

Reduced Naive CD4 T Cell Numbers and Impaired Induction of CD27 in Response to T Cell Receptor Stimulation Reflect a State of Immune Activation in Chronic Hepatitis C Virus Infection

Nicole L. Yonkers,¹ Scott Sieg,^{1,2} Benigno Rodriguez,² and Donald D. Anthony^{1,2}

Department of; ¹Pathology and ²Medicine, Divisions of Infectious and Rheumatic Diseases, University Hospitals Case Medical Center, Veterans Administration Medical Center, and Case CFAR, Case Western Reserve University, Cleveland, Ohio

Background. Chronic hepatitis C virus (HCV) infection is characterized by reduced numbers of functional HCV-specific T cells. In addition, chronically HCV-infected individuals have reduced response to vaccine. Alterations in naive CD4 T cell phenotype or function may contribute to these immune impairments.

Methods. Using flow cytometric analysis and enzyme-linked immunospot assay, we examined peripheral naive CD4 T cell phenotype and function in chronically HCV-infected patients and control subjects.

Results. We observed significantly lower absolute cell numbers of naive CD4 T cells in HCV-infected patients, localized to the $CD127^+ CD25^{low/-}$ and $CD31^+$ (RTE) subsets. Moreover, we found greater percentages of naive cells expressing CD25 and KI67 in HCV-infected patients, consistent with immune activation, further supported by higher plasma sCD27 levels. Functional analysis revealed an intact interferon- γ response to allogeneic B cell stimulus. However, after direct TCR stimulation, naive CD4 T cells from HCV-infected patients had altered up-regulation of KI67 and CD25 and less CD27 expression. The latter was associated with elevated baseline activation state. In addition, naive CD4 T cells from HCV-infected patients were more susceptible to cell death.

Conclusions. These numerical and functional defects may contribute to inadequate formation of virus and neoantigen-specific T cell responses during chronic HCV infection.

During acute hepatitis C virus (HCV) infection, broad and robust antiviral T cells with expression of IL-7R α (CD127) are thought to facilitate viral clearance [1, 2]. During chronic HCV infection, virus-specific T cells lack effector function and express inhibitory molecules (PD-1/TIM-3) [3, 4, 5, 6, 7]. Although little is known about

naive T cell function during chronic HCV infection, reduced responsiveness to vaccination with neoantigen has been observed [8, 9, 10]. Whether impaired naive T cell function or numbers contribute to formation of a defective memory T cell pool or to the lack of control of viral infection has not been defined.

Naive CD4 T cells emigrate from the thymus expressing CD31 and undergo homeostatic proliferation in response to self-peptide-MHC and IL-7 [11, 12]. After proper activation, long-lasting memory T cells with effector potential form. During the process of aging, thymic involution is thought to contribute to impaired output of $CD31^+$ -naive CD4 T cells [13]. Although homeostatic expansion may in part compensate for reduced output, one consequence is a larger $CD31^-$ -naive T cell pool with a more restricted TCR repertoire [14, 15]. This increase in $CD31^-$ -naive T cells is thought to

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Reprints or correspondence: Dr Donald D. Anthony, Biomedical Research Bldg 1028, Case Western Reserve University, 2109 Adelbert Rd, Cleveland, OH 44106 (dda3@case.edu).

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contribute to age-related immune impairment [14, 15]. In agreement, we reported that CD31⁻ are less likely than CD31⁺ naive T cells to proliferate after TCR stimulation [16]. Therefore, CD31 expression reflects functional potential.

To generate effective, long-lasting immune responses, naive CD4 T cell activation requires signaling through both TCR and costimulatory molecules, including CD27. Naive CD4 T cells constitutively express CD27 but transiently up-regulate expression further after activation. CD70, the CD27 ligand, is up-regulated on antigen-presenting cells during maturation and B and T cells after stimulation [17, 18]. CD27 ligation during naive T cell activation enhances TCR-dependent expansion and generation of long-lived memory cells [19, 20, 21]. At the same time, enhanced CD27 engagement, as modeled in CD70 transgenic mice, leads to effector memory T cell accumulation with B cell and naive T cell depletion [22, 23, 24]. Therefore, tight regulation of CD27-CD70 interactions is thought to be crucial for immune response formation and retraction [22, 23, 24].

To determine whether altered naive CD4 T cell phenotype or function are present during chronic HCV infection, we compared CD4 T cell subset numbers and function in HCV-infected patients and age-comparative healthy control subjects. We focused on CD31 expression, ability of T cells to up-regulate CD27, CD25, and Ki67 in response to TCR stimulation, and ability to secrete interferon (IFN)- γ in response to alloantigen.

MATERIAL AND METHODS

Participants

Study participants signed informed consent approved by the institutional review board for human studies at University Hospitals Case Medical Center or the Cleveland VA Medical Center (Cleveland, OH). HCV-infected patients ($n = 40$) were chronically infected (HCV serum antibody-positive for >6 months and detectable plasma HCV RNA) and not receiving treatment. Clinical characteristics for HCV-infected patients are shown in Table 1. Duration of HCV infection was imputed based on first year of reported risk factor (intravenous drug use, blood transfusion before 1992, and Vietnam service) [25]. Control subjects ($n = 25$) were recruited in comparable age range (mean age, 51 years; range, 35–62 years). Sex and racial distribution for control subjects were 36% male, 16% black, and 84% white.

Cell Isolation

CD45RO-depleted PBMCs were prepared using the bead method (Miltenyi Biotech). Naive CD4 T cells were isolated using negative selection (CD45RO/CD8/CD14/CD15/CD16/CD19/CD34/CD36/CD56/CD123/anti-TCR γ/δ /HLA-DR/CD235a depletion; Miltenyi Biotech).

CD4 T Cell Subset Frequency and Absolute Cell Count

PBMCs were stained with anti-CD3PERCP/CD4Pacific Blue/CD45ROFITC/CD25PE-CY7 (IL-2R)/CD127PE (IL-

Table 1. Hepatitis C Virus (HCV)-Positive Patient Clinical Parameters

Variable	HCV-infected patients
Plasma HCV bDNA level, IU/mL	631,572 (2332–5,384,560)
Serum albumin level, g/dL	3.8 (3.5–4.6)
Total serum bilirubin level, mg/dL	0.8 (0.4–2.0)
Blood International Normalized Ratio level	0.98 (0.9–1.23)
Platelet count, k/cmm³	223 (56–342)
Serum AST level, UL	48 (19–436)
Serum ALT level, UL	54 (16–434)
APRI, AST/PLT index	
<0.4	37
0.4–1.5	48
>1.5	15
HCV genotype, %	
1	83
2/3	12
Unknown	5
Duration of HCV infection, %	
5–10 years	5
>10 years	87.5
Unknown	7.5
Age, years	55 (47–69)
Male sex, %	97.5
Race, %	
Black	47.5
White	42.5
Unidentified	10

NOTE. Data are median (range), unless otherwise indicated ALT, alanine aminotransferase; APRI, AST to platelet ratio index (calculated as described [32]); AST, aspartate aminotransferase.

7R)/CD31APC (BD Biosciences) and CD27APC-CY7 (ebioscience). Absolute cell counts were obtained using Trucount absolute counting tubes (BD Biosciences). Flow cytometric analysis was performed on a BD LSRII.

Naive CD4 T Cell Activation, Proliferation, and Cell Death

CD45RO-depleted PBMCs were cultured (10^6 cells/mL) in the presence and absence of plate-bound anti-CD3 (10 μ g/mL) and soluble anti-CD28 (20 μ g/mL) agonistic antibodies at 37°C for 3 days, unless otherwise indicated. Cells were washed and stained with CD3/CD4/CD27/CD25, CD14/CD16/CD56/CD19APC, and KI67PE or AnnexinVFITC (BD Biosciences). Naive CD4 T cells were identified based on expression of CD4 and CD3 and lack of expression of monocyte (CD14), NK cell (CD16, CD56), or B cell (CD19) markers. CD27, CD25, and KI67 expression on CD4 T cells was evaluated at baseline and after culture. AnnexinV expression was evaluated within 30 min after staining by flow cytometry. Cell culture supernatants were analyzed for soluble CD27 (sCD27) by enzyme-linked immunosorbent assay (ELISA; Pelikine, RDI).

Naive CD4 T Cell IFN- γ

B cell lines from 3 separate healthy donors were carried in culture with NIH3T3 CD40L expressing fibroblast and IL-4 stimulation as described elsewhere [26, 27]. Naive CD4 T cells (200,000 cells/well) were stimulated for 3 days at 37°C in the presence and absence of activated allogeneic B cell lines (50,000 cells/well) in IFN- γ enzyme-linked immunospot assay plates. The maximal naive CD4 T cell IFN- γ -producing frequency in response to any of 3 B cell stimulator lines was compared between groups.

Plasma sCD27

Plasma sCD27 was measured by ELISA (Pelikine, RDI).

Statistics

All statistical analyses were performed using SPSS for Windows (SPSS). To compare continuous variables across and within groups, we used Mann-Whitney's *U* test. Associations between continuous variables were evaluated using Spearman's rank correlation coefficient. All tests of significance were 2-sided, and *P* values < .05 were considered to be statistically significant.

RESULTS

Naive CD4 T Cell Lymphopenia and Relative Increase in Central Memory CD4 T Cell Frequency during Chronic HCV Infection

We first evaluated CD4 T cell subset distribution in HCV-infected patients ($n = 34$) and age-comparative control subjects ($n = 23$). As shown in Figure 1A, 3 subsets of CD4 T cells were defined by CD27 and CD45RO expression: naive (CD27⁺CD45RO⁻), central memory (CD27⁺CD45RO⁺), and effector memory (CD27⁻CD45RO⁺) [28, 29]. We observed lower proportions of naive CD4 T cells in HCV-infected patients (35.3% vs 55.4%; $P < 0.001$) (Figure 1B). In addition, we found greater proportions of CD4 T cells with central memory phenotype in HCV-infected patients (48.9% vs 32.4%; $P < 0.001$) (Figure 1C), and no difference was observed in effector memory CD4 T cell frequency (10.9% vs 8.9%; $P = 0.4$) (Figure 1D). To further delineate CD4 T cells, we evaluated expression of CD25 (IL-2R α) and CD127 (IL-7R α) on CD4 T cell subsets. We adopted the gating strategy described by Dunham et al: CD127⁺CD25^{low/-} (IL-2-producing naive and central memory cells), CD127⁻CD25⁻ (effector cells expressing perforin), and CD127^{low}CD25^{hi} (mainly FoxP3⁺ T regulatory cells) [30]. With use of this method, the reduced CD4 T cell frequency in the HCV-infected group was localized to the CD127⁺CD25^{low/-} naive CD4 T cell compartment (32% vs 48.8%; $P < 0.001$). In contrast, greater proportions of central memory CD4 T cells in HCV-infected patients were distributed among all CD127/CD25 subsets (CD127⁺CD25^{low/-}, 41% vs 24.2%; $P < .001$; CD127^{low}CD25^{hi}, 3.9% vs 2.9%; $P = .07$; and CD127⁻/CD25⁻, 2.7% vs 1.2%; $P = .005$).

To further characterize CD4 T cell subset skewing, we measured absolute cell counts. We observed lower absolute numbers of naive CD4 T cells in the HCV-infected group (242 cells/mm³ vs 378 cells/mm³; $P = .002$) (Figure 1E). In contrast, absolute numbers of central memory and effector memory cells did not significantly differ between groups ($P = .1$ and $P = .6$, respectively) (Figures 1F and 1G), and total CD4 cell numbers were similar ($P = .7$). Intragroup analyses of sex and race did not reveal statistical differences in naive CD4 T cell number ($P = .8$ for each). In addition, intergroup comparisons revealed similar differences in absolute naive CD4 T cell counts when stratifying the analysis by sex and race ($P = .03$ and $P = .06$). These results indicate that increased frequencies of central memory CD4 T cells in HCV-infected patients are likely to be reflective of subset preservation in the context of decreased naive CD4 T cell numbers.

Lower Numbers of Recent Thymic Emigrants, Serum Alanine Aminotransferase Level, and Plasma HCV Level are Associated with Naive CD4 Lymphopenia

Naive CD4 T cell CD31 expression correlates with T cell receptor excision circle levels, providing a measure of recent thymic emigrants (RTE) [31]. IL-7 signaling, via CD127, influences CD31 expression on naive CD4 T cells [12]. Therefore, we measured CD31 expression on naive CD4 T cells. As shown in Figure 2A, we observed lower frequencies of CD31⁺-naive CD4 RTE in HCV-infected patients (49.5% vs 62.6%; $P = .001$), consistent with either reduced thymic output, redistribution, or peripheral depletion of this population. Absolute number of CD31⁺-naive CD4 T cells was also lower in HCV-infected patients (96.5 vs 192.5 cells/mm³; $P = .004$).

Comparing clinical parameters (HCV level, alanine aminotransferase (ALT) level, aspartate aminotransferase (AST) level, total bilirubin level, duration of infection, and platelet count) with CD4 cell subset frequency, we observed negative correlations between absolute number of naive CD4 T cells and HCV level ($r = -0.6$; $P = .008$) and serum ALT level ($r = -0.56$; $P = .02$) (Figures 2B and 2C). APRI has been shown to indicate HCV-related liver fibrosis [32]. APRI tended to negatively correlate with naive CD4 T cell absolute count ($r = -0.4$; $P = .07$). Together, these associations suggest that naive CD4 T cell lymphopenia is related to high HCV level, increased liver inflammation (ALT level), and potentially, disease stage (APRI).

Naive CD4 T Cells from HCV-Infected Patients Have Lower Surface CD27 Expression after TCR Stimulation

Naive CD4 T cells constitutively express CD27 and transiently further up-regulate expression after TCR activation [33]. We measured CD27 expression after stimulation with anti-CD3 and anti-CD28 agonistic antibodies. CD45RO-depleted

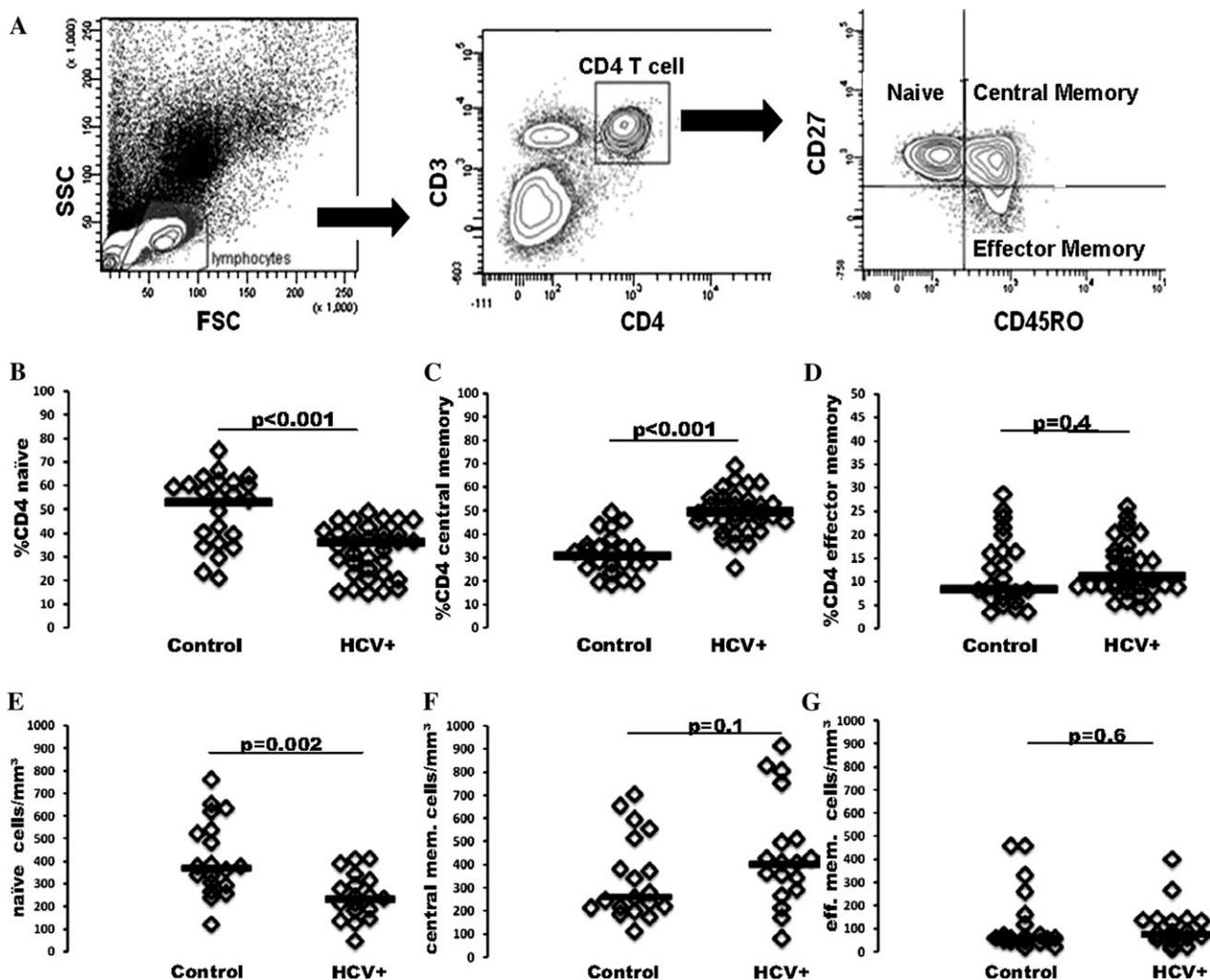


Figure 1. Naive CD4 T cell lymphopenia in persons chronically infected with hepatitis C virus (HCV). Freshly isolated PBMCs were stained for surface expression of CD3, CD4, CD27, and CD45RO. CD3⁺CD4⁺ T cell subset frequencies were identified by flow cytometric analysis as follows: naive CD27⁺CD45RO⁻, central memory CD27⁺CD45RO⁺, and effector memory CD27⁻CD45RO⁺. A representative sample from 1 individual is shown (A). CD4 T cell subset frequencies are shown in healthy control subjects ($n = 23$) and HCV-infected patients ($n = 34$) as naive (B), central memory (C), and effector memory (D); black lines represent median values. Fifty microliters of whole blood samples were stained for expression of CD3, CD4, CD27, and CD45RO, in BD Trucount absolute counting tubes. CD4 T cell subset absolute counts (cells/mm³) are shown in healthy control subjects ($n = 19$) and HCV-infected patients ($n = 18$) as naive (E), central memory (F), and effector memory (G); black lines represent median values.

PBMCs were cultured in the presence and absence of TCR stimulation for 3 days, and frequencies of naive CD4 T cells in the CD27LO (P3) and CD27HI (P4) gates were determined (Figure 3A). After TCR stimulation, the percentage of naive CD4 T cells in the CD27HI gate was lower in the HCV-infected group (44.6% vs 73.3%; $P = .006$) (Figure 3B). Kinetic analyses revealed frequencies of CD27HI-naive CD4 T cells in the HCV-infected group was nonsignificantly lower at day 1 after TCR stimulation (7.7% vs 17%; $P = .5$). The control group maintained peak levels of CD27 expression during days 3–5 with TCR stimulation, and 5 of 8 HCV-infected patients had peak expression at day 3, with a subsequent decrease in expression by day 5 (Figure 3C), resulting

in significantly fewer CD27HI cells in the HCV-infected group at day 5 (40.5% vs 83.9%; $P = .006$) (Figure 3C).

CD27 can be shed from the cell surface upon cell activation [34]. Therefore, we tested whether lower CD27 expression in response to TCR stimulation is attributable to increased shedding. We measured soluble CD27 in cell culture supernatants (Figure 3D). Both control and HCV-infected groups had increased levels of sCD27 in the presence, compared with absence, of TCR stimulation. However, we observed less sCD27 in HCV-infected patients' T cell culture supernatants (51.6 U/mL vs 73.3 U/mL; $P = .02$) (Figure 3D). Therefore, lower CD27 cell surface expression up on TCR stimulation in the HCV-infected group is unlikely attributable to increased shedding.

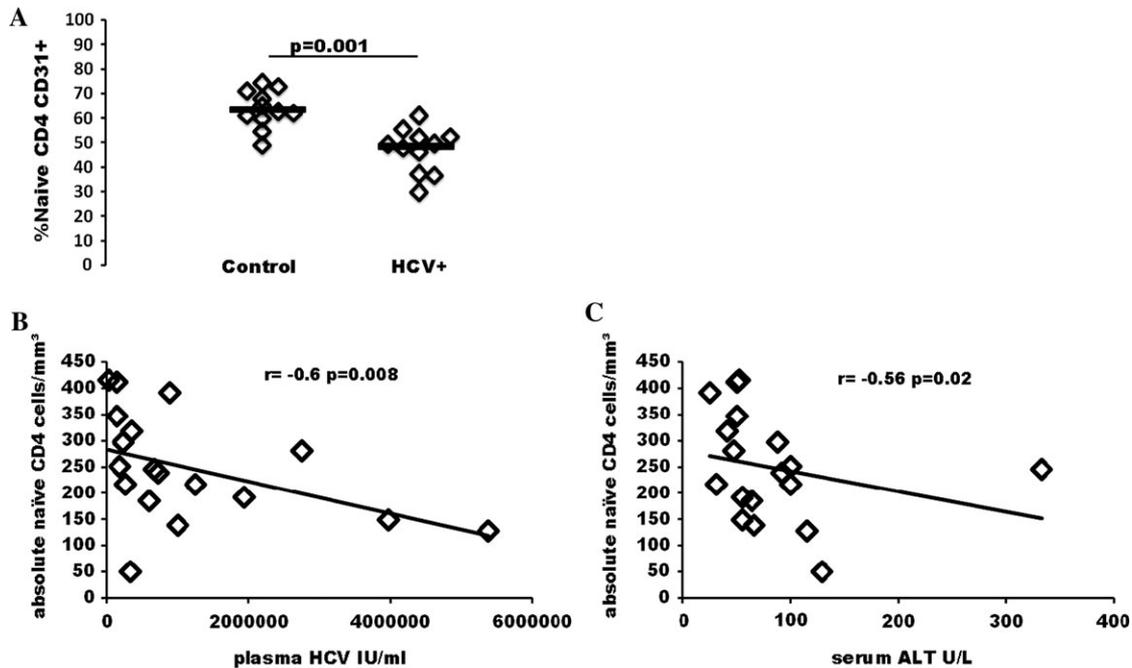


Figure 2. Fewer thymic emigrants, plasma hepatitis C virus (HCV) level, and liver inflammation are associated with reduced naive CD4 T cells in HCV-infected patients. Fifty microliters of whole blood samples were stained for expression of CD3, CD4, CD27, CD45RO, and CD31 in BD Trucount absolute counting tubes and cell numbers, and frequencies were analyzed by flow cytometric analysis. The percentage of naive CD4 T cells expressing CD31 is shown in healthy control subjects ($n = 11$) and HCV-infected patients ($n = 11$) (A); black lines represent median values. Negative correlations between absolute count of naive CD4 T cells and plasma HCV level (IU/mL) and serum alanine aminotransferase (ALT) level (U/mL) in HCV-infected patients ($n = 18$) is shown in B and C, respectively.

Naive CD4 T Cells in HCV-Infected Patients Have Greater Baseline Expression of CD25 and KI67 and Altered Kinetics in Up-regulation after TCR Stimulation, while IFN- γ Production in Response to Allo-antigen Remains Intact

In addition to up-regulation of CD27, naive CD4 T cells also increase surface expression of CD25 during TCR activation, allowing for proper IL-2 signaling [35, 36]. We measured CD25 and cycle entry marker (KI67) expression in naive CD4 T cells at baseline and after stimulation. We found greater percentages of naive CD4 T cells expressing CD25 (11.6% vs 3.3%; $P = .007$) (Figure 4A) and KI67 (6.2% vs 2.8%; $P = .03$) (Figure 4B) at baseline in HCV-infected patients.

Figure 4C shows CD25 and KI67 expression on naive CD4 T cells in the absence and presence of TCR stimulation at day 3 in a representative control subject. Expression of CD25 and KI67 on naive CD4 T cells after 3 days of TCR stimulation tended to be lower in HCV-infected patients (81.9% vs 94.2%; $P = .08$; 69.7% vs 83.4%; $P = .1$) (Figures 4D and 4F). Kinetic analyses performed in a subset of patients revealed no statistically significant differences in expression of CD25 on naive CD4 T cells after days 1, 3, or 5 of TCR stimulation ($P = .7$, $P = .08$, and $P = .7$, respectively) (Figure 4E). However, peak levels of CD25 were observed in the control group by day 3, but not until day 5 in most HCV-infected patients (Figure 4E). In addition, KI67 expression tended to be greater in the HCV-infected group at

day 1 of TCR stimulation (22.4% vs 4%; $P = .08$) (Figure 4G), although with little induction at day 1, this likely reflects greater baseline expression (Figure 4B). The KI67 expression on naive CD4 T cells peaked by day 3 but diminished by day 5 in all but one control subject and 4 of 8 HCV-infected patients. The remaining samples from HCV-infected patients had delayed up-regulation of KI67 expression, with peak expression at day 5 (HCV-infected group (69.5%) vs control subjects (54%); $P = .07$) (Figure 4G). Therefore, it appears that despite greater baseline expression of CD25 and KI67, at least half of the HCV-infected patients have delayed up-regulation of CD25 and KI67 in response to in vitro stimulation.

We also evaluated isolated naive CD4 T cell IFN- γ production in response to allo-antigen. We observed similar frequencies of IFN- γ -producing cells in response to allogeneic B cell lines comparing groups, indicating intact IFN- γ production in response to alloantigen (median 17 vs 17 spot forming units/well; $n = 15$ per group; $P = .8$).

Greater Expression of CD25 and KI67 on Naive CD4 T Cells at Baseline in HCV-Infected Patients is Associated with Lower CD27 Expression after TCR Stimulation, Higher Immune Activation, and Disposition to Undergo Cell Death

Proper activation of naive CD4 T cells requires integration of multiple signals initiated by engagement of the TCR,

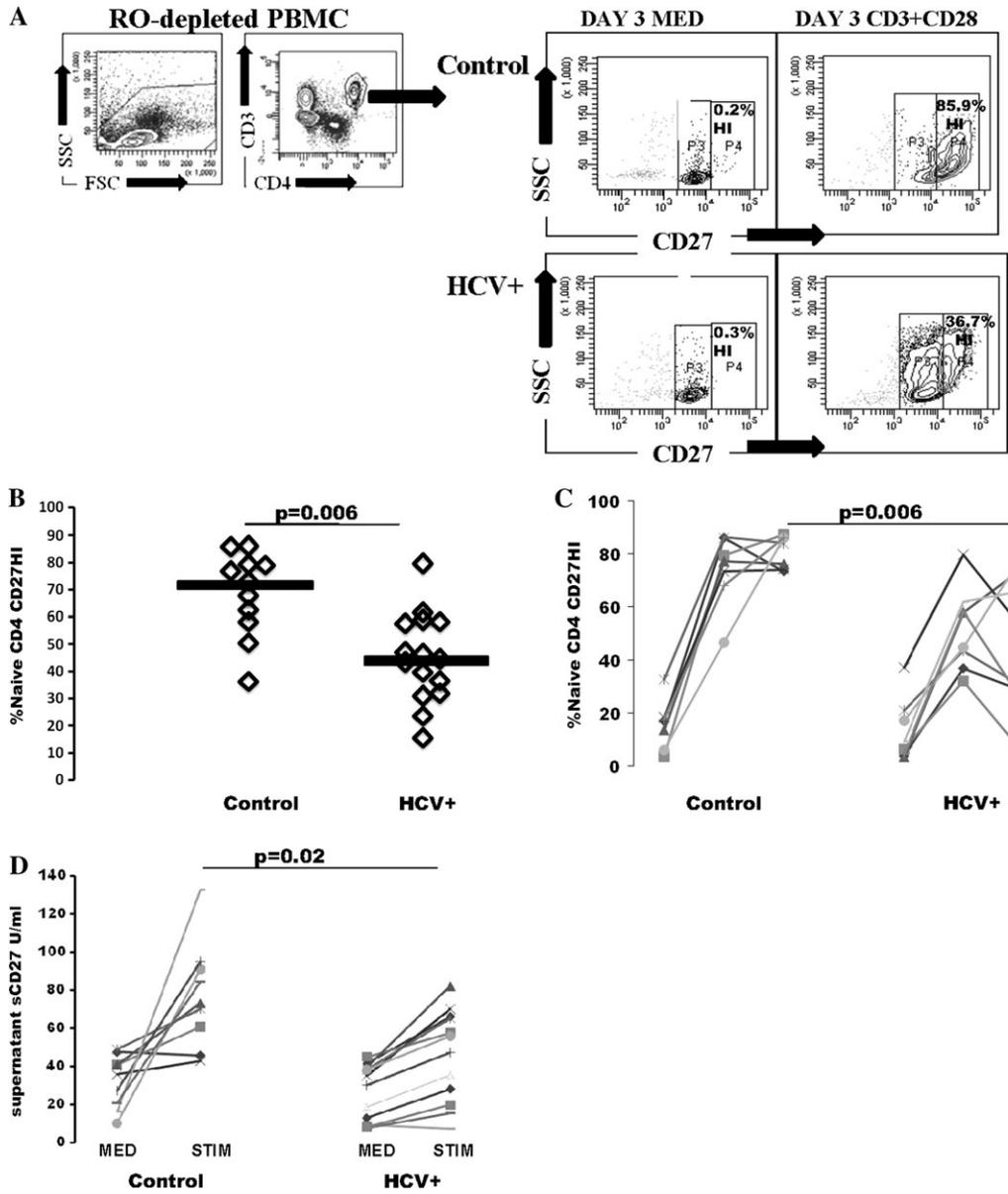


Figure 3. Pronounced defect in CD27 expression on naive CD4 T cells after TCR stimulation is observed in HCV-infected patients. CD45RO-depleted PBMCs were stimulated in the presence or absence of plate-bound CD3 (10 $\mu\text{g}/\text{mL}$) and soluble CD28 (20 $\mu\text{g}/\text{mL}$) agonistic antibodies for 3 days at 37°C. CD27 expression on CD3⁺CD4⁺ T cells was determined as low (gate P3) or high (gate P4). A representative gating example is shown for 1 healthy control subject and 1 HCV-infected patient in the absence (medium) or presence of TCR stimulation (CD3⁺CD28) at day 3; the percentage of CD3⁺CD4⁺ cells expressing high levels of CD27 is given in gate P4 (A). The percentage of CD3⁺CD4⁺ cells expressing high levels of CD27 after day 3 of TCR stimulation is given for control subjects ($n = 11$) and HCV-infected patients ($n = 15$) (B); black lines represent median values. Kinetic analysis of CD27 up-regulation was performed in a subset of control subjects ($n = 7$) and HCV-infected patients ($n = 8$). Percentage of CD3⁺CD4⁺ T cells expressing high levels of CD27 after TCR stimulation is shown at day 1, day 3, and day 5 (C). Soluble CD27 level (U/mL) was measured in cell culture supernatants after 3 days of culture in the absence (medium) or presence of TCR stimulation from control subjects ($n = 9$) and HCV-infected patients ($n = 12$) by enzyme-linked immunosorbent assay (D).

costimulatory molecules, and cytokine receptors [37]. Therefore, expression levels of various cell surface markers on naive CD4 T cells may influence whether activation results in a successful effector response and establishment of a viable memory cell pool. In particular, CD27 expression during naive T cell activation is important in antigen specific memory formation [19]. In

addition, CD27 is important for T cell survival after activation [20]. Therefore, we assessed the relationship between percentage of CD27HI cells after 3 days of TCR stimulation and percentage of naive cells expressing CD25 and KI67 at baseline (Figures 5A and 5B). In the HCV-infected group, we found a negative correlation between CD27HI expression on TCR activated naive

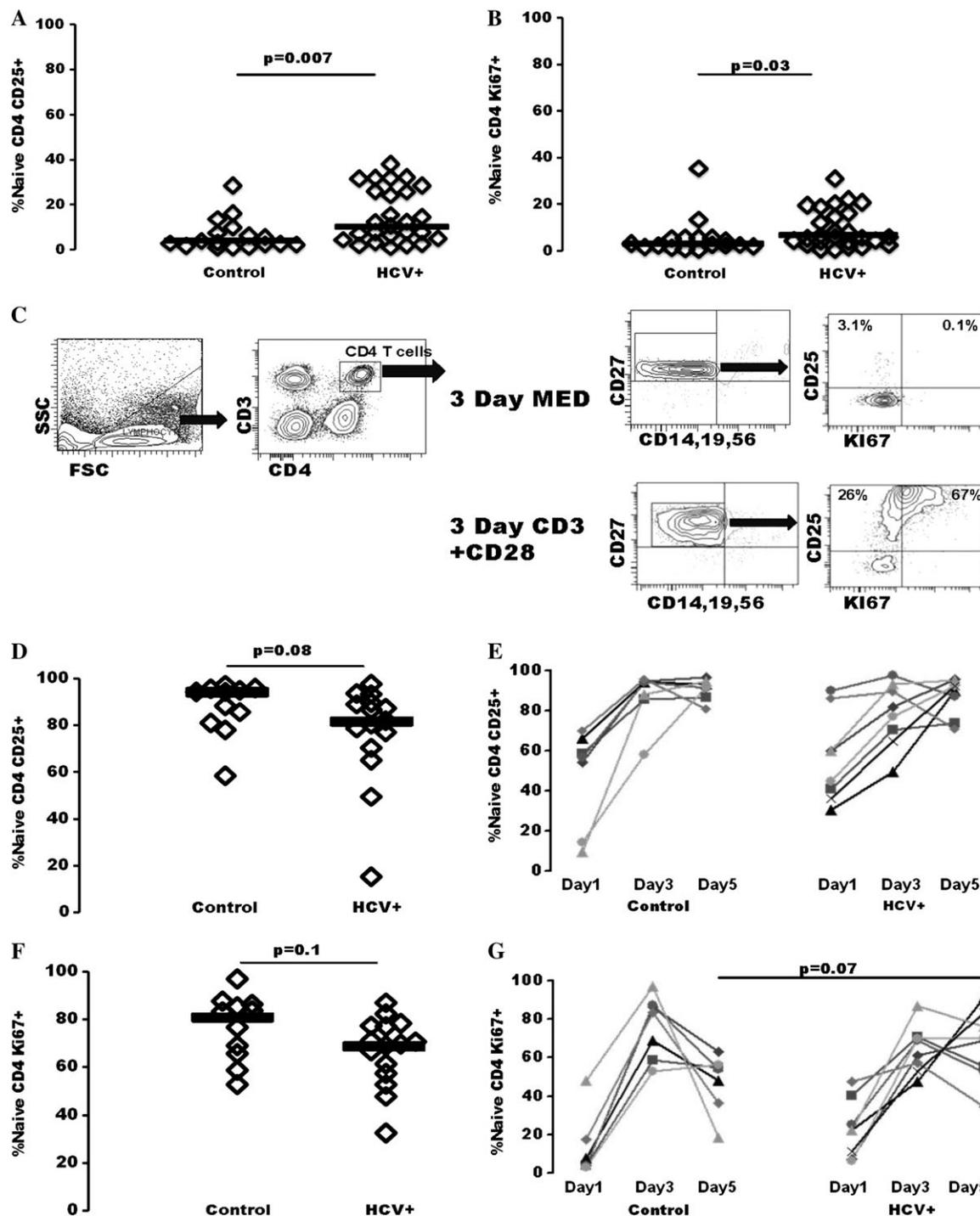


Figure 4. Naive CD4 T cells from hepatitis C virus (HCV)-infected patients have higher baseline expression of CD25 and Ki67 and altered up-regulation after TCR stimulation. CD45RO-depleted PBMCs stained for expression of CD3, CD4, CD27, CD25, Ki67, and CD14-16-19-56 (dump gate) at baseline and after stimulation in the presence or absence of plate-bound CD3 (10 $\mu\text{g}/\text{mL}$) and soluble CD28 (20 $\mu\text{g}/\text{mL}$) agonistic antibodies. CD25 and Ki67 expression on CD4⁺CD3⁺CD27⁺ T cells was determined at baseline and after TCR stimulation. The percentage of CD3⁺CD4⁺CD27⁺ cells expressing CD25 and Ki67 is shown in control subjects ($n = 17$) and HCV-infected patients ($n = 26$) (A and B, respectively); black lines represent median values. The gating strategy for CD25 and Ki67 expression on CD3⁺CD4⁺CD27⁺ cells is shown for 1 healthy control subject in the absence (medium) and presence of TCR stimulation at day 3 (C). The percentage of CD3⁺CD4⁺CD27⁺ cells expressing CD25 and Ki67 after 3 days of TCR stimulation is given for control subjects ($n = 11$) and HCV-infected patients ($n = 15$) (D and F, respectively); black lines represent median values. Kinetic analysis of CD25 and Ki67 up-regulation was performed in a subset of control subjects ($n = 7$) and HCV-infected patients ($n = 8$). Percentage of CD3⁺CD4⁺CD27⁺ T cells expressing CD25 and Ki67 after TCR stimulation is shown at day 1, day 3, and day 5 (E and G, respectively).

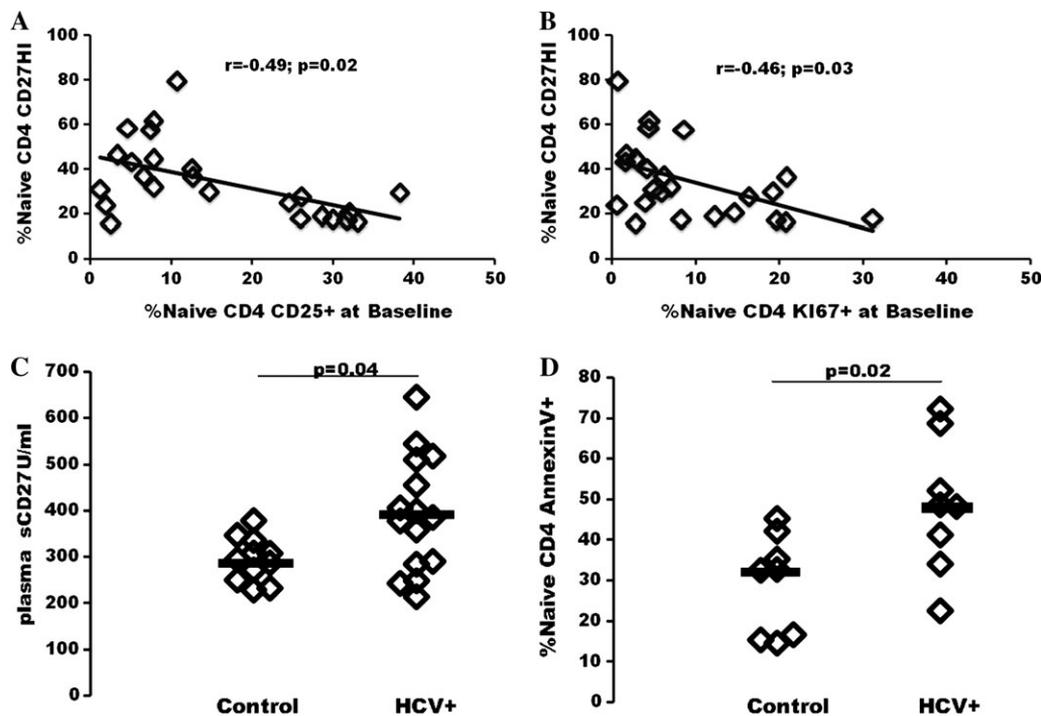


Figure 5. Higher naive CD4 T cell activation state is associated with reduced CD27 expression after TCR stimulation, increased plasma sCD27, and susceptibility to cell death. CD45RO-depleted PBMCs were cultured and stained as described in figure 4. Negative correlations between percentage of naive CD4 T cells expressing CD25 and KI67 at baseline and percentage of CD4⁺CD3⁺ cells expressing high levels of CD27 after TCR stimulation for 3 days are shown for HCV-infected patients ($n = 24$) (A and B, respectively). Plasma samples were obtained from control subjects ($n = 11$) and HCV-infected patients ($n = 15$). Plasma levels of sCD27 (U/mL) were determined by enzyme-linked immunosorbent assay (C); black lines represent median values. CD45RO-depleted PBMCs were stained for CD3, CD4, CD27, and Annexin V at baseline and after 3 days of culture in absence (medium) or presence of TCR stimulation. Flow cytometric analysis was used to determine the percentage of CD3⁺CD4⁺ cells staining annexin V positive in control subjects ($n = 8$) and HCV-infected patients ($n = 8$). D, Percentage of CD3⁺CD4⁺ annexin V–positive cells after 3 days of TCR stimulation.

CD4 T cells and baseline CD25 ($r = -0.49$, $P = .02$) and KI67 ($r = -0.46$, $P = .03$) expression. Therefore, greater naive T cell activation and cell cycling at baseline are associated with lower CD27 expression in response to TCR stimulus, suggesting that immune activation in vivo may contribute to impaired ability of naive CD4 T cells to respond to TCR stimulus.

Plasma sCD27 is the result of shedding from a number of cell types, including B cells and T cells, and is a marker of immune activation in the setting of B cell malignancy, HIV infection, and Systemic Lupus Erythematosus [38–40]. To determine whether evidence of in vivo systemic immune activation is present, we measured plasma sCD27 level. We observed higher sCD27 levels in HCV-infected patients (385.5 U/mL vs 295 U/mL; $P = .04$) (Figure 5C).

Finally, because CD27 signaling has been shown to facilitate memory T cell survival [19], we evaluated cell death after TCR stimulation by measuring AnnexinV staining after 3 days of culture in presence and absence of TCR stimulation. We observed a trend toward greater intrinsic cell death in media culture of samples from HCV-infected patients (40% vs 29%; $P = .07$). In response to TCR stimulation, significantly higher proportions of AnnexinV-positive cells were observed in HCV-infected patients (48% vs 33%; $P = .02$) (Figure 5D). Of interest,

we did not observe any cells staining AnnexinV positive in the CD27HI fraction, indicating that cells expressing high levels of CD27 are not dying. Therefore, naive CD4 cells from HCV-infected patients appear to be more susceptible to cell death upon TCR stimulation.

DISCUSSION

In the present study, we observed skewing in CD4 T cell subset distribution during chronic HCV infection, with fewer numbers of naive CD4 T cells localized to the CD127⁺CD25^{low/-} and CD31⁺ RTE subsets, and a relative increase in central memory CD4 T cell frequency. Further analysis revealed higher baseline naive CD4 activation, and cell cycling (greater CD25 and KI67 expression) was associated with lower CD27 expression after TCR stimulation (Figure 6). Although IFN- γ production in response to allo-antigen remained intact, these cells were prone to undergo apoptosis, consistent with impairment in effective memory cell formation. Finally, naive CD4 numerical and functional skewing was associated with plasma HCV level and liver inflammation (ALT).

Absolute naive CD4 T cell lymphopenia may be attributable to reduced thymic output, anatomic redistribution, or depletion.

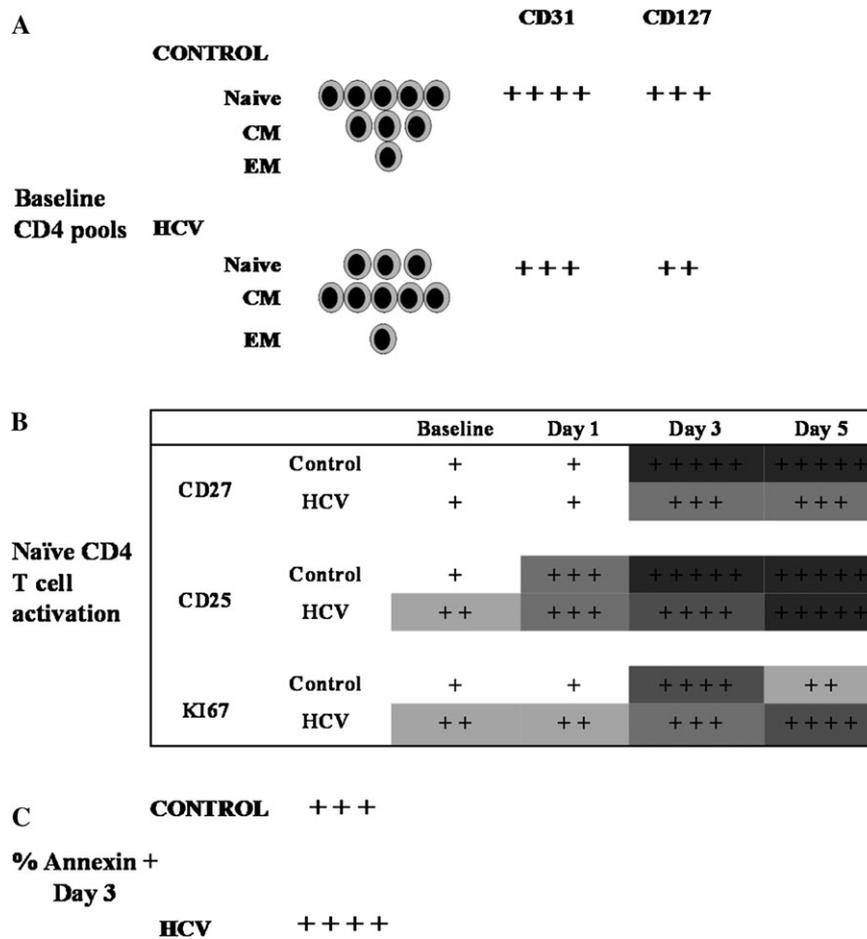


Figure 6. Summary of naive CD4 T cell phenotype and function in chronic hepatitis C virus (HCV) infection. *A*, CD4 T cell pool distribution and naive CD4 T cell CD31 and CD127 expression profile. *B*, TCR-induced naive CD4 T cell activation and cell cycle entry as function of time. *C*, TCR-induced naive CD4 T cell death after 3 days of stimulation.

Although our data do not address contributions of thymic output or anatomic redistribution, our observation of enhanced naive CD4 T cell activation (CD25 expression) and cell cycling (KI67 expression) is consistent with a stimulus contributing to depletion. The observed increase in plasma sCD27 levels also supports a state of immune activation, although the source of sCD27 may derive from many cell types, including CD4 T cells. Because only a small overlapping group of participants were studied for both naive CD4 enumeration and plasma sCD27 assays, we were unable to adequately assess a correlation between the plasma sCD27 and naive T cell activation. However, these findings suggest that immune activation contributes to naive CD4 T cell depletion. CD4 lymphopenia has been described in HIV-uninfected individuals with liver cirrhosis, 50% of whom had HCV-related cirrhosis [41]. The HCV-infected patients described here had less advanced stage liver disease (APRI), lower total bilirubin levels (mean \pm SD 0.83 mg/dL \pm 0.35 vs 2.4 mg/dL \pm 2.3) and greater platelet counts (mean \pm SD 202.6 k/cmm \pm 65.1 vs 95.1 k/cmm \pm 54.7), compared with the HCV-infected group reported by McGovern et al [41].

Therefore, the preserved total CD4 cell counts observed here are expected. However, the naive CD4 lymphopenia that we observed may be partly related to these previous findings. In particular, APRI scores for our participants tended to be associated with naive CD4 cell count, indicating that liver disease stage may have an effect on naive CD4 cell numbers. Although splenomegaly has been proposed to potentially contribute to this finding [41], our observed associations between naive CD4 T cell number and HCV level and ALT level suggest that other factors of HCV-related disease likely contribute.

A lymphopenic environment changes the dynamic of naive T cell homeostasis and has been termed lymphopenia-induced proliferation [42, 43]. Normally, basal signals from IL-7 and self-peptide-MHC drive slow homeostatic division of naive CD4 T cells [11, 12]. However, in lymphopenic mice, IL-7 independent, rapid, spontaneous proliferation induces memory T cell markers on naive T cells [44]. At the same time, CD31⁺ naive CD4 T cells are particularly responsive to IL-7 [12]. Therefore, reduced IL-7 receptor (CD127) and CD31 expression on naive CD4 cells likely renders them less responsive to IL-7

mediated homeostatic signaling. In addition, IL-7 signaling is implicated in sustaining CD31 expression on naive CD4 T cells [12]. Therefore, fewer numbers of CD31⁺-naive cells may result from reduced expression of CD127, the latter finding also previously observed by others [2, 45]. Although the factors underlying these perturbations are not known, our results confirm a state of immune activation (increased plasma sCD27 level), with specific increased activation of naive CD4 T cells (CD25 and KI67 expression) in HCV-infected patients. These findings are consistent with alterations in homeostasis akin to that observed during lymphopenia-induced proliferation.

Reduced naive CD4 T cell CD31 expression has been observed during aging, HIV infection, and multiple sclerosis [31, 46, 47]. Loss of less differentiated naive T cells is thought to dampen the generation of an effective immune response and even contribute to autoimmunity [14, 15]. We previously found that CD31⁺-naive CD4 T cells are more likely to enter cell cycle on TCR stimulation, which is associated with greater CD27 up-regulation on CD31⁺ than CD31⁻ cells [16]. Therefore, altered kinetics of naive CD4 T cell activation (CD25) and cell cycle entry (KI67) and lower CD27 expression after TCR stimulation may be partly attributable to decreased numbers of CD31⁺ cells.

CD27-CD70 interactions have a pivotal role in controlling T cell activation, expansion, and retraction. Tightly regulated expression of both molecules provides proper signaling in effective T cell response generation and survival [48]. This concept has been explored in mice [19, 22, 24]. Excessive CD70-CD27 signaling, as modeled in CD70tg mice, leads to naive T cell depletion, effector memory cell accumulation, and eventual death due to opportunistic infection (*Pneumocystis carinii*) [24]. In contrast, absence of CD27 expression in CD27^{-/-} mice leads to reduced primary and secondary immune response to influenza infection [19]. Of note, CD4 and CD8 T cell IFN- γ production in these mice was found to be intact. In general, these data are consistent with our findings that naive CD4 T cells from HCV-infected patients are defective in CD27 expression and are more susceptible to death after TCR stimulation, but IFN- γ production was intact. Lower CD27 expression after TCR stimulation may result from reduced up-regulation, cell surface shedding, or death of the CD27HI-expressing cells. We do not think that cell surface shedding or cell death substantially contribute, because we found lower levels of sCD27 in naive CD4 cell cultures from HCV-infected patients, and the CD27HI cells did not stain AnnexinV positive. Therefore, we favor impairment in up-regulation of CD27. Although the consequences of reduced CD27 expression on TCR stimulation are yet to be determined, mouse models predict impairment in effective memory T cell pool formation and/or maintenance.

Taken together, these results show that numbers of peripheral blood naive CD4 T cells are reduced in HCV-infected patients,

with selective depletion of CD31⁺ and CD127⁺CD25^{low/-} subsets. The naive CD4 T cells that remain are increased in activation state, which is associated with impaired response to TCR stimulus (lower CD27 expression). The decreased survival of naive cells after TCR stimulation is consistent with results observed in CD27-deficient mice, in which impaired CD27 signaling is known to contribute to lack of a sustained memory T cell pool. These findings may directly contribute to the known impaired virus specific effector memory T cell pool [4–7] and reduced response to vaccination [8–10] observed in chronically HCV-infected persons.

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