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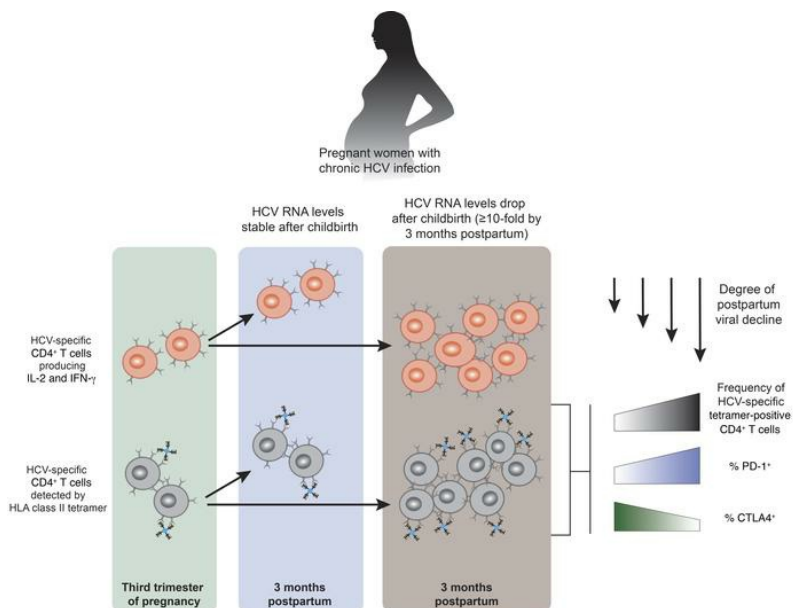
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CD4⁺ T cell restoration and control of hepatitis C virus replication after childbirth

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Chronic hepatitis C virus (HCV) infection is characterized by persistent high-level viremia and defective cellular immunity, including a lack of functional HCV-specific CD4⁺ T cells. We previously described an exceptional period of viral control that occurs in some chronically infected women after childbirth. Here, we investigated whether reduced HCV replication after pregnancy is associated with recovery of CD4⁺ T cell immunity. Class II tetramer analysis revealed significantly greater frequencies of circulating HCV-specific CD4⁺ T cells at 3 months postpartum in women with concurrent declines in viremia compared with those with stable viremia. These HCV-specific CD4⁺ T cells had an effector-memory phenotype. Inhibitory coreceptor expression on these cells corresponded to the degree of viral control. Circulating CD4⁺ T cells produced IL-2 and IFN- γ after HCV antigen stimulation, demonstrating Th1 functionality. These data provide direct evidence that the profound loss of HCV-specific CD4⁺ T cell help that results in chronic infection is reversible following pregnancy, and this recovery of CD4⁺ T cells is associated with at least transient control of persistent viral replication.

Introduction

Hepatitis C virus (HCV) infections persist indefinitely in approximately three-quarters of infected individuals, predisposing to late complications, including hepatic cirrhosis and hepatocellular carcinoma (1). One of the strongest predictors of persistent HCV infection is early failure of the virus-specific CD4⁺ helper T cell response (2). In observational studies, individuals who successfully resolve infection have highly functional CD4⁺ helper T cells that broadly target the virus and then contract to form long-lived memory populations (2–6). Those who progress to persistent infection may also initially prime broad CD4⁺ T cell responses. However, these cells show early defects in proliferation, and functional CD4⁺ T cell responses are soon lost from the circulation (4–8). By the chronic phase of infection, HCV-specific CD4⁺ T cells are not readily visualized in peripheral blood using HLA class II tetramers (3, 4, 7, 9).

Whether functional CD4⁺ T cell responses can be restored in individuals with chronic HCV infection remains an important unanswered question. Spontaneous restoration of functional T cell immunity is exceedingly rare after chronic HCV replication is established (10). Interestingly, some persistently infected women who become pregnant achieve substantial declines in HCV viral load after childbirth (11–13). In our cohort, approximately one-third of chronically infected pregnant women experienced postpartum viral declines of at least 1 log₁₀, sometimes exceeding 3 log₁₀ (11).

This degree of viral load variation is otherwise rarely observed in chronic hepatitis C (14). We previously found postpartum viral control to be associated with restored T cell function as measured by an IFN- γ ELISpot assay (11), and analysis of viral evolution in 2 mothers indicated increased CD8⁺ T cell-driven selection pressure after delivery (15). Whether CD4⁺ helper T cell immunity is reconstituted in chronically infected women after pregnancy has not been established. We therefore compared HCV-specific CD4⁺ T cell frequency, phenotype, and cytokine production in women with and without postpartum control of chronic HCV replication. Our study provides direct evidence that defects in CD4⁺ T cell immunity caused by chronic hepatitis C are potentially reversible within the context of pregnancy and parturition, and that CD4⁺ T cell recovery is associated with suppression of persistent HCV replication.

Results and Discussion

Our primary study cohort consisted of 32 pregnant women with chronic HCV, many of whom were recently described (11). Ten of these women achieved a decline in viremia of at least 1 log₁₀ between the third trimester of pregnancy (T3) and 3 months postpartum (3PP) and were designated as controllers (Figure 1A). Twenty-two did not achieve this degree of viral decline and were designated as noncontrollers (Figure 1A). Controllers were more likely to carry the favorable *IFNL3* rs12979860 CC genotype and *HLA-DPBI* polymorphisms associated with high expression of HLA-DP ($P = 0.049$ and $P = 0.019$, respectively, Fisher's exact test), as previously described (11). The 2 groups did not differ significantly in terms of age, estimated duration of infection, gestational age at delivery, viral load during pregnancy, or HCV genotype, as shown in Supplemental Table 1; supplemental material available online with this article; <https://doi.org/10.1172/JCI123623DS1>.

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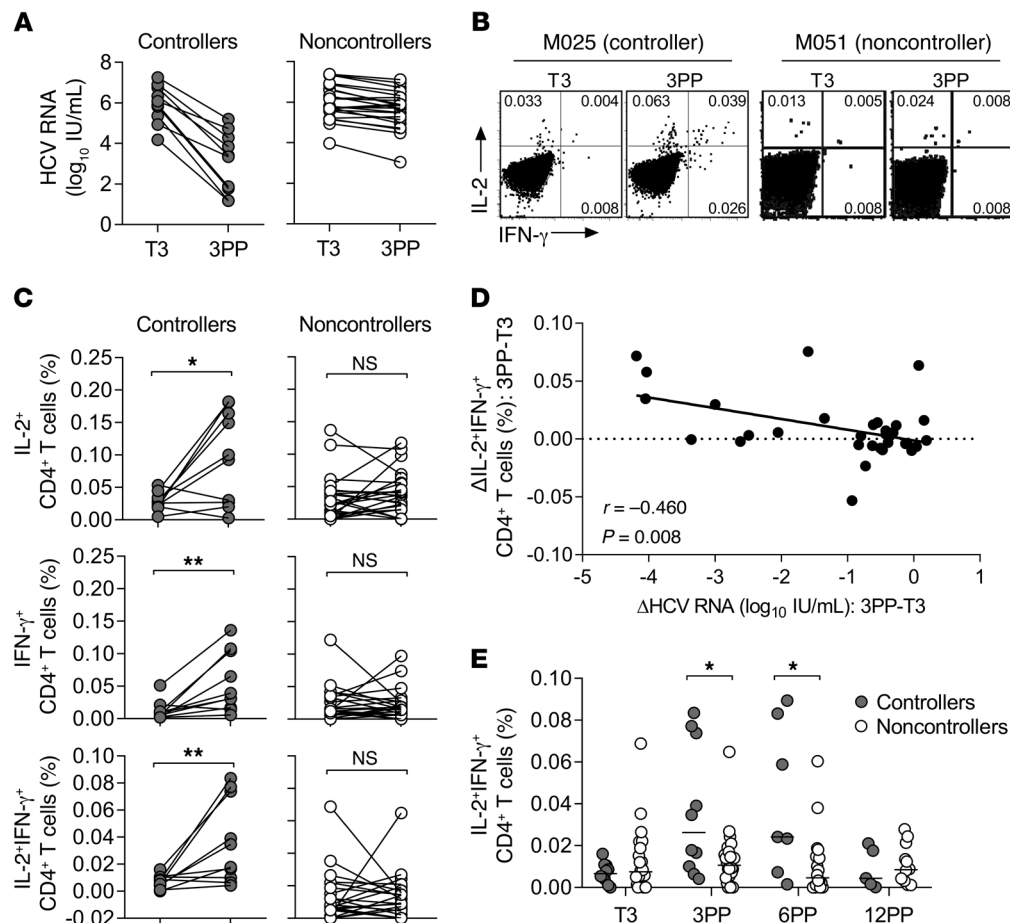


Figure 1. Function of HCV-specific CD4⁺ T cells in women with and without postpartum viral control. (A) Plasma HCV RNA levels at the third trimester (T3) and 3 months postpartum (3PP) for 10 women with (controllers) and 22 women without (noncontrollers) postpartum viral load reductions of at least 1 log₁₀ IU/mL. (B) Example HCV-specific CD4⁺ T cell cytokine responses of 1 controller and 1 noncontroller assessed by intracellular cytokine stain following PBMC stimulation with 3 separate peptide pools spanning HCV NS3-NS4. (C) Background-subtracted frequencies of HCV-specific cytokine-producing CD4⁺ T cells at T3 and 3PP for 10 controllers (left) and 22 noncontrollers (right) (Wilcoxon matched-pairs signed rank test). (D) Pearson's correlation of changes in viral load and HCV-specific IL-2⁺IFN-γ⁺ CD4⁺ T cell frequencies from T3 to 3PP. (E) HCV-specific CD4⁺ T cell IL-2⁺IFN-γ⁺ coproduction of controllers and noncontrollers at T3, 3PP, 6PP, and 12PP (Mann-Whitney U test). Horizontal lines indicate median values. *P < 0.05; **P < 0.01.

To assess the potential role of HCV-specific CD4⁺ T cell immunity in postpartum viral control, cryopreserved peripheral blood mononuclear cells (PBMCs) from controllers and noncontrollers were stimulated with genotype-matched peptide pools corresponding to the HCV proteins NS3, NS4A, and NS4B. These nonstructural proteins are dominant targets of the CD4⁺ T cell response during acute hepatitis C (4). Intracellular cytokine staining (ICS) was then performed. Example responses from one controller and one noncontroller at T3 and 3PP are shown in Figure 1B; responses from the entire cohort are provided in Supplemental Figure 1. As a group, controllers demonstrated significantly increased frequencies of IL-2⁺, IFN-γ⁺, and IL-2⁺IFN-γ⁺ HCV-specific CD4⁺ T cells at 3PP as compared with T3 ($P = 0.037$, $P = 0.006$, and $P = 0.010$, respectively; Figure 1C). This is in contrast to noncontrollers, in whom cytokine production did not significantly increase postpartum ($P = 0.290$, $P = 0.949$, $P = 0.949$, respectively; Figure 1C). A relationship between postpartum viral control and improved CD4⁺ T cell IL-2⁺IFN-γ⁺ coproduction was also evident when viral control was considered as a continuous rather than categorical variable ($P = 0.008$; Figure 1D).

Direct comparison of controllers versus noncontrollers revealed that frequencies of IL-2⁺IFN-γ⁺ coproducing HCV-specific CD4⁺ T cells were similar between the 2 groups during the third trimester, rose significantly in controllers as compared with noncontrollers at 3PP and 6PP ($P = 0.035$ and $P = 0.020$, respectively),

and then fell to similar levels among the subset of controllers and noncontrollers studied at 12 months postpartum (Figure 1E). The ICS assay also measured IL-10, IL-17a, and IL-21 production, but it failed to detect significant frequencies of HCV-specific CD4⁺ T cells producing these cytokines in either controllers or noncontrollers (data not shown). Together these data suggest that HCV-specific Th1 responses are restored in some women after delivery, in contrast to the typical absence of functional CD4⁺ T cell populations in chronic HCV infection.

We next compared HCV-specific CD4⁺ T cell frequencies in the peripheral blood of controllers ($n = 6$) and noncontrollers ($n = 5$) using HLA class II tetramers listed in Supplemental Table 2. This direct visualization allowed us to discern whether the augmented postpartum Th1 response observed in controllers but not noncontrollers (Figure 1C) reflected differences in the frequency or the function of circulating HCV-specific CD4⁺ T cells. Example plots for 2 controllers and 2 noncontrollers with shared HLA-DRB1 alleles are shown in Figure 2A, with the remainder of plots shown in Supplemental Figure 2. As a group, controllers demonstrated significantly higher tetramer-positive frequencies in the postpartum period compared with noncontrollers ($P = 0.004$; Figure 2B). Class II tetramer-positive frequencies also correlated strongly with viral load changes analyzed as continuous variables ($P = 0.002$; Figure 2C). These tetramer data suggest that the greater HCV-specific Th1 responses observed in controllers as compared

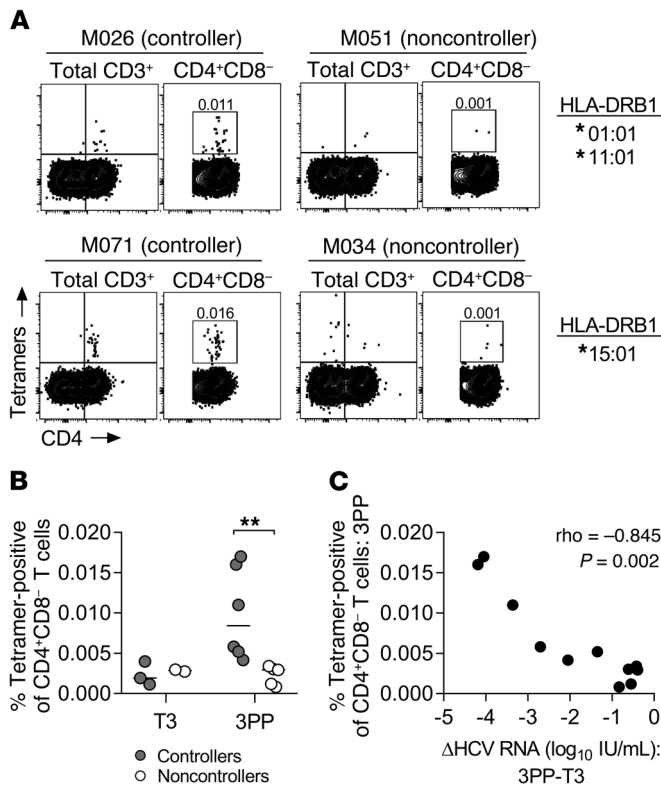


Figure 2. HCV-specific CD4⁺ T cell frequencies by class II tetramer staining. (A) Examples of tetramer-positive populations among total CD3⁺ T cells (left) or CD4⁺CD8⁻CD3⁺ T cells (right) for 2 controllers and 2 noncontrollers with matched HLA class II tetramers. (B) Frequencies of tetramer-positive CD4⁺ T cells per CD4⁺CD8⁻CD3⁺ T cells at T3 and 3PP. Up to 4 tetramers were pooled for each subject based on HLA-type and tetramer availability. Frequencies were compared for 6 controllers and 5 noncontrollers at 3PP using the Mann-Whitney *U* test. Horizontal lines indicate median values. (C) Spearman's correlation of change in viral load from T3 to 3PP with the frequency of tetramer-positive CD4⁺ T cells at 3PP. **P* < 0.05; ***P* < 0.01.

with noncontrollers at 3PP (Figure 1E) reflect higher frequencies of circulating virus-specific cells rather than enhanced functionality of individual virus-specific cells.

There have been few descriptions of HCV-specific CD4⁺ T cells in chronic hepatitis C (8), in part because these cells decline to frequencies near or below the detection threshold in blood following the acute phase of infection (4, 7, 9, 16). Direct visualization of CD4⁺ T cells in 6 women with postpartum viral control provided a unique opportunity to assess expression of markers of T cell differentiation (CD45RA, CCR7, and CD127) and exhaustion (PD-1 and CTLA-4). As noted previously, class II tetramer-positive T cell frequencies were highest in subjects with the sharpest postpartum viral load declines (Figure 3A, columns 1 and 2). The tetramer-positive CD4⁺ T cells were predominately CD45RA⁺CCR7⁺, characteristic of an effector memory population with prior antigen exposure and lack of lymph node homing potential (Figure 3A, column 3). CD4⁺ T cells from 2 subjects with the most efficient suppression of virus replication (M026 and M071) also coexpressed CD127 (Figure 3A, column 4), perhaps indicating a memory population that is maintained by homeostatic IL-7-driven proliferation.

PD-1 and CTLA-4 expression on HCV-specific CD4⁺ T cells is higher from subjects with chronic versus resolved HCV infection (8). Moreover, in some chronically infected individuals, in vitro blockade of PD-1 and/or CTLA-4 signaling can improve proliferation and cytokine production of exhausted HCV-specific CD4⁺ and CD8⁺ T cells (2, 8). We therefore examined expression of these coinhibitory receptors on CD4⁺ T cells at 3PP when control of virus replication was most pronounced. Tetramer-positive CD4⁺ T cell populations from all 6 controllers were predominantly PD-1⁺ at 3PP (Figure 3A). Surprisingly, higher PD-1 expression frequency was associated with better postpartum viral control (Figure 3B, left). PD-1 expression in this setting is therefore most likely a marker of CD4⁺ T cell activation rather than exhaustion, as has been described in acute primary HCV infections that spontaneously resolved (9).

Unlike PD-1, the coinhibitor receptor CTLA-4 was expressed on less than half of the tetramer-positive HCV-specific CD4⁺ T cells (Figure 3A, column 5), and its expression was lowest in women with the greatest viral declines (Figure 3B, center). Moreover, the frequency of tetramer-positive CD4⁺ T cells expressing PD-1 but not CTLA-4 was strongly associated with the degree of postpartum viral control (Figure 3B, right). These divergent patterns of PD-1 and CTLA-4 expression suggest that they may be differentially regulated in CD4⁺ T cells during chronic HCV infection (8, 17) and have distinct implications for postpartum CD4⁺ T cell recovery. Indeed, our data suggest that CTLA-4 is a critical negative regulator of HCV-specific CD4⁺ T cell activity in the postpartum period, whereas PD-1 expression may be more indicative of CD4⁺ T cell activation than exhaustion in this setting.

To more directly test the relation of PD-1 expression and CD4⁺ T cell functional status, we performed ICS after stimulation with peptides corresponding to class II epitopes used in the tetramer studies (summarized in Supplemental Table 2). Three women with substantial viral control and high frequencies of tetramer-positive CD4⁺ T cells at 3PP were studied. Using PMBCs from either 3PP or 6PP, depending on sample availability, the class II epitopes stimulated CD4⁺ T cell responses in all 3 women, as measured by production of IL-2, IFN-γ, and TNF-α (Figure 4A). These responses were predominately polyfunctional in subjects M071 and M026 (Figure 4B). Both subjects had deep viral suppression and concomitant high PD-1 expression on tetramer-positive cells (>90%) at the corresponding time point, further supporting the conclusion that PD-1 expression on these CD4⁺ T cells reflected activation rather than exhaustion. Responses were of lower frequency and less polyfunctional in subject M025 (Figure 4, A and B), who had experienced a rebound in viral titers and actually had lower PD-1 expression on tetramer-positive cells (63%) by the time of the assay at 6PP.

In summary, our study provides a detailed characterization of HCV-specific CD4⁺ T cell function and differentiation when virus replication is controlled after childbirth. Postpartum control of persistent HCV viremia was linked to an increased frequency of circulating effector memory virus-specific CD4⁺ T cells capable of producing multiple Th1 cytokines. A PD-1⁺CTLA-4⁻ phenotype predominated in women with the greatest viral declines. Conversely, failure to control viremia corresponded to a persistent low or undetectable frequency of circulating HCV-specific CD4⁺ T cells after delivery.

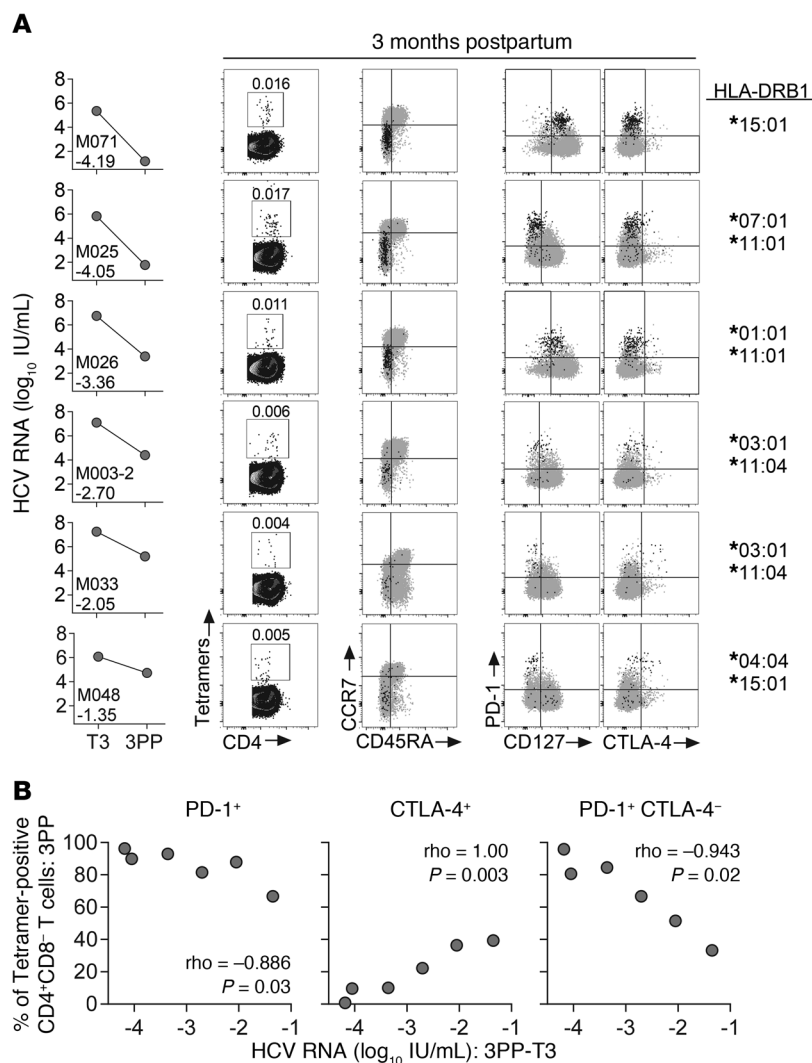


Figure 3. Tetramer-positive CD4⁺ T cell phenotypes of 6 women with postpartum viral control. (A) The viral load (column 1), tetramer-positive CD4⁺ T cell frequency (column 2), and phenotypic marker expression of tetramer-positive (black) and bulk (gray) CD4⁺ T cells (columns 3–5). All phenotypic analyses were performed at the 3PP time point, when tetramer-positive frequencies were highest. Relevant HLA alleles for class II tetramer studies are listed in the right column. (B) Spearman's correlation of the change in viral load from T3 to 3PP with the frequency of tetramer-positive CD4⁺ T cells expressing PD-1 (left), CTLA-4 (middle), and PD-1 but not CTLA-4 (right) at 3PP.

Considering the usual difficulty identifying HCV-specific CD4⁺ T cells in chronic infection, detection of functional CD4⁺ T cells in chronically infected women after childbirth is notable. It indicates that restoration of previously failed T helper responses is sometimes possible in chronic hepatitis C and that postpartum immunomodulation is sufficient to overcome even the pervasive influence of high antigen burden. The importance of CD4⁺ T cells in postpartum viral control is consistent with past studies describing the central role of CD4⁺ T cells in controlling acute HCV infection (2, 3, 5, 6). The present study may thus provide insight into characteristics of immune responses required for successful resolution of HCV outside of the peripartum period. Alternatively, childbirth does herald an immense immunologic transition (18), and there may be fea-

tures of postpartum HCV control that are unique to this context.

Identification of predictors of robust postpartum CD4⁺ T cell responses could provide valuable insight into mechanisms of immune restoration. Genetic polymorphisms in *IFNL3* and *HLA-DPBI* were previously linked to postpartum viral control in this cohort (11), but neither locus was directly associated with postpartum HCV-specific CD4⁺ T cell function in the present study (Supplemental Figure 3, A and C, Supplemental Table 3). An exploratory analysis for other factors associated with postpartum CD4⁺ T cell responses identified a potential effect of viral load at T3 (Supplemental Table 3). Interestingly, T3 viral load was previously linked with *IFNL3* genotype in this cohort (11). After adjusting for T3 viral load, *IFNL3* 12979860 CC genotype was in fact significantly predictive of postpartum HCV-specific CD4⁺ T cell IL-2⁺IFN- γ ⁺ frequencies ($\beta = 0.56$, $P = 0.004$), whereas *HLA-DPBI* genotype was not (Supplemental Figure 3, B and D, Supplemental Table 4). These data could be interpreted to suggest that *IFNL3* 12979860 CC genotype promotes postpartum Th1 restoration, but this effect is dampened by countervailing forces related to higher viral burden prior to delivery. A multivariable linear regression model combining Th1 responses with *IFNL3* and *HLA-DPBI* genotypes better predicted postpartum viral control (adjusted $r^2 = 0.36$) than genetic factors alone (adjusted $r^2 = 0.21$). *IFNL3* retained significance in the model of viral control that incorporated Th1 responses, but its effect as measured by the magnitude of the standardized β coefficient was weakened (Supplemental Table 5). Larger studies are needed to formally assess whether the association of *IFNL3* 12979860 CC with postpartum viral control is at least partially mediated via a net favorable effect on HCV-specific T cell help, as suggested in some studies of acutely infected nonpregnant adults (19).

This study does have limitations. First, its observational design limits our ability to infer causality in the association of CD4⁺ T cell function with viral decline. However, lack of HCV-specific CD4⁺ T cell recovery following pharmacological cure of chronic HCV suggests that antigen reduction alone is not sufficient to reverse CD4⁺ T cell exhaustion (4, 20, 21). Second, the function and phenotype of CD4⁺ T cell responses observed in the peripheral blood after delivery likely do not precisely match those of liver resident T cells (22). Nevertheless, the observed frequency, function, and phenotype of peripheral HCV-specific CD4⁺ T cells correlated with viral control in the postpartum period, and we would expect these trends to be magnified at the site of infection. Finally, although the frequency of circulating HCV-specific CD4⁺ T cells in postpartum controllers exceeded that which is typically observed in chronically infected individuals, the phenotypic and functional

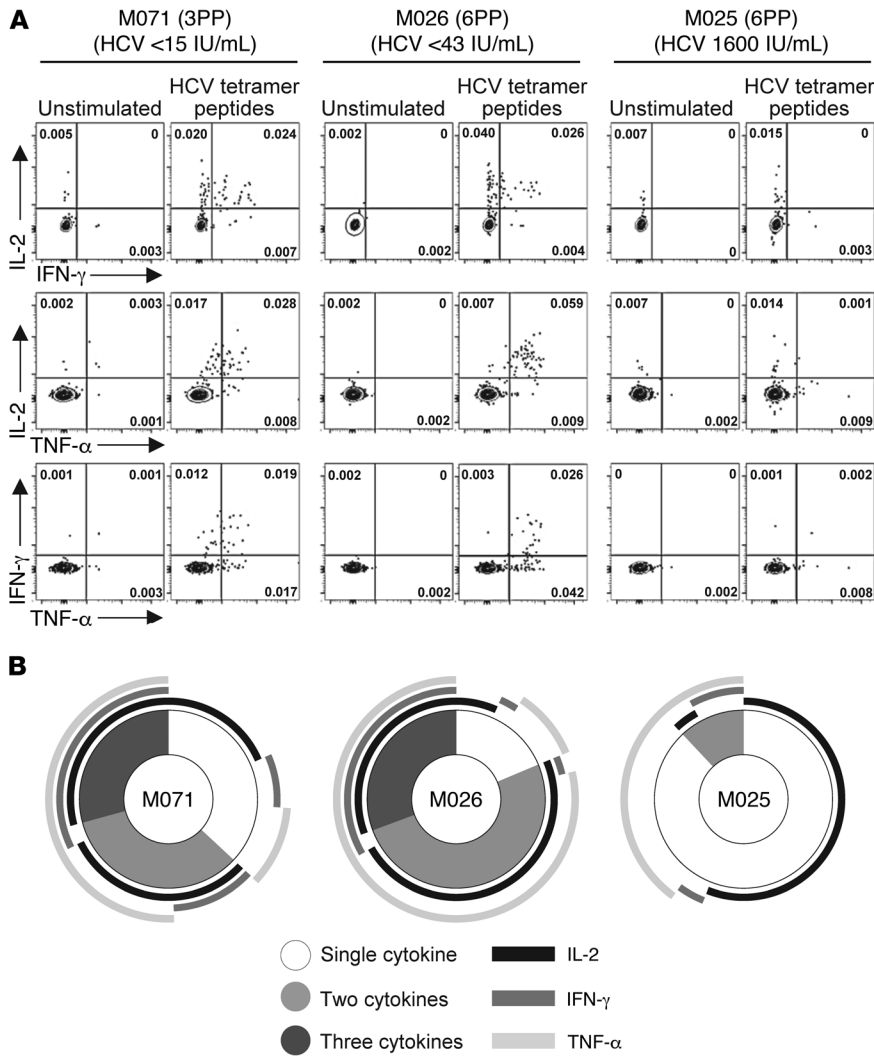


Figure 4. Function of CD4⁺ T cells targeting tetramer epitopes in 3 women with robust postpartum viral control. (A) Production of IL-2, IFN- γ , and TNF- α measured by intracellular cytokine staining following challenge of PBMCs with peptides corresponding to the epitopes used in class II tetramer studies (see Supplemental Table 2 for list of tetramers for each subject). (B) Polyfunctionality of CD4⁺ T cell responses. Pie charts represent populations of CD4⁺ T cells secreting a single cytokine (either IL-2, IFN- γ , or TNF- α ; white pie slices), 2 cytokines (medium gray pie slices), or all 3 cytokines (dark gray pie slices). Arcs represent production patterns of the individual cytokines: IL-2 (inner black arcs), IFN- γ (middle medium gray arcs), and TNF- α (outer light gray arcs).

characterization of these cells was based on relatively rare populations observed in a small subset of our cohort and warrants verification in additional studies.

How recovery of functional HCV-specific CD4⁺ T cells contributes to postpartum viral control remains to be determined. We previously demonstrated that the drop in viremia after childbirth is accompanied by renewed selection pressure on class II epitopes (15). It is possible that CD4⁺ T cells provide help for restoration of the CD8⁺ T cell response, but we also cannot rule out a helper effect for B cells. Future studies will be needed to assess the downstream effects of improved HCV-specific CD4⁺ T cell immunity after childbirth.

In conclusion, we found that postpartum reduction in HCV replication is associated with a boost in functional effec-

tor memory HCV-specific CD4⁺ T cell responses. This unexpected finding underscores the value of studying hepatitis C in the context of pregnancy as a unique model of natural immune restoration against persistent viral pathogens and the potential plasticity of T cell exhaustion.

Methods

See Supplemental Methods for detailed methods.

Statistics. Statistical differences between groups were assessed with 2-tailed, nonparametric tests for paired (Wilcoxon signed-rank test) and unpaired (Mann Whitney U test) data. Bivariate associations were measured by Pearson’s or Spearman’s correlation. Independent effects of determinates of viral control and postpartum T helper responses were assessed by multivariable linear regression. Statistical significance was defined as P less than 0.05.

Study approval. This study was approved by the IRBs of The Ohio State University and Nationwide Children’s Hospital.

Author contributions

JRH, CMW, AG, and MRP designed the cohort study. JRH, SLC, CMW, GML, and AG designed the experimental plans. SLC, ATC, and JRH conducted the experiments. SLC, ATC, MMC and JRH analyzed the data. JRH and MRP recruited subjects. SLC, JRH, and CMW wrote the manuscript. All authors provided critical review of the manuscript.

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