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Induction of Primary Human T Cell Responses against Hepatitis C Virus-Derived Antigens NS3 or Core by Autologous Dendritic Cells Expressing Hepatitis C Virus Antigens: Potential for Vaccine and Immunotherapy

Wen Li,* Deepa K. Krishnadas,* Jie Li,* D. Lorne J. Tyrrell, † and Babita Agrawal2*

Hepatitis C virus (HCV) infection is a serious health problem with an estimated 175 million chronically infected people worldwide. The majority of infected patients progress to persistent or chronic infection state. However, 15–20% of the infected individuals get acute infections, followed by clearance. Substantial evidence has accumulated to suggest the role of adaptive host immune responses in viral clearance in HCV infection.

Currently, the most effective treatment for chronic HCV is the combination of peg-IFN-α and ribavirin, but <50–60% of treated people have sustained benefit from antiviral therapy (1). In Western countries, genotype 1, which infects 70–80% of patients, is associated with a poor response to IFN-α therapy (2, 3). There are several side effects associated with IFN-α treatment, such as psychiatric disturbances, flu-like symptoms, leucopenia, or thrombocytopenia, and the most frequent side effect of ribavirin is hemolytic anemia (4). These data indicate the urgent need for alternate treatment strategies.

Patients with HCV infection fail to initiate, maintain, and sustain a strong Th1 response that is targeted against several immunodominant proteins (5). Although HCV genome is very variable with hundreds of serotypes and six genotypes, several structural (Core) and nonstructural proteins are highly conserved among genotypes and subtypes (6). Interestingly, a vigorous multispecific CD4+ T cells response against some of these conserved protein epitopes have been suggested to be correlative of viral clearance (7). Generally, CD8+ cytotoxic T cell responses against viral or tumor Ag-derived peptide epitopes are recognized to play major role in antiviral and antitumor immunity (8). However, in the case of HCV, from studies with humans and chimpanzees, CD4+ T cell responses and IFN-γ produced by CD4+ T cells have been suggested to be important in viral clearance (9–13).

Interestingly, in a study with chimpanzees, it was shown that animal that most rapidly cleared circulating HCV displayed the most vigorous and sustained response of IFN-γ-producing and proliferating CD4+ T cells in the blood (14). Even in the peg-IFN-α- and ribavirin-treated chronic HCV patients, sustained response has been shown to be associated with significant increase in frequency, strength, and breadth of type-1 CD4+ T cells (15). However, the potential of normal uninfected human donors’ T cells to respond against HCV Ags has not yet been explored. It is possible that, in the majority of HCV-infected patients, development of chronicity is, in fact, a reflection of inefficient presentation of HCV Ags, inefficient activation, and maintenance of T cell responses and/or exhaustion of activated T cells in the early phase of infections (16). The protective T cells immunity reported to date is, therefore, a representation of 15–30% of the patients who have cleared the virus.
Dendritic cells (DCs) have been shown to be the most potent APCs in the immune system, expressing high levels of MHC molecules, costimulatory molecules, and adhesion molecules, to efficiently stimulate T cells (17). DCs also have been shown to successfully prime naive T cells in vitro against several known tumor Ags (18–24). By examining T cell responses of healthy individuals against tumor or viral Ags, one can determine not what a cancer- or viral-infected patient recognizes but rather what a healthy immune system recognizes and responds strongly to. This information would be potentially very significant in the design and development of vaccine candidates for various diseases.

In a number of studies, DCs pulsed with synthetic peptides have been used (25); however, in humans, this approach is limited to a relatively small numbers of previously identified peptides in the context of a specific MHC class I (MHC-I) or class II (MHC-II) molecules. The use of whole recombinant proteins Ag may be limiting due to the fact that it may not be efficiently uptaken and processed or presented by the APCs (26). In contrast, expression of viral or tumor Ags in DCs eliminates these limitations and leads to efficient processing and presentation of various peptides in context of both MHC-I and MHC-II molecules (27), displaying a complete repertoire of presentable peptides to the T cells.

In our earlier studies (28), we have demonstrated that DCs obtained from normal healthy donors’ PBMCs, upon infection with recombinant adenoviral vectors containing HCV Core or NS3 genes express these proteins in the cells and still have normal phenotype and functions. In the present study, we have examined whether DCs expressing HCV Core or NS3 Ags are able to prime/stimulate CD4+ and CD8+ T cell responses from normal healthy HCV-naive individuals in vitro. We have obtained preliminary but first conclusive evidence of in vitro priming of mostly CD4+ T cell responses against HCV Ags Core and NS3, as determined by cytokine production, T cell proliferation, phenotype analysis, and secondary T cell proliferative response of in vitro primed T cells against relevant recombinant proteins Ags and immunodominant peptide epitopes derived from these Ags.

### Materials and Methods

#### Cell line and culture

Monolayer of 293A cell line (Qbiogene), an adenovirus-transformed human embryonic cell line that provides phenotypic complementation of the E1 genes, was used for recombinant adenovirus plaque assays, amplification, and virus titration (Fig. 1). QBI-293A cells were grown at 37°C and 5% CO2 in high-glucose DMEM (Invitrogen Life Technologies) containing 4.5 g/L glucose and 110 mg/L NaPyruvate supplemented with 2 mM glutamine and FBS (Invitrogen Life Technologies). The percentage of serum varies from 2 to 10% to adapt the speed of cell growth to the experimental requirements.

#### Plasmid construction

The Core (aa 1–191) and NS3 (aa 1027–1657) genes of HCV-1 strain (genotype 1a) were PCR amplified from the full-length clones of HCV (29). pCVH77C was provided by Dr. J. Bukh (National Institutes of Health, National Institute of Allergy and Infectious Diseases, Bethesda, MD). Primers used in this study for Core and NS3 contain a BamHI site. The PCR products were cloned into the commercial pCR 2.1 vector (Invitrogen Life Technologies) to create pCR 2.1 Core and pCR 2.1 NS3. Cloned fragments were verified by sequencing. Both plasmids were digested with BamHI, and the purified cDNA fragments were cloned into AdenoVator Transfer vector (pAdenoVator-CMV5-IRES-GFP; Qbiogene) generating CMV5/GFP/Core and CMV5/GFP/NS3.

#### Recombinant adenovirus vectors

Recombinant adenoviruses were propagated, purified, and stored as per the standard method provided in the manual (Qbiogene). pAdenoVator ΔE1/ΔE3 is a replication-deficient adenovirus vector based on the adenovirus serotype 5 (Ad5), E1/E3 deletion mutant. The transfer vector CMV5/GFP/Core and CMV5/GFP/NS3 were linearized with PmeI. Cotransformation was performed with each linearized transfer vector and pAdenoVator ΔE1/ΔE3 DNA into BJ5183-competent cells. One of the best positive recombinants was selected for the transfer and propagation in DH5α cells. The recombinant DNA was purified with the Qiagen Plasmid Midi kit according to the manufacturer’s instructions. Both AdenoVator recombinants of rAd5/ΔCore and rAd5/ΔNS3 were digested with PacI and were transected to 293A cells using Effectene transfection reagent (Qiagen). Virus plaques were isolated and amplified in 293A cells. The recombinant adenoviral vectors were stored in aliquots at −80°C. Viral particles of Ad5/CMV-LacZ (with no gene insert) were provided by QBiogene and used as a control adenoviral vector (denoted as CV throughout the manuscript). We

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**FIGURE 1.** A, The cDNA of Core or NS3 of HCV and its coding region are shown. The end positions of the amino acids also are indicated. B, The scheme of recombinant adenovirus with gene insertion. The control vectors lacking the insert were used as control vectors (CV).
have previously reported efficient expression of HCV Core and NS3 Ags in 293 A as well as human dendritic cells as detected by Western blotting and immunofluorescence staining (28).

Preparation and infection of human PBMC-derived DCs

DCs were generated from human PBMCs. Briefly, PBMCs were isolated from peripheral blood of healthy individuals by Ficoll-Hypaque (Amer sham Biosciences) density gradient centrifugation (30) and resuspended at 5 × 10^6 cells/ml in RPMI 1640 medium (Invitrogen Life Technologies), supplemented with 1-glutamine, 1% human AB serum (Sigma-Aldrich), 1% sodium pyruvate (Invitrogen Life Technologies), 0.5 μM penicillin-streptomycin (Invitrogen Life Technologies), 50 U/ml ulinid, and 50 μM 2-mercaptoethanol. The PBMCs were plated in six-well plates (3 ml/well) and incubated at 37°C (5% CO₂) for 4 h for adherence. After 2-h incubation, the nonadherent cells were removed, and fresh RPMI medium containing recombinant human GM-CSF (50 ng/ml) (PeproTech) and recombinant human IL-4 (10 ng/ml) (PeproTech) was added to the adherent cells. The adherent cells were incubated for 5 days. In flow cytometry experiments, the coexpression of CD11c and HLA-DR, further providing the evidence of generation of DCs. In the experiments, where immature DCs were used, LPS was not added. After 24-h stimulation, the LPS (Sigma-Aldrich) was added at 100 ng/ml 24 h postinfection and allowed to further incubate for 24 h to mature DCs. In the experiments, where immature DCs were used, LPS was not added.

Infection with adenovirus

DCs harvested on days 5–7 of the culture with GM-CSF and IL-4 were infected with recombinant adenoviruses expressing HCV Core or NS3 or control LacZ gene at a multiplicity of infection (MOI) of 100, unless otherwise mentioned in the figures. In the experiments where LPS stimulation was performed, the LPS (Sigma-Aldrich) was added at 100 ng/ml 24 h postinfection and allowed to further incubate for 24 h to mature the DCs. In the experiments, where immature DCs were used, LPS was not added.

RNA isolation, cDNA synthesis, and reverse transcription

Total RNA was prepared (Roche), according to the manufacturer’s instructions, from 1 to 2 × 10^7 T cells. cDNA was synthesized from 0.5 to 1 μg of total RNA. One microtiter of oligo(dT)12–18 (500 μM) (Invitrogen Life Technologies) was added, incubated for 10 min at 70°C, and chilled on ice. After mixing with 4 μl of first-strand buffer, 2 μl of 0.1 M DTT, and 1 μl of 10 mM dNTP mix, it was incubated for 2 min at 42°C. One microtiter of Superscript II reverse transcriptase (200 U/ml; Invitrogen Life Technologies) was added to the samples and incubated for 50 min at 42°C. The reaction was inactivated by heating at 70°C for 15 min.

Real-time PCR for cytokines

Cytokine gene expression was quantified by real-time PCR on the Lightcycler (Roche). Real-time PCR for cytokines was performed in a total volume of 20 μl in the presence of 2 μl of 10× reaction buffer (Taq polymerase, dNTPs, MgCl₂, and SYBR Green (Roche Diagnostics)), and 2 μl of cDNA (or water as negative control, which was always included). MgCl₂ was added to a final concentration of 2.5–4 mM, and 0.3–1 pmol of each oligo(dT) primer was added. To determine cytokine induction by T cells, iDCs were infected with 100 MOI of recombinant adenoviral vectors for 12 h. These were added with (4-h incubation with 100 ng/ml LPS) or without maturation to autologous T cells in 24-well plates (DCs (10^5/well), and T cells (2 × 10^5/well) for 24 h. At this time, cells were harvested and mRNA extracted followed by real-time RT-PCR analyses of various cytokines. In experiments with purified cells, cells were harvested after 24-h stimulation, and CD4⁺ or CD8⁺ T cells were purified (99%) by magnetic bead columns (MACS columns; Miltenyi Biotech) following the manufacturer’s protocol, and mRNA was extracted followed by real-time RT-PCR analyses of IFN-γ or TNF-α.

Antibody staining and FACscan analysis

The following mAbs conjugated to FITC, PE, or quantum red (QB) were used to assess the cell surface phenotype of T cells. Control IgG1-PE (BD Pharmingen), control IgG2a-FITC (BD Biosciences), CD4-Qu (IgG1; clone Q4120; Sigma-Aldrich), CD8-Qu (IgG 2a, clone UCHT-4; Sigma-Aldrich), CD69-PE (IgG1; BD Pharmingen), CD25-PE (IgG1; BD Pharmingen), and CD3-FTTC (IgG1, clone UCHT-1; Sigma-Aldrich). Corresponding isotype-matched control mAbs were used to establish background fluorescence.

T cells were harvested on day 5 after coculture with virus-infected DCs. Approximately 3–10 × 10^6 cells were washed in FACS wash buffer (1% FBS and 1% sodium azide in PBS) and incubated on ice with conjugated Ab in dark. After 30 min, cells were washed with FACS wash buffer and resuspended in 500 μl of FACS wash solution. One hundred microliters of FACs fixation solution (1% sodium azide and 2% paraformaldehyde amino acids in PBS) was added to the cell suspension. The cells were analyzed using a FACScan flow cytometer (BD Biosciences). Isotype control Ab stained <3% of cells.

Recombinant HCV proteins

All recombinant HCV proteins, control superoxide dismutase (SOD) protein, SDS lystate, and Escherichia coli lystate were provided by Dr. M. Houghton (Chiron). The HCV proteins were genotype 1a and had >99% homology with H77 sequence. These proteins were c200(aa 1192–1993, NS3), c33c (aa 1192–1457, NS3), c22–3 (aa 2–120, Core), and c100–3 (aa 1569–1931, part of NS3 and NS4).

T cell proliferation assay

Proliferative responses of T cells were measured in triplicate cultures in flat-bottom 96-well microtiter plates (Costar). A total of 2 × 10⁵ autologous T cells was cultured with different concentrations of infected or noninfected DCs (10⁴ to 2 × 10⁵) in 200 μl of AIM-V medium (Invitrogen Life Technologies) at 37°C for 5 days. Nonadherent cells were removed by using 70°C for 15 min.

Table I. Primer sequences for human cytokines for real-time PCR

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<th>mRNA Target</th>
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<th>Product Size (bp)</th>
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<td>147</td>
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</tbody>
</table>

FW, forward primer; RV, reverse primer.

⁵ Previously described in 2002 (31).

⁶ Previously described in 2001 (32).

of >80% CD3+ T cells (data not shown). The assay included negative (no Ag) and positive (phytohemagglutinin, 1 μg/ml) controls. The cells were pulsed with 0.5 μCi/well [3H]thymidine (Amersham Biosciences) for 16 h and harvested on filter papers (PerkinElmer). The levels of [3H]thymidine incorporation into the cellular DNA were counted in a liquid scintillation counter (MicroBeta Trilux; PerkinElmer). Tests were run in replicates of three to five wells. To determine the IFN-γ secreted in the supernatant of cultured T cells, supernatant was collected before adding [3H]thymidine and used to perform ELISA using commercial ELISA kits (BioSource International). For the Ab blocking experiments, anti-class I (W6/32) or anti-class II (HB151) Abs were added to the wells at 1 μg/ml final concentrations.

To determine the secondary T cell responses against recombinant protein Ags, replica plating assays were performed. Initially, ~21 wells of 96-well plates were plated with NS3, Core, or control adenoviral-infected DCs (10⁴/well) together with 2 × 10³ autologous T cells in total 200 μl of AIM-V medium for 6 days. On day 6, each well was split into three equal wells on three different 96-well plates. On the first plate, no Ag was added; on the second plate, the relevant recombinant protein (NS3 protein for NS3 group and Core protein for Core group) were added at 20 μg/ml. On the third plate, irrelevant Ag (Core for NS3 group and NS3 for Core group), control Ags, SOD, SDS extract, and E. coli extract, etc., were added in five to six replicates. Each well was fed with irradiated autologous PBMCs (1 × 10⁵/well) and cultured for another 5 days. At the end of the 5 days, 0.5 μCi/well [3H]thymidine was added, followed by harvesting the cells on the day 6 and counting the levels of [3H]thymidine incorporation into the cellular DNA.

To determine the immunodominant peptide-specific responses, T cells from primary cultures were restimulated with immunodominant peptides Core 21–40 (DYKFPGGGQIVGGVYLLPRR) or NS3 1248–1271 (GYKVLVLNPVAAATLGFAGYMSKA) (Dalton Chemical) at 20 μg/ml in the presence of autologous irradiated PBMCs (2 × 10⁵/well) as APCs in 96-well plates for 5 days. The proliferation of T cells was measured by [3H]thymidine incorporation assay.

**Statistical analyses**

Statistical analyses were done by Tukey’s test using SPSS software (version 11.5; SPSS).

**Results**

**DCs expressing HCV-derived Core or NS3 Ags stimulate the induction of various cytokines in autologous T cells from HCV-naive individuals**

We have previously reported normal function and phenotype of human DCs expressing HCV-Core or NS3 Ags (28). In these studies, we also demonstrated that, by infection with recombinant adenoviral vectors, we can achieve ~100% transduction efficiency without toxicity to the cells (28). The DCs expressing NS3, Core, control vector, or uninfected were used in limiting numbers to stimulate autologous T cells from HCV-naive individuals to examine in vitro priming of T cells against HCV Ags. After 24-h coculture, the cells were harvested and used to isolate mRNA followed by reverse transcription to cDNA, which was then used to run real-time PCR (Fig. 2) to quantitate the message for various cytokines such as IFN-γ, IL-2, IL-12-p40, IL-6, TNF-α, IL-4, and IL-10. The DCs plus T cell cocultures were performed in two conditions: immature DCs stimulated or mature DCs stimulated (by 100 ng/ml LPS). Data from three representative donors are shown in Fig. 2. In the first donor, strong IFN-γ and TNF-α mRNA were induced in T cells upon stimulation with mature DCs expressing HCV-NS3 or HCV-Core Ags.

**FIGURE 2.** A, Induction of cytokine mRNA in T cells primed in vitro by autologous DCs expressing HCV-NS3, Core, or no Ag. For these experiments, iDCs were infected with 100 MOI of recombinant adenoviral vectors for 12 h. These were added with (4-h incubation with 100 ng/ml LPS) or without maturation to autologous T cells in 24-well plates (DCs 10⁴/well, and T cells 2 × 10⁵/well) for 24 h. At this time, cells were harvested and mRNA extracted, followed by real-time RT-PCR analyses of various cytokines. The x axis represents the following DC-stimulating groups: one to four immature DC-stimulated T cells (1, uninfected DCs; 2, rAd-NS3, 100 MOI; 3, rAd-Core, 100 MOI; 4, rAd-LacZ, 100 MOI) and five to eight mature DC-stimulated (LPS-matured DCs) T cells (5, uninfected DCs; 6, rAd-NS3, 100 MOI; 7, rAd-Core, 100 MOI; 8, rAd-LacZ, 100 MOI). B, Induction of cytokine mRNA in purified CD4+ or CD8+ T cells primed in vitro by autologous DCs expressing HCV-NS3, Core, or control vector. For these experiments, iDCs were infected with 100 MOI of recombinant adenoviral vectors for 12 h. These were added after maturation (4-h incubation with 100 ng/ml LPS) to autologous nonadherent cells in 24-well plates (DCs 10⁴/well, and non adherent cells 2 × 10⁵/well) for 24 h. At this time, cells were harvested, CD4+ or CD8+ T cells were purified by magnetic bead columns and mRNA was extracted followed by real-time RT-PCR analyses of IFN-γ or TNF-α.
HCV Core or NS3 but not against uninfected or control vector-infected DCs. IL-4 mRNA was not detected above background. Similarly, in the second donor’s T cells, strong IFN-γ and TNF-α mRNA were identified in response to mature DCs expressing HCV Core or NS3 Ags, but IL-4 message was shown moderately (~200 copies in response to HCV Core or NS3 Ags, compared with ~50 copies for control). In contrast, in the third donor, low IFN-γ or TNF-α mRNA (50–150 and 45–70 copies, respectively) were induced, whereas high IL-4 message (~4000–7000 copies) was identified in response to NS3 or Core Ags (Fig. 2). In all three donors’ T cells, IL-6 message was induced to a significantly high degree, and IL-12p40 was induced at a lower level (3–30 copies) in HCV Ag-specific manner, as the control vector-infected DCs induced significantly lower levels of IL-12p40 and IL-6 (p < 0.05). Induction of IL-2 message was observed only in T cells stimulated with immature DCs expressing HCV Core or NS3 but not by mature DCs (Fig. 2, donors 1–3) in all three donors. In contrast, with all other cytokines, stronger mRNA level was induced upon stimulation with mature DCs expressing HCV Core or NS3, compared with immature DCs. In donors 2 and 3, significant HCV-Ag-dependent IL-10 mRNA was induced, whereas in the donor 1, moderate levels of IL-10 was induced (~350 copies) in response to HCV Core, compared with ~200–250 copies in response to HCV NS3 and control vector-infected DCs. To determine the source of induced IFN-γ and TNF-α mRNA, CD4+ or CD8+ T cells were purified after 24-h coculture of non adherent cells with autologous DCs expressing control vector, HCV NS3 or Core (Fig. 2B), followed by mRNA purification and real-time RT-PCR analyses of cytokines. Both IFN-γ and TNF-α mRNA were induced by CD8+ T cells in response to HCV Core as well as NS3; however, the response was higher in Core-stimulated T cells. In comparison, IFN-γ and TNF-α mRNA produced by CD8+ T cells was ~10-fold lower than that produced by CD4+ T cells. We also determined the levels of IFN-γ being secreted in the supernatant by T cells stimulated for 4 days with autologous DCs expressing HCV Core or NS3 Ags. In three donors, the level of IFN-γ secreted in the supernatant corresponded to the pattern of IFN-γ mRNA (Table II). In two donors, high IFN-γ levels were detected in response to Core or NS3, whereas in one donor, low levels of IFN-γ was detected. The DCs cultured alone at similar numbers did not produce detectable cytokines in the supernatant (data not shown).

Normal donors’ T cells proliferate upon stimulation with autologous DCs endogenously expressing HCV-derived Core or NS3 Ags

To examine whether naïve T cells from normal donors can be stimulated to proliferate against HCV-derived NS3 or Core Ags, the immature or mature (with 100 ng/ml LPS) DCs expressing NS3 or Core Ags were cultured with autologous T cells for 5 days at various DC:T cells ratios (200:1 to 20:1). As controls, uninfected DCs or control vector-infected DCs were used as Ag-negative DCs (Fig. 3). T cell proliferation was determined as a measure of T cell stimulation. The data from three representative donors are shown individually in Fig. 3. Even after 5–6 days of culture, in vitro Core-specific proliferation seemed to be significantly high in all of the donors, compared with negative control groups (uninfected DCs or control vector infected). Upon stimulation with mature DCs expressing Core, proliferation was higher than upon stimulation with immature DCs expressing HCV Core. However, with both mature and immature DCs, the proliferation against Core was significantly higher than against control vector-infected DCs (p = <0.05). The Ag-specific proliferative response against NS3 appeared to be much lower than that against Core Ag. In most instances, NS3-specific proliferative response was not apparent when immature DCs were used to stimulate T cells. However, upon using NS3 expressing mature DCs, T cell proliferation was observed above background but was not statistically significant (p = >0.1 at all DC:T ratios).

### Both CD4+ and CD8+ T cells are stimulated in response to autologous DCs expressing HCV-derived Core or NS3 Ags

To determine whether DCs expressing HCV Core or NS3 Ags are leading to selective CD4+ or CD8+ T cell activation, we examined the expression of CD4 or CD8 along with activation molecules CD25 or CD69 (Fig. 4). In these flow cytometry experiments, mature DCs were used to stimulate naïve T cells for 5 days followed by staining with a combination of Abs. In primary 5-day cultures, both CD4+ and CD8+ T cells were present at ~65–70 and 17–18% of the total T cell population, respectively. However, in response to both NS3 and Core, selective activation of CD4+ T cells was observed as determined by CD25 or CD69 expression. In the Core expressing DC activated T cells, ~21 and 14% of the CD4+ T cells expressed CD25 or CD69, respectively, whereas in the NS3-DC expressing DC-activated cells, 15.7 and 11% of the CD4+ T cells were expressing CD25 or CD69. However, in the CD8+ T cells, only 5.7 or 6.1% of the total CD8+ T cells were positive for CD25 or CD69 expression in Core Ag-stimulated groups. In the control groups with uninfected DC-stimulated T cells or the control vector-expressing DC-stimulated T cell cultures, 13–14 or 10–11% of the CD4+ T cells were CD25+ or CD69+, respectively. Among the CD8+ T cells in the control vector or uninfected DCs groups, 2–5% CD8+ T cells showed CD25 or CD69 expression (Fig. 4). To determine the type of cells proliferating in the cultures stimulated with HCV Ags expressing DCs, blocking experiments were performed (Fig. 5). Both anti-MHC-I and anti-MHC-II Abs partially blocked HCV Core- or NS3-specific proliferative responses (Fig. 5).

### T cells primed in vitro by autologous DCs expressing HCV Core or NS3 Ags proliferate in Ag-specific manner in secondary cultures

The T cells primed in vitro in limiting dilution cultures by autologous DCs expressing HCV Core or NS3 were restimulated with autologous irradiated PBMCs and the respective Core or NS3 recombinant protein Ags. These experiments were done in replica plating manner, so we were able to examine the Core- or NS3-specific response, compared with no Ag or irrelevant Ags or the vehicle controls. In addition, recombinant Core protein was used as negative specificity control Ag in NS3-stimulated cultures and vice versa. As shown in Fig. 6, the second restimulation showed Core- or NS3-specific responses in individual wells, which was statistically significant for both NS3 and Core Ags (p = <0.05). As controls, we also stimulated T cells with untransduced or control vector-treated DCs in the first week, followed by restimulation
FIGURE 3. HCV Ags NS3- and Core-specific primary T cell proliferative response upon stimulation with autologous DCs expressing Core or NS3 Ags. The DC T cells were cocultured for 5 days, followed by addition of $[^{3}H]$thymidine (0.5 μCi/well) for 18 h and harvesting the cells, followed by counting the $[^{3}H]$thymidine incorporation. The data are shown with three individual donors’ T cells. A and B, Core-specific response with immature and mature DCs. C and D, NS3-specific responses with immature and mature DCs, respectively. A and B, Uninfected DCs (○) and Core (▲; panels A and B). C and D, NS3-expressing DCs stimulated T cells, and control vector-infected DCs stimulated T cells (●).
with no Ag, Core, NS3, or control Ags (data not shown). In both of these control cultures, the proliferation in response to HCV Core or NS3 recombinant proteins were lower than the control no Ag or vehicle control wells, and the average cpm of all of the wells was also lower than the no Ag or vehicle control groups. As controls, we also stimulated naive T cells in primary cultures with DCs loaded with recombinant proteins Ags and did not observe Ag-specific proliferation (data not shown).

In addition to determining Ag-specific T cell response against recombinant protein Ags, we determined the specificity of response against immunodominant permissive T cell peptide epitopes for HCV Core and NS3 Ags 21–40 and 1248–1271, respectively. Both of these peptides have been shown previously to be permissive TH epitopes, i.e., they are able to be presented in context of multiple HLA class II molecules to CD4+ T cells (7, 10). As shown in Fig. 7, the T cells primed with HCV Core or NS3-expressing DCs showed peptide-specific proliferation response against Core-21–40 and NS3-1248-1271, respectively. The peptide-specific responses were statistically significant for both Core and NS3 Ags \( p = <0.05 \). As controls, the control vector-infected DC-primed T cells also were cultured with Core or NS3 peptide, but no proliferative response above background was observed (data not shown).

### FIGURE 4
Flow cytometry analyses of T cell cultured with DCs expressing control, HCV-NS3, or HCV-Core for 5 days, followed by staining for CD3/CD4, CD8/CD25, or CD69 in triple color formats. For the analyses, CD3+ T cells were gated and analyzed for CD25 or CD69 expression on CD4+ or CD8+ T cells. The table summarizes the percentage of CD4+ or CD8+ T cells expressing CD25 or CD69 as activation markers. The data are representative of three repeated experiments.

<table>
<thead>
<tr>
<th>Groups</th>
<th>CD25%</th>
<th>%CD4+</th>
<th>CD25%</th>
<th>%CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC+T cells</td>
<td>14.0</td>
<td>10.0</td>
<td>5.0</td>
<td>4.0</td>
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<tr>
<td>CVDC+ T cells</td>
<td>13.0</td>
<td>11.0</td>
<td>2.5</td>
<td>4.9</td>
</tr>
<tr>
<td>Ad-NS3 DC + T cells</td>
<td>15.7</td>
<td>11.1</td>
<td>2.0</td>
<td>4.7</td>
</tr>
<tr>
<td>Ad-Core DC + T cells</td>
<td>21.2</td>
<td>14.0</td>
<td>5.7</td>
<td>6.1</td>
</tr>
</tbody>
</table>

* The data represent % of CD4 or CD8 T cells expressing CD25 or CD69.
FIGURE 5. Blocking of HCV Core- or NS3-specific primary T cell responses by Abs against MHC-I and MHC-II. The primary HCV-NS3 or Core-specific T cell cultures were established (as described in Fig. 3) but in the presence or absence of anti-MHC-I or anti-MHC-II Abs and isotype control Abs at 1 μg/well. Shown are Core-expressing DCs (top panel) and NS3-expressing DCs (bottom panel). Both anti-class I (left) and anti-class II (right) Abs partially block the primary T cell proliferative responses.

Discussion

In this paper, we describe the in vitro induction of HCV Ags NS3 and Core reactive human T cell responses from HCV-naive (uninfected) individuals. These studies have advantages over previous reports (9–13) in that these seek to explore anti-HCV T cell responses that can be generated in healthy, immunocompetent individuals to HCV Ags presented by professional APCs. This study eliminates the use of T cells from HCV-infected people, which could be defective and modulated (33). In addition, by using T cells and DCs from healthy individuals, we are examining the potential for T cell repertoire that has not been affected by long-term presence of HCV in the body. These studies have huge potential for the investigation and development of prophylactic as well as immunotherapeutic vaccines for HCV infection.

CD4+ T cell responses have been suggested to be important in HCV viral clearance (13). In addition, IFN-γ production by CD4+ T cells in response to HCV Ags has been suggested to correlate with HCV clearance in chimpanzees that cleared HCV (14). In our earlier studies, we have reported efficient expression of HCV NS3 and Core proteins in human DCs (28). In the present study, these HCV Ags expressing DCs were used to stimulate autologous T cells in vitro. As an initial measure of T cell response, we examined the mRNA induction of various cytokines (IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α and IL-12p40) by real-time RT-PCR (Fig. 2). Significant numbers of cytokines mRNA copies were detected after 24 h stimulation of T cells with immature or mature autologous DCs expressing HCV NS3 or Core. Interestingly, the immature DCs did not stimulate high quantities of cytokines’ mRNA except IL-2. In all of the donors’ T cells, mRNA for IL-2 was induced in response to HCV Core, and in two donors’ T cells, in response to NS3 upon stimulation with autologous DCs expressing these Ags. With the control vector-infected or uninfected control immature DCs, no or very little IL-2 was induced. Surprisingly, message for mRNA for IL-2 was not detected upon stimulation of T cells with mature DCs expressing NS3 or Core. It is possible that, with immature DCs expressing HCV NS3 or Core, the naive T cells are moderately stimulated to produce low levels of IL-2 (only 12–25 copies of mRNA), whereas upon stimulation with mature DCs expressing HCV Ags lineage committed T cells lost the ability to produce IL-2. With all of the cytokines tested, there were clearly more cytokines induced in response to HCV Core- or NS3-containing DCs, compared with uninfected or control vector-expressing DCs, despite the fact that they were all used at the same MOI.

The induction of IFN-γ, TNF-α, and IL-4 in response to HCV Core or NS3 Ags provided a very interesting pattern. In two of three donors, high IFN-γ and TNF-α were induced against both Core and NS3 Ags (Fig. 2), whereas no or modest IL-4 was induced. In the third donor, clearly a significantly high amounts of IL-4 mRNA was induced with very low levels of IFN-γ and TNF-α mRNA. These results demonstrate an inherent ability of individuals to respond to HCV Core and NS3 Ags in Th1/Th0 and Th2 type of responses. In these studies, a quantitative difference in the amount of cytokine mRNA was observed upon stimulation with NS3 or Core Ags expressing DCs, but in almost all cases, both NS3 and Core Ags induced qualitatively similar cytokine response, suggesting that Core does not differentially stimulate Th2 cytokine patterns, or suppress T cells from expressing cytokine mRNA. Upon examining the IFN-γ secreted in the supernatant of T cells stimulated with DCs expressing HCV Core or NS3 Ags after 5-day culture, a pattern similar to mRNA was observed, i.e., two donors induced significant IFN-γ production, whereas the third donor showed very low levels of IFN-γ secretion specific to HCV Core or NS3 Ags (Table II). In the control groups where uninfected or control vector-infected DCs were used to stimulate autologous T cells, this specific pattern of cytokine response was not seen (Fig. 2). These results suggested that HCV Ags presented by DCs have an inherent ability to induce Th1/Th0 or Th2 type responses in normal T cells. It is not yet clear whether it is related to HLA diversity, previous or ongoing status of donors, or an inherent property of human T cells to respond to HCV Ags in two distinct patterns. Also, it is not clear whether two distinct response patterns of naïve T cells to HCV Ags is what determines the outcome of immune response and disease status upon infection with HCV. From studies of HCV patients, however, Th1 and Th0 responses against various HCV Ags have been suggested to provide a favorable outcome to viremia and disease, whereas Th2 responses have been correlated to persistent viremia (34). HCV Core- and NS3-induced expression of IL-12p40, IL-6, and IL-10 was not much different in different individuals, exemplifying their status as neither Th1 nor Th2 cytokines. However, induction of IL-10 in response to Core and NS3 does suggest stimulation of Treg-like cells. For these cytokines as well, we observed HCV Ags
Core- or NS3-specific response distinct from uninfected or control vector-infected DC-stimulated T cell responses.

The induction of cytokine mRNAs in T cells upon stimulation with HCV Core or NS3 expressing autologous DCs was related to increased T cell proliferation (Fig. 3). Overall HCV Core Ag-expressing DC-stimulated T cells showed increased proliferation compared with NS3-stimulated T cells (Fig. 3). Mature DC-stimulated T cells proliferate more than immature DCs expressing HCV Ags Core- or NS3-stimulated T cells. The differential proliferation of T cells in response to HCV Core- or NS3-expressing immature or mature DCs corresponded to inflammatory cytokines being induced in T cells (Fig. 3). In all three donors, we observed higher HCV Core-specific proliferation compared with NS3-specific proliferation. HCV-derived NS3 has been shown previously to alter IRF-3 activation downstream of TLR signaling, leading to alteration of Ag presentation, NF-κB activation, and inefficient activation of T cells. It is possible that a lower primary proliferation of T cells in response to NS3 could be a reflection of reduced innate response due to expression of NS3 in the DCs. The blocking experiment using anti-MHC-I and anti-MHC-II Abs suggested the role of both CD4+ and CD8+ T cells in the observed proliferation.

Interestingly, upon examining the phenotype of T cells responding in the primary cultures, it was observed that more CD4+ T cells show activated phenotype, compared with CD8+ in both NS3- and Core-stimulated T cell cultures (Fig. 4). The percentage of CD4+ T cells expressing CD25 or CD69 was significantly higher in the HCV Ag-stimulated T cells, compared with the no Ag or control vector-expressing DC-activated T cells. This observation is contradictory to other reports with tumor Ags where tumor Ags expressing DCs were shown to primarily stimulate CD8+ CTL from naive T cells (35–37). However, our observations may explain the previously reported CD4+ T cell responses in HCV-infected individuals and support the suggestion that CD4+ T cells are important for viral clearance (13). It is not clear why, in the primary cultures, we did not see high CD8+ T cell activation. Our previous studies demonstrated that the DCs expressing Core or NS3 Ags express normal levels of class I and class II molecules (28), suggesting that the differential expression of class I vs class II molecules in HCV Core- or NS3-expressing DCs did not lead to the observed differences in CD4+ vs CD8+ T cell activation. It is possible that NS3- and Core-expressing DCs selectively suppress the activation of CD8+ T cells or, in fact, naturally stimulate less CD8+ T cells from normal individuals. It is possible that, after several rounds of in vitro stimulation, a significantly higher number of activated CD8+ T cells will be observed. More detailed phenotype and functional analysis would help clarify this question.

**FIGURE 6.** NS3 or Core-specific proliferation in secondary cultures. Initially, 21 wells of a 96-well plates were plated with NS3-, Core-, or control adenovirus-infected DCs (10^6/well) together with 2 × 10^5 autologous T cells in total 200 μl of AIM-V medium for 6 days. On day 6, each well was split into three wells on three different 96-well plates. On the first plate, no Ag was added; on the second plate, the relevant recombinant protein (NS3 protein for NS3 group and Core protein for Core group) were added at 20 μg/ml. On the third plate, irrelevant Ag (Core for NS3 group and NS3 for Core group), control Ags, SOD, SDS extract, and E. coli extract, etc., were added in five to six replicates. Each well was fed with irradiated autologous PBMCs (1 × 10^6/well) and cultured for another 5 days. The bottom two panels are average cpm from 21 wells of the top two graphs. The data are representative of two repeated experiments from two different donors.
In our next experiments, we determined whether we can identify Ag-specific T cell proliferation in secondary cultures (Fig. 6 and 7). For these experiments, the DCs expressing HCV Core or NS3 Ags were used in the priming cultures and the irradiated autologous PBMCs along with recombinant proteins or control Ags were used as APCs in the secondary cultures. Because, it is expected that the frequency of Ag-specific T cells would be very low in HCV-naive individuals, we performed these experiments in replica plating cultures (38). The format of replica plating experiments allows one to identify individual Ag-specific proliferation responses in individual wells and at the same time examine the response of those cells against no Ag or irrelevant Ag-stimulated cultures. For the first week in the priming cultures all of the T cells in multiple wells were stimulated with limiting number of DCs expressing Core or NS3 Ags. Before the second stimulation with irradiated PBMCs as APCs with or without Ags, each well of the primary culture was split into three identical (replica) wells. The first replica well was stimulated with APCs without Ag, the second replica well was stimulated with APCs plus the respective protein Ag, and the third replica well was stimulated with APCs plus control Ags. The results of this experiment are shown in Fig. 6. The proliferative responses of T cells in replica plating experiments provided conclusive evidence of HCV Ag Core- and NS3-specific T cell proliferation in the primary in vitro cultures and also in vitro priming of T cells against HCV Ags Core and NS3 using autologous DCs expressing these HCV Ags. These results were further corroborated by the proliferative response of HCV Core or NS3-primed T cells against immunodominant peptides from both Core and NS3 Ags (Fig. 7).

In conclusion, our studies demonstrated that, by using DCs expressing HCV Ags, it is possible to prime and stimulate naïve T cells against HCV Ags. HCV is a worldwide health problem, and therefore protective and immunotherapeutic vaccines need to be developed as soon as possible (39, 40). By using the DCs expressing HCV Ags endogenously to stimulate T cells, a complete repertoire of class I- and class II-restricted peptides from HCV Ags can be presented to T cells. This system can be used to elucidate the role of various HCV proteins in the induction of T cell responses that are involved in virus control or clearance. Experiments are underway in our laboratory to identify epitopes derived from HCV Ags Core and NS3, which are recognized by T cells obtained from healthy individuals. Also detailed phenotypic and functional characterization of the responding primary T cells is currently underway. An added advantage in studying the T cell repertoire of healthy individuals to identify HCV Ag-specific responses is that in HCV-infected individuals, the T cells may be suboptimally primed in vivo with tolerizing DCs, or the hepatocytes expressing HCV Ags, rather than professional activated and mature DCs. In addition, because the DCs expressing HCV Ags are able to prime the Ag-specific T cells in vitro, they will have the potential to be used as cellular vaccines or as T cell adoptive transfer therapy in vivo in both prophylactic and therapeutic setting.

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Disclosures
The authors have no financial conflict of interest.

References