## Title

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# Memory T Cell Proliferation before Hepatitis C Virus Therapy Predicts Antiviral Immune Responses and Treatment Success

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**The contribution of the host immune system to the efficacy of new anti-hepatitis C virus (HCV) drugs is unclear. We undertook a longitudinal prospective study of 33 individuals with chronic HCV treated with combination pegylated IFN-α, ribavirin, and telaprevir/boceprevir. We characterized innate and adaptive immune cells to determine whether kinetics of the host response could predict sustained virologic response (SVR). We show that characteristics of the host immune system present before treatment were correlated with successful therapy. Augmentation of adaptive immune responses during therapy was more impressive among those achieving SVR. Most importantly, active memory T cell proliferation before therapy predicted SVR and was associated with the magnitude of the HCV-specific responses at week 12 after treatment start. After therapy initiation, the most important correlate of success was minimal monocyte activation, as predicted by previous in vitro work. In addition, subjects achieving SVR had increasing expression of the transcription factor T-bet, a driver of Th1 differentiation and cytotoxic effector cell maturation. These results show that host immune features present before treatment initiation predict SVR and eventual development of a higher frequency of functional virus-specific cells in blood. Such host characteristics may also be required for successful vaccine-mediated protection.** ***The Journal of Immunology*****, 2018, 200: 000–000.**

Treatment of hepatitis C virus (HCV) infection will change significantly in the coming years because new all-oral treatment options are available with a shorter duration of treatment and more manageable side effects. Drug therapy eliminates the infection in most cases, with success indicated by sustained aviremia. Sustained virologic response (SVR) is defined as an absence of detectable HCV RNA in blood ≥12 wk after completion of antiviral therapy when using a U.S. Food and Drug Administration–approved nucleic acid test having a sensitivity ≤ 25 IU/ml (1, 2). In >99% of patients, achievement of SVR after completion of therapy has been shown to be durable for ≥5 y (3).

Combination therapy with pegylated IFN-α (PEG–IFN-α) and ribavirin (RBV) served for more than a decade as standard treatment for chronic HCV (4), although the mechanisms by which these drugs work are not fully understood. Variables predictive of treatment success include polymorphisms near the *IFNL3* (*IL28B*) gene (5, 6) and HCV genotype (2, 7). However, the host immune mechanisms mediating these associations have not been clearly elucidated. Certain immune functions, including greater frequency of HCV-specific $CD4^+$ T cells (8, 9) and higher anti-HCV Ab titers at pretreatment time points (10), are also indicators of success, suggesting that adaptive immunity is critical for elimination of virus; however, many longitudinal studies have reported data that fail to support this conclusion. Barnes et al. (11) reported a profound decline in the frequency of IL-2– and IFN-γ–secreting HCV-specific T cells following the start of treatment.

IFN-α and RBV have a variety of immune effects that may contribute to treatment-mediated clearance (12–16). IFN-α alters the surface phenotype and functional capacity of dendritic cells. We previously demonstrated a robust association between SVR and lower levels of monocyte activation in response to in vitro IFN-α stimulation (12). Type I IFN signaling in $CD8^+$ T cells is critical for the generation of effector and memory cells (13); therefore, IFN-α treatment might permit an expansion of these cells. Thus, IFN-based therapies may have lasting immunologic effects that differ

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Abbreviations used in this article: AUC, area under the curve; CM, central memory; DAA, direct-acting antiviral; HCV, hepatitis C virus; mDC, myeloid dendritic cell; PacBlue, Pacific Blue; PD-1, programmed cell death protein 1; pDC, plasmacytoid dendritic cell; PEG–IFN-α, pegylated IFN-α; RBV, ribavirin; STRIDE, Study of Treatment Response and Immunologic Determinants; SVR, sustained virologic response.

from those of direct-acting antiviral (DAA)-only regimens. DAAs are molecules that disrupt viral replication and infection by targeting specific nonstructural proteins of the virus, thus presumably having lesser or even negligible effects on immunity. Therefore, it is possible that HCV recurrence rates are different after exclusive DAA therapy; if so, host immunity may contribute to the initial success of IFN-based therapy, as well as to subsequent long-term antiviral immunity (17–20). In patients with hepatocellular carcinoma, the use of DAA-only regimens may lead to more rapid tumor occurrence or to recurrence of HCV-associated hepatocellular carcinoma, possibly due to reduced anti-HCV immune surveillance (21, 22).

Only a few small studies have focused on immune dynamics during the critical 12 wk after initiation of therapy, a time period during which most eventual responders are first able to reduce viral loads to undetectable levels in peripheral blood. Understanding pretreatment immune variation and early host immune responses that may impact treatment outcomes could inform future DAA-only strategies, which are now focused on reducing the duration of therapy to ≤8 wk. This study may also prove unique, because newer DAA regimens achieve nearly universal clearance; thus, opportunities to evaluate host immune responses in successful and unsuccessful scenarios will be very limited. Our results show that outcomes can be predicted by baseline immune factors, as well as by differences in innate and adaptive immune responses occurring with treatment. Understanding these factors may enable more appropriate and efficient selection of therapy for HCV-infected people.

## Materials and Methods

### *Study design*

Anti-HCV Ab-positive genotype 1 viremic adults with chronic infection were recruited into a multicenter San Francisco cohort (Study of Treatment Response and Immunologic Determinants [STRIDE]). Subjects were recruited at University of California, San Francisco–affiliated hospitals and clinics, including Moffit-Long Hospital, the Zuckerberg San Francisco General Hospital, and the San Francisco Veterans Affairs Medical Center. Chronicity was established by the presence of anti-HCV Ab and/or persistent viremia for ≥6 mo prior to the start of the study. Forty-four subjects gave informed consent to participate in the study; this article includes the analysis of 33 subjects who completed ≥12 wk of therapy and whose final treatment outcome was determined. All subjects were hepatitis B virus surface Ab negative and HIV-1 negative. All subjects received treatment with PEG–IFN-α-2a plus weight-based RBV and DAA. One patient received boceprevir, 31 received telaprevir, and 1 was started on telaprevir and switched midcourse to boceprevir because of development of a rash. The total treatment duration was 4–48 wk for telaprevir- or boceprevir-inclusive regimens (per response-guided therapy guidelines). Blood samples were drawn and processed within 1 wk prior to initiation of treatment and then at 3 and 7 d and at 2, 4, 12, and 24 wk posttreatment. Those who had treatment stopped because of futility rules during response-guided therapy or who had HCV RNA that was detectable 12 wk after the end of treatment were designated treatment nonresponders (non-SVR). Subjects with undetectable HCV RNA 12 wk after treatment completion were designated as having achieved SVR. This study was conducted in accordance with international guidelines on good clinical practice and was approved by institutional review boards at all participating institutions (Committee on Human Research number 10-00267). All subjects provided written informed consent to participate in the study and provide specimens.

### *Viral genotyping and viral load*

HCV genotyping was performed by INNO-LiPA assay (Roche). Plasma viral loads were measured by quantitative PCR using Roche HCV COBAS TaqMan v2.0 (25 IU/ml limit of detection).

### *Flow cytometry*

PBMCs were stained in four panels containing anti-CD3–Pacific Blue (PacBlue), anti-CD3–Alexa Fluor 700, anti-CD25–PE–Cy7, anti-CD38–PE, anti-HLA–DR–PE–Cy7, anti-CCR5–allophycocyanin, anti-CD123–PerCP–Cy5.5, anti-CD16–PacBlue, anti-CD80–FITC, anti-CD83–PE, anti-CD86–allophycocyanin, anti-PD1–FITC, anti-PD-L1–PE, anti-HLA class I–allophycocyanin, anti-CD69–allophycocyanin–Cy7 (BD Biosciences), anti-CD4–Qdot655, anti-CD8–PE–Cy5.5, anti-CD14–Qdot605 (Invitrogen), anti-CD45RA–ECD, anti-CD127–PE, anti-HLA-DR–ECD, anti-CD20–ECD (Beckman Coulter), anti-CD11c–Alexa Fluor 700 (eBioscience), and anti-CD27–allophycocyanin–Cy7 (BioLegend). A staining reagent for dead cells (Invitrogen Aqua Live/Dead Fixable Stain) was included. Cells were washed and fixed in PBS containing 1% paraformaldehyde or permeabilized using a FOXP3 Fix/Perm Kit (BioLegend), according to the manufacturer's instructions, intracellularly stained with anti-Ki67–Alexa Fluor 488 (BD Biosciences), anti-FOXP3–PacBlue, anti-T-bet–BV711 (BioLegend), and anti-Eomes–eFluor 660 (eBioscience), fixed, and analyzed with an LSR II cytometer (Becton Dickinson) and FlowJo.

### *Cytokine flow cytometry assay*

Cytokine-producing cells were determined by intracellular staining after in vitro stimulation with PMA and ionomycin or with overlapping HCV peptides divided into subgenomic pools (core/E1, NS3, and NS4; BEI Resources). One million PBMCs were incubated for 16 h at 37°C with an HCV peptide pool (1 μg/ml) and anti-CD28 + anti-CD49d costimulation (2 μg/ml BD FastImmune) or controls (50 ng/ml PMA and 1 μg/ml ionomycin) containing GolgiPlug (5 μg/ml). After incubation, the cells were washed and stained with anti-CD3–PacBlue, anti-CD4–Qdot655, anti-CD8–PE–Cy5.5, anti-CD45RA–ECD, and anti-CD27–allophycocyanin–Cy7. Cells were then washed, permeabilized using Cytofix/Cytoperm (BD Biosciences), and stained with anti-IFN-γ–PE–Cy7, anti-TNF-α–Alexa Fluor 700, anti-IL-10–PE (BD Biosciences), anti-IL-17–PE (eBioscience), and anti-IL-4–FITC (BioLegend).

### *Statistical analysis*

Statistical analysis was performed in the R programming environment. Boruta R package provides feature selection from variable importance measures, such as those output by a random forest. In our case, the precursor random forest was output by the R package randomForest (23). Boruta compares the original attributes' importance, with importance achievable at random and estimated using permuted copies of the variables. Attributes with significantly worse importance than shadow ones are consecutively dropped, whereas those significantly better than shadows are labeled "confirmed." Default parameter settings in the Boruta R package were used (e.g., $p$ value = 0.01, and multiple-comparisons adjustment by the Bonferroni method was applied). For the calculation of slopes, we first fit linear regressions and then extracted intercepts and slopes. The $p$ values for different slopes (or intercepts) according to treatment outcome were calculated using the $t$ test.

## Results

### *Subject characteristics and treatment outcomes*

Thirty-three subjects were enrolled in the STRIDE prospective study and followed throughout anti-HCV drug therapy. Table I shows the baseline features of enrolled subjects, categorized according to SVR versus non-SVR status. Twenty-four (72.7%) subjects achieved SVR and nine (27.3%) did not. No statistical differences in baseline variables were observed between SVR and non-SVR subjects, with the exception of the frequency of the *IL28B* rs12979860 CC genotype, which was associated with SVR (Table I) (5, 6).

### *Baseline T cell proliferation (Ki-67) predicts SVR*

We first tested whether any immunologic parameters measured prior to treatment might be associated with SVR using a random forest approach. All clinical variables represented in Table I were first included in the analysis. The attributes confirmed as relevant by the Boruta algorithm were IL-28B genotype and viral subgenotype (Fig. 1A), in agreement with previous studies (5–7). Next, we examined the association of baseline immunologic parameters with later achievement of SVR. In each subject, we assessed >350 immunophenotypes derived from cytometry panels including B, T, NK, and APC markers. These phenotypes

Table I. Subjects characteristics at baseline

| Variable | SVR ($n$ = 24) | Non-SVR ($n$ = 9) | $p$ Values |
|---|---|---|---|
| Age (y; mean ± SD) | 51 ± 12.6 | 59 ± 6.07 | 0.15 |
| Gender ($n$ [% of subjects]) | | | |
| Female | 10 (41.6) | 5 (55.5) | 0.54 |
| Male | 13 (54.1) | 4 (44.4) | |
| Unknown | 1 (4.1) | 0 (0) | |
| Ethnicity ($n$ [% of subjects]) | | | |
| White | 10 (41.6) | 5 (55.5) | 0.64 |
| Black | 4 (16.6) | 2 (22.2) | |
| Asian | 4 (16.6) | 0 (0) | |
| Native American | 1 (4.1) | 0 (0) | |
| Latino | 3 (12.5) | 2 (22.2) | |
| Unknown | 2 (8.3) | 0 (0) | |
| HCV genotype ($n$ [% of subjects]) | | | |
| 1a | 13 (54.1) | 9 (100) | 0.06 |
| 1b | 7 (29.1) | 0 (0) | |
| Subtype unavailable | 3 (12.5) | 0 (0) | |
| Subtype unknown | 1 (4.1) | 0 (0) | |
| IL-28B genotype ($n$ [% of subjects]) | | | |
| CC | 11 (45.8) | 0 (0) | 0.01 |
| CT or TT | 12 (50.0) | 9 (100) | |
| Unknown | 1 (4.1) | 0 (0) | |
| Intravenous drug user ($n$ [% of subjects]) | | | |
| Yes | 8 (33.3) | 6 (66.6) | 0.18 |
| Unknown | 4 (16.6) | 0 (0) | |
| Log baseline viral load (IU/ml; mean ± SD) | 13.95 ± 1.56 | 15 ± 0.73 | |
| Diabetes at baseline ($n$ [% of subjects]) | | | |
| Yes | 1 (4.1) | 1 (11.1) | 0.48 |
| Unknown | 1 (4.1) | 0 (0) | |
| Hypertension at baseline ($n$ [% of subjects]) | | | |
| Yes | 12 (50.0) | 2 (22.2) | 0.12 |
| Unknown | 1 (4.1) | 0 (0) | |
| Dyslipidemia at baseline ($n$ [% of subjects]) | 2 (8.3) | 1 (11.1) | 0.83 |
| Cirrhosis at baseline ($n$ [% of subjects]) | 1 (4.1) | 0 (0) | 0.53 |

(including activation, proliferation, T-bet and Eomes, nonvirus-specific and virus-specific T cell responses, regulatory cells, and APC-related variables), together with the IL-28B type and viral subgenotype, were included in the random forest analysis. The 30 most important variables rated are shown in Fig. 1B. Two host immune parameters measured before therapy were found to be particularly important by the algorithm (asterisks in Fig. 1B). Both were related to Ki-67 expression in T cell subsets, suggesting that maintenance of T cell proliferation contributes to eventual SVR achievement. IL-28B genotype was rated of lower importance than these immunophenotypes. Baseline levels of $CD4^+$ and $CD8^+$ memory T cells expressing Ki-67 were higher in SVR subjects (Fig. 1C, 1D). Ki-67 expression in these subsets did not differ by subject IL-28B type.

### *Treatment failure is associated with higher expression of activation markers in monocytes*

We previously observed that monocyte responsiveness to IFN-α treatment in vitro is associated with failure to achieve SVR, suggesting that innate immune responsiveness may influence clearance (12). To test whether restrained APC activation might also be associated with treatment response in vivo, the markers CD80, CD83, CD86, HLA class I, CD69, and PD-L1 were used to assess activation of monocytes, myeloid dendritic cells (mDCs), plasmacytoid dendritic cells (pDCs), and B cells. Our new longitudinal data corroborate the previous result: subjects achieving SVR manifested little or no monocyte activation after treatment initiation, whereas those failing to achieve SVR demonstrated consistent monocyte activation in the first week of treatment (Fig. 2A, 2B). Non-SVR subjects manifested higher frequencies of $CD80^+CD83^+CD86^+$ monocytes (Fig. 2A), with significant differences at 3 and 7 d (Fig. 2A, middle and right panels). Changes in individual activation markers (e.g., CD83) were also observed (Fig. 2B). A trend toward similar increases in B cell and mDC activation was observed in subjects not achieving SVR (Fig. 2C, 2D), but these increases were not statistically significant. No significant correlation was observed between greater Ki-67 expression in T cells before treatment and reduced monocyte activation posttreatment.

### *Higher T-bet expression in $CD4^+$ memory T cells from subjects achieving SVR*

We examined whether T-bet and Eomes expression was related to treatment-mediated clearance (24). $CD4^+$ and $CD8^+$ T cells were classified into naive ($CD27^+CD45RA^+$), central memory (CM; $CD27^+CD45RA^-$), effector memory ($CD27^-CD45RA^-$), and terminally differentiated effector ($CD27^-CD45RA^+$) subsets. Representative flow cytometry plots for T-bet and Eomes expression within $CD4^+$ and $CD8^+$ CM T cells from one non-SVR and one SVR subject are shown in Supplemental Fig. 1. There was a tendency toward increased T-bet expression among $CD4^+$ CM T cells from subjects achieving SVR: both groups started with similar means at baseline, but SVR subjects manifested subsequent increases (Fig. 3A). The slopes of T-bet expression were statistically different in SVR versus non-SVR groups (mean slope of 0.099 and −0.096, respectively), indicating increased T-bet only in those achieving SVR. Analysis of the area under the curve (AUC) for T-bet expression on $CD4^+$ CM T cells also revealed greater frequencies of $T\text{-bet}^+$ cells in SVR subjects (Fig. 3A). T-bet expression among $CD8^+$ CM T cells was also higher in SVR subjects (Fig. 3B), with apparent failure of T-bet expression in individuals not achieving SVR. The AUC for T-bet expression in $CD8^+$ T cells was statistically higher in SVR subjects (Fig. 3B). In contrast, the trajectory of Eomes expression in $CD4^+$ and $CD8^+$ CM T cells did not differ after treatment initiation (Fig. 3C, 3D), although SVR subjects demonstrated greater frequencies of $Eomes^+$ T cells when considered as AUC, which was only significant among $CD4^+$ CM T cells (Fig. 3C).

### *Similar virus-specific T cell responses in SVR and non-SVR groups*

The differences in Ki-67 expression, T-bet, and Eomes described above suggest possible differences in virus-specific effector T cell functions. Accordingly, we assessed the magnitude and kinetics of $CD4^+$ and $CD8^+$ T cell responses to three HCV peptide pools (core/E1, NS3, and NS4). Th1 and Th2 responses were defined by the proportion of cells producing IFN-γ, IL-2, and/or TNF-α (Th1) or the proportion producing IL-4 and/or IL-10 (Th2). Representative gating for $CD4^+$ and $CD8^+$ cells expressing IFN-γ, IL-2, TNF-α, IL-10, and IL-4 is shown in Supplemental Fig. 2. Surprisingly, no difference in the magnitude of Th1 responses was found in SVR versus non-SVR subjects during the first 12 wk after treatment when considering the total population of $CD4^+$ or $CD8^+$ T cells (Fig. 4A, left and right panels, respectively). Interestingly, however, within the $CD4^+$ CM T cell compartment, SVR subjects displayed higher responses to all Ags at week 12 ($p$ = 0.07, 0.02,

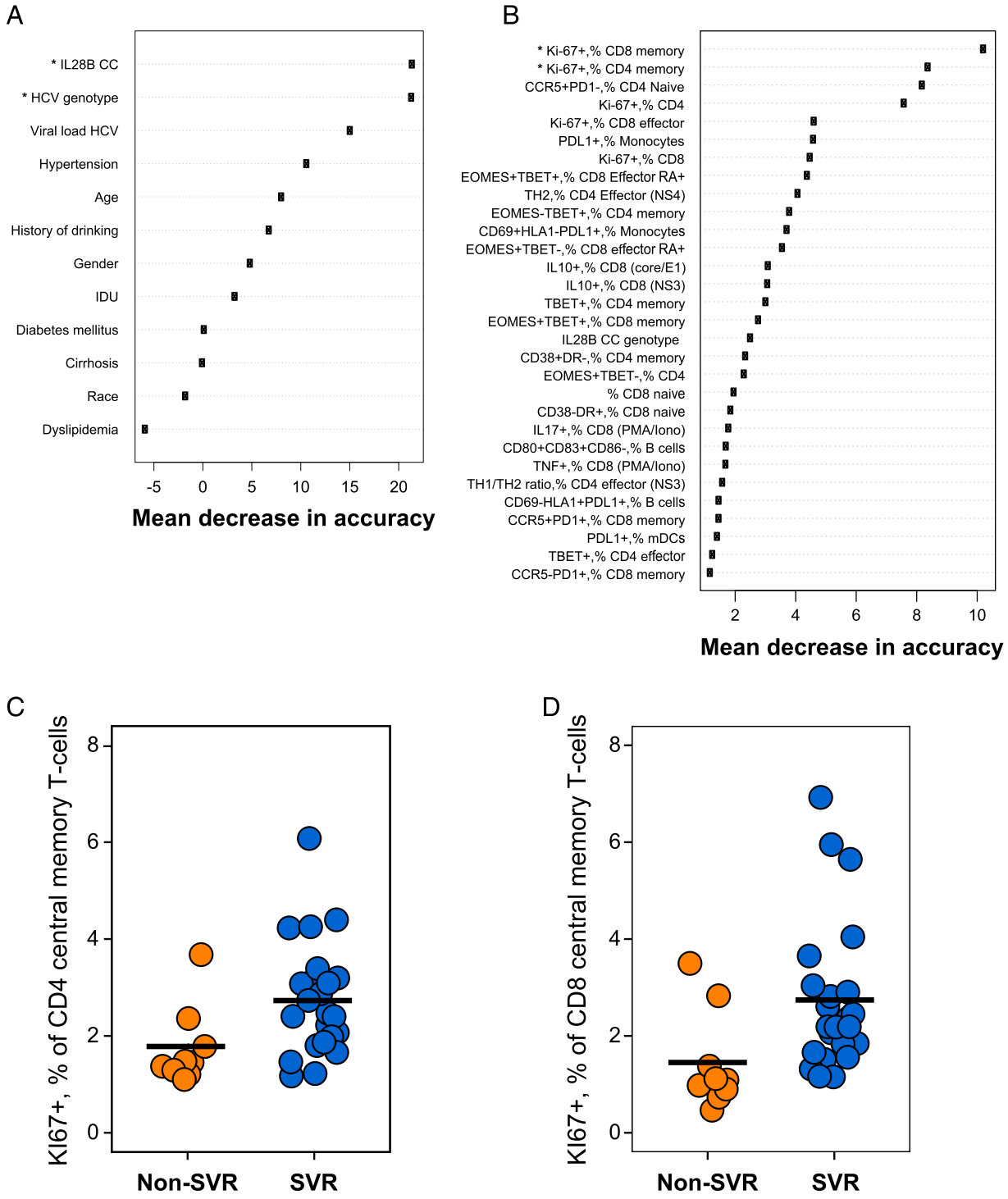


**FIGURE 1.** Important predictors of SVR selected by random forest. (**A**) Importance of clinical and demographic variables to SVR status, as determined by random forest. IL-28B CC genotype and HCV genotype are marked with asterisks (*) to indicate confirmed importance (superior to that of permuted variables) by Boruta. (**B**) Importance of immune variables measured by flow cytometry at baseline, together with the IL-28B CC type and HCV subgenotype. Ki-67 expression on $CD4^+$ and $CD8^+$ CM T cells was rated as more important than the IL-28B CC genotype (17th position). Expression of Ki-67 by $CD4^+$ (**C**) and $CD8^+$ (**D**) CM T cells in SVR and non-SVR groups.

and 0.02 for core/E1, NS3, and NS4, respectively, Fig. 4B). A positive association between the frequency of $CD4^+$ CM T cells expressing Ki-67 at baseline (Fig. 1C) and the magnitude of Th1 responses to core/E1 at week 12 is shown in Fig. 4C. In contrast, HCV-specific Th2 responses to core/E1 Ags were lower in SVR subjects (Fig. 4D), although the difference was not statistically significant when considering frequency or AUC (Fig. 4D, right panel). Similar trends were observed when considering responses to NS3 and NS4 Ags (data not shown).

### *Nonresponse to HCV treatment correlates with increased $CD8^+$ memory T cell activation*

We hypothesized that the monocyte activation differences observed might lead to different levels of T cell activation (i.e., to higher

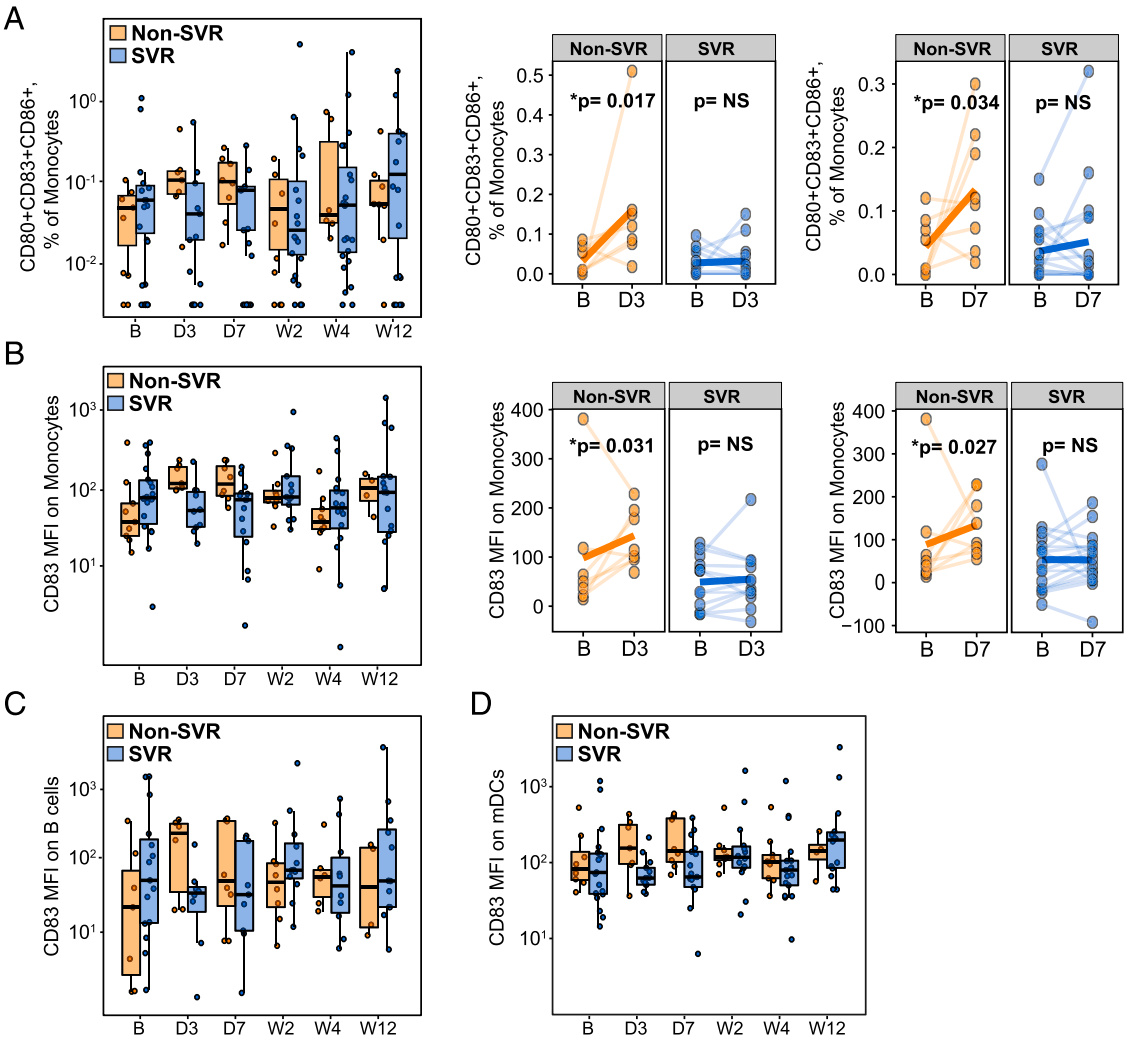


**FIGURE 2.** Upregulation of activation markers on monocytes after treatment initiation correlated with failure to achieve SVR. The expression of activation markers on monocytes, B cells, and mDCs is shown longitudinally at baseline, as well as at days 3 and 7 and weeks 2, 4, and 12 after treatment initiation. Monocytes were identified as HLA-DR$^+$CD20$^-$CD14$^+$, mDCs were identified as HLA-DR$^+$CD16$^-$CD14$^-$CD20$^-$CD11c$^+$, pDCs were identified as HLA-DR$^+$CD16$^-$CD14$^-$CD20$^-$CD123$^+$, and B cells were identified as HLA-DR$^+$CD14$^-$CD20$^+$. (**A**) Coexpression of CD80, CD83, and CD86 on monocytes longitudinally in SVR and non-SVR groups (left panel). Highlighted changes in coexpression of CD80, CD83, and CD86 between baseline and day 3 (middle panel) or baseline and day 7 (right panel). (**B**) Mean fluorescence intensity (MFI) of CD83 on monocytes. MFI of CD83 on B cells (**C**) and mDCs (**D**). The *p* values were calculated using the Friedman rank-sum test to compare baseline and day 3 and baseline and day 7 data in non-SVR versus SVR.

generalized T cell activation in the non-SVR group). Therefore, we evaluated the effect of anti-HCV treatment on CD38 and HLA-DR expression by CD4$^+$ and CD8$^+$ T cells. Total CD8$^+$ and CD8$^+$ CM T cells from non-SVR subjects exhibited increased expression of these activation markers during treatment (Fig. 5A, 5B, respectively), with statistically significant differences when the slope and AUC were calculated (Fig. 5C, 5D, respectively).

## Discussion

The host immune mechanisms that contribute to treatment-mediated HCV clearance are still unclear. Similar host mechanisms may also be needed for effective prophylactic vaccination. The present study shows evidence that T cell proliferation before therapy is strongly associated with achievement of SVR, being rated of higher importance than other established predictive factors, including *IL28B* type and HCV subgenotype (6, 7). In turn, T cell proliferation before therapy is linked to development of more robust Th1-type responses to virus by 12 wk after treatment initiation.

Effective T cell immunity requires maintenance of a memory compartment that, following reactivation, generates a large pool of short-lived terminal progeny while replenishing the long-lived memory pool. HCV-infected subjects who spontaneously resolve the infection develop long-lived memory T cell responses (25) that can be protective upon re-exposure (26). We found that subjects achieving SVR exhibited higher proliferative capacity among CD4$^+$ and CD8$^+$ CM T cells before treatment. This condition may be important, because Ag-specific CD4$^+$ and CD8$^+$ memory T cells include effector memory populations (27) that may synergize with IFN-inclusive HCV therapy, which harnesses the immune system by releasing it from chronic Ag exposure. In the case of PEG–IFN-α/RBV therapy, it was also found that patients presenting a better HCV-specific CD8$^+$ T cell proliferative potential at baseline were more likely to achieve SVR (28). Although it is difficult to reconcile this view with apparent similarities in circulating Ag-specific T cells in SVR and non-SVR subjects early in treatment, when viral loads are dropping rapidly in SVR subjects, it remains possible that liver-resident Ag-specific T cells in SVR

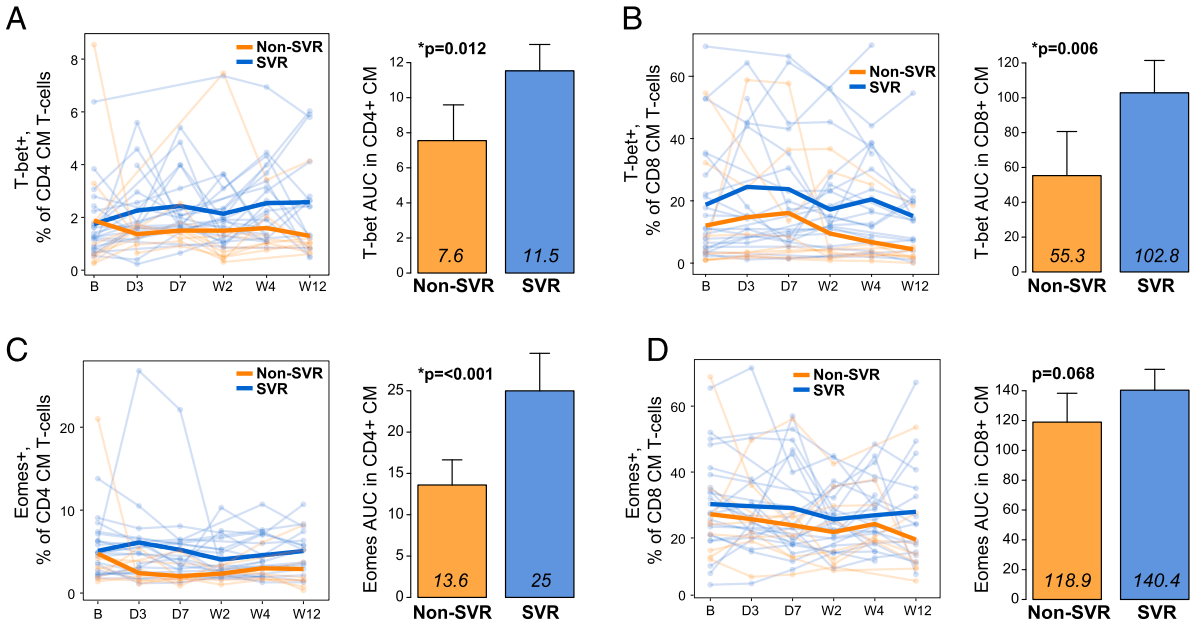


**FIGURE 3.** Longitudinal analysis of T-bet and Eomes expression in memory T cells. Longitudinal representation of T-bet (**A** and **B**) and Eomes (**C** and **D**) expression in $CD4^+$ and $CD8^+$ memory T cells. AUCs were calculated using the *PK* package in R and are shown in the bar graphs.

subjects are more numerous and/or more effective. Greater efficacy may be reflected broadly in greater T-bet expression and lesser generalized (nonspecific) activation of circulating cells.

T-bet was originally defined as the master regulatory transcription factor involved in promoting Th1-type $CD4^+$ T cell development in mouse models (16). T-bet is known to modulate genes involved in T cell mobilization (*CXCR3*), cell signaling (*IL12RB1*), and effector function (*IFNG*) (29). Additionally, T-bet expression is associated with cytotoxic $CD8^+$ T cell effector differentiation and function, including the upregulation of perforin and granzyme B (30, 31). T-bet has been implicated in sustaining memory subsets; however, T-bet levels decline as cells become more memory-like (32). Like T-bet, Eomes can positively influence the expression of IFN-γ in $CD8^+$ T cells (33, 34). However, Eomes expression increases as cells become more memory-like (30, 32, 33), and Eomes-knockout mice are deficient in long-term memory formation and fail to undergo homeostatic renewal (33, 35), highlighting its critical role in memory differentiation. Our longitudinal data demonstrate higher T-bet expression within memory $CD4^+$ and $CD8^+$ T cells in SVR subjects. T-bet levels rapidly increase after therapy initiation, with the rise being more pronounced and better maintained among SVR subjects. This rise may be indicative of superior T cell function among SVR subjects during the critical early weeks after treatment initiation, when viremia is first brought under control. Eomes expression among $CD4^+$ memory T cells increased in SVR subjects, but it declined in those not achieving SVR. Like T-bet, Eomes can induce Th1 differentiation and IFN-γ expression in mouse $CD4^+$ T cells (36).

After long exposure to persistent antigenic stimulation (e.g., in chronic HCV), $CD8^+$ T cells become dysfunctional, exhausted, and fail to properly differentiate into effective memory $CD8^+$ T cells (32). Such exhaustion is normally accompanied by expression of inhibitory molecules, like programmed cell death protein 1 (PD-1) (37). One recent study of mice chronically infected with LCMV showed that PD-1 expression can be repressed by T-bet, improving the fitness of exhausted $CD8^+$ T cells (38). In addition, continuous antigenic stimulation can lead to loss of T-bet expression (14, 38). Our observation of increased T-bet among $CD4^+$ and $CD8^+$ CM T cells in treatment responders suggests a detrimental effect of T cell exhaustion on SVR. However, in our study, no differences in PD-1 levels were observed between SVR and non-SVR patients (data not shown), and studies of conventional PEG–IFN-α/RBV therapy have similarly demonstrated minor or no differences (39).

Because our data suggested an association between greater T-bet expression and SVR, we further investigated the impact of therapy on HCV-specific T cell responses. In agreement with most, but not all, previously published data (26, 40–44), we found weak T cell responses after HCV pharmacologic therapy. Despite the impaired response overall, Th1 responses to HCV Ags (e.g., core/E1, NS3, and/or NS4) were higher in SVR subjects at 12 wk after therapy, suggesting that therapy-induced viral clearance may be associated with the induction or expansion of HCV Ag-specific T cells (8, 45). SVR subjects also showed a trend for low IL-4 and IL-10 production among HCV-specific $CD4^+$ CM T cells. Tsai et al. (46) reported that levels of IL-4 and IL-10 were elevated in chronic hepatitis C infection (47). Recently, Wan et al. (48) showed that, although Th1 and Th2 cytokines were markedly increased in serum, IL-4 and IL-10 were likely to be responsible for inhibition of an effective cell-mediated antiviral immune response. The overall low frequencies of HCV-specific $CD8^+$ T cells observed in our study may reflect a selective compartmentalization of HCV-specific T cells in the liver.

We previously demonstrated a strong association between SVR and lower levels of monocyte activation in response to in vitro IFN-α stimulation (12). The longitudinal in vivo data presented in this article showed an early increase (3 and 7 d after treatment initiation) in monocyte activation markers only in non-SVR subjects. Increased monocyte activation was positively associated with T cell activation ($CD38^+HLA\text{-}DR^-$) at days 3 and 7. These activation levels continued to rise in non-SVR subjects throughout treatment.

Restrained monocyte and T cell activation may be linked to viral clearance through greater virus-specific T cell number or function, but we observed only minor differences in HCV-specific T cells only after SVR establishment (12 wk). One possibility is that cells producing Th1 cytokines are indeed important contributors to clearance but that their efficacy is counteracted by an overabundance

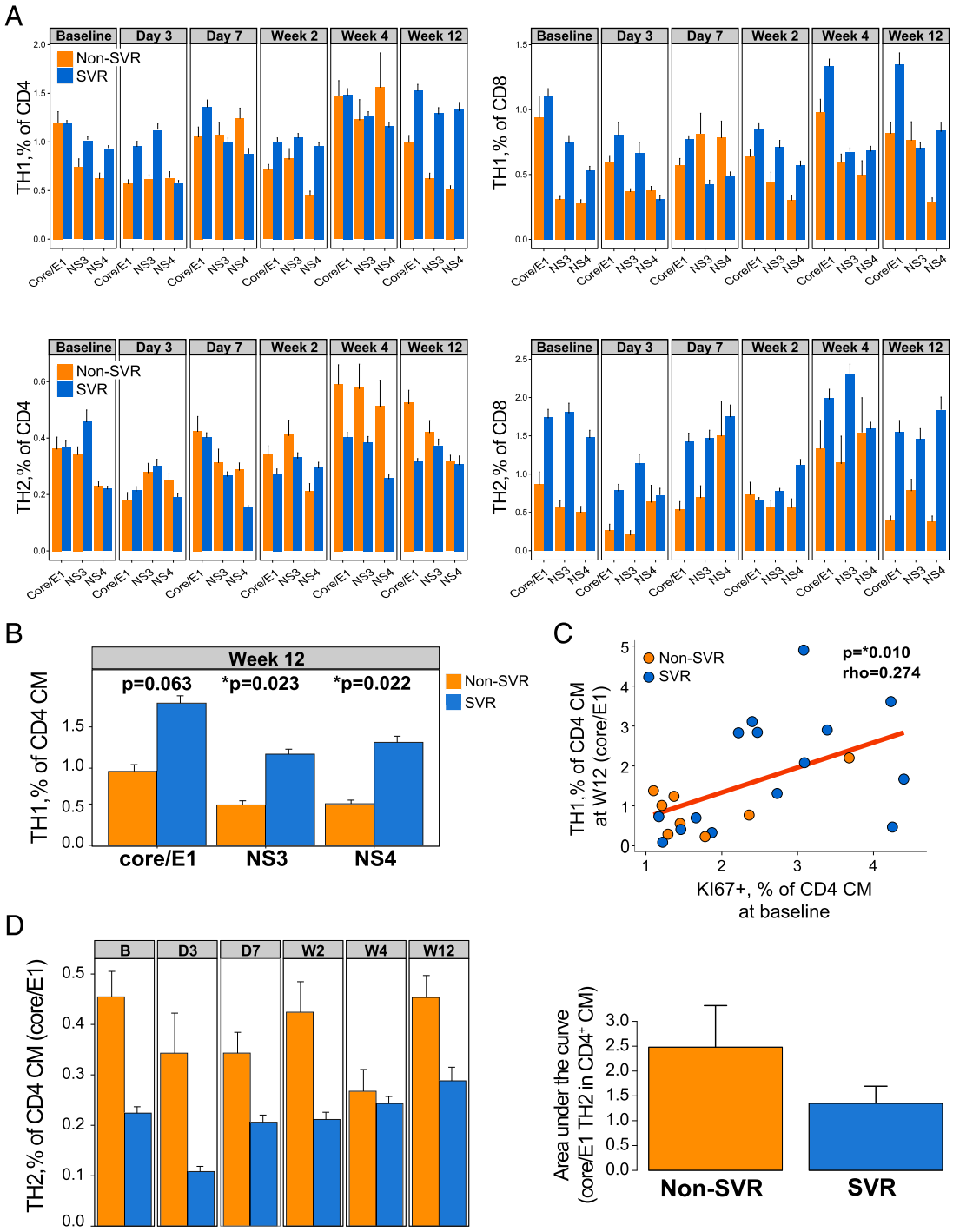


**FIGURE 4.** Ag-specific T cell responses throughout HCV treatment. (**A**) Overall Th1-type responses (upper panels; IFN-γ, IL-2, and/or TNF-α) and overall Th2 responses (lower panels; IL-4 and/or IL-10) among $CD4^+$ T cells (left panels) and $CD8^+$ T cells (right panels). (**B**) Th1 responses among $CD4^+$ memory T cells at week 12 in response to stimulation with core/E1, NS3, or NS4 peptides. (**C**) Correlation between the frequency of Th1 responses among $CD4^+$ CM T cells in response to core/E1 peptide stimulation at week 12 and the levels of $CD4^+$ CM T cells expressing Ki-67 before treatment initiation. (**D**) Th2 responses among $CD4^+$ CM T cells in response to core/E1 peptide stimulation during the treatment period. Area under the Th2 responses versus time curve (AUC) among $CD4^+$ CM T cells in subjects achieving or not achieving SVR is shown in the bar graph. The *p* values were calculated using a Wilcoxon rank-sum test.

of Th2 cytokines in patients not reaching SVR (Fig. 4D). It is also plausible that the functioning of innate immune cells is a primary determinant of treatment outcome (i.e., that monocyte activation in non-SVR patients contributes directly to HCV persistence, counteracting the effects of effector T cell expansion). Soluble HCV core has been shown to directly activate peripheral blood monocytes, inducing exuberant expression of TNF-α and IL-10 and diminishing TLR-induced expression of monocyte-derived IFN-α (49). This skewed generation of TNF-α and IL-10 has direct detrimental effects on the survival, number, and functions of pDCs (49). Not surprisingly, cytokine-driven pDC apoptosis leads to markedly diminished levels of IFN-α, because pDCs are the predominant source of IFN-α during acute viral infection. Thus, monocyte activation during HCV therapy might reflect an

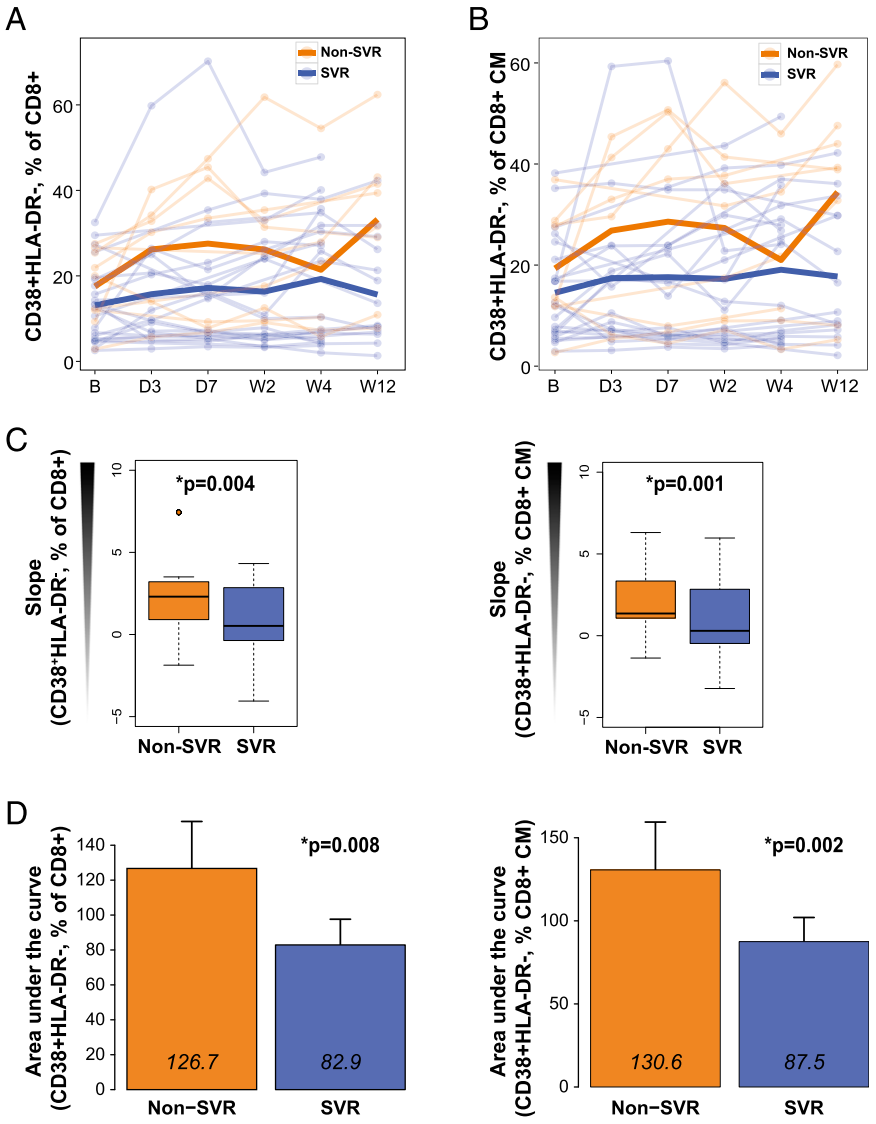


**FIGURE 5.** Longitudinal analysis of T cell activation markers. (**A** and **B**) Longitudinal representation of the $CD38^{+}HLA\text{-}DR^{-}$ phenotype among total and $CD8^{+}$ CM T cells. (**C**) Calculated slopes of the frequency of $CD38^{+}HLA\text{-}DR^{-}$ cells among total $CD8^{+}$ T cells (left panel) and $CD8^{+}$ CM T cells (right panel), indicating a greater positive slope (increase) in non-SVR subjects. (**D**) AUC for total $CD8^{+}$ T cells (left panel) and $CD8^{+}$ CM T cells (right panel). The *p* values for slope differences between treatment outcomes were calculated using *t* tests, whereas the *p* values for differences between the two AUCs (non-SVR versus SVR) were calculated using the *PK* package in R.

increased abundance of HCV core protein and/or could lead directly to reduced intrahepatic elaboration of type I IFN by host T cells.

Collectively, our data suggest development of a chronically activated and possibly exhausted effector phenotype in non-SVR subjects before treatment begins. In contrast, subjects achieving SVR have vigorously proliferating memory T cell compartments in place before treatment. After treatment begins, these subjects experience minimal monocyte activation, along with proliferation of T-bet–expressing T cells, and they eventually manifest a higher frequency of circulating HCV-specific T cells by 12 wk. Thus, it appears that the status of the host immune system prior to treatment is determinative of treatment outcome, signaling the immunologic capacity to augment antiviral drug effects. The predictive immune parameters noted in this article might be important determinants of successful immunity to HCV, and they may inform efforts to develop candidate HCV vaccines. In addition, maintenance of memory T cell populations via proliferation might be determinative of outcome for other chronic viral infections in humans (50).

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## Disclosures

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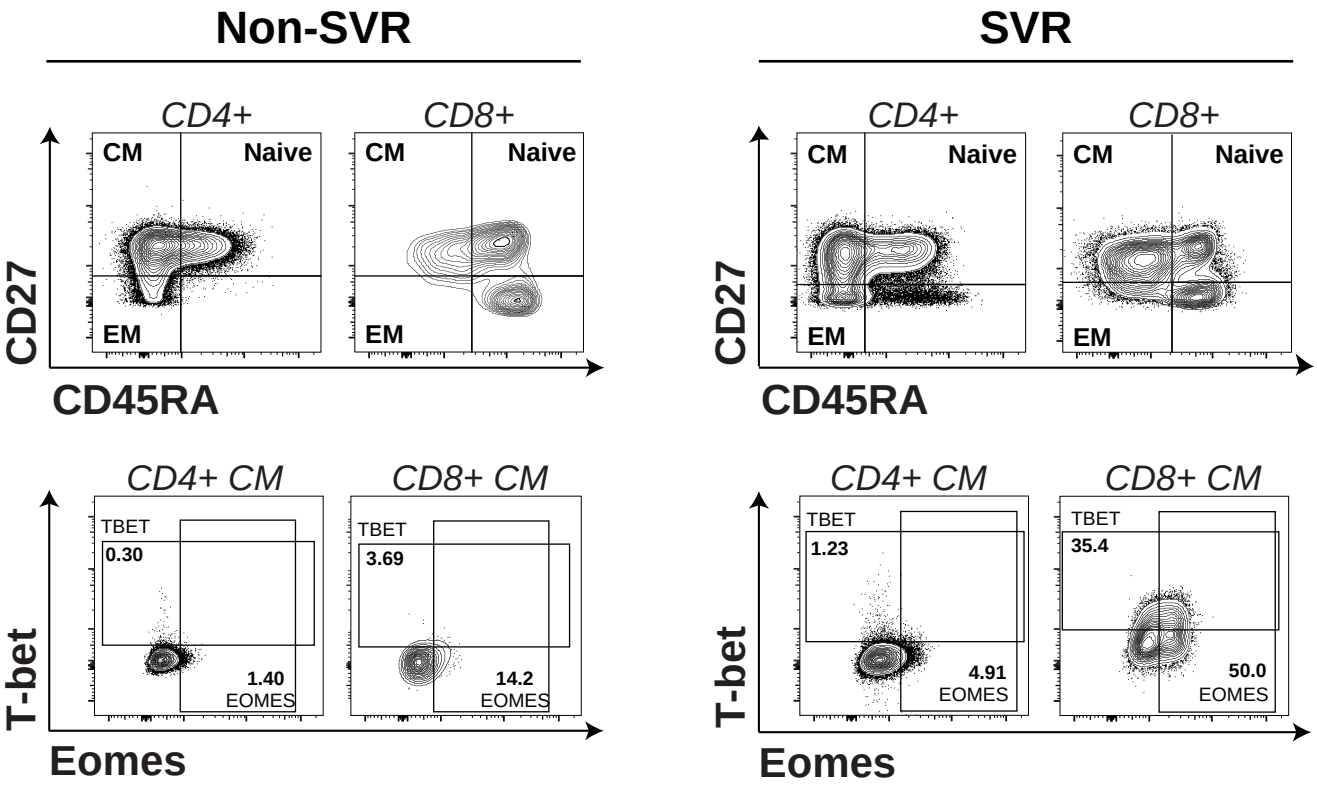
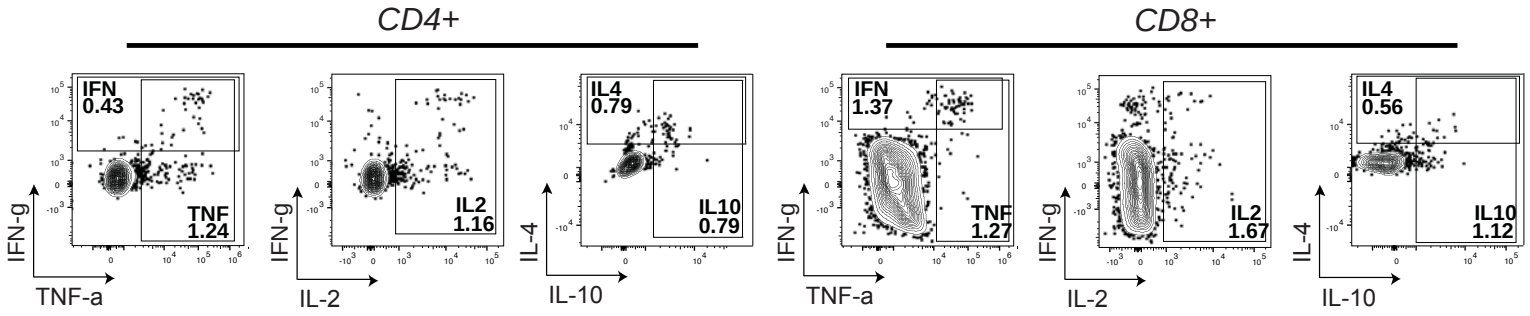



**Supplementary Figure 1.** Gating strategy for T-bet and Eomes expression (lower panels) on $CD4^+$ and $CD8^+$ memory T-cells (upper panels) from one non-SVR subject and one SVR subject. Gates were set using $T\text{-}bet^+Eomes^-$ cells (e.g., 1.23% in $CD4^+$ CM cells from SVR) across the dataset to define the boundary between $Eomes^-$ and $Eomes^+$ cells, and vice versa.




**Supplementary Figure 2.** Representative flow cytometry plots demonstrating expression of the different cytokines analyzed (IFN-γ, IL-2, TNF-α, IL-10, and IL-4) among $CD4^+$ T-cells (left plots) and $CD8^+$ T-cells (right plots). Gates for cytokine expression were set using unstimulated negative controls and PMA/ionomycin-stimulated positive controls to define boundaries between negative and positive cells.