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## Peripheral virus-specific T-cell Interleukin-10 responses develop early in acute hepatitis C infection and become dominant in chronic hepatitis

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### Abstract

**Background & Aims**—Interleukin-10 (IL-10) has been ascribed pro-viral but anti-fibrotic properties in chronic hepatitis C virus (HCV) infection. In this study, we examined the role of HCV-specific T-cell IL-10 response in patients with acute and chronic HCV infection.

**Methods**—Peripheral HCV-specific T-cell IL-10 and IFN $\gamma$  responses were measured in cytokine Elispot assay using overlapping HCV-derived peptides in patients with chronic (n=61), resolved (n=15) and acute (n=8) hepatitis C, looking for their onset, quantity, breadth and durability relative to clinical and virological outcomes. The source and effect of HCV-specific IL-10 response were determined in depletion and IL-10 neutralization experiments.

**Results**—Both HCV-specific IL-10 and IFN $\gamma$  responses were detected early within 1–2 months of acute clinical hepatitis C. However, only HCV-specific IL-10 response correlated with elevated liver enzymes, increased viremia and suppressed HCV-specific CD4<sup>+</sup> T-cell proliferation in acute infection. While these associations were lost in established chronic infection, HCV-specific IL-10 responses were increased in patients without cirrhosis while IL-10 blockade enhanced antiviral effector IFN $\gamma$  responses.

**Conclusions**—HCV-specific IL-10 Tr1 responses may play a dual role in HCV infection, dampening effector T-cells to promote viral persistence in acute infection but also protecting against progressive fibrosis in chronic infection.

### Keywords

hepatitis C; interleukin-10; interferon-gamma; regulatory T-cells; CD8<sup>+</sup> T-cells; IL-10; HCV

### INTRODUCTION

T-cells play a critical role in natural hepatitis C virus (HCV) clearance. While HCV is spontaneously cleared with a vigorous and broad virus-specific effector T-cell response, such

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responses are impaired in persistent infections (1–5). While most studies have focused on the antiviral effector cytokine IFN $\gamma$ , a role for the immune regulatory cytokine interleukin-10 (IL-10) has been suggested by enhanced HCV titers and reduced hepatic fibrosis during exogenous IL-10 therapy in HCV-infected patients (6) as well as associations between IL-10 promoter polymorphisms and clinical outcomes (7,8). IL-10 is an important immune regulatory cytokine with pleiotropic effects (9,10). T-cells that predominantly secrete IL-10 (but not IFN $\gamma$ , IL-4 or IL-5) with immune regulatory properties have been termed 'Tr1' cells, defining a subset of antigen-specific regulatory T-cells (Tregs) (11) distinct from thymic-derived, naturally occurring CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs (12). Relevant for HCV pathogenesis, circulating CD4<sup>+</sup> T-cells with HCV-specific IL-10 production have been described in patients with chronic HCV infection (5,13,14). Furthermore, HCV-specific IL-10<sup>+</sup> CD8<sup>+</sup> Tr1 cells have been detected in the liver of HCV-infected patients with IL-10-dependent effector T-cell suppression (15,16).

In this study, we report that a broadly specific HCV-specific Tr1 response is induced early during acute hepatitis C associated with reduced HCV-specific CD4<sup>+</sup> T-cell proliferation and increased HCV viremia. Both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells (but not CD25<sup>+</sup> Tregs) contributed to HCV-specific Tr1 response that was also associated with reduced fibrosis in established chronic HCV infection. Collectively, these results suggest that HCV-specific Tr1 response may suppresses antiviral effector response, promoting chronic evolution in acute infection while limiting progressive liver damage in established chronic infection (17).

## MATERIALS AND METHODS

### Patients

Subjects were recruited from the Gastroenterology Clinics at the Philadelphia Veterans Affairs Medical Center and the Clinical Translational Research Center at the University of Pennsylvania following informed consent according to protocols approved by respective institutional review boards. All patients were assessed for baseline demographic, clinical and virological parameters, including serum HCV RNA by Roche COBAS qualitative, quantitative COBAS, or TaqMan reverse-transcriptase polymerase chain reaction (RT-PCR) assay (Roche Diagnostics, Branchburg, NJ) and HCV genotype by InnoLIPA (Innogenetics, Gent, Belgium). They included 15 spontaneously 'recovered' (Group R: HCV Ab<sup>+</sup>/HCV RNA<sup>-</sup>) and 11 uninfected control (Group N: HCV Ab<sup>-</sup>/HCV RNA<sup>-</sup>) (Table 1) and 61 'chronic' patients with chronic genotype 1 infection (Group C: HCV Ab<sup>+</sup>/RNA<sup>+</sup>). Eight patients with acute genotype 1 hepatitis C were also enrolled prior to antiviral therapy (18), including 3 spontaneous resolvers, 3 with chronic evolution, and 2 with early viral control and interferon treatment-associated resolution (Table 2). Exclusion criteria included HIV and/or HBV co-infection, immunosuppressive therapy, and conditions precluding research blood donation. Cirrhosis was diagnosed in 8 based on liver histology (Modified Ishak F4-6) and in 7 with clinical and/or radiological evidence of portal hypertension (encephalopathy, ascites and/or varices). Thirty-one patients were defined as non-cirrhotic based on histology with Modified Ishak score F0-3.

### Recombinant HCV proteins

Recombinant genotype 1a-derived HCV core, NS3/4, and NS5 and control superoxide dismutase (SOD) proteins known to stimulate CD4<sup>+</sup> T-cells were generously provided by Dr. Michael Houghton (Chiron Corporation, Emeryville, CA). (3, 19–21)

### Overlapping HCV peptides

361 overlapping 15mer peptides (offset by 6 amino acid residues) spanning the entire HCV core and NS3-NS5 proteins based on HCV-H sequence (genotype 1a) were synthesized (Genemed, San Francisco, CA) and mixed into 12 separate pools containing 20–31 consecutive

peptides/pool or 36 pools with 10–11 peptides/pool as previously described.(22,23) These peptides were immunogenic for CD4<sup>+</sup> and CD8<sup>+</sup> T-cells.(22,23)

### Peripheral blood mononuclear cells (PBMC)

PBMC were isolated from blood using Ficoll-Histopaque (Sigma, St. Louis MO) density centrifugation.(22,23) Cramp 2000

### HCV-specific CD4<sup>+</sup> proliferative T-cell response

CD4<sup>+</sup> proliferation assay was performed as previously described (3,22,23) with  $2 \times 10^5$  cells/well stimulated with recombinant HCV and control SOD proteins (10 $\mu$ g/mL) for 7 days, or phytohemagglutinin (PHA) at 2  $\mu$ g/ml for day 4 days, with 16 hours of <sup>3</sup>H-thymidine uptake (1 $\mu$ Ci/well) (Dupont NEN, Boston, MA). The results were expressed as a stimulation index (SI) with mean counts per minute (cpm) in stimulated wells divided by the mean cpm in control wells.(23)

### Cytokine Elispot Assay

IL-10 and IFN $\gamma$  Elispot assay was performed with  $2 \times 10^5$  PBMC/well in triplicates as described previously.(23) HCV-specific CD4<sup>+</sup> T-cell responses were examined using recombinant HCV and control proteins (10 $\mu$ g/ml). HCV-specific total T-cell IL-10 and IFN $\gamma$  responses were examined using overlapping HCV-derived 15mers (5 $\mu$ M/peptide) with positive controls including PHA (2 $\mu$ g/mL), tetanus toxoid (0.5 $\mu$ g/mL) and *Candida albicans* (20 $\mu$ g/mL).(22, 23) IL-10 and IFN $\gamma$  spot forming units (SFU) were counted using Elispot reader (Hitech Instruments, Media, PA). HCV-specific Tr1 or Th1 frequency was calculated by subtracting the mean SFU in negative control wells from mean SFU in antigen-stimulated wells and expressed as SFU/10<sup>6</sup> PBMC for each antigen or combined for total HCV-specific Tr1 or Th1 response (22,23). The cut-off for a positive response for individual peptide pools was defined as >50 SFU/10<sup>6</sup> PBMC which was the 95% percentile for each peptide pool in 11 healthy controls. The validity of IL-10 SFU counts was confirmed in a subset of assays at multiple sensitivity settings.

### Cell subset depletion and isolation

CD4 and CD8 T-cell depletion of PBMC was performed using CD4- and CD8-dynabeads (Dyna/Invitrogen, Carlsbad CA). CD4<sup>+</sup>CD25<sup>hi</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T-cells were separated by the CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cell Isolation Kit (Miltenyi Biotech, Auburn CA) (22). Purity of isolated cell subset was confirmed by flow cytometry.

### IL-10 blockade

IFN $\gamma$  Elispot was performed with the addition of 10-mcg/ml mouse anti-human IL-10 (MAB217, R&D Systems, Minneapolis MN) or isotype control during antigenic stimulation of whole, CD4<sup>+</sup>-depleted or CD8-depleted PBMC. The effect of anti-IL-10 on nonspecific IFN $\gamma$  response was first determined by subtracting the IFN $\gamma$  SFU in media control wells without anti-IL-10 from the IFN $\gamma$  SFU in media control wells with anti-IL-10. The effect of anti-IL-10 specifically for antigen-specific IFN $\gamma$  response was assessed by subtracting the IFN $\gamma$  SFU in media control wells from antigen-stimulated wells, both with added anti-IL-10.

### Flow cytometry

Cells were stained with fluorescent antibodies, acquired on FACSCalibur or FACSCanto (Becton Dickinson, Franklin Lakes, NJ), and analyzed by FlowJo (Tree Star Inc., Ashland OR). The cut-off for each marker was based on the isotype antibody. All antibodies were purchased

from Becton Dickinson (Becton Dickinson, Franklin Lakes, NJ) except for anti-Foxp3 (eBioscience, San Diego CA).

### Multiplex cytokine quantification

Culture supernatant was harvested after 24-hour stimulation of cell subsets 0.04 mcg/ml phorbol myristate acetate (PMA) plus 0.8 mcg/ml ionomycin (Sigma, St Louis MO) or media alone and examined using the BD Cytokine Bead Array Human Th1/Th2 Cytokine Kit (Becton Dickinson, San Jose CA) per manufacturer's instructions.

### Statistical analysis

The median values for clinical and immunologic parameters were compared using the nonparametric Kruskal-Wallis ANOVA, Wilcoxon Rank Sum or Mann-Whitney U test. Frequency data were compared using  $\chi^2$  or Fisher's exact test based on sample size. Spearman rank correlation was used for bivariate correlation of variables with log transformation of skewed variables to attain normalization. The correlations were confirmed with Generalized Estimate Equation linear regression to adjust for patient clustering when appropriate. All data were analyzed with JMP 5.1 (SAS Institute Inc, Cary NC). P-values below 0.05 were considered significant.

## RESULTS

HCV-specific Tr1 response is associated with increased viremia and liver inflammation but reduced HCV-specific CD4<sup>+</sup> proliferative T-cell response in acute hepatitis C. We began by determining the kinetics of HCV-specific IL-10<sup>+</sup> Tr1 and IFN $\gamma$ <sup>+</sup> Th1 responses during acute hepatitis C relative to HCV-specific CD4<sup>+</sup> T-cell proliferation, serum alanine aminotransferase (sALT) activity and HCV RNA titer as well as virological outcome. As shown in Figure 1A, HCV-specific IL10<sup>+</sup> Tr1 response was detected within the first 6 months of clinical onset regardless of virological outcomes: AR06 (week 2, SFU 330) AR04 (week 6, SFU 1,884), AR03 (week 11, SFU 2,508), A16 (week 2, SFU 2214), A14 (week 15, 303), AC13 (week 1, SFU 17,278), AC10 (week 15, SFU 871) and AC12 (week 19, SFU 1,008). Self-limited infections were associated with early reductions of HCV-specific IL-10<sup>+</sup> Tr1 response (SFU<400) relative to strong CD4<sup>+</sup> T-cell proliferation (sum SI 10–100), a parameter associated with HCV clearance (18, 21, 24). By contrast, at all time points earlier than 24 weeks, chronic evolution was associated with marked HCV-specific IL-10<sup>+</sup> Tr1 response (SFU>1000) and suppressed CD4<sup>+</sup> T-cell proliferation (sum SI<10). For all observations within the first 24 weeks, weighting to account for multiple observations per subject, there was a significant difference in the median of IL-10 SFU (AC 4548 vs. AR 105, p=0.0013, Figure 1B) and CD4 stimulation index (AC 3.3 versus AR 42.9, p=0.0034), but not IFN $\gamma$  SFU. For entire cohort, HCV-specific Tr1 response was inversely associated with HCV-specific CD4<sup>+</sup> T-cell proliferation (Figure 1C). Accordingly, HCV-specific Tr1 response was directly associated with HCV RNA titers and sALT activity. By contrast, HCV-specific IFN $\gamma$  response did not associate with HCV-specific CD4<sup>+</sup> T-cell proliferation, viral titer, sALT activity or HCV-specific Tr1 response (data not shown). These results suggest that early and prolonged induction of HCV-specific Tr1 response in acute HCV infection may contribute to antiviral CD4<sup>+</sup> T-cell suppression and chronic evolution.

### HCV persistence is associated with increased frequency and scope of HCV-specific Tr1 response

We then examined HCV-specific Tr1 response in patients with established chronic HCV infection. HCV-specific IL-10<sup>+</sup> T-cells could be detected at ten-fold higher frequencies in the Chronic than the Recovered or Normal subjects (Figure 2A)(median C659 vs. R65 SFU/10<sup>6</sup> PBMC, p=0.0022). Similarly, HCV-specific Tr1 response in the Chronic subjects was broader

than the Recovered or Normal subjects (C 25% vs. R 0% vs. N 0%,  $p=0.0001$ ). These differences were HCV-specific since IL-10 responses to the tetanus toxoid and *C. albicans* (data not shown) were similar between the three groups (Figure 2A, right). The opposite was true for HCV-specific IFN $\gamma$  response, which was greater among the Recovered than the Chronic, or Normal subjects both in frequency (median C 258 vs. R 723 SFU/ $10^6$  PBMC,  $p=0.0006$ ) and scope (C 0% vs. R 42% vs. N 0%,  $p=0.004$ ) (Figure 2B). Interestingly, HCV peptide pools eliciting increased IL-10 responses in Chronic patients were also highly antigenic for IFN $\gamma$  responses among the Recovered subjects (Figure 2C, black bars), resulting in a significant correlation in the relative immunogenicities of the HCV peptide pools for IL-10 responses among the Chronic patients and IFN $\gamma$  responses among the Recovered subjects ( $p=0.035$ ) (Figure 2D). Thus, HCV-specific T-cells were maintained at similar frequencies and antigenic hierarchy in patients with chronic and recovered HCV infection, but polarized towards IL-10 production in chronic HCV infection and to IFN $\gamma$  in HCV clearance.

### **HCV-specific Tr1 response correlates with reduced fibrosis in established chronic HCV infection**

While HCV-specific Tr1 response correlated with immunological, virological and clinical parameters during acute hepatitis C, HCV-specific Tr1 response was not associated with HCV-specific CD4<sup>+</sup> T-cell proliferation, which was generally suppressed among Chronic patients. Similarly, HCV-specific Tr1 response among the Chronic patients did not correlate with HCV titers, sALT activity (Figure 3B/C) or histological activity (data not shown). However, HCV-specific Tr1 response was significantly lower in 15 patients with histological (n=8) and/or clinical (n=7) cirrhosis, compared to 31 patients without cirrhosis on liver biopsy (median IL-10 SFU: Cirrhotics 418 vs. Non-cirrhotic 899 SFU,  $p=0.037$ ) (Figure 3D), suggesting an inverse relationship between HCV-specific Tr1 response and cirrhosis.

### **Both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells contribute to the peripheral HCV-specific IL-10 Tr1 response**

The contribution of CD8<sup>+</sup> T-cells in HCV-specific IL-10 response was examined in whole and CD8-depleted PBMC samples in IL-10 Elispot assay. As shown in Figure 4A, HCV-specific Tr1 response was variably influenced by CD8<sup>+</sup> T-cell depletion, increasing in 4/6 patients in response to HCV Core (pool 1, aa1-195) and 3/6 to C-terminal NS5B (pool 12, aa2827-3015), while decreasing in 4/6 patients in response to HCV core (pool 2). By contrast, HCV-specific IL-10 response was significantly reduced by CD4<sup>+</sup> depletion in overall circulating frequency ( $p=0.006$ ) and in 15/17 patients (Figure 4B). These results suggest that both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells may contribute to HCV-specific IL-10 response, although to a greater magnitude by the CD4<sup>+</sup> T-cells.

We then examined which CD4<sup>+</sup> subsets contribute to IL-10 response. As shown in Figure 4C, IL-10 production was significantly greater in both CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> subsets compared to whole PBMC, consistent with the notion that IL-10 is secreted by CD4<sup>+</sup> T-cells. However, there was no difference in IL-10 production by CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T-cells, suggesting that IL-10 production is not specifically mediated by CD25<sup>+</sup> Tregs. Furthermore, HCV-specific Tr1 response did not correlate with CD4<sup>+</sup>CD25<sup>+</sup> and/or Foxp3<sup>+</sup> Treg frequency cross-sectionally in patients with established chronic infection (Figure 4D) or longitudinally during acute evolving hepatitis C (Figure 4E), suggesting that CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs are not the primary sources of HCV-specific Tr1 response.

### **IL-10 receptor (IL-10R) blockade results in enhanced HCV-specific IFN $\gamma$ + effector T cell response**

The functional impact of IL-10 on antigen-specific IFN $\gamma$  response was examined in PBMC following antigen-specific stimulation with and without IL-10R blockade in IFN $\gamma$  Elispot. As shown for 3 representative patients in Figure 5A, IL-10 blockade in PBMC with and without



CD4 or CD8-depletion led to variable increases in background IFN $\gamma$  production in some patients. However, IL-10 blockade further increased in IFN $\gamma$  response to HCV-derived peptides in whole, CD4-depleted and CD8-depleted PBMC (Figure 5B) which was statistically significant despite the small sample size for the responses to HCV Core 127–195 peptides. For example, in CD4-depleted PBMC, IL-10R blockade enhanced IFN $\gamma$  response in 4/6 patients for C-terminal Core 127–195 and in 3/6 patients for N-terminal NS3 1027–1095. HCV-specific IFN $\gamma$  response was also augmented in CD8-depleted PBMC in 6/6 patients for HCV Core 127–195 and in 3/6 patients for NS3 1027–1095. The immune augmentation induced by IL-10 blockade in CD4-depleted PBMC suggests that IL-10 produced by non-CD4 T-cells also contributes to HCV-specific effector T-cell suppression ex-vivo. These data collectively suggest that IL-10 can reversibly suppress some antiviral effector T-cell responses in HCV infection, perhaps favoring chronic evolution in acute infection but also limiting liver inflammation during chronic infection.

## DISCUSSION

IL-10<sup>+</sup> Tr1 cells represent a subset of Tregs, distinct from CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells and with suppressive or regulatory properties (25–27). IL-10 is produced by many cell types including dendritic cells (28,29), macrophages, monocytes, NK (30,31) and NKT-cells (32) with pleiotropic effects. For example, IL-10 can downregulate antigen processing by transporter-associated with antigen processing, MHC expression and IL-12 production by antigen-presenting cells (APC) (9,10), resulting in impaired T-cell proliferation, effector function and memory (33). Virus-specific Tr1 responses have been linked to chronic hepatitis B and C (5,13,34–38). Relevant to HCV, HCV-specific IL-10<sup>+</sup> CD8 T-cell frequency in the liver correlated inversely to histological activity (16,39). Additionally, changes in serum or T-cell IL-10 levels have been associated with the efficacy of antiviral therapy in some studies (32,34,40–46).

While antigen-specific Tr1 response has been detected in patients with acute and chronic hepatitis C (13,34,47,48), this response begins early within 1–2 months of acute clinical hepatitis in our subjects, contrasting their detection only after 24 weeks of infection in a study of genotype 4 infection(48). HCV-specific Tr1 response was also detected regardless of subsequent virological outcome in our study, similar to Urbani et al (47). However, in the present study the strength of HCV-specific Tr1 response (but not IFN $\gamma$  response) correlated with clinical, virological and immunological parameters during acute infection. The direct associations between HCV-specific Tr1 response and sALT levels and viremia as well as suppressed antiviral CD4 T-cell proliferation during acute infection suggests that IL-10 response is induced as a compensatory mechanism to dampen the ongoing inflammation (49), leading to antiviral CD4 T-cell suppression and increased viremia with chronic evolution.

One notable finding in our study is that HCV-specific Tr1 response was broadly directed with a similar magnitude and scope as the robust HCV-specific IFN $\gamma$  response observed with resolved HCV infection. This suggested that HCV-specific T-cells are maintained at similar frequency, scope and antigenic hierarchy in chronic HCV infection as those in recovered HCV infection but are polarized toward IL-10 production rather than the IFN $\gamma$  production characteristic of spontaneous HCV clearance. Possible explanations for this finding include altered T-cell maturation due to virus-induced dysfunction of antigen-presenting cells (29,50, 51), viral epitope mutation (52), or altered T-cells costimulation (53–55). In the chronic phase, peripheral HCV-specific Tr1 response did not correlate with CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cell frequency, distinguishing Tr1 cells from CD4<sup>+</sup>CD25<sup>+</sup> Tregs (26,27). Although HCV-specific Tr1 response did not correlate with sALT activity in established chronic HCV infection, it correlated inversely with cirrhosis with the caveat that histological or clinical evidence of cirrhosis could only be assessed in 46 of the 61 patients. Based on antifibrotic effect reported

by exogenous IL-10 therapy (6), HCV-specific Tr1 response may exert a protective effect that is lost in patients with cirrhosis.

As for the cell subset(s), CD4<sup>+</sup>CD25<sup>-</sup> T-cells contributed to peripheral HCV-specific Tr1 response in HCV-infected patients contrasting with previous studies focusing on CD4<sup>+</sup>CD25<sup>+</sup> IL-10<sup>+</sup> T-cells (56,57). Interestingly, IL-10 blockade, similar to results reported by Alatrakchi et al.(58), enhanced HCV-specific IFN $\gamma$  response to HCV Core peptides in CD4-depleted as well as CD8-depleted PBMC, suggesting that IL-10 production by CD4<sup>-</sup> and/or CD8<sup>-</sup> cells (including non-T-cells such as monocytes, B-cells or NK cells) participate in HCV-specific immune regulation. Enhancement of HCV-specific effector T-cell response by IL-10R blockade has interesting therapeutic implications, particularly in light of virus control with restoration of effector T-cell function in murine LCMV infection demonstrated by IL-10 blockade in-vivo (59,60). However, the inverse association between HCV-specific Tr1 response and cirrhosis in our study suggests further caution in applying IL-10 blockade to HCV infection.

It is important to acknowledge some of the potential limitations in this study. First, peripheral HCV-specific Tr1 cells in our study could represent only a subpopulation of dysfunctional cells without functional relevance to the liver, the site of infection. However, the inverse association between peripheral HCV-specific Tr1 response and cirrhosis suggests that they have a prognostic relevance at the very minimum. Second, because we relied primarily on the Elispot assay to detect IL-10<sup>+</sup> cells, we cannot exclude the contribution of non-T-cells in IL-10 production. Nevertheless, HCV-specific nature of the IL-10 responses strongly suggested TCR-mediated signaling using HCV-derived peptides. Third, a third of the subjects did not undergo liver biopsy for histological assessment of cirrhosis. However, we believe that it is justified to assign the 7 clinically cirrhotic patients to the cirrhotic group, as it is neither indicated nor ethical to biopsy such subjects who most likely have had histological cirrhosis for 7–10 years (61). Furthermore, the exclusion of subjects without a liver biopsy still yielded a strong trend towards a difference (p=0.08 with only n=8 in cirrhotic group).

In conclusion, HCV-specific Tr1 response occurred early in acute hepatitis C infection in the setting of active inflammation and viremia with suppressed HCV-specific CD4 T-cell response. During established chronic infection, IL-10<sup>+</sup> Tr1 cells persisted at high frequency with a broad specificity. While IL-10 blockade could enhance antiviral effector IFN $\gamma$ <sup>+</sup> T-cell responses *ex vivo*, HCV-specific Tr1 response was also inversely associated with liver cirrhosis in patients with chronic infection, suggesting an important immune regulatory effect that limits liver disease progression. Thus, our data suggest an association of HCV-specific Tr1 responses with both HCV viral persistence and disease control. These findings have important implications in HCV immune pathogenesis and immunotherapeutic development.

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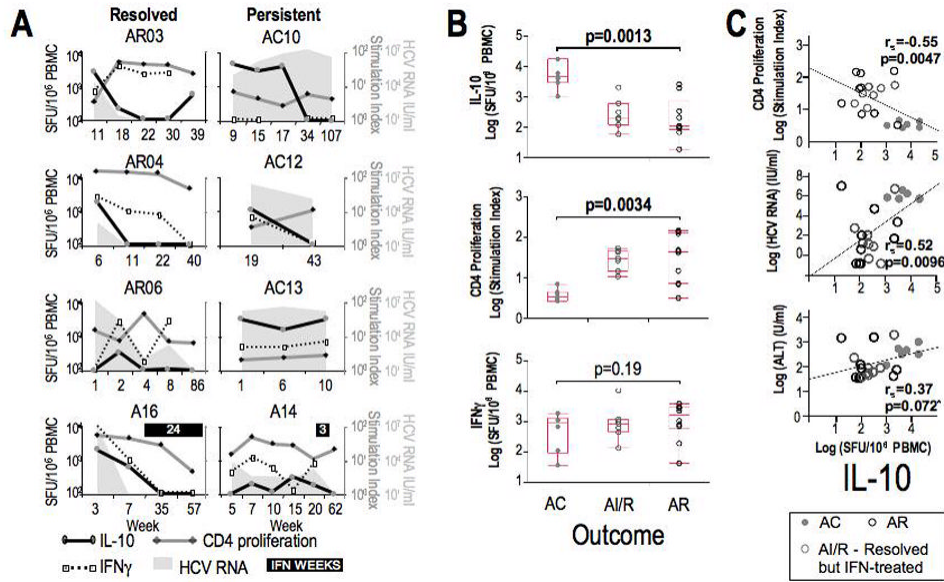
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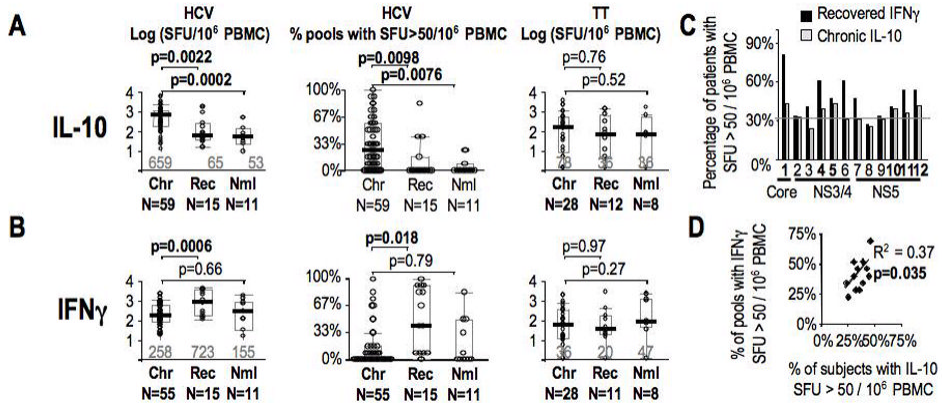
## Abbreviations

HCV, hepatitis C virus  
 IFN, interferon  
 PEG-IFN $\alpha$ , pegylated interferon-alpha  
 PBMC, peripheral blood mononuclear cells  
 PCR, reverse-transcriptase polymerase chain reaction  
 PHA, phytohemagglutinin  
 SD, standard deviation  
 SFU, spot-forming unit  
 SOD, superoxide dismutase  
 SVR, sustained virologic response  
 Th1, type 1 helper T-cell response  
 Th2, type 2 helper T-cell response  
 Tr1, type 1 regulatory T-cell response  
 Treg, classical regulatory T-cell (CD4<sup>+</sup>CD25<sup>hi</sup> or CD4<sup>+</sup>foxp3<sup>+</sup>)



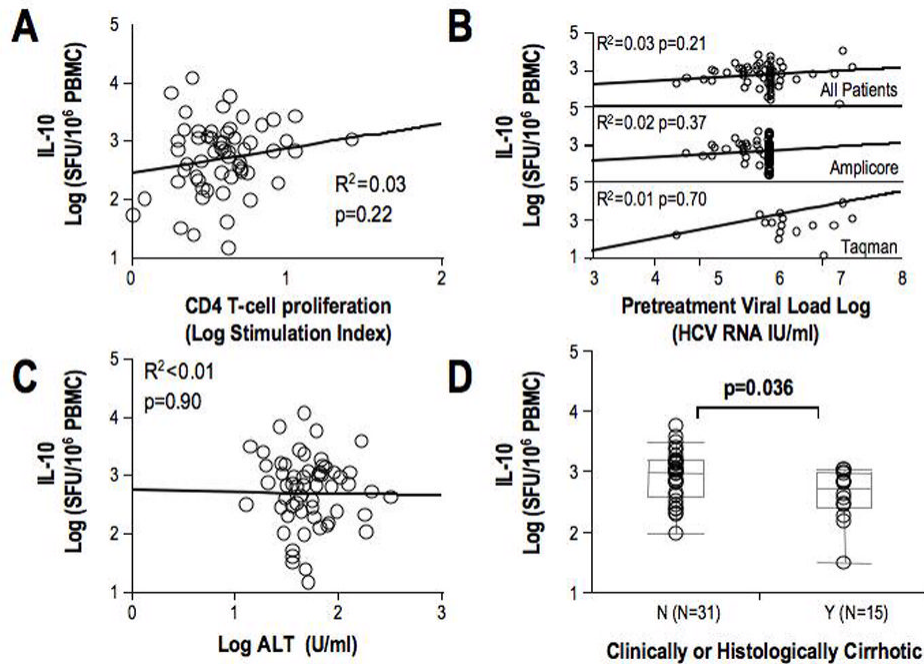
**Figure 1. Evolution of IL-10 responses in acute hepatitis C relative to clinical, immunological and immunophenotypical parameters**

**A.** Temporal evolution IL-10 SFU/10<sup>6</sup> PBMC (black line), CD4 T-cell proliferation (expressed as stimulation index) (grey line) and IFN $\gamma$  SFU/10<sup>6</sup> PBMC (dotted line) relative to HCV viral titer IU/ml (grey background) in 3 acute resolving (AR03, AR04, and AR06), 3 acute with chronic evolution (AC10, AC12, and AC13), and 2 acute patients who resolved but received interferon (AI/R: A14 - received only 3 PEG-IFN injections, A16 - was RNA-negative before started interferon). **B.** Weighted comparison of all observations within the first 24 weeks for IL-10 SFU/10<sup>6</sup> PBMC, CD4 T-cell proliferation and IFN $\gamma$  SFU/10<sup>6</sup> PBMC relative to clinical outcome. P-value given for AC versus AR subjects obtained by Wilcoxon Test. **C.** Weighted Spearman correlations of CD4 proliferation (log stimulation index), HCV RNA titer (log IU/ml), and ALT (log U/ml) with HCV-specific IL-10 SFU/10<sup>6</sup> PBMC for all patient observations within the first 24 weeks after presentation (n=8 patients, mean 3 observations per patient). Data from AC (black circles), interferon-treated (grey circles) and AR (grey dots) are shown. Spearman  $r_s$  and p-values are shown for all data. By GEE linear regression modeling, IL-10 SFU and CD4 stimulation index remained highly significant (p=0.0012), as did the association with HCV RNA titer (p=0.0108). \*Exclusion of single outlier value from AR06 week 1 increased strength of correlation of IL-10 with ALT ( $r_s=0.53$ , p=0.0088).



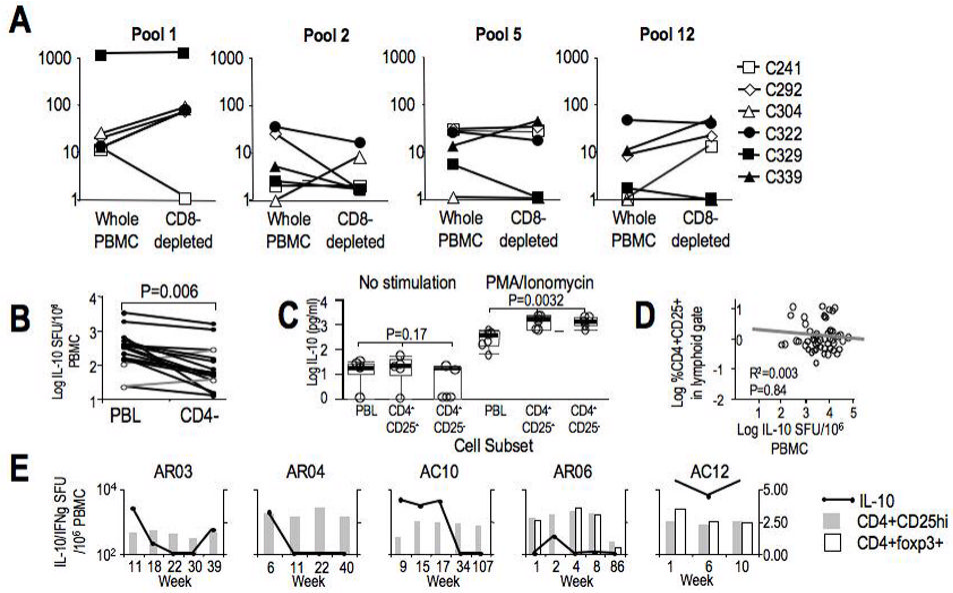
**Figure 2. Magnitude and breadth of HCV-specific IL-10 Tr1 responses in chronic hepatitis C**  
 A. Total HCV-specific IL-10 SFU/10<sup>6</sup> PBMC, percentage of pools with IL-10 SFU/10<sup>6</sup> PBMC > 50, and Tetanus toxoid-specific IL-10 SFU/10<sup>6</sup> PBMC by Elispot assay are shown for chronic genotype 1 HCV patients (Chr), recovered controls (Rec) and healthy donors (Nml). Median values are shown in grey with box plots indicating median (black bars), 25<sup>th</sup> and 75<sup>th</sup> percentiles. B. Total HCV-specific IFN $\gamma$  SFU/10<sup>6</sup> PBMC, percentage of pools with IFN $\gamma$  SFU/10<sup>6</sup> PBMC > 50, and Tetanus toxoid-specific IFN $\gamma$  SFU/10<sup>6</sup> PBMC by Elispot assay are shown for chronic HCV patients (Chr), recovered controls (Rec) and healthy donors (Nml). C. To demonstrate Tr1 regional response hierarchy in chronic patients relative to the effector IFN $\gamma$  regional hierarchy, for each of the 12 peptide pools (annotated by region within the polyprotein), the percentage of positive IL-10 response in chronic HCV patients (N=61) (grey bars) are compared to the percentage of positive effector IFN $\gamma$  responses in recovered patients (N=15)(black bars) are plotted. Grey line indicates 33%. D. Pearson correlation of chronic IL-10 and recovered IFN $\gamma$  responses for the 12 peptide pools showing that regional specificities of IL-10 responses in chronic patients resembles specificities for effector response in recovered subjects.





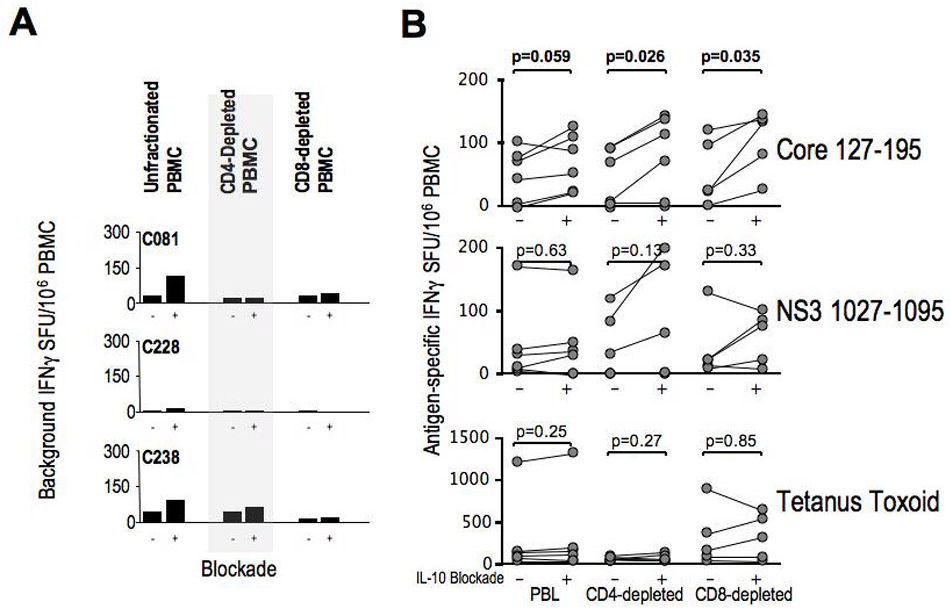
**Figure 3. Correlation of HCV-specific IL-10 responses with immunologic, virologic and clinical parameters in chronic hepatitis C**

**A.** Spearman correlation of HCV-specific IL-10 responses and CD4 T-cell proliferation and IFN $\gamma$  responses in 59 chronic patients. **B.** Spearman correlation of IL-10 responses with HCV RNA titer in all chronic HCV patients using both Roche COBAS Amplicore (maximum reported value 850,000 IU/ml) and Taqman PCR (top), Amplicore only (middle) and Taqman only (bottom). **C.** Spearman correlation of HCV-specific IL-10 responses and ALT in 59 chronic HCV patients. **D.** IL-10 Tr1 responses in patients with or without cirrhosis. Cirrhosis was defined histologically (<F4/6 fibrosis, N=31 versus >F4/6 N=8) or clinically (evidence of portal hypertension or onset of decompensated cirrhosis, N=7). In 15 patients there were inadequate histological or clinical data to determine presence or absence of cirrhosis.



**Figure 4. Cell subsets and IL-10**

**A.** HCV-specific (to Pools 1 (Core) and 2 (NS3)), *C. albicans*-specific, and PHA-induced IL-10 SFU/ $10^6$  PBMC in unfractionated PBMC and CD8<sup>+</sup> T-cell-depleted PBMC in 6 chronic HCV patients. Depleted subset SFU were corrected for changes in T-cell frequency by multiplying by [%CD3<sub>PBMC</sub>]/[%CD3<sub>CD8-</sub>]. **B.** Summed HCV-specific IL-10 SFU/ $10^6$  PBMC to 12 pools of 15mer overlapping peptides spanning Core, NS3-NS5 with unfractionated and CD4<sup>+</sup> T-cell-depleted PBMC in 17 chronic HCV patients. **C.** IL-10 secretion measured by cytokine bead array (expressed in log IL-10 pg/ml) in 24 hour unstimulated and PMA/ionomycin stimulated cultures of unfractionated PBMC, bead-selected CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> subsets in 5 chronic HCV patients. **D.** Correlation of frequency of peripheral CD4<sup>+</sup>CD25<sup>-</sup>FITC<sup>+</sup> T-cells and IL-10 SFU/ $10^6$  PBMC in 59 chronic HCV patients. **E.** CD4<sup>+</sup>CD25<sup>hi</sup> and CD4<sup>+</sup>foxp3<sup>+</sup> T-cell frequency in acute HCV patients. CD25<sup>+</sup> cutoff was defined by 99.9% isotype in the lymphoid gate, while CD25<sup>hi</sup> was defined by 99.9% of CD8<sup>+</sup> T-cells. An example of the gating strategy for CD4<sup>+</sup>CD25<sup>hi</sup> population and CD4<sup>+</sup>foxp3<sup>+</sup> gating is shown in **Supplementary Figure 1**. For 3 AR and 2 AC subjects CD4<sup>+</sup>CD25<sup>hi</sup> (grey bars) and CD4<sup>+</sup>foxp3<sup>+</sup> (white bars) are plotted relative to IL-10 SFU/ $10^6$  PBMC (black line).



**Figure 5. Effect of IL-10 blockade on IFN $\gamma$  effector responses**

**A.** Unstimulated (media-control) IFN $\gamma$  SFU/10<sup>6</sup> PBMC with IL-10R blocking antibody or isotype control in PBMC, CD4-depleted and CD8-depleted PBMC subsets. 3 representative chronic HCV patients' data shown. **B.** IFN $\gamma$  SFU/10<sup>6</sup> PBMC after stimulation with HCV Core 127–195 15mer peptides (5 $\mu$ M), NS3 1027–1095 peptides (5 $\mu$ M) and tetanus toxoid (0.1  $\mu$ g/ml) in PBMC, CD4-depleted/CD8-enriched and CD8-depleted/CD4-enriched PBMC subsets from 6 Chronic HCV subjects. To correct for the effect of IL-10 blockade on background IFN $\gamma$  production, mean SFU values for stimulated wells reflect subtraction of the mean SFU of unstimulated wells. P-values were obtained by matched-pair Wilcoxon sign-rank testing.

Table 1

## Chronic, Recovered and Normal Control Subjects

|                                  | Chronic N=61    |                  | Normal (HCV Ab-/HCV RNA-) N=11 |         | Recovered (HCV Ab+/HCV RNA-) N=15 |         | p(Recovered vs. Chronic) |
|----------------------------------|-----------------|------------------|--------------------------------|---------|-----------------------------------|---------|--------------------------|
|                                  | Median          | Range            | Median                         | Range   | Median                            | Range   |                          |
| Male/Female<br>White/Black/Other | 61/0<br>15/43/3 |                  | 5/6<br>7/3/1                   |         | 13/2<br>8/7/0                     |         | p=0.037<br>p=0.08        |
| Age (years)                      | 52              | 35-69            | 44                             | 24-46   | 51                                | 42-64   | p=0.030                  |
| HCA RNA (IU/ml)                  | 773500          | 8,620-18,900,000 |                                |         |                                   |         | <b>p&lt;0.0001</b>       |
| ALT (U/ML)                       | 47              | 13-326           | 22                             | 13-43   | 22                                | 12-39   | <b>p&lt;0.0001</b>       |
| Albumin (g/dl)                   | 4.1             | 3.4-5.3          | 4.2                            | 3.8-4.7 | 4.4                               | 3.9-4.9 | p=0.052                  |
| Total Bilirubin (mg/dl)          | 0.8             | 0.2-1.9          | 0.4                            | 0.2-1.4 | 0.7                               | 0.2-1.1 | p=0.11                   |
| INR                              | 1.0             | 0.9-1.12         | 1.0                            | 1.0-1.2 | 1.0                               | 0.9-1.2 | p=0.47                   |
| Platelet (× 1000/ml)             | 235             | 96-456           | 243                            | 203-346 | 244                               | 154-305 | p=0.54                   |

Table 2

## Acute Hepatitis C Cohort

| ID                 | Gender | Ethnicity        | Genotype       | Age | BMI (kg/m <sup>2</sup> ) | Diabetic    | Interferon-treated | 1st HCV RNA (IU/ml) | Peak ALT (U/ml) | Peak Total Bilirubin (mg/dl) |
|--------------------|--------|------------------|----------------|-----|--------------------------|-------------|--------------------|---------------------|-----------------|------------------------------|
| AR03 <sup>1/</sup> | M      | Asian            | 1a             | 57  | 26.5                     | N           | N                  | 23700               | 75              | 1.1                          |
| AR04 <sup>1/</sup> | F      | African-American | 1b             | 48  | 29.6                     | N           | N                  | 1100                | 42              | 0.7                          |
| AR06               | M      | Caucasian        | 1a             | 24  | 29.1                     | N           | N                  | 20100000            | 1517            | 7.6                          |
| AC10 <sup>1/</sup> | M      | Caucasian        | 1b             | 64  | 42.1                     | Y (Type II) | N                  | 133000              | 40              | 0.5                          |
| AC12               | F      | Caucasian        | 1b             | 23  | 27.3                     | N           | N                  | 2370000             | 115             | 0.8                          |
| AC13               | M      | Caucasian        | 1b             | 39  | 29.8                     | N           | N                  | 1750000             | 998             | 1.1                          |
| A14 <sup>3/</sup>  | F      | African-American | 1a             | 54  | 24.7                     | Y (Type I)  | Y                  | 7780                | 227             | 1.5                          |
| A16 <sup>2/</sup>  | M      | Caucasian        | 1 <sup>4</sup> | 68  | 30.5                     | N           | Y                  | 11900000            | 1910            | 10.4                         |

<sup>1/</sup> Patient included in cohort described in Kaplan, DE et al. Gastroenterology 2007; 132:654-666

<sup>2/</sup> Patient was HCV RNA-negative prior to initiation of pegylated-interferon alpha/ribavirin therapy at week 8

<sup>3/</sup> Patient maintained HCV RNA titer <10<sup>4</sup> IU/ml until week 26, at which point patient was initiated on pegylated-interferon alpha/ribavirin therapy. She tolerated only 3 injections but still cleared infection

<sup>4/</sup> Not subtypeable by InnoLiPA assay