGIT), PD-1 is the main inhibitory receptor dampening TCR signaling. Once this axis is hampered, blocking other receptors that have complimentary inhibitory mechanisms (such as effects on proliferation, cytokine production or interference with co-stimulatory pathways\(^9\)) could lead to a synergistic effect.

In summary, exertion of effector functions of exhausted CD8 T cells is strongly and actively inhibited in vivo by potent inhibition of TCR signaling. In their absence, exhausted CD8 T cells are able to resume function and, above all, their cytotoxic potential.

**Methods**

**Bulk RNAseq.** P14 cells were sorted (BD FACS Aria, BD Biosciences) from animals with either acute or chronic LCMV infection. After 4 h of in vitro restimulation in the presence of anti-CD3 (clone 145-2C11, Biolegend) and anti-CD28 (clone 37.51, Biolegend) antibodies, RNA was extracted using TRIzol.
Gene expression was analyzed using the DEseq2 package in R. The enriched mRNA was reverse transcribed and sequenced on the Illumina platform. RNA was selected for messenger RNA (mRNA) transcripts using polyA-tail cap-trapping. One of three experiments is shown (n = 3 mice). Each dot represents an individual mouse. See also Supplementary Figs. 4 and 5. Source data are provided as a Source Data File.

Mice. Wild-type male CD45.2 C57BL/6J mice purchased from Janvier Elevation, Nr4a1-GFP mice expressing GFP under the control of the NUR77 promoter, Tcf7-GFP mice expressing GFP under the control of Tcf7 (encoding TCF1) promoter, P14 transgenic (CD45.1) mice expressing a TCR specific for LCMV clone 13, and organs (spleen, bone marrow, lymph nodes, lung, liver, and kidney) were taken from the heart or vena cava. The mice were then perfused with 20 ml PBS, and cells were harvested. All animal experiments were conducted according to the Swiss federal regulations for animal experimentation permissions 147/2014, 115/2017. Infections. In all, 104 transgenic cells (P14 or P14-Nr4a1-GFP) were adoptively transferred into naive hosts 1 day prior high-dose LCMV clone 13 infection. Twenty-one days post infection, one group received one dose of anti-PD-L1 blocking antibody intravenously. The mice were sacrificed 3 h after treatment.

Cell sorting. Spleen samples were depleted of CD4 and CD8 cells by incubating splenocytes suspensions in enrichment buffer (PBS, 1% FCS, 2 mM EDTA) with biotinylated α-CD4 and α-B220 antibodies (1/100 dilution) at room temperature for 20 min, followed by incubation with streptavidin-conjugated beads (Mojol, Biolegend) (4%) for 5 min at room temperature. Cells were then placed on a magnetic separator (StemCell) for 3 min at room temperature, followed by collection of supernatant. Enriched samples from the spleen or cell suspensions from other tissues of interest (see "Cell isolation from tissues") were stained with α-CD8 and α-Nr4a1 to sort P14 cells (ARIA cell sorter, BD Biosciences).

Flow cytometric analysis. Spleen cells were stained with antibodies specific for P14-Nr4a1-GFP cells. The stained cells were acquired on a BD FACSCanto II. The data was analyzed using FlowJo 10 software.

Cell isolation from tissues. Mice were sacrificed with carbon dioxide. Blood was taken from the heart or vena cava. The mice were then perfused with 20 ml PBS, and organs (spleen, bone marrow, lymph nodes, lung, liver, and kidney) were isolated. Lungs, liver, and kidney were cut into pieces and digested in complete RPMI (Bioconcept) (RPMI-1640 containing 10% fetal bovine serum (Omnilab), streptomycin (Gibco), 1 mM sodium pyruvate (Gibco), 20 mM HEPS (Gibco)) supplemented with 2.4 mg/ml collagenase type I (Roche Diagnostics, Rotkreuz, Switzerland) for 30 min at 37 °C. Bone marrow was flushed with a syringe containing 5 ml of complete RPMI. Spleens and lymph nodes were washed through 70 μm filter with a syringe (1 ml) plunger. Cell suspension from lungs, liver, and kidney were enriched for lymphocytes by centrifugation over Percoll density centrifugation (30% (v/v) syringe (1 ml) plunger. Cell suspension from lungs, liver, and kidney were enriched for lymphocytes by centrifugation over Percoll density centrifugation (30% (v/v) Percoll in PBS) at 4 °C (500 x g) for 30 min. Cell suspensions were filtered (70 μm) and treated with ammonium-chloride-potassium buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA in water) to lyse erythrocytes for 5 min at room temperature.

In vitro restimulations. Restimulations of sorted virus-specific P14-Nr4a1-GFP cells were performed in 96-well flat-bottom plates precoated with anti-CD3 and anti-CD28 at 1 μg/ml. Sorted cells were incubated in complete RPMI at 37 °C for 6 h at 37 °C in presence of 2 μM monensin A, 10 μg/ml brefeldin, and α-CDC107a antibody.

**Fig. 6** Increase in TCR signaling after short-term PD-L1 blockade. a P14-Nr4a1-GFP were adoptively transferred into naive hosts 1 day prior high-dose LCMV clone 13 infection. Twenty-one days post infection, one group received one dose of anti-PD-L1 blocking antibody intravenously. The mice were sacrificed 3 h after treatment. b Representative plots showing Nr4a1-GFP signal in two groups, control (ctr) and treated (α-PD-L1). c Median of frequency of Nr4a1-GFP+ cells out of P14 cells. d and e (gated on alive single CD8+ CD45.1+ CD45.1 GFP+ cells) are shown for cells isolated from the spleen. Bar plots represent mean ± SD. Asterisk (*) represents p-value = 0.05 (Mann–Whitney one-tailed-test). One of three experiments is shown (n = 3 mice). Each dot represents an individual mouse. See also Supplementary Figs. 4 and 5. Source data are provided as a Source Data File.
was performed in GraphPad Prism 8.2.0 (La Jolla, California, USA). Gating strategy is shown in Supplementary Fig. 5. In order to facilitate comparison of GFP medians between different experimental time points, GFP values were normalized by the values of a reference population (GFP median of non transgenic CD8 T cells). GFP values shown in Figs. 2c, 4c, 6d, 7e and Supplementary Figs. 1d, 5d, 6c are normalized and represent fold increase compared to the reference population.

**In vivo blocking.** One dose of blocking antibodies (200 µg of α-PD-L1 (clone 10F.9G2), α-TIM-3 (clone RMT3-23), α-LAG-3 (clone C9B7W), α-CTLA-4 (clone UC10-4F10-11), α-TIGIT (clone 1B4))69) were administered IV 3, 6, or 12 h prior sacrifice.

**In vivo killing assay.** Splenocytes were incubated at 37 °C for 1 h with or without 0.1 µg/ml gp33–41 in RPMI, labeled with cell proliferation dye (eFluor 450 or Cell Trace Yellow, Thermo Fisher) according to manufacturer’s protocols. The reference population and the target population (pulsed or unpulsed) were mixed (15 × 10^6 splenocytes per population) and transferred IV into chronically infected mice. After 3 h, the animals were sacrificed and target cell abundance was quantified by flow cytometry.

**In vitro killing assay.** CD45.1^+^ P14-Tcfl7^−^ GFP (transgenic P14 cells expressing GFP under the control of the TCF1 promoter, encoded by Tcfl7) cells were transferred into CD45.2^+^ hosts 1 day prior chronic LCMV clone 13 infection. P14 cells were sorted from organs (lungs, liver, and spleen) of chronically infected mice 21 dpi based on CX3CR1, CXCR6, and TCF1-GFP into different subsets. Target cells (EL4 cells) were incubated with IFN-γ at 100 U/ml (PeproTech) for 6 h prior pulsing to induce PD-L1 upregulation and pulsed with gp33–41 peptide at 37 °C for 1 h, then washed with medium. For one condition, PD-L1 was blocked with 30 µg/ml α-PD-L1 (clone 10F.9G2) for 1 h hour at 37 °C. Sorted populations were incubated with unpulsed or pulsed (in the presence or absence of α-PD-L1). EL4 target cells at 5:1 E:T ratio. Counting beads (CalibRITE, BD Biosciences) were added to the samples stained for flow cytometry.

**Statistical analysis.** Graphpad prism 8.2.0 software or R was used to calculate significance between the samples. p-values ≤0.05 were considered significant. Statistical test is indicated in each figure.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** RNA sequence data are deposited in the European Nucleotide Archive under accession code PRJEB38896. The flow cytometry files supporting the findings are available from the corresponding author upon request. Source data are provided with this paper.
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