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Signatures of Protective Memory Immune Responses During Hepatitis C Virus Reinfection

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BACKGROUND & AIMS: Development of a vaccine against hepatitis C virus (HCV) has been hindered by our limited understanding of immune correlates of protection during real-life exposure to the virus. We studied the immune response during HCV reinfection. **METHODS:** We analyzed blood samples from participants in the Montreal Acute Hepatitis C Injection Drug User Cohort Study who were reinfected with HCV from 2009 to 2012. Five patients spontaneously resolved their second infection and 4 developed chronic infections. We monitored the phenotypic and functional dynamics of HCV-specific memory T cell responses in all subjects during natural re-exposure and reinfection. **RESULTS:** Populations of $CD4^+$ and $CD8^+$ T cells with HCV-specific polyfunctional memory were expanded in all 5 individuals who resolved 2 successive HCV infections. We detected $CD127^{hi}$ HCV-specific memory $CD8^+$ T cells before reinfection regardless of a subject's ability to clear subsequent infections. Protection against viral persistence was associated with the expansion of a $CD127^{neg}$, $PD1^{lo}$ effector memory T cells at the peak of the response. We also observed broadening of T-cell response, indicating generation of de novo T-cell responses. The 4 individuals who failed to clear their subsequent infection had limited expansion of HCV-specific $CD4^+$ and $CD8^+$ memory T cells and expressed variable levels of the exhaustion marker PD1 on HCV-specific $CD8^+$ T cells. Dominant epitope regions of HCV strains isolated from patients with persistent reinfection had sequence variations that were not recognized by the pre-existing memory T cells. **CONCLUSIONS:** Protection from persistent HCV reinfection depends on the magnitude, breadth, and quality of the HCV-specific memory T-cell response. Sequence homology among viruses and ability of T cells to recognize multiple strains of HCV are critical determinants of protective memory.

Keywords: Cytokines; Protective Immunity; Immune Regulation; Viral Infection.

Despite the recent introduction of potent antivirals against hepatitis C virus (HCV), there is an urgent need for an effective prophylactic vaccine. A first step is defining correlates of protective immunity during real-life exposure among high-risk populations, such as injection drug users (IDUs).[1] CD4 and CD8 HCV-specific T-cell responses are induced during acute HCV and mediate spontaneous resolution. An effective response leading to viral clearance is typically of high magnitude, broad, polyfunctional (ie, producing more than one cytokine or function) and sustained.[2] Individuals who spontaneously resolve acute HCV develop long-lived memory T cells.[3,4] Chimpanzees who have resolved one HCV infection were protected from chronic infection upon re-exposure, but protection was less efficient on heterologous viral rechallenge (reviewed in Abdel-Hakeem and Shoukry[5]). Accelerated viral clearance was associated with rapid recall of memory T-cell responses[4] and CD4 T-cell help was critical to maintain an efficient memory T-cell response.[6] In humans, high-risk IDUs who have already resolved one HCV infection were less likely to be reinfected than HCV-naïve individuals.[1,7] Osburn et al[8] demonstrated that reinfections were characterized by reduced peak and duration of viremia as compared with primary infection and associated with broadened cellular immune responses that facilitated viral clearance. However, the detailed phenotype and function of HCV-specific T cells during reinfection in a real-life exposure setting remain poorly defined.

Here, we examined longitudinally the breadth, phenotype, and effector functions of the HCV-specific memory T-cell response and variations in viral sequence during HCV reinfection in a group of IDUs who have previously resolved a primary HCV infection.

Materials and Methods

Hepatitis C Virus RNA, Genotype, and Hepatitis C Virus Antibody Testing

Qualitative HCV-RNA was tested using COBAS Ampliprep/COBAS Amplicor HCV Test, version 2.0 (Roche Molecular

Abbreviations used in this paper: **ELISPOT, enzyme-linked immunospot assay; HCV, hepatitis C virus; HIV, human immunodeficiency virus; ICS, intracellular cytokine staining; IDUs, injection drug users; IFN, interferon; PBMC, peripheral blood mononuclear cell; SFC, spot-forming cell; TNFα, tumor necrosis factor alpha; Treg, regulatory T cell.**

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Systems, Branchburg, NJ). HCV genotyping was performed as described previously.[9] Anti-HCV antibodies were assessed by the AxSym HCV Assay (Abbott GMBH & CO, Saint Laurent, Quebec, Canada).

Peptides and HLA Class I Tetramers

Peptides were synthesized by Sheldon Biotechnology Centre, McGill University (Montreal, QC, Canada). Major histocompatibility complex class I tetramers were synthesized by the National Immune Monitoring Laboratory (Montréal, QC, Canada) or the NIH Tetramer Core Facility (Emory University, Atlanta, GA) and are as follows: HLA-A1–restricted HCV NS3 peptide amino acids (aa) 1436–1444 (ATDALMTGY) [A1/NS3-1436], HLA-A2–restricted HCV NS3 peptide aa 1073–1081 (CINGVCWTV) [A2/NS3-1073], HLA-B27–restricted HCV peptide NS5B peptide aa 2841-2849 (ARMILMTHF) [B27/NS5B-2841].

Flow Cytometry–Based Assays

All assays were performed on frozen peripheral blood mononuclear cells (PBMCs) using a standard BD LSR II instrument with FACSDiva software version 6.1.3 (BD Biosciences, San Jose, CA). Data files were analyzed using FlowJo software version 9.5 for Mac (Tree Star, Inc., Ashland, OR). Tetramer staining coupled with phenotypic analysis, intracellular cytokine staining (ICS) and CD107a degranulation assay were performed as described previously[10] in response to HCV peptide pools (1 μg/mL) or HCV minimum peptide (10 μg/mL). Polyfunctionality was assessed by exporting flow cytometry standard data as Boolean gates using FlowJo and SPICE software.[11] Carboxyfluorescein succinimidyl ester (CFSE) 6-day proliferation assays were performed as described previously[10] with or without HCV minimum peptide or peptide pool.

Hepatitis C Virus Epitope Sequencing

HCV RNA was extracted from EDTA plasma using AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen, Union City, CA), reverse transcribed, polymerase chain reaction amplified, cloned, and sequenced as described previously[10] at the Molecular Biology and Functional Genomics service of the Institut de Recherches Cliniques de Montréal (Montreal, QC).

Results

Identification of Hepatitis C Virus Reinfection Cases

This study was conducted among participants of the Montreal Acute Hep C IDU Cohort Study (HEPCO)[12] and approved by the Institutional Ethics Committee (Protocol SL05.014). Primary HCV infection was identified in cohort participants who were initially negative for both HCV RNA and anti-HCV antibodies for at least 6 months of follow-up, then had a positive HCV RNA and/or antibody test as described previously.[10,13] Participants who have resolved their primary HCV infection or participants who tested HCV RNA negative and HCV antibody positive at recruitment were enrolled in the reinfection study and followed every 3 months. HCV reinfection was defined by an HCV-RNA–positive test after 2 negative tests at least 60 days apart. The day of the first positive RNA test was defined as day 0 post detection of reinfection. This study includes 9 cases of reinfection identified between 2009 and 2012, for which clinical data documenting the primary infection and longitudinal blood samples during the reinfection episode were available. Samples before the reinfection episode were available for 6 patients. Samples from time points during or right after clearance of the primary infection were available for 4 patients. Five patients spontaneously resolved their second infection and 4 patients became chronically infected and are referred to hereafter as the SR/SR group and the SR/CI group, respectively. Both groups had comparable exposure risk as measured by the number of injections during the past month.[14] Patients' demographics, clinical characteristics, and infection history are listed in Supplementary Table 1.

Spontaneous Resolution of Hepatitis C Virus Reinfection Is Associated With an Increase in the Magnitude and Breadth of the Hepatitis C Virus–Specific T Cells

We examined the magnitude and breadth of the HCV-specific T cells using an interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) assay against overlapping peptide pools representing the HCV genotype 1a H77 polyprotein. We chose this reference sequence for the preliminary screening because all second infections were of genotype 1, and subtype 1a is the most common subtype in our cohort. The genotype of the primary infecting HCV viral strain could not be determined in many subjects because of rapid viral clearance, low viral loads, or unavailability of samples. Wherever samples were available, we monitored the ELISPOT response before and during the second infection in the SR/SR (Figure 1*A*) and SR/CI (Figure 1*B*) patients. The magnitude of the HCV-specific cell response during reinfection was higher in the SR/SR group as compared with the SR/CI group. The mean frequency at the earliest time point tested after reinfection (mean, 8 weeks) was 7440 spot-forming cells (SFC)/million PBMCs in the SR/SR group vs 1760 SFC/million PBMCs in the SR/CI group. The response to pools representing the structural and nonstructural regions of HCV was higher for the SR/SR group (mean, 1470 and 5970 SFC/million PBMCs, respectively) as compared with the SR/CI group (mean, 160 and 1600 SFC/million PBMCs) (Figure 1*C*). The breadth of the immune response, measured by the number of peptide pools targeted by the immune response, was also higher in the SR/SR group at the earliest time point during reinfection. A mean of 10 (of 11) peptide pools were targeted in the SR/SR group, vs 7 pools for the SR/CI group (Figure 1*A* and *B* and data not shown).

Expansion of the HCV-specific memory T cells was associated with viral clearance on reinfection in the SR/SR group. One exception was patient SR/SR-3, who successfully cleared his reinfection within 4 weeks, despite no change in the magnitude and breadth of the response against the HCV genotype 1a peptides (Supplementary Figure 1*A*). Because the infecting subtype could not be determined, we tested his response to a panel of peptides corresponding to genotype 1b J4 reference sequence and observed an increase in the