Hepatitis C virus–specific CD4+ T cell phenotype and function in different infection outcomes

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CD4+ T cell failure is a hallmark of chronic hepatitis C virus (HCV) infection. However, the mechanisms underlying the impairment and loss of virus-specific CD4+ T cells in persisting HCV infection remain unclear. Here we examined HCV-specific CD4+ T cells longitudinally during acute infection with different infection outcomes. We found that HCV-specific CD4+ T cells are characterized by expression of a narrower range of T cell inhibitory receptors compared with CD8+ T cells, with initially high expression levels of PD-1 and CTLA-4 that were associated with negative regulation of proliferation in all patients, irrespective of outcome. In addition, HCV-specific CD4+ T cells were phenotypically similar during early resolving and persistent infection and secreted similar levels of cytokines. However, upon viral control, CD4+ T cells quickly downregulated inhibitory receptors and differentiated into long-lived memory cells. In contrast, persisting viremia continued to drive T cell activation and PD-1 and CTLA-4 expression, and blocked T cell differentiation, until the cells quickly disappeared from the circulation. Our data support an important and physiological role for inhibitory receptor–mediated regulation of CD4+ T cells in early HCV infection, irrespective of outcome, with persistent HCV viremia leading to sustained upregulation of PD-1 and CTLA-4.

Introduction

Hepatitis C virus (HCV) infection presents a unique human disease model to define determinants of acute versus chronic infection because its natural history includes both completely resolving and persistent viremia. CD4+ T cells play a critical role in orchestrating immune responses during acute infection. Indeed, functional CD4+ T helper responses are required in the control of primary and secondary HCV infection in chimpanzees and humans (1–4). The importance of CD4 responses for the outcome of HCV infection is further supported by the finding that several SNPs in the HLA class II locus show strong associations with infection outcome, second only to the dominant IL28B signal (5). Having said that, even subjects progressing to chronic infection usually develop vigorous and broadly directed CD4+ T cell responses in the earliest phase of HCV infection (2). However, in individuals unable to clear HCV, CD4+ T cell responses are transient and proliferate poorly (2, 3).

T cell exhaustion is a key mechanism mediating CD8+ T cell failure in chronic viral infections including HIV-1 and HCV in humans (6, 7), SIV in rhesus macaques (8), and lymphocytic choriomeningitis virus (LCMV) in mice (9). Exhausted virus-specific CD8+ T cells typically exhibit reduced proliferation and diminished effector functions and are characterized by sustained expression of multiple inhibitory receptors, such as CD160, CTLA-4, TIM-3, 2B4, KLRG1 and PD-1 (10), and absence of expression of CD127 (11). High levels of T cell inhibitory receptors are also observed on virus-specific CD4+ T cells (12, 13), suggesting they are subjected to similar exhaustion mechanisms. However, several questions remain: whether coexpression of multiple T cell inhibitory receptors is comparable between CD4+ T cells and CD8+ T cells, how these inhibitory mechanisms impact early T cell responses and, more importantly, if early CD4 responses are differentially regulated between progressors and resolvers.

Here, we examined HCV-specific CD4+ T cell responses using HLA class II multimers in patients with distinct virological outcomes. Most previous HCV or HIV studies defined virus-specific CD4+ T cells through antigenic stimulation with peptide pools or proteins, which limits analysis to functional T cell populations targeting multiple epitopes. In contrast, the use of tetramers allows assessment of both the overall presence of individual T cell populations as well as their phenotype, including that of nonfunctional cells.

Results and Discussion

We used 12 different class II tetramers to study 55 acutely HCV-infected individuals with different HCV infection outcomes and examined HCV-specific CD4+ T cell responses longitudinally (Supplemental Tables 1 and 2, Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI126277DS1). Overall, 27 of 33 (81%) progressors and 22 of 22 (100%) resolvers had at least one multimer response detectable during the first 6 months of infection. As previously reported (2), HCV-specific CD4+ T cells rapidly declined and soon became completely undetectable in blood with progression to

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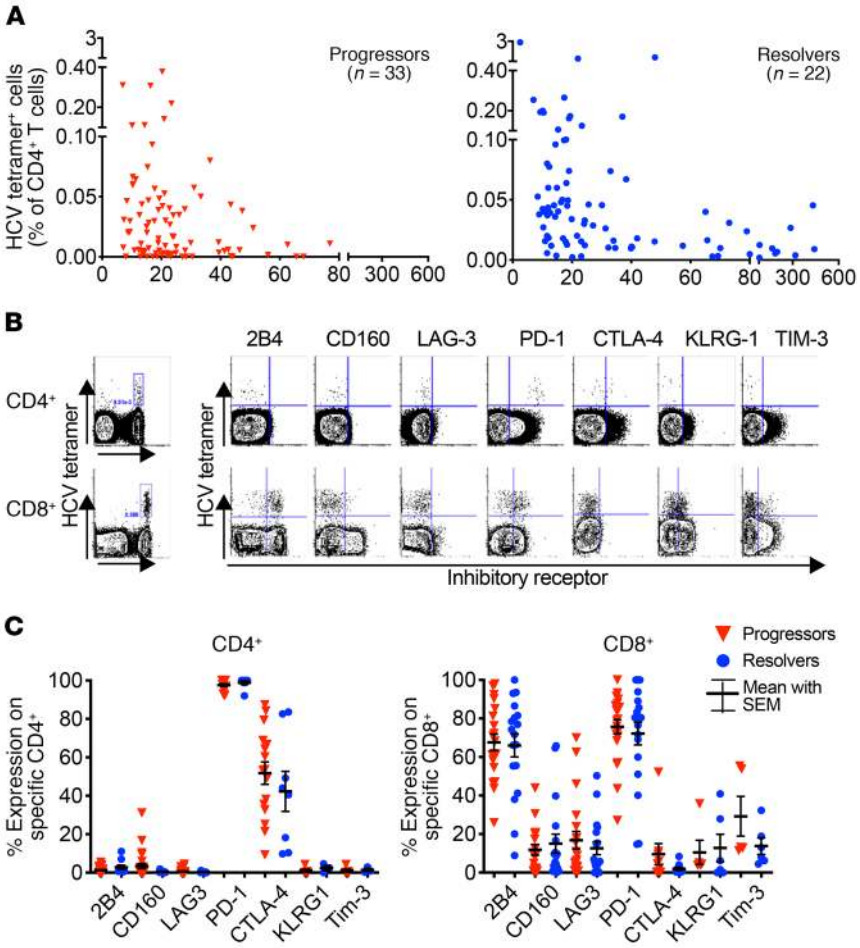

Figure 1. Frequencies of HCV-specific CD4⁺ T cells over time and inhibitory receptor expression on HCV-specific CD4⁺ versus CD8⁺ T cells. (**A**) HCV-specific CD4⁺ T cell frequencies from longitudinally sampled PBMCs of 33 patient progressors (top) and 22 resolvers (bottom). At least one HCV-specific CD4 response was detected in 27 of 33 (81%) progressors and 22 of 22 (100%) resolvers during the earliest phase of infection. However, HCV-specific CD4⁺ T cells quickly disappeared in blood of progressors, whereas a small population of these cells remained detectable directly ex vivo in resolvers years after resolution of infection. (**B**) Representative flow contour plots demonstrating expression of inhibitory receptors on HCV-specific CD4⁺ (top) or CD8⁺ T cells (bottom). (**C**) Cumulative data of the percentages of HCV-specific CD4⁺ and CD8⁺ T cells expressing each indicated inhibitory receptor. CD4⁺ T cell inhibitory receptor phenotypes were analyzed from a total of 15 progressors and 8 resolvers, while HCV-specific CD8⁺ T cells were examined from a total of 11 progressors and 8 resolvers. Each patient was analyzed at 2 to 3 time points.

chronic infection. In contrast, HCV-specific CD4⁺ T cells persisted long-term in resolving infection, with detectable responses in 11 of 12 patients with samples after week 60 (Figure 1A).

Simultaneous expression of multiple inhibitory receptors, such as PD-1, CTLA-4, CD160, LAG-3, TIM-3, KLRG1, and 2B4 is implicated in diminished CD8⁺ T cell effector functions and CD8⁺ T cell death in chronic HCV infection (7). We determined whether these inhibitory receptors are also expressed by CD4⁺ T cells (Figure 1, B and C). PD-1 and CTLA-4 were detected by flow cytometry on HCV-specific CD4⁺ T cells in virtually all subjects regardless of infection outcome, as previously shown (14), while CD160, LAG-3, TIM-3, KLRG1, and 2B4 were not detected at significant levels (Figure 1C). In contrast, almost all HCV-specific CD8⁺ T cells (for specificities see Supplemental Table 3) coexpressed 2B4 with PD-1, in addition to varied combinations including LAG-3, CD160, TIM-3, and KLRG1. CTLA-4 was rarely detected on CD8⁺ T cells. Thus, HCV-specific CD4⁺ T cells express a distinct and narrower repertoire of T cell inhibitory receptors compared with CD8⁺ T cells. While additional unmeasured receptors may function in CD4⁺ T cells, the data demonstrate that these cells exhibit a distinct expression pattern from HCV-specific CD8⁺ T cells.

Next, we assessed PD-1 and CTLA-4 expression in HCV-specific CD4⁺ T cells during early infection by disease outcome. We analyzed progressors and resolvers with a first PBMC sample available no later than 16 weeks into infection, when viral loads usually fluctuate significantly, irrespective of infection outcome. Additionally, we studied the activation marker CD38 and T cell memory markers CD127, CD45RA, and CCR7 (Figure 2A). In the early phase, all HCV-specific CD4⁺ T cells expressed PD-1 and many coexpressed CTLA-4 and CD38, though with more variability (Figure 2B). Similarly, there was no significant difference in CD127 expression or their memory phenotype. These cells were either central or effector memory cells (Figure 2B). Relative expression levels measured by median fluorescence intensity (MFI, Figure 2C and not shown) were consistent with the analysis using the percentage of positive cells. These data demonstrate a surprisingly similar CD4 phenotype during the first months of infection despite different infection outcomes.

To determine whether these similar phenotypes prevail beyond the initial phase of infection, we examined the subsequent evolution of HCV-specific CD4⁺ T cells longitudinally. In progressors, we observed persistent high levels of PD-1 expression before cells finally disappeared from circulation. Interestingly, 6 of 10 resolvers analyzed beyond 48 weeks after infection also displayed continued PD-1 expression after years without HCV viremia (Figure 3A). However, PD-1 expression levels (by MFI) decreased substantially with decreasing levels of viremia, in contrast to progressors (Figure 3A). Similarly, the majority of specific cells in persisting infection remained CTLA-4–positive. In resolving infection, most T cells expressed little to no CTLA-4 beyond

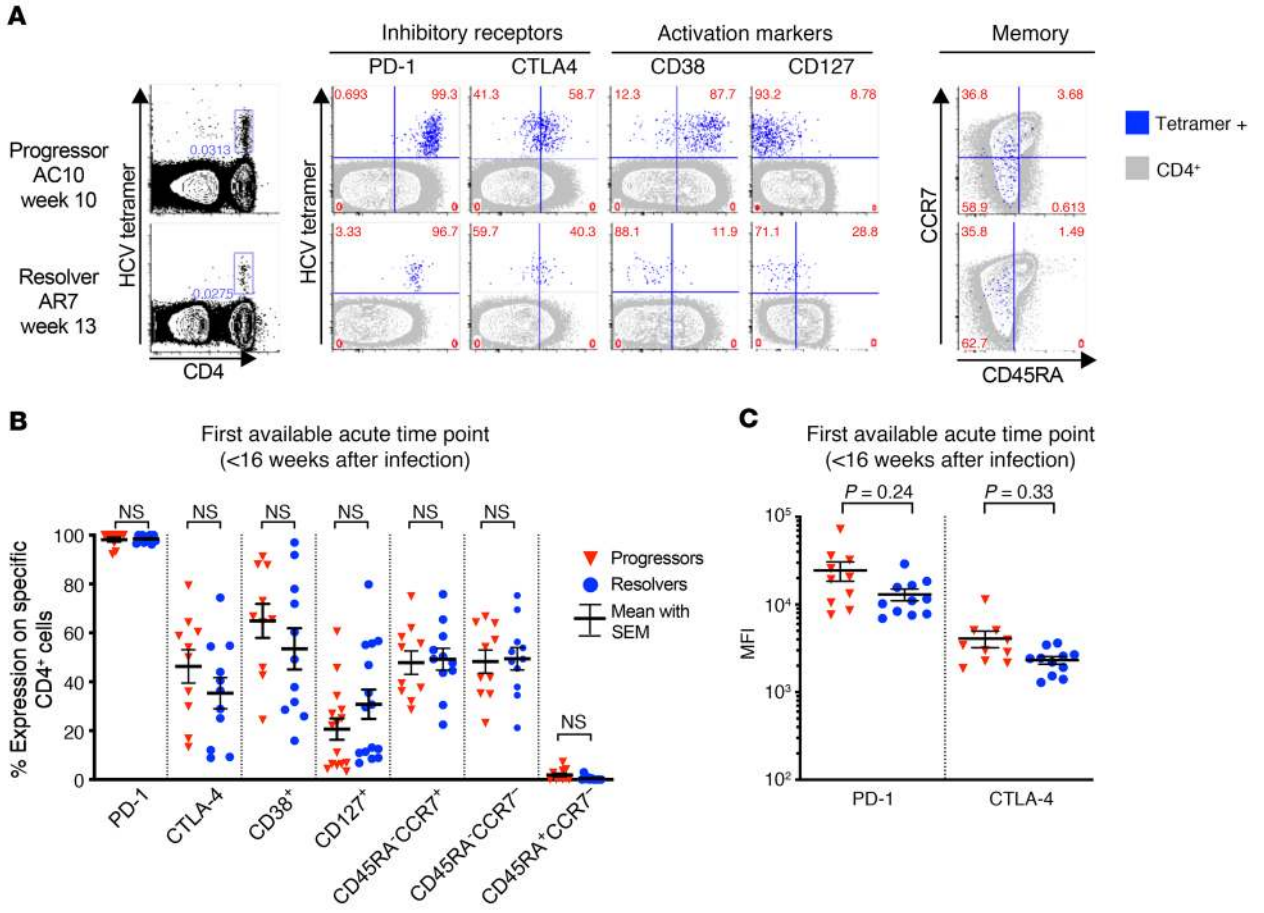

Figure 2. HCV-specific CD4⁺ T cell phenotype during the initial 16 weeks of infection. (**A**) Ex vivo phenotypic analysis of HCV-specific CD4⁺ T cells (dot plot) or total CD4⁺ T cells (contour plot) from the first available time point of resolvers and progressors. (**B**) Percentages of HCV-specific CD4⁺ T cells expressing each indicated immune markers are plotted for 10 progressors and 11 resolvers. No significant difference was found between the 2 groups of patients for any given marker. (**C**) PD-1 and CTLA-4 expression levels were also analyzed by median MFI. Statistical analysis was performed using the Kruskal-Wallis test.

24 weeks (Figure 3B). In parallel, specific cells in persisting, but not in resolving, infection continued to express CD38 (Figure 3C), suggesting sustained T cell activation before their disappearance. These activated cells also continued to express higher levels of the apoptosis receptor CD95 (Fas) (Figure 3D), which could contribute to apoptosis and therefore loss of the HCV-specific CD4 populations in persisting infection. A similar preapoptotic phenotype (PD-1^{hi} CD95^{hi} CD127^{lo} BCL-2^{lo}), including translocation of PD-1 into the area of CD95/Fas-capping, an early necessary step of CD95/Fas-induced apoptosis, was previously described for HIV-specific CD8⁺ T cells (15). Finally, the memory phenotype of HCV-specific CD4⁺ T cells in progressors did not evolve, remaining central or effector memory with low CD127 expression (Figure 3E and Supplemental Figure 2A). In contrast, viral control led to memory differentiation toward mostly CD127⁺ central memory populations.

All together, this analysis revealed 2 distinct phases within the first year of HCV infection. In the initial phase of infection (<16 weeks), T cell inhibitory receptor expression, cellular activation status, and memory phenotype are similar in most patients, despite distinct results from standard lymphoproliferation assays that have been universally observed in persisting versus acute infection at this stage (2, 3). Only in the subsequent phase, after HCV control is firmly established in the resolver group, did we observe significant memory differentiation, suggesting that downregulation of inhibitory receptors and development of CD127-expressing central memory cells is a consequence, not a cause, of successful viral control. Throughout our observation period, viral titers and both CD38 and PD-1/CTLA-4 inhibitory receptor expression correlated strongly in both outcomes (Supplemental Figure 2, B and C). In contrast, CD127 was negatively correlated with viral load and T cell activation in resolvers but not in progressors (Supplemental Figure 2D), despite significant fluctuations in viral load with occasionally full control in progressors. This finding suggests that episodes of transient viral control immediately impact CD38, PD-1, and CTLA-4 expression, but are either too short for T cell differentiation to occur or that memory differentiation is generally blocked in progressors.

We next sought to investigate whether CD4⁺ T cells differ in functionality by infection outcomes. Unlike CD8⁺ T cells, CD4⁺ T cells cannot be assessed by multimer staining combined with intracellular cytokine studies due to low cell frequencies and rapid loss of HLA class II tetramer binding after peptide stimulation. However, our previous tetramer analyses allowed us to select samples with proven presence of HCV-specific CD4⁺ T cells. Using pools of HCV proteins to define the total HCV-specific CD4⁺ T cell

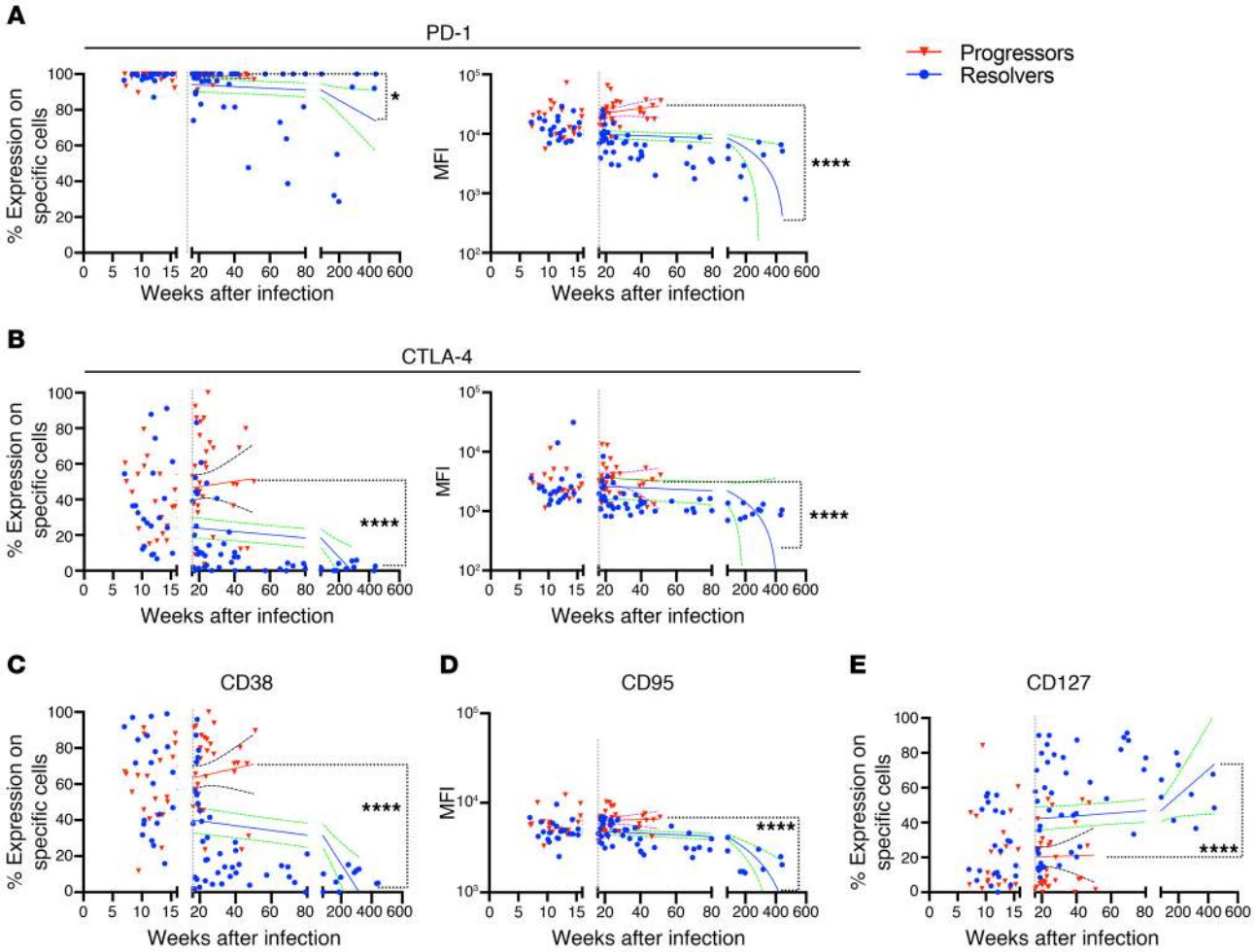

Figure 3. HCV-specific CD4⁺ T cells diverge in phenotypes once viral loads diverge. Immune phenotypes of HCV-specific CD4 cells were longitudinally examined in 15 progressors and 20 resolvers. Expression levels of PD-1 (**A**), CTLA-4 (**B**), CD38 (**C**), CD95 (**D**), and CD127 (**E**) in percentages or MFI in HCV-specific CD4⁺ cells from different infection outcomes are plotted against weeks after infection. Differences in slopes or elevation between progressors and resolvers (after 16 weeks) were calculated based on linear regression analysis. *$P < 0.05$; ****$P < 0.001$.

population by upregulation of CD40L, we analyzed IFN-γ, TNF-α, and IL-2 (Figure 4A) production during different time points that had positive tetramer staining results. We found lower numbers of CD40L-expressing cells after stimulation in progressors (Figure 4B), consistent with the rapid decline of tetramer-positive cells detected by tetramer staining (Figure 1A). However, we observed no significant differences between infection outcomes in the proportion of CD40L positive cells secreting individual cytokines or in the polyfunctionality of CD4⁺ T cell populations (Figure 4C and Supplemental Figure 3A). About half of CD40L⁺ cells did not express any cytokines, similar to HCV-specific CD8⁺ T cells (16), and less than one-fourth of cells (median) were polyfunctional in either outcome. We also tested whether HCV-specific CD4⁺ T cells secrete IL-21, but in contrast to a previous study using in vitro expansion, we failed to detect significant populations of such cells in our direct ex vivo assays (17). Our results do not contradict previous data demonstrating polyfunctional CD4⁺ T cells as key to HCV control (18). Here, functional assays were performed on samples that had previously confirmed presence of tetramer-positive HCV-specific CD4⁺ T cells, whereas the functional assays without concomitant tetramer data cannot distinguish between a lack of functionality and the physical absence of specific CD4⁺ T cells. Thus, the data here demonstrate that the reduced detection of functional CD4⁺ T cells in chronic HCV infection observed in previous studies is mostly a consequence of vastly diminished frequencies of HCV-specific CD4⁺ T cells, and not a result of these cells losing the ability to secrete cytokines.

Next, we examined the proliferative capacity of HCV-specific CD4⁺ T cell populations in the context of differential expression of T cell inhibitory receptors. We selected PBMCs that we had previously phenotyped, and assessed proliferative capacity of HCV-specific CD4⁺ T cells after stimulation with cognate peptide in the presence or absence of rIL-2. Presence of proliferating HCV-specific CD4⁺ T cells was assessed at day 14 (Figure 4D). In the absence of rIL-2, CellTrace tetramer-positive cells were detectable in 14 of 34 assays, indicating inhibition of proliferation in the majority of early samples, with a trend toward more proliferating cells in resolvers (61%) versus progressors (29%, $P = 0.08$, Supplemental Figure 3B). In contrast, all samples exhibited proliferation in the presence of rIL-2. Thus, this single epitope response

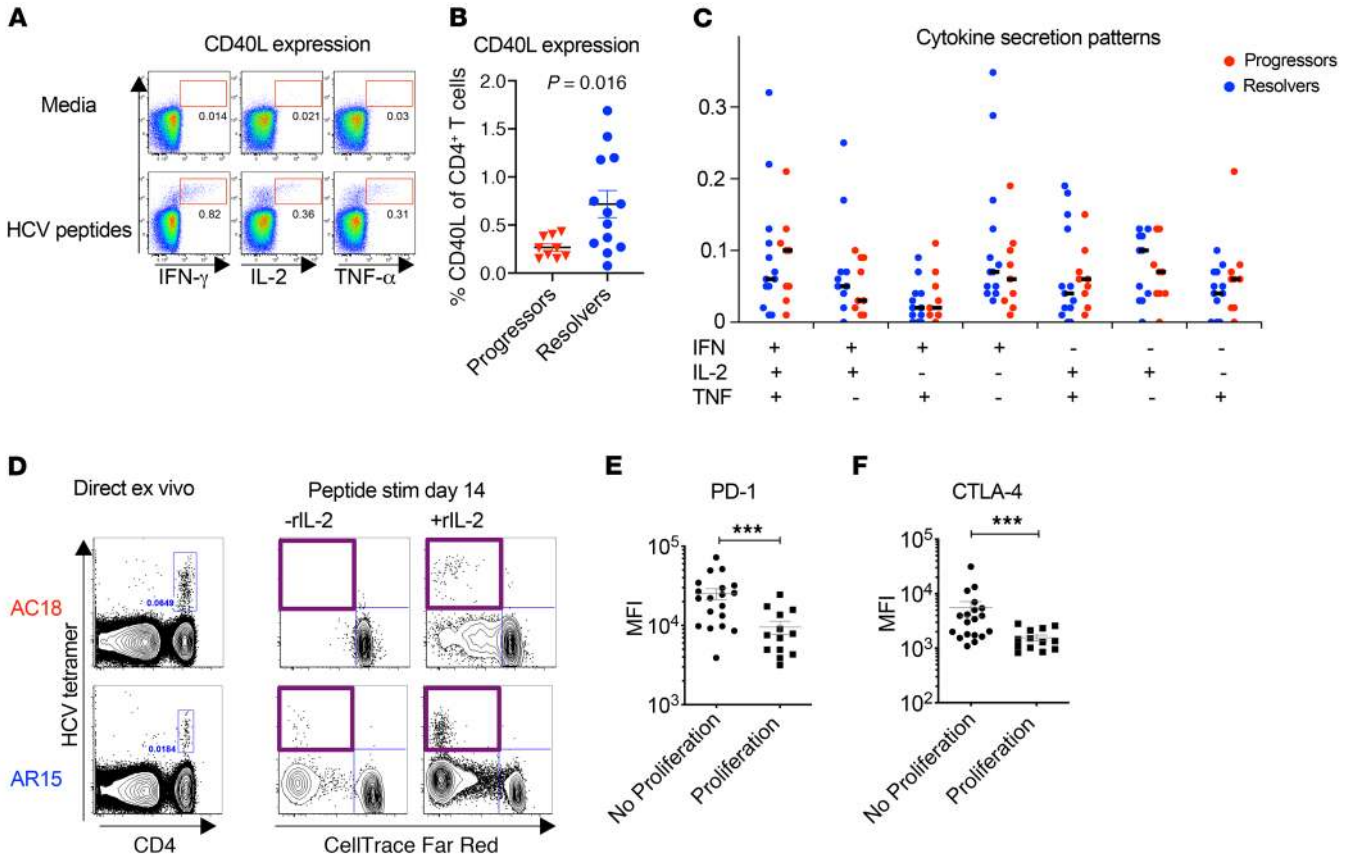

Figure 4. Functional analysis of HCV-specific CD4⁺ T cells. (**A**) Representative flow cytometry plots for Th1 cytokine secretion by HCV-specific CD4⁺ T cells after stimulation with HCV proteins. Antigen-specific CD4 responses were identified as CD40L⁺CD4⁺ T cells. (**B**) Higher frequency of CD40L⁺ expressing CD4⁺ T cells (of total CD4⁺ cells) in response to HCV protein stimulation in 13 patients with resolving versus 9 with progressing infections ($P = 0.016$, 2-tailed Mann-Whitney *U* test). (**C**) (Poly)functional profiles of the CD40L-expressing cells. Progressors, red; resolvers, blue. (**D**) The mean number of CD40L⁺ CD4 populations secreting 0, 1, 2, or 3 cytokines. Representative flow cytometry plot demonstrating detection of tetramer-positive CD4⁺ T cells directly ex vivo and proliferation capacity of these cells upon cognate peptide stimulation in the absence or presence of rIL-2. After 14 days of culture, proliferating antigen-specific CD4⁺ T cells were identified as CD3⁺CD4⁺ Class II tetramer⁺ CellTrace Far Red by flow cytometry. Expression levels of PD-1 (**E**) and CTLA-4 (**F**) in samples that proliferated versus those that did not in the absence of rIL-2 (***$P < 0.001$, 2-tailed Mann-Whitney *U* test).

assay paints a more nuanced and granular picture compared with the standard proliferation assay using whole HCV proteins that measures CD4 proliferation more broadly (2, 3). Importantly, cells that did not proliferate in either outcome expressed significantly higher levels of PD-1 and CTLA-4 than those that proliferated (Figure 4, E and F). Therefore, high levels of PD-1 or CTLA-4 expression may be associated with inhibition of T cell proliferation in all patients regardless of outcome. This observation highlights the often underappreciated physiological role of inhibitory pathways for fine-tuning and controlling T cell responses and preventing immunopathologies. Activity of PD-1 beyond chronic infection aligns with our previous observation that in vitro PD-1 blockade improves proliferation of a PD-1–expressing CD8⁺ T cell response in a previously resolved infection (11). This finding implies that only sustained upregulation of PD-1 and CTLA-4, as observed in persisting infection, has a deleterious effect on CD4 populations and may underlie their disappearance.

Together, our analyses provide important insights into the regulation and function of virus-specific CD4⁺ T cells in a human infection with both acute and chronic outcomes. HCV-specific CD4⁺ and CD8⁺ T cells exhibit distinct inhibitory receptor expression profiles, with only PD-1 as a shared and almost universally expressed receptor. This finding suggests that different checkpoint blockade targets will have varying impacts on CD4 versus CD8 responses, presenting a possible explanation for varying efficacy across therapies as well as observed synergies in multimodal approaches. This could be especially relevant for cancer, where antigen-specific CD4 responses have not been studied in great detail. Since the primary role of the inhibitory pathways is not to facilitate cancer and chronic infection, but to tune an adequate T cell response, it is not surprising that PD-1 and CTLA-4 were operative in the first months of infection in both acute and chronic HCV infection. Elucidating the critical roles of these receptors in developing effective T cell memory necessitates additional detailed studies, given the increasing number of patients receiving checkpoint blockade, but also illustrates the challenges in developing an effective HCV vaccine.

Surprisingly, we did not identify major differences in cytokine secretion by infection outcome. No individual cytokine was expressed at a higher level in resolving infection, and numbers of polyfunctional cells did not differ. Technical constraints prevented us from directly correlating cytokine data with PD-1 and CTLA-4

expression, so we cannot exclude some impact of inhibitory receptors on cytokine secretion. Nevertheless, the parallel tetramer data did enable us to differentiate between dysfunctional and absent $CD4^+$ T cell populations, suggesting that limited proliferation and subsequent CD4 loss, not impaired cytokine secretion, is the main driver of $CD4^+$ T cell failure in persistent infection.

Finally, our data suggest that the disappearance of HCV-specific $CD4^+$ T cells could be a result of prolonged PD-1 and CTLA-4 expression and the associated negative impact on T cell proliferation, driven by sustained viremia and resultant TCR activation. This could be potentiated by continued expression of the death receptor Fas, which has been shown to interact with PD-1 in the initiation of apoptosis (19). Further work must determine whether these CD4 responses are truly lost in long-term chronic infection, or whether changes in a patient's immune status, antigen loss through HCV antiviral therapy, or anti–PD-1 and CTLA-4 checkpoint blockade can resuscitate functional HCV-specific CD4 immunity. In addition, while these studies in humans cannot fully establish causality, the data suggest that mechanisms other than T cell inhibitory pathways may initially determine the degree of HCV control and lay the foundation for subsequent PD-1– and CLA-4–mediated $CD4^+$ T cell exhaustion in viral persistence.

Methods

Study approval. All individuals gave written informed consent and were recruited within 6 months of HCV infection. This study was approved by the Institutional Review Boards of Massachusetts General Hospital and the Oswaldo Cruz Institute. Detailed methods are presented in the Supplemental Material.

Author contributions

DC, JA, PSFS, CAF, AYK, LLX, and GML designed and executed the cohort study. DC and GML designed experimental plans. DC, LM, BR, and GH conducted experiments. DC, DW, JA, SS, RCH, and GML analyzed data. DC, DW, RCH, and GML wrote the manuscript. All authors provided critical review of the manuscript.

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