A functional subset of CD8\(^+\) T cells during chronic exhaustion is defined by SIRP\(\alpha\) expression

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Prolonged exposure of CD8\(^+\) T cells to antigenic stimulation, as in chronic viral infections, leads to a state of diminished function termed exhaustion. We now demonstrate that even during exhaustion there is a subset of functional CD8\(^+\) T cells defined by surface expression of SIRP\(\alpha\), a protein not previously reported on lymphocytes. On SIRP\(\alpha\)+ CD8\(^+\) T cells, expression of co-inhibitory receptors is counterbalanced by expression of co-stimulatory receptors and it is only SIRP\(\alpha\)+ cells that actively proliferate, transcribe IFN\(\gamma\) and show cytolytic activity. Furthermore, target cells that express the ligand for SIRP\(\alpha\), CD47, are more susceptible to CD8\(^+\) T cell-killing in vivo. SIRP\(\alpha\)+ CD8\(^+\) T cells are evident in mice infected with Friend retrovirus, LCMV Clone 13, and in patients with chronic HCV infections. Furthermore, therapeutic blockade of PD-L1 to reinvigorate CD8\(^+\) T cells during chronic infection expands the cytotoxic subset of SIRP\(\alpha\)+ CD8\(^+\) T cells.
Key effectors in host immune responses to intracellular pathogens are CD8+ cytolytic T lymphocytes (CTL). CTLs become activated in a pathogen-specific manner, undergo extensive expansion, and function to locate and kill infected cells. While the destructive capacity of CTLs is essential for their activity, it also provides the potential to cause immunopathological damage. Thus the immune system has evolved multilayered mechanisms to control the duration and magnitude of CTL responses. For example, the contraction of the CD8+ T cell response is hardwired and not dependent on pathogen clearance. Thus, even in circumstances where a virus is not cleared, the CTL population nevertheless contracts. Furthermore, prolonged antigen stimulation during chronic infections causes a diminished state of T cell function known as exhaustion. Such dysfunction not only protects the host from immunopathology but also contributes to the failure to clear infections.

T cell exhaustion was first discovered in mice chronically infected with lymphocytic choriomeningitis virus (LCMV)3, but it is now known to also occur in humans chronically infected with viruses such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV). Exhausted CD8+ T cells have increased expression of co-inhibitory receptors whose breadth and level of expression have been correlated with dysfunction. While the destructive capacity of CTLs is essential for their activity, extensive expansion, and function to locate and kill infected cells. Indeed, the SIRPα- subset had high expression of inhibitory molecules, but this was counter-balanced by high expression of co-stimulatory molecules. Furthermore, the SIRPα- subset had high levels of cytotoxic granules, displayed evidence of recent cytolytic activity (CD107α+), and were more cytotoxic ex vivo than the SIRPα- subset. In vivo CTL experiments indicated that SIRPα interactions with CD47 were important for optimal cytolytic activity. Thus SIRPα marks the subset of PD-1+ CD8+ T cells that retains antiviral activity during chronic FV infection.

**Results**

**SIRPα is expressed on CD8+ T cells during LCMV infection.** To identify cell surface markers that could mark unique subsets of exhausted CD8+ T cells, an analysis of publicly available microarray data was performed on T cell receptor (TCR) transgenic LCMV-specific CD8+ T cells that had been adoptively transferred into wild-type (WT) mice infected with either the Armstrong (Arm) strain of LCMV (causes only acute infections) or the Cl13 strain (progresses to chronic infections). We identified Sirpa as a gene of interest because it showed an expression pattern similar to PD-1 over time and had sustained upregulation during Cl13 chronic infection compared to more transient expression with Arm infection (Fig. 1a, b). Twenty thousand seven hundred and seventy-six genes were analyzed for correlated expression with Pdcd1 and Sirpa ranked in the 97th percentile during acute and chronic infection (Supplementary Fig. 1). Sirpa was of special interest because it had been shown to be important in innate immunity but was not known to be expressed on CD8+ T cells or other adaptive immune cells. Furthermore, the sustained expression of Sirpa on CD8+ T cells late after infection with Cl13 suggested that it might identify an interesting subset of cells during exhaustion. Protein expression was verified by flow cytometry on LCMV-specific, transgenic CD8+ T cells at 42 days post-infection when CD8+ T cell responses to Arm would have contracted but responses to Cl13 would be largely exhausted and express PD-1. Over 90% of the transgenic CD8+ T cells remaining after Arm infection were PD-1 low and SIRPα− (Fig. 1c). In contrast, over 95% of the transgenic CD8+ T cells remaining after Cl13 infection were PD-1 high and a significant subset expressed SIRPα (Fig. 1d). The mean fluorescence intensity of SIRPα expression was significantly higher on the CD8+ T cells responding to the chronic Cl13 strain compared to acute Arm (Fig. 1e).

SIRPα upregulation during acute and chronic FV infection. To further examine SIRPα expression on CD8+ T cells during chronic infection, we analyzed naive controls (Fig. 1f) and mice infected with FV during early acute infection (7 days post-infection (dpi) (Fig. 1g), late acute infection when T cell responses peak (14 dpi) (Fig. 1h), and chronic infection (>6 wpi) (Fig. 1i) when T cells are exhausted17, FV-specific CD8+ T cells were stained with dextramers specific for the immunodominant CD8+ T cell epitope, gag3,33, and with the activation marker CD11a33 (Fig. 1f-i). Subpopulations gated for these markers as indicated by quadrants with arrows were then analyzed for the expression of PD-1 and SIRPα. Consistent with previous reports, almost all CD8+ T cells from naive mice were SIRPα− and did not stain...
Wild-type C57/BL6 mice were adoptively transferred with 1000 T cell receptor transgenic lymphocytic choriomeningitis virus (LCMV)-speciﬁc CD8+ T cells from the spleens of naive P14 mice and then infected with either LCMV Arm or CL13. The cells were then analyzed at multiple time points by flow cytometry for CD11a expression and FV-Db gagL dextramer staining. A representative flow cytometric gating strategy is shown in supplementary Fig. 6a. CD8+ gated T cells at 42 days postinfection with Arm (c) or CL13 (d) are shown. Numbers in the upper right quadrant are mean percentages of SIRPα+ cells (n = 4 Arm, n = 3 CL13). P = 0.0029 by unpaired, two-way t test. Average mean fluorescence intensity of SIRPα expression (P = 0.0088 by unpaired two-way t test) (e). CD8+ splenocytes from naive (f), 7 dpi (g), 14 dpi (h), or chronic (i) Friend virus (FV)-infected mice were analyzed by flow cytometry for CD11a expression and FV-Db gagL dextramer staining. A representative flow cytometry plot is shown. Dextramer+ CD11a+ (j, k) and dextramer− CD11a− subsets (l–o) were further analyzed for PD-1 and SIRPα expression during the course of FV infection. Arrows originate in the quadrant further analyzed and point to the analysis. The percentage in each quadrant depicts the means from eight mice at each time point, with standard deviations in parentheses. The flow cytometric gating strategy is shown in supplementary Fig. 6a–d. Not significant (ns), p > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (unpaired, two-way t tests).
with FV-specific dextramers (Fig. 1f, l). At 7 dpi, there were still very few dextramer+ cells, but by 14 dpi there was a distinct subpopulation of activated, dextramer+ cells (Fig. 1h) that expressed PD-1 and a large majority of which (mean = 72.3%) also expressed SIRPα (Fig. 1j) albeit at lower levels than macrophages (Supplementary Fig. 2). During chronic infections, dextramer+ cells were preserved (Fig. 1i), expressed PD-1 (Fig. 1k), and about one third of them also expressed SIRPα (mean = 34.5%) (Fig. 1k). Cells high in SIRPα proportion expression were generally also high in PD-1 expression (Fig. 1j, k). SIRPα was also expressed on activated (CD11a+) CD8+ T cells responding to other FV peptides (Supplementary Fig. 3). Thus SIRPα was expressed on activated CD8+ T cells during both acute (Fig. 1j) and chronic FV infection (Fig. 1k and Supplementary Fig. 3) while non-activated cells remained predominantly negative (Fig. 1l–o).

SIRPα upregulation after cell division. To examine the kinetics of SIRPα upregulation during FV infection, adoptive transfer experiments were performed using labeled, FV-specific TCR transgenic CD8+ T cells35 carrying the Thy1.1+ genetic marker. Naïve donor cells were adoptively transferred into Thy1.2+ mice that were either acutely (7 dpi) or chronically (>6 wpi) infected with FV. Such cells adoptively transferred into acutely infected mice are highly functional, whereas they rapidly become dysfunctional upon transfer into chronically infected recipients37. Three days after transfer, the donor cells were analyzed for the expression of SIRPα, PD-1, and proliferation (dilution of fluorescent signal). Pearson correlation analyses showed highly significant correlations between the expression of PD-1 and SIRPα in both acutely and chronically infected mice (Fig. 2a, e). Both PD-1 and SIRPα expression were induced during cell division (Fig. 2b, c, f, g) and the expression data were quantified for multiple mice at each cell division (Fig. 2d, h). In chronically infected mice, SIRPα expression was rapidly induced in about 20% of the transferred cells and slightly but significantly increased to about 35% (Fig. 2h), similar to the endogenous subset (Fig. 1k). By contrast, cells transferred into acutely infected mice showed increasing levels of SIRPα expression throughout all divisions (Fig. 2d). Thus donor cells from the same pool of naïve SIRPα− cells had much different levels and kinetics of SIRPα induction dependent on whether they were transferred into acutely infected or chronically infected mice.

Distinct phenotype of SIRPα+ CD8+ T cells. To determine whether the FV-specific PD-1/SIRPα double-positive CD8+ T cells from chronically infected mice comprised a subset of cells with a distinct phenotype, the expression of additional markers was examined by flow cytometry. The PD-1+ SIRPα+ CD8+ T cells from chronically infected mice also expressed high levels of the co-inhibitory receptors Tim3 and Lag3, CD95 (Fas), which leads to apoptosis upon ligand binding, and IL-2Rβ chain (CD122), which helps drive a PD-1+ phenotype36 (Fig. 3a–d). This expression pattern would suggest an exhausted phenotype except that these cells also expressed high levels of the activation/co-stimulatory molecules, CD43, CD44, CD40, and CD278 (inducible T cell co-stimulator) (Fig. 3e–h). Neither the SIRPα− cells nor the SIRPα+ subsets showed high expression of the terminal
differential marker, KLRG1, but mean expression was twice as high on the SIRPa⁺ subset as the SIRPa⁻ subset (Fig. 3). The expression of CD62L (L-selectin lymphoid homing receptor) on SIRPa⁺ CD8⁺ T cells, which is downregulated during activation but returns on memory T cells, was intermediate between SIRPa⁻ CD8⁺ T cells and naive CD8⁺ T cells (Fig. 3). SIRPa⁺ CD8⁺ T cells expressed high levels of CD47, the ligand for SIRPa, and CD278 (inducible T cell co-stimulator), (i) KLRG1, (j) CD62L, (k) CD47, and (l) CX3CR1. A representative off-set histogram overlay is displayed for each marker as well as the average geometric mean fluorescence intensity (MFI) from one experiment is given (n = 4 mice). CD8⁺ dextramer⁻ cells (non-DbgagL-specific cells from infected mice) are shown in dashed gray, PD-1⁺/SIRPa⁻ CD8⁺ dextramer⁺ cells are shown in solid line gray, and PD-1⁺/SIRPa⁺ CD8⁺ dextramer⁺ are shown in black. The vertical dashed line delineates positivity relative to the FMO control). Results are from one of three independent experiments with similar results (with n = 8 additional mice). ns, p > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (unpaired, two-way t tests). The flow cytometric gating strategy is shown in supplementary Fig. 6h-m.

**SIRPa⁺ CD8⁺ T cells have a unique transcriptional profile.** To gain a broad perspective of the differences between CD8⁺ T cells expressing SIRPa or not, whole-transcriptome shotgun sequencing (RNA-SEQ) was performed on cell-sorted populations of splenic SIRPa⁻ and SIRPa⁺ FV-specific TCR transgenic CD8⁺ T cells that had been adoptively transferred into FV chronically infected mice 2 weeks earlier. A total of 325 transcripts were differentially expressed at a significant level between the subpopulations, and 82% of the differentially expressed transcripts were upregulated in the SIRPa⁺ population (Supplementary data 1). Granzyme B (GzmB), Ki-67 (Mki67) IFN-γ (IFNG), and the inflammatory chemokines CCL3 and CCL4 were significantly upregulated in the SIRPa⁺ subset (Fig. 4, Supplementary data 1), which is consistent with these cells expressing markers of activation and co-stimulation (Fig. 3). Analysis of the top 100 most differentially upregulated genes by gene set enrichment analysis (ToppFun) revealed that the top biological process upregulated by SIRPa⁺ CD8⁺ T cells was positive regulation of the immune system followed by active proliferation (Supplementary Table 1). Intriguingly, the next highest biological process was negative immune regulation. Thus the SIRPa⁺ T cells transcribed
numerous genes capable of both immune activation and inhibitory functions with a skewing toward activation. Comparison of the genes that correlated with SIRPa expression in LCMV-specific CD8$^+$ T cells with the genes significantly upregulated in FV-specific PD1$^+$ SIRPa$^+$ CD8$^+$ T cells identified 158 genes that were shared (Supplementary data 2 and Supplementary Figure 4). The most downregulated gene in the SIRPa$^+$ subset was Perm1, an inducer of mitochondrial biogenesis and oxidative phosphorylation typically utilized by exhausted T cells, whereas effector T cells downregulate mitochondrial biogenesis in favor of the glycolytic pathway. Another highly downregulated gene was an inhibitor of T cell activation, Pik3ip1. Thus the phenotyping (Fig. 3) and transcriptional profiling (Fig. 4) results indicated that SIRPa identified a unique subset within the exhausted population of CD8$^+$ T cells that preserved effector function.

**SIRPa expression associated with in vivo effector function.** To determine whether the function of the SIRPa$^+$ subset differed from the SIRPa$^-$ subset, FV-specific (dextram larger) CD8$^+$ T cells from acutely (Fig. 5a) and chronically (Fig. 5b) infected mice were stained for intracellular expression of granzyme B and surface expression of CD107a, an indicator of recent cytolytic activity. Both the SIRPa$^+$ and SIRPa$^-$ subsets had cells expressing granzyme B (Fig. 5c–g). Importantly, almost no SIRPa$^-$ cells expressed CD107a while more than half of the SIRPa$^+$ cells were CD107a$^+$, indicating that they had recently undergone exocytosis (Fig. 5c–f, h). Similar results were found from both acutely and chronically infected mice. In acutely infected mice, a large percentage of both the SIRPa$^+$ and SIRPa$^-$ subsets had recently proliferated (Ki-67$^+$), although the proportion in the SIRPa$^+$ subset was significantly higher (Fig. 5i). In chronically infected mice, a very few SIRPa$^-$ cells were Ki-67$^+$, whereas a mean of approximately 35% of the SIRPa$^+$ subset was Ki-67$^+$ (Fig. 5i). Thus the SIRPa$^+$ subset appeared more functional in both cytolytic activity and proliferative capacity than the SIRPa$^-$ subset, confirming the transcriptional profile results provided by the RNA-SEQ analysis and ToppFun analysis (Fig. 4, Supplementary data 1, and Supplementary Tables 1).

**In vitro CTL killing by SIRPa$^+$ CD8$^+$ T cells.** A direct test of cytolytic activity was done using an in vitro killing assay to compare the SIRPa$^+$ and SIRPa$^-$ subsets. To obtain sufficient cells for the assay and to avoid stimulating sorted cells by crosslinking with dextramers, we performed adoptive transfers of genetically labeled (Thy1.1$^+$), FV-specific, TCR transgenic CD8$^+$ T cells specific for the immunodominant FV gag peptide. The cells were adoptively transferred into chronically infected recipients where they were allowed to proliferate and become exhausted for 13–15 days. They were then harvested and fluorescence-activated cell sorted into SIRPa$^-$ and SIRPa$^+$ subpopulations and co-cultured with either FV-gag peptide-loaded target cells or control cells. As expected for CD8$^+$ T cells from a chronic infection, the in vitro CTL activity was low but significantly more FV-specific killing was observed with the SIRPa$^+$ CD8$^+$ T cells than with the SIRPa$^-$ subset (Fig. 5j). For comparison, SIRPa$^+$ and SIRPa$^-$ CD8$^+$ T cell effectors taken from acutely infected mice displayed much higher killing frequencies than cells from chronic infections (Fig. 5k), but consistent with the chronic infection results, more killing was observed with the SIRPa$^+$ subset compared to the SIRPa$^-$ subset. Thus, during both acute and chronic FV infections, the expression of SIRPa correlated with enhanced cytolytic activity (Fig. 5j, k) and proliferative capacity (Figs. 4 and 5i), suggesting that SIRPa identified cells that sustained an antiviral response during chronic infection. Such a role has been associated with the transcription factor, T cell factor-1 (TCF-1) and we observed significantly higher intracellular TCF-1 expression in the SIRPa$^+$ CD8$^+$ T subset than in the SIRPa$^-$ subset (Fig. 5l, m).

**CD47$^+$ targets are more efficiently killed in vivo.** To confirm that cytolytically active CTLs were present in chronically infected mice and to ascertain whether SIRPa was playing a functional role in that activity, an in vivo CTL killing experiment was performed using viral peptide-loaded target cells that either expressed CD47, the ligand for SIRPa, or had a gene inactivation of CD47. Target cells from both WT and CD47 null genotypes, either FV peptide-loaded or control-treated, were differentially labeled with fluorescent stains (Fig. 6a), and all four types of target cells were adoptively transferred at equivalent numbers (Fig. 6b) into naive or chronically infected mice (Fig. 6c, d). Spleens were harvested 6 h after transfer and analyzed by flow cytometry for killing. CD47 null target cells were susceptible to macrophage-mediated phagocytosis regardless of loading with cognate peptide but no virus-specific killing of targets was observed in naive mice as both control targets and peptide-loaded targets remained at the starting ratio of 50:50 (Fig. 6c). In contrast, virus-specific killing was observed in chronically infected mice (Fig. 6d), which was quantified in two separate experiments. In the first experiment, four of the six chronically infected mice tested displayed CD8$^+$ CTL activity, and the virus-specific killing was significantly greater in WT targets than in CD47 null targets (Fig. 6e). In the second experiment, all 14 mice displayed CTL activity, which was again significantly greater against the WT targets compared to the CD47 null targets (Fig. 6f). Interestingly, compared to uninfected cells from a FV-infected mouse, the infected cells significantly upregulated expression of CD47 (Fig. 7). Thus SIRPa-CD47 ligation was not required for cytolysis in vivo, but it significantly enhanced cytolyis.

**CD8$^+$ T cells from human HCV patients upregulate SIRPa.** To determine whether SIRPa expression could also be found on human T cells during a chronic viral infection, CD8$^+$ T cells from healthy controls or patients with chronic HCV (Table 1) were examined using CyTOF, flow cytometry that uses heavy metal ion-tagged antibodies. In CD8$^+$ T cells from both HCV uninfected and infected patients, the main subset was SIRPa negative (Fig. 8a, b). However, in HCV patients there was a subpopulation of CD8$^+$ T cells with increased expression of SIRPa (Fig. 8a, b and Supplementary Fig. 5a, b). We analyzed CD57 and CD28 markers because chronic antigenic stimulation of human CD8$^+$ T cells is associated with the upregulation of CD57 and downregulation of costimulatory CD28. The CD57$^+$ CD28$^-$ subset is increased in HCV patients. Although this subset is heterogenous, it is generally associated with a reduced state of function and proliferation. SIRPa expression was significantly higher on CD8$^+$ T cells from HCV-infected individuals compared to controls in both the functional CD57$^+$ CD28$^-$ subset as well as the CD57$^+$ CD28$^-$ subset (Fig. 8c). Samples from one patient were also tested by flow cytometry and an example of the data and comparison with SIRPa expression on macrophages is shown (Supplementary Fig. 5c). Thus SIRPa is also expressed on human CD8$^+$ T cells and is upregulated during chronic HCV infections. SIRPa$^+$ cells from both CD57$^+$ and CD57$^-$ subtypes also had higher levels of phosphorylated signal transducer and activator of transcription factor 3 (STAT3), CD244/2B4, and HLA-DR, indicating a higher activation status compared to their SIRPa$^-$ counterparts (Fig. 8d–f). These results are consistent with SIRPa$^+$ marking a subset of functional CD8$^+$ T cells. Furthermore, stimulation of
human peripheral blood mononuclear cells (PBMCs) with anti-CD3 and anti-CD28 for 5 days led to significant upregulation of SIRPα on proliferating CD8+ T cells in comparison to unstimulated controls (Supplementary Fig. 5d, e).

Programmed cell death ligand 1 (PD-L1) blockade expands SIRPα+ CD8+ T cells. Of interest in treating chronic infections and cancer are immune checkpoint inhibitors, such as anti-PD-1 or anti-PD-L1, which can reinvigorate exhausted T cell
responses. We treated FV chronically infected mice with anti-PD-L1 and observed a significant expansion of FV-specific (Dextramer+) recently cytolytic (CD107a+) CD8+ T cells (Fig. 9a). An average of 80% of the recently cytolytic CD8+ T cells were also SIRPα+ (Fig. 9b), indicating that either the SIRPα+ subset specifically expanded or that the expanded subset of cytolytic cells upregulated SIRPα. Thus the expression of SIRPα can be used to determine whether...
immune checkpoint inhibitor therapy successfully expanded functional CD8\(^+\) T cells.

**Discussion**

Until now, SIRP\(\alpha\) has been considered to primarily be an inhibitory signaling receptor expressed predominantly on myeloid cells in the hematopoietic compartment\(^{21,48}\). The results presented here present a more complex picture, both in terms of cell-specific expression and perhaps the nature of the signaling. We confirm that SIRP\(\alpha\) has little or no expression on naive T cells, as previously shown in mice\(^{49}\), rats\(^{50}\), and humans\(^{51}\). However, we now show that SIRP\(\alpha\) is expressed on activated CTL during viral infection. Such expression may have previously been missed owing to examination of only naive cells. In addition to the expanded cell-specific expression profile, it is possible that SIRP\(\alpha\) signaling in CD8\(^+\) T cells may not be negative. During both acute and chronic FV infections, almost all CD8\(^+\) T cells that showed evidence of recent cytolytic activity (CD107a\(^+\)) were also SIRP\(\alpha\)\(^+\) (Fig. 5h). Compared to the SIRP\(\alpha\)\(^-\) subset, the SIRP\(\alpha\)\(^+\) subset was also significantly more proliferative (Ki-67\(^+\)) (Fig. 5i), expressed higher levels of TCF-1 (Fig. 5m), had higher expression of IFN-\(\gamma\) message (Fig. 4, Supplementary data 1), and transcribed significantly more genes indicative of immune activation (Fig. 4, Supplementary data 1). Furthermore, cell-sorted SIRP\(\alpha\)\(^+\) CD8\(^+\) T cells from chronically infected mice had greater in vitro cytotoxicity than the SIRP\(\alpha\)\(^-\) cells from the same mice (Fig. 5j). However, SIRP\(\alpha\) might simply mark the active CTL subset rather than positively regulating CD8\(^+\) T-cell functions. CTL targets lacking CD47 were nevertheless killed by CTL in vivo, albeit to a significantly lower level than targets expressing CD47 (Fig. 4e, f). These results indicate that SIRP\(\alpha\)-CD47 interactions are involved in the cytolytic process but do not address whether SIRP\(\alpha\) acts as a positive or negative regulator of functional CTL development. Given the high level of co-stimulatory molecules expressed by SIRP\(\alpha\)\(^+\) CD8\(^+\) T cells (Fig. 3), it is possible that negative signaling by SIRP\(\alpha\) could be overcome by positive regulatory signaling. Further studies will be required to determine how SIRP\(\alpha\) expression, specifically in CD8\(^+\) T cells, impacts development and function. What is most evident and novel from the data is that cell-specific SIRP\(\alpha\) expression is not as limited as previously thought, that it is expressed on the most proliferative and functionally active subset of CD8\(^+\) T cells in both acute and chronic infections, and that it is involved in the cytolytic process. As such, SIRP\(\alpha\) will allow scientists and clinicians to follow the expansion or contraction of the functional subset during immunotherapy with relevance not only to infections but also cancer and autoimmune diseases. We find that SIRP\(\alpha\) protein surface expression is increased not only in activated mouse T cells but also in activated human T cells, suggesting that this may be a conserved marker of active CD8\(^+\) T cells. The elevated SIRP\(\alpha\) levels on CD8\(^+\) T cells from patients with chronic HCV infection was most pronounced on the CD57\(^-\) CD28\(^+\) subset.

It is worth considering how SIRP\(\alpha\)-CD47 interactions might be involved in the cytolytic process in vivo since cognate target cells that did not express CD47 were killed less effectively than WT target cells in multiple in vivo CTL assays (Fig. 6e, f). One possibility is that SIRP\(\alpha\) delivered activating rather than inhibitory signals, which is not unprecedented. It has previously been shown that SIRP\(\alpha\) signaling in macrophages could activate the production of nitrous oxide and reactive oxygen species via Janus-activated kinase/STAT and phosphoinositide-3 kinase/Rac1/NOX/H\(\mathbf{2}\)/O\(\mathbf{2}\) pathways\(^{52}\). SIRP\(\alpha\)-mediated positive signaling in macrophages is also required for migration and chemotaxis\(^{22,53}\). In addition, T cells from SIRP\(\alpha\) mutant mice exposed to autoimmune antigens\(^{54,55}\) or flagellin\(^{56}\) showed reduced cytokine production, which was attributed to decreased numbers of DCs in the lymphoid tissues of such mice\(^{23}\). However, it may also have been an intrinsic T cell property since expression of SIRP\(\alpha\) on T cells was neither known at the time nor tested. Thus SIRP\(\alpha\) is capable of delivering activating as well as inhibitory signals depending on the context including the presence of adapter proteins such as Skap\(^{53}\) and GRB\(^{56}\) and phosphatases such as SHP1\(^{57}\) and SHP2\(^{58}\). No evidence of differential expression of Shp1 or Shp2 was found in the RNA-SEQ analysis (data not shown), but Skap2 transcription was increased 2.8-fold in the SIRP\(\alpha\)\(^+\) subset (Supplementary data 1). Interestingly, one of the most highly overexpressed genes in the SIRP\(\alpha\)\(^+\) subset (20x increase, Supplementary data 1) was Lyn. Lyn is a tyrosine protein kinase with a role in regulating cell activation, and like SIRP\(\alpha\), it has an inhibitory role in myeloid cells\(^{59}\). In B cells, Lyn phosphorylation initiates an activation cascade\(^{60}\), indicating that like SIRP\(\alpha\) it can deliver either inhibitory or activating signals in a context-dependent manner. Alternatively, it is possible that SIRP\(\alpha\)-CD47 interactions simply stabilize cell-to-cell contacts and the cytolytic synapse. The spanning distance of end-to-end bound CD47–SIRP\(\alpha\) complexes (~14 nm, similar to TCR–MHC, CD28–CD86, and CD40–CD40L) suggests that significant binding between a target and effector cell would take place predominantly in immunological synapses where abundant bulkier cell surface proteins such as CD43 and CD45 that can sterically hinder more short range interactions are redistributed outside of the cytolytic synapse\(^{61}\). The strength of the interactions between cells is influenced not only by the affinity between the receptors and ligands but also by their density. Thus the high expression of SIRP\(\alpha\) on CTL combined with upregulated CD47 on infected
targets (Fig. 7) could have a significant impact on the strength and duration of interactions within the immunological synapse as has been previously suggested62.

Prior to these experiments, it was known that virus-specific CD8+ T cells were sustained in FV chronically infected mice, albeit at low numbers (~1–3% of CD8+ T cells). It is now apparent that the FV-specific CD8+ T cells in mice with chronic FV are heterogeneous with respect to function and that there is a cytolytically active subset that can be identified by cell surface expression of SIRPα. As discussed, this active subset displays high expression of both co-stimulatory and co-inhibitory molecules (Fig. 3a–h). Immune checkpoint inhibition by anti-PD-L1 produced an expanded population of CTL, the large majority of which expressed SIRPα (Fig. 9).

**Fig. 6** Enhanced in vivo cytolytic activity against target cells expressing CD47, the ligand for signal-regulatory protein alpha. Naive and mice chronically infected with Friend virus (FV) were adoptively transferred with differentially fluorophore-labeled target cells as outlined in a and in Methods. Briefly, wild-type C57/BL6 splenocytes were differentially labeled with two concentrations of carboxyfluorescein succinimidyl ester and CD47−/− splenocytes were differentially labeled with two concentrations of CellTrace™-violet. The brighter of each subset was peptide-loaded with 25 μM FV-DbGagL peptide, while the lower intensity subset was sham-loaded in dimethyl sulfoxide media. a The gating strategy identifying the target cell populations and representative dot plots showing target cell populations (b) before injection and retrieved from (c) naive and (d) chronic recipients 6 h post-transfer. The percentages given are the means for each cell subset combining data from two independent experiments. e, f The percentage of killing comparing C57/BL6 WT and CD47−/− target cells as described in Methods. The data points showing the killing of each type of target cell within the same recipient mouse were connected with a line and the differences were statistically significant as indicated by two-way paired t tests. Data from the two independent experiments include a total of 20 mice. Virus-specific killing is defined as the percentage of killing of each population of FV peptide-pulsed cells calculated as follows: 100 - ([% peptide pulsed in infected/% un-pulsed in infected]/[% peptide pulsed in uninfected/% unpulsed in uninfected]) × 100). The flow cytometric gating strategy is shown in supplementary Fig. 6n–p.
levels of co-inhibitory receptors indicating a different phenotype than previously described for exhausted or functional cells.

We have shown that the expression of SIRPa on CD8+ T cells is diagnostic for the presence of active CTLs, even in exhausted settings. Thus SIRPa+ CD8+ T cells are interesting targets for immunotherapy, especially if they can be specifically expanded or further activated to kill chronically infected cells and/or tumors. The fact that target cells without CD47 expression were still targets for CTL killing, albeit to a lesser extent than targets with CD47 expression indicates that it is important when considering using CD47 blockade to treat cancer. CD47 is overexpressed by human cancer cells, allowing them to evade macrophage-mediated phagocytosis, and CD47 blockade has been shown to potentiate macrophage-mediated phagocytosis of tumor cells in numerous models and is currently in several clinical trials to treat various cancers. The current results suggest that, while activating macrophage antitumor phagocytosis, CD47 blockade might also inhibit CD8+ T cell antitumor activity. However, such inhibitory effects might be overcome using PD-1 blockade, a cancer immunotherapy currently thought to function primarily via CD8+ T cell activation. Interestingly, it was recently shown that, similar to tumor-infiltrating T cells, tumor-associated macrophages also express PD-1 co-inhibitory receptors. Thus cancer therapies targeting PD-1 or its ligand could be activating macrophage functions as well as T cells. A combination of PD-1 and CD47 blockade could have synergistic effects by potentiating the antitumor activity by both macrophages and T cells.

Methods

Mice, viruses, infection, and tissue harvest. For LCMV studies, female 4–6-week-old C57BL/6 J mice from NCI and Thy-1.1+ P14 TCR transgenic mice that recognize the H-2Db gp33 epitope were used where indicated. Mice were intraperitoneally (i.p.) infected with 2 × 10^9 plaque-forming units (p.f.u.) LCMV Armstrong (Arm)—which causes a acute infection—or intravenously (i.v.) with 2 × 10^6 p.f.u. LCMV Clone 13 (Cl13)—which causes a chronic infection. The use of all animals was conducted in accordance with Yale University IACUC guidelines. For LCMV studies, mice were female (C57BL/10 × A.BY) F1 (Y10) (H-2b,b, Fvβ1, Fv3β3, Fv2β3) and FV-specific Thy1.1+ C57B16/Tg mice between 12 and 24 weeks of age at the beginning of the experiments and were bred at the Rocky Mountain Laboratories. The FV stock had been passaged in mice for more than three decades and contains three separate viruses: (1) B-tropic Friend murine leukemia helper virus (F-MuLV), which is a replication competent retrovirus; (2) polyomavirus-induced spleen focus-forming virus, which is a defective retrovirus that is packaged by F-MuLV-encoded virus particles; and (3) lactate dehydrogenase-elevating virus, an endemic murine nidovirus related to coronaviruses. Mice were infected by i.v. injection of 0.2 mL phosphate-buffered saline (PBS) containing 1500 spleen focus-forming units of FV complex. Mice were treated in accordance with IACUC-approved animal use protocols following the regulations and guidelines of the Animal Care and Use Committee of the Rocky Mountain Laboratories and the National Institute of Health Office of Laboratory Animal Welfare.

LCMV Affymetrix. Affymetrix arrays from GSE41867 were obtained as CEL files, MassArray normalized using the "affy" package in Bioconductor, mapped to NCBI Entrez gene identifiers using a custom chip definition file, and converted to MGI gene symbols. Gene expression values were mean-and-log2-normalized prior to analysis.

Flow cytometry. For flow cytometric analysis, live lymphocytes were gated using a SSC-A and FSC-A gate. Cells were then gated by time to exclude artifacts caused by erratic sample flow and by FSC-H and FSC-A to exclude doublets. Gating strategies are shown in Supplementary Figure 6. The antibodies used for surface staining were: A700-anti-CD8 (53-6-7, Biolegend 600-081-82, lot E08952-1633; 1/800) or PacBlue-anti-CD8 (53-6-7, BD Pharmigen 558106, lot 38114; 1/400); fluorescein isothiocyanate (FITC)-anti-CD11a (2D7, BioLegend 101006, lot B1658666; 1/400); PE-CF594-anti-PD-1 (4D9, 1/1000; BD Pharmingen); PE-CF594-anti-CD11c (10F10, 1/1000; BD Pharmingen); PE-FITC-anti-CD107a (1D4B, BD Pharmingen 553793, lot 02482; 1/1000); phycoerythrin (PE)-anti-Tim3 (8B.2C12, Biolegend 655188, lot 1400103; 1/400); PE-anti-Lag3 (C9B7W, BD

Table 1 HCV patient characteristics

| ID | Previous IFN | Genotype | Liver transplant waitlist | Sex |
|----|--------------|----------|--------------------------|-----|
| 2  | Yes          | 1        | No                       | Male|
| 4  | Yes          | 1        | No                       | Female|
| 7  | No           | 2        | No                       | Female|
| 8  | Yes          | 2        | No                       | Female|
| 9  | Yes          | 1        | No                       | Male |
| 12 | No           | 2        | No                       | Female|
| 13 | No           | 1        | Yes                      | Female|
| 14 | Yes          | 1        | No                       | Male |
| 20 | Yes          | 1        | No                       | Male |
| 22 | Yes          | 1        | No                       | Female|
| 27 | Yes          | 1        | No                       | Male |
| 29 | Yes          | 1        | Yes                      | Male |
| 30 | No           | 4        | No                       | Female|
| 35 | No           | 1        | No                       | Female|
| 38 | Yes          | 1        | No                       | Male |

HCV hepatitis C virus, IFN interferon

expression of co-inhibitory receptors is not specific to exhausted cells and occurs during T cell activation as well. Thus it is not possible to differentiate dysfunctional T cells from activated T cells based only on the expression of co-inhibitory receptors. Recently, there has been shown to be a great deal of heterogeneity in the level of dysfunction of CD8+ T cells in an exhausted setting such as within a tumor or in a chronic infection. A detectable level of function and virus control persists in chronic viral settings as evidenced by the fact that CD8+ T cell-escape variants arise in chronic HIV infections and that viral titers increase following depletion of CD8+ T cells in simian immunodeficient virus-infected macaques. Depletion of CD8+ T cells in mice with chronic FV infections does not produce virus relapse, but this is likely due to compensatory mechanisms by antiviral CD4+ T cells and does not indicate that the residual CD8+ T cells exert no control over chronic infection. The SIRPa-positive and -negative CD8+ T cell subsets during exhaustion did not have significantly different expression levels of Thet, EOMES, CTLA4, or Bcl2 (Supplementary data 1). Although the SIRPa+ subset displayed higher levels of CD44, Ki67, TCF-1, and CD62L suggesting a functional memory phenotype, they also displayed high

Fig. 7 Uprogulation of CD47 on Friend virus (FV)-infected splenocytes. Mice were infected with FV or left naive and the splenocytes were analyzed by flow cytometry to compare the mean fluorescence intensity of CD47 on naive or FV-infected (Virus+) and uninfected (Virus−) total splenocytes at 7 days post-infection (dpi) with significance indicated by one-way analysis of variance. Virus was detected by cell surface expression of glycosylated gag antigen with mAb 34 as described in Methods. Data from one of four independent experiments are shown. ns, ****p ≤ 0.0001 (unpaired, two-way t tests)
**Fig. 8** Increased signal-regulatory protein alpha (SIRPα) expression on CD8+ T cells from hepatitis C virus (HCV)-infected patients identifies a more activated phenotype. 

(a) Representative plots of CyTOF analysis of the median healthy donor and (b) the median HCV patient donor in regards to SIRPα expression levels. CD8+ CD57− CD28+ (circles) and CD8+ CD57+ CD28− (squares) peripheral blood mononuclear cells from healthy controls (open symbols) and HCV-infected patients (closed symbols) were analyzed by CyTOF for SIRPα expression. For CyTOF analyses, fluorescence intensity data are commonly transformed to arcsinh for analysis and display.81 The SIRPα+ and SIRPα− subsets of CD8+ CD57− CD28+ (circles) and CD8+ CD57+ CD28− (squares) from HCV-infected patients were further analyzed by CyTOF for the expression of (d) phosphorylated STAT3, (e) CD244, and (f) HLADR. Median expression levels (Arcsinh transformed) for each subset are represented by corresponding symbols, where each symbol represents an individual sample and the bar represents the mean. Differences between samples were statistically significant as shown by two-way unpaired t test.

Pharmigen 552380, lot (0000054474; 1/50); FITC-anti-Fas (Jo2, BD Pharmigen 15404, lot M045159; 1/100); PE-Cy7-anti-CD43 (1B11, BioLegend 121218, lot B132711; 1/1000); BV605-anti-CD44 (IM7, BD Horizon 563058, lot 7177869; 1/100); BV711-anti-CD40 (3/23, BD Biosciences 740700, lot 6326576; 1/400); PE-Cy7-anti-CD278 (C398.4A, BioLegend 313520, lot B135805; 1/200); PE-Cy7-anti-CD62L (MEL-14, eBioscience 25-0621-82, lot E07577-943; 1/1000); PE-anti-CD122 (TM-b1, BD Biosciences 553362, lot 24161; 1/200); FITC-anti-KLRG1 (2F1, eBioscience 11-5893-82, lot E09834-484; 1/800); PE-Cy7-anti-CX3CR1 (SA011F11, BioLegend 149016, lot B216575; 1/200); APC-anti-CD47 (miap301, eBioscience 17-0471-82, lot 4301458; 1/100); and PerCP-Cy5.5-anti-SIRP α (P84, BioLegend 144010, lot B252132; 1/100). Monoclonal antibody (mAb) P84 specificity is based on the following: signal regulatory proteins (SIRPα and SIRPβ in the mouse) are expressed on neurons, hematopoietic stem cells, and myeloid cells including macrophages, monocytes, granulocytes, and DCs25. DCs, macrophages, and monocytes from mice with targeted SIRPα gene disruptions completely lose reactivity with mAb p84 (anti-SIRPα) even though their SIRPβ expression is normal. These results indicate specificity of p84 for SIRPα without cross-reactivity for SIRPβ.77 For FV-specific H-2Db/Abu-Abu-L-Abu-LTVFL staining, allophyco-cyanin (APC)– or PE-Dp gagL–MHC Dextramer (Immudex, Copenhagen, Denmark) was used at 1/25. For intracellular staining, cells were surfaced stained and
Fig. 9 Expansion of cytolytic Friend virus (FV)-specific SIRPα+ CD8+ T cells during programmed cell death ligand 1 (PD-L1) blockade. As described in Methods, mice chronically infected with FV were injected every other day with anti-PD-L1 blocking antibody and analyzed the second day following the final injection for the number of dextramer+ CD107α+ (a) and dextramer+ SIRPα+ CD107α+ (b) T cells in the spleen. Bars represent the mean and symbols represent individual mice (n = 6–7) pooled from 2 independent experiments, with statistical differences analyzed by two-way unpaired t tests.

fixed prior to permeabilization. The antibodies used for intracellular staining were PE-anti-EOMES (Dan1IgM, Ebioscience 12-4785-72, lot E10466-16341/1/200); PE-Cy7-anti-Tbet (eB40t210, Ebioscience 25-5825-82, lot 4277988; 1/200); A700-anti-Ki67 (B56, BD Pharmigen 561277, lot 7073537; 1/200); PE-anti-TCF-1 (533-966, BD Biosciences 864217, lot 8081983; 1/200), and APC-human-gramycan B (GB005, eBioscience, Cat. # 1908524; 1/50). To stain for intracellular granzyme B, cells were fixed overnight in 0.5% paraformaldehyde (PFA) and then permeabilized with 0.1% saponin/PBS containing 0.1% sodium azide, 0.5% bovine serum albumin, and 50 mM glucose. To stain for all other intracellular markers, intracellular staining was performed using the Ebioscience Foxp3 Kit, following the company's recommendation. To detect FV-infected cells, cells were stained with tissue culture supernatant containing mAb (MAB 34), which is specific for F-MuLV glycosylated Gag protein (mAb34 was produced at RML/NIAID/NIH as a culture supernatant). MAB 34 binding was detected with FITC-labeled goat anti-mouse IgG2b (R12-3, BD Pharmigen 553395, lot 328851; 1/800).

Methods, mice chronically infected with FV were injected every other day with anti-PD-L1 blocking antibody and analyzed the second day following the final injection for the number of dextramer+ CD107α+ (a) and dextramer+ SIRPα+ CD107α+ (b) T cells in the spleen. Bars represent the mean and symbols represent individual mice (n = 6–7) pooled from 2 independent experiments, with statistical differences analyzed by two-way unpaired t tests.
well and stimulated (or left unstimulated in T cell media of RPMI containing supplementation with 50 units/ml IL-2 from Peprotech) for 5 days. Cells were then stained and analyzed by flow cytometry.

**Linear regression modeling.** For the estimation of regression coefficients, we iteratively conducted multiple linear regressions with the scalar-dependent variables set as age, sex, history of previous IFN treatment, interferon-alpha therapy. Genes Cells 15, 1189–1200 (2010).

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