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# Prospective analysis of effector and regulatory CD4$^+$ T cells in chronic HCV patients undergoing combination antiviral therapy☆

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***Background/Aims*: The role of HCV-specific CD4$^+$ T cells and regulatory T cells in influencing the outcome of antiviral therapy is incompletely defined.**

***Methods*: CD4$^+$ IFN-γ ELISPOT assays ($n$ = 58) and flow cytometric analysis of FoxP3-expressing T regulatory cells ($n$ = 62) were performed on patients from the Virahep-C study at baseline, during and after cessation of antiviral therapy.**

***Results*: Total HCV-specific IFN-γ CD4$^+$ T cell ELISPOT responses did not increase with therapy, but rather decreased by 8 weeks and remained below baseline 24 weeks after cessation of therapy. There were no statistically significant differences with respect to viral kinetics, race and virologic outcome. In contrast, viral relapse after treatment was associated with a three-fold increase in HCV-specific responses. The frequency and phenotype of regulatory T cells during therapy were not significantly different in terms of race, viral kinetic groups or virologic outcome.**

***Conclusions*: A contraction of HCV-specific CD4$^+$ T cell responses was found during treatment with recovery of responses in patients experiencing virologic relapse after treatment. The levels of FoxP3-expressing regulatory T cells did not vary by race and were not predictive of virologic outcome. Work is ongoing to explore the contribution of mechanisms independent of CD4$^+$ T cells in therapy-induced viral clearance.**

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***Keywords*: Hepatitis C virus; Antiviral therapy; T cell immunity; T regulatory cells; Interferon-γ**

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## 1. Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis in the world affecting approximately 175 million people [1]. The prevalence of cirrhosis from chronic HCV infection and the incidence of its complications are expected to increase in the United States over the next 10 to 20 years [1,2]. Despite significant advances in treatment, approximately one-half of HCV genotype 1-infected patients fail to eradicate infection with standard combination therapy using pegylated interferon (peginterferon) and ribavirin [3]. Elucidation of the factors that determine the differential response rates to therapy is important to understanding the causes of antiviral resistance and to identify potential therapeutic targets in the future. Because strong and multispecific cellular immune responses are indispensable for spontaneous recovery from HCV infection [4,5], it has been suggested that $CD4^+$ T cell responses also mediate therapy-induced recovery. Theoretically, antiviral therapy might reverse the functional exhaustion of HCV-specific T cells by a number of mechanisms, e.g., rapid reduction of viral titer [6], upregulation of soluble factors involved in T cell activation, maturation of dendritic cells [7,8], or elimination of viral proteins known to inhibit innate immune responses [9–12].

To date, studies examining the effect of antiviral therapy on HCV-specific immunity have provided conflicting results [13–17]. Studies have shown that patients with chronic HCV have elevated circulating frequencies of regulatory T cells (Tregs) that impair HCV-specific responses [18–21]; however, to date, no study has examined their potential role during antiviral therapy. As a part of a prospective, multi-centered NIH-funded study of viral resistance to antiviral therapy of hepatitis C (Virahep-C), immune responses to HCV were assessed for their association with outcome of therapy [22]. In an effort to better understand the role of immunity in determining response to antiviral therapy, particularly with regards to race and early viral kinetics, patients from the Virahep-C cohort were studied longitudinally. For this analysis we examined changes in HCV-specific and non HCV-specific Th1 immune responses during combination therapy and whether the proportional frequency and phenotype of Tregs are associated with race and ultimate virologic response.

## 2. Patients and methods

### 2.1. Study patients

Patients were from the Virahep-C cohort which has been previously described [22]. Briefly, this was a multi-center collaborative treatment study using combination therapy (peginterferon alfa-2a and ribavirin, Roche Pharmaceuticals, Nutley, NJ) for treatment naïve participants [196 African Americans (AA) and 205 Caucasian Americans (CA)] with chronic HCV genotype 1 infection. The primary outcome was sustained virological response (SVR), defined as lack of detectable HCV RNA by qualitative assay 24 weeks following the end of therapy. The subset of patients in this study was selected such that they were evenly distributed by race (AA or CA) and viral kinetic groups. (See Supplementary Materials).

### 2.2. Cell preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood as described previously [23]. Blood samples were obtained in the second screening visit (baseline, prior to starting antiviral therapy), at treatment week 8 (TW8), treatment week 24 (TW24) and 24 weeks after completing therapy (FU24). Responders (HCV RNA $<$50 IU/ml at 24 weeks) had blood samples obtained at treatment week 48 (TW48), whereas in non-responders (detectable virus 24 weeks following treatment initiation) treatment was halted and follow-up continued for an additional 24 weeks. Since TW48 only included responders to antiviral therapy and was drawn chronologically at the same time as FU24 in non-responders these data are not shown. Cell viability did not vary across the 8 clinical sites. (See Supplementary Materials).

### 2.3. HCV antigens and controls

Purified recombinant HCV genotype 1-derived proteins and controls were used as described previously [23]. The same lot of antigens was used for baseline and longitudinal analyses. (See Supplementary Materials).

### 2.4. Interferon-$\gamma$ ELISPOT assay

ELISPOT assays were performed as previously described [23]. Cryopreserved PBMCs were used throughout. However, because only a subset of patients were studied prospectively according to racial and viral outcome, ELISPOTs for baseline time points were performed earlier than all treatment time points (TW8, TW24, TW48) and follow-up time points (FU24), which were all done simultaneously. Only patients who had viable PBMCs that produced IFN-$\gamma$ following stimulation with PHA or SEB were included in the analyses. In addition, CMV-specific responses were not statistically different ($p > 0.05$) from baseline, during, and after treatment, suggesting that the effect of cyropreservation was minimal. (See Supplementary Materials).

### 2.5. Flow cytometric analysis of cell surface and intracellular antigens

Four-color multiparameter flow cytometry was performed using a BD FACSCalibur instrument (BD Biosciences, San Jose, CA) compensated with single fluorochromes and CellQuest™ software (BD Biosciences). Cell surface monoclonal antibodies consisted of CD3, CD4, CD8, CD25, CD69, CD62L, CD45RO (all from BD Biosciences), anti-lymphocyte activation gene-3 (LAG-3) (Alexis Biochemicals, San Diego, CA) and intracellular monoclonal antibodies for cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) (BD Sciences) and FoxP3 (eBioscience, San Diego, CA). Cryopreserved PBMCs ($2.5–5.0 \times 10^5$) were stained for cell surface antigen expression at 4 °C in the dark for 30 min, then washed twice in 2 ml phosphate-buffered saline containing 1% bovine serum albumin and 0.01% sodium azide and fixed in 200$\mu$l of 1% paraformaldehyde. Intracellular staining for CTLA-4 was performed following membrane permeabilization of fixed cells with 0.02% saponin (Sigma–Aldrich, St. Louis, MO). FoxP3 staining was performed according to manufacture's staining kit instructions. Isotype-matched control antibodies were used to determine background levels of staining.