Expression of hepatitis C virus-derived core or NS3 antigens in human dendritic cells leads to induction of pro-inflammatory cytokines and normal T-cell stimulation capabilities

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The majority of hepatitis C virus (HCV)-infected individuals become chronically infected, which can result in liver cirrhosis and hepatocellular carcinoma. Patients with chronic HCV are unable to prime and maintain vigorous T-cell responses, which are required to rid the body of the viral infection. Dendritic cells (DCs) are the professional antigen-presenting cells that probably play a dominant role in priming and maintaining vigorous T-cell responses in HCV infection. Furthermore, inefficient DC function may play an important role in HCV chronicity. In order to determine the effect of HCV NS3 and core proteins on phenotype and function of human DCs, recombinant adenoviral vectors containing NS3 or core genes were used to infect human DCs. HCV NS3- or core-protein expression in DCs was confirmed by Western blotting and immunofluorescence staining. The DCs expressing HCV NS3 or core proteins expressed several inflammatory cytokine mRNAs, had a normal phenotype and effectively stimulated allogeneic T cells, as well as T cells specific for another foreign antigen (tetanus toxoid). These findings are important for rational design of cellular-vaccine approaches for the immunotherapy of chronic HCV.

INTRODUCTION

Hepatitis C virus (HCV) is a major health problem as it frequently results in chronic infection, which may lead to liver cirrhosis and hepatocellular carcinoma. Infection with HCV results in persistent viral infection in 70–85% of patients, presumably due to the ability of the virus to incapacitate the activation of the host immune mechanisms that are capable of clearing the infection. Therefore, identifying the factors of HCV persistence and understanding the mechanisms by which HCV modulates the immune system are of utmost importance for preventing chronic infections and developing novel immunotherapeutic strategies that can promote efficient cellular immune responses in chronically infected patients.

Dendritic cells (DCs) play an essential role in triggering primary antiviral cellular immune responses and may also contribute to the propagation of viral infection and pathogenesis of a disease. Hepatocytes have low expression of major histocompatibility complex molecules and do not present costimulatory molecules that are important for full T-cell activation. DCs are the most potent antigen-presenting cells (APCs) and are crucial for inducing a primary antiviral T-cell response. DCs, after uptaking antigens, migrate to the draining lymph nodes, where they mature and efficiently present viral antigens as class I- and class II-restricted epitopes to naive T lymphocytes. The role of HCV antigen-specific T cells in viral clearance or persistence has been investigated extensively in both humans and chimpanzees (Battegay et al., 1995; Cerny et al., 1995; Koziel et al., 1992; Lauer et al., 2002; Lechner et al., 2000; Rehermann et al., 1996; Shirai et al., 1994; Shoukry et al., 2003; Thimme et al., 2001; Urbani et al., 2002). These studies suggest that acute HCV infections followed by viral clearance are associated with high frequencies of HCV-specific CD4+ and CD8+ T-cell responses that can persist (Lechner et al., 2000; Thimme et al., 2001). On the other hand, chronic HCV infections are characterized by weak and restricted CD4+ and CD8+ T-cell responses that are not sustained (Rehermann et al., 1996; Thimme et al., 2001). During initial HCV infection, the interaction between the virus, virus-derived proteins and DCs may contribute to the priming and maintenance of CD4+ and CD8+ T-cell responses, which result in viral clearance or persistence. Therefore, examination of DC function in the context of HCV infection and HCV-derived antigens is essential to understanding the immune response and its regulation in HCV infection.

A supplementary table showing synthetic oligonucleotide primers used for cytokine analysis by real-time RT-PCR is available in JGV Online.
There is some evidence to suggest that, in chronic HCV infection in humans, DC function is impaired (Auffermann-Gretzinger et al., 2001; Bain et al., 2001; Kakumoto et al., 2000; Kanto et al., 1999). Mouse DCs transfected with HCV-derived structural genes were shown to have low stimulatory capacity (Hiasa et al., 1998). These studies suggest that HCV-derived structural proteins have a modulatory effect on DC functions. However, there have been contrary reports, showing a lack of impairment of DC function in chronic HCV infection in both chimpanzees and humans (Larsson et al., 2004; Longman et al., 2004). These contradictory findings may be due to technical aspects, patient selection or disease status.

HCV-derived core and NS3 proteins have been selected for the present studies. There is significant experimental evidence to suggest that NS3 is an immunodominant protein and that T cells reactive against NS3-derived peptides are very important in clearing the virus (Diepolder et al., 1995, 1997). In contrast, core protein and core protein-derived peptides have been implicated in inducing T-cell suppression or downregulation (Langhans et al., 2000; Large et al., 1999). Core protein has also been shown to facilitate induction of Fas-mediated apoptosis in JURKAT cells (Hahn et al., 2000).

In the present study, we have examined the phenotype and function of human DCs expressing HCV core or NS3 proteins in order to determine the role of these HCV proteins in modulating DC function. Recombinant adenoviral vectors containing NS3 or core of HCV were prepared and used to infect DCs obtained from normal human donors’ peripheral blood-derived monocytes. The recombinant adeno-viral infection led to efficient expression of HCV NS3 and core in both immature and mature DCs, as shown by Western blotting and immunofluorescence. The expression of HCV NS3 and core proteins in DCs led to the expression of various cytokine mRNAs. The phenotype and function (as determined by expression of various DC surface markers and costimulatory molecules, allo-T-cell stimulation and processing and presentation of a foreign antigen) of DCs expressing HCV NS3 or core were similar to those of the uninfected or control vector-infected DCs, suggesting that the HCV NS3 or core protein-expressing DCs are phenotypically and functionally normal and stimulate T cells efficiently.

**METHODS**

**Cell lines and culture.** A monolayer of cell line 293A (QBiogene Inc.), an adenovirus-transformed human embryonic cell line that provides phenotypic complementation of the E1 genes, was used for recombinant adenosivirus plaque assays, amplification and virus titration. 293A cells were grown at 37°C and 5% CO2 in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) containing 4.5 g glucose l⁻¹ and 110 mg sodium pyruvate l⁻¹, supplemented with 2 mM glutamine and fetal bovine serum (FBS) (Gibco). The percentage of serum varied from 2 to 10% in order 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 H77 cDNA (Yanagi et al., 1997). pCV-H77C was kindly provided by Dr Jens Bukh. Primers used in this study for core (forward, 5’-CGGATCCATGAAGGAACGAGCAG-3’; reverse, 5’-CGGATCCATTGGTTGAGGCGACAC-3’) and NS3 (forward, 5’-CGGATCCATGGCGGCACTCCAGGCTAC-3’; reverse, 5’-CGGATCCATTGGTTGAGGCGACAC-3’) contained a BamHI site (italicized). Start and stop codons are shown in bold type. The PCR products were cloned into the commercial pCR 2.1 vector (Invitrogen) to create pCR2.1core and pCR2.1NS3. Cloned fragments were verified by sequencing. Both plasmids were digested with BamHI and the purified cDNA fragments were cloned into the AdenoVator transfer vector (pAdenoVator-CMV5-IRES-GFP, QBiogene), generating CMV5/GFP/core and CMV5/GFP/NS3.

**Recombinant adenosivirus.** Recombinant adenosviruses were propagated, purified and stored by using the standard methods provided in the manual (QBiogene). pAdenoVator ΔE1/ΔE3 is a replication-deficient adenosivirus vector based on the adenovirus serotype 5 (Ad5) E1/E3 deletion mutant. The transfer vectors CMV5/GFP/core and CMV5/GFP/NS3 were linearized with Pmel. Co-transformation 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 transfer and propagation in Esherichia coli DH5α cells. The recombinant DNA was purified with a Qiagen plasmid midi kit according to the manufacturer’s instructions. Both AdenoVator recombinants of ΔE1/core and rAd/NS3 were digested with PstI and were transfected into 293A cells by using Effectene transfection reagent (Qiagen). Virus plaques were isolated and amplified in 293A cells. The recombinant adeno-viral 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 adeno-viral vector (denoted as CV throughout the manuscript). Structures of recombinant adenosviruses are shown in Fig. 1.

**Preparation and transduction of human peripheral blood monocyte-derived DCs.** Peripheral blood samples were obtained from normal, healthy donors (30–55 years of age) upon informed consent. This work was approved by the Health Research Ethics Board at the University of Alberta, Canada. DCs were generated from human peripheral blood mononuclear cells (PBMCs). Briefly, PBMCs were isolated from healthy individuals by Ficoll-Hypaque (Amersham Biosciences) density-gradient centrifugation (Iwata et al., 1982) and resuspended at 5×10⁶ cells ml⁻¹ in RPMI 1640 (Gibco) supplemented with L-glutamine, 1 % human AB serum (Sigma), 1 % sodium pyruvate (Gibco), 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ (Gibco). The PBMCs were plated in six-well plates (5 ml per well) and incubated at 37°C (5 % CO2) for 2 h for adherence. After 2 h incubation, the non-adherent cells were removed and fresh RPMI medium containing recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (50 ng ml⁻¹; Peprotech Canada Inc.) and recombinant human interleukin 4 (IL-4) (10 ng ml⁻¹; Peprotech) was added to the adherent cells. The adherent cells were incubated for 5 days. In flow-cytometry experiments, the coexpression of HLA-DR with CD11c was determined in the DC populations obtained from the 5-day cultures of adherent cells in the presence of GM-CSF and IL-4 and it was observed that approximately 90 % of the cells obtained from these cultures were double-positive for CD11c and HLA-DR, providing the evidence of generation of a DC population in the cultures.

**Infection with adenosivirus.** DCs harvested on day 5 of culture with GM-CSF and IL-4 were infected with recombinant defective adenosviruses expressing HCV core, NS3 or control lacZ gene at an m.o.i. of 100 unless mentioned otherwise in the figures. Cells were harvested 24 or 48 h later, washed once and used for Western blot analysis,
Dendritic cells in HCV infection

Western blot analysis. Western blotting of HCV core and NS3 proteins was carried out by using cell extracts from DCs or 293A cells infected with rAd/core, rAd/NS3 and rAd/LacZ. Forty-eight hours after infection, cells were rinsed with PBS buffer, lysed in 1x Laemmli buffer [50 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 0-0.1% bromophenol blue, 100 mM DTT] and boiled for an additional 10 min. Cell lysate from each sample was loaded on 15% (for core) and 10% (for NS3) polyacrylamide gel, separated by SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad) by using a Trans-Blot apparatus. Western blot analysis.

RNA isolation, cDNA synthesis and reverse transcription. To evaluate cytokine induction, DCs were infected with adenoviral vectors as described above. After infection, cells were incubated for 24 h followed by addition of LPS for 4 or 24 h. Cells were harvested and total RNA was prepared from between 1x10^6 and 2x10^6 DCs, according to the instructions of the manufacturer (Roche Diagnostics). cDNA was synthesized from 0-5 μg total RNA. One micro-litre of oligo dT(12-18) (500 μg ml^-1; Invitrogen) was added, incubated 50 min at 42°C and chilled on ice. After mixing with 2 μl first-strand buffer, 2 μl 0-1 M dithiothreitol (DTT) and 1 μl 10 mM dNTP mix were added and incubated at 42°C for 2 min. One micro-litre of Superscript II reverse transcriptase (Invitrogen) was added at 200 U μl^-1. The samples were incubated for 50 min at 42°C and the reaction was inactivated by heating at 70°C for 15 min.

Indirect immunofluorescence. DCs (3-5x10^5 cells) were grown on slides and infected with Ad/Core or Ad/NS3 for 24 h, followed by incubation with or without LPS for 24 h. The slides were washed twice with PBS and fixed in 3-5% paraformaldehyde solution in PBS for 15 min at room temperature. After washing with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min. The cells were washed once in PBS and blocked with 2% BSA (Sigma-Aldrich) in PBS. A 1:100 dilution of a primary mAb to HCV core or NS3 (Research Diagnostics Inc.) was prepared in PBS containing 1% BSA and incubated with the fixed cells for 1 h at room temperature to detect core- or NS3-protein expression. Following two additional washes with PBS, incubation with HLA-DR-fluorescein isothiocyanate (FITC) for 30 min, washing twice with PBS and incubation with secondary antibody for 1 h, the cells were washed twice with PBS, counter-stained with DAPI (4',6-diamidino-2-phenylindole) (Sigma-Aldrich) and mounted in mounting medium. Stained cells were viewed with appropriate filters under a fluorescence microscope. Alexa 564-conjugated goat anti-mouse IgG diluted 1:2000 in PBS was used as the secondary antibody (Molecular Probes).

Real-time PCR for cytokines. Cytokine-gene expression was quantified by real-time PCR on a LightCycler (Roche Diagnostics) according to the manufacturer’s instructions. Primers used in this study are shown in the Supplementary Table, available in JGV Online. Real-time PCR was performed in a total volume of 20 μl in the presence of 2 μl 10× reaction buffer (Taq polymerase, dNTPs, MgCl2 and SYBR Green; Roche Diagnostics) and 2 μl 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 oligonucleotide primer was added.

Antibodies and flow-cytometry analysis. The following mAbs conjugated to FITC, phycoerythrin (PE) or quantum red (QR) were used to assess the cell-surface phenotype of DCs by FACscan or immunofluorescence staining: control IgG1-PE, control IgG2a-FITC, CD11c-PE (IgG2b), CD83-PE (IgG1), CD86-PE (IgG1), CD14-PE (IgG1), CD40-PE, HLA-DR-FITC or -PE (BD Biosciences).
Pharmingen) and W6/32–FITC (IgG1, Sigma-Aldrich). Corresponding isotype-matched control mAbs were used to establish background fluorescence. The marker was set to exclude 99% of isotype-control antibody-stained cells.

DCs were harvested 48 h after adenovirus-vector infection (with or without LPS stimulation) and approximately $3 \times 10^5$–$10^6$ cells were washed in FACS wash buffer (1% FBS, 1% sodium azide in PBS) and incubated with 0.5–1 μg conjugated antibody at 4°C. After 30 min, cells were washed with FACS wash buffer, then resuspended in 500 μl FACS wash solution and 100 μl FACS fixation solution (1% sodium azide, 2% paraformaldehyde in PBS) was added. Stained DCs were analysed by using a FACScan flow cytometer (Becton Dickinson). Isotype-control antibody stained <3% of cells. The cells were gated on the basis of side and forward scatter to select DCs. As shown in Fig. 5, >95% of cells were positive for CD11c, confirming the DC phenotype of the preparation.

**T-cell proliferation assay.** Proliferative responses of T cells were measured in triplicate cultures in flat-bottom 96-well microtitre plates (Corning Inc.). Allogeneic, non-adherent cells (2 $\times$ 10³; obtained during removal of adherent cells) were co-cultured with different concentrations of infected or non-infected DCs (10⁵–10³) in 200 μl AIM-V medium (Gibco) at 37°C for 5 days as described by Iwatani et al. (1982). The assay included negative (medium)- and positive [phytohaemagglutinin (PHA), 1 μg ml⁻¹]-control wells for T-cell function. The cells were pulsed with 0.5 μCi (18.5 kBq) [³H]thymidine per well (Amersham Biosciences) for 12–18 h and harvested on filter papers (Perkin-Elmer). The levels of [³H]thymidine incorporation into cellular DNA were counted in a MicroBeta Trilux liquid scintillation counter (Perkin-Elmer). For the tetanus toxoid (TT) antigen-presentation assay, the transduced DCs were cultured with TT (0.5 μg ml⁻¹) and then added to autologous non-adherent cells, similar to the allogeneic T-cell proliferation assay.

**RESULTS**

**Endogenous expression of HCV-derived NS3 or core proteins in human PBMC-derived DCs**

The recombinant adenovirus vectors containing HCV-derived NS3, core or the control (Ad/LacZ) were incubated at 50–200 m.o.i. with human monocyte-derived immature DCs for 24 h followed by stimulation with 100 ng LPS ml⁻¹ to mature the DCs. Initially, the cells were observed visually under a fluorescent microscope to determine the efficiency of gene expression by green fluorescent protein (GFP) expression, whereas almost 100% of cells were dimly positive for GFP (data not shown). HCV protein expression in the DCs was then determined by Western blotting (Fig. 2). The molecular masses of the NS3 and core proteins were 67 and 21 kDa, respectively, corresponding to the putative molecular masses for HCV NS3 and core. In three repeated Western blot experiments, we observed consistent protein expression at 100 and 200 m.o.i. adenoviral vector. This provided the basis for using 100 m.o.i. for the following experiments.

In order to determine the percentage of DCs expressing HCV NS3 or core in our experiments, multicolour immunofluorescence staining was performed (Fig. 3) using mAbs against NS3 or core labelled indirectly via a secondary antibody conjugated to Alexa red, HLA-DR antibody labelled directly by FITC (green) and DAPI staining for nucleus staining (blue). GFP expression was significantly dimmer than HLA-DR–FITC staining (data not shown). In these experiments, the immature (Fig. 3a) as well as mature (Fig. 3b) DC populations were stained. As controls, control adenoviral-vector-infected DCs were used, which were not stained by anti-NS3 or anti-core antibodies (data not shown), but did show similar staining with anti-HLA-DR and DAPI. As shown in Fig. 3, among both mature and immature DC populations, the DAPI staining corresponded almost completely with HLA-DR staining and overlapped totally with anti-core or anti-NS3 staining. Suggesting that approximately 100% of DCs were infected with the recombinant adenovirus vectors to express HCV NS3 or core that was recognized by anti-HCV antibodies. Upon infection by 200 m.o.i. recombinant adenoviral vectors, similar HCV protein and HLA-DR expression was observed and cell viability was also 100% (data not shown).

**Cytokine induction in human DCs expressing HCV-derived core or NS3 proteins**

DCs are sentinels of the immune system and respond to external stimuli, antigens, viruses and bacteria by inducing cytokine expression to stimulate or anergize the naïve T cells...
against specific antigens. Induction of the Th1-related cytokines IFN-γ, TNF-α, IL-6, IL-12 p40 and IL-2 or the Th2 cytokine IL-10 was determined by real-time PCR assays from both immature DCs (Fig. 4, left panel) and mature DCs (Fig. 4, right panel) expressing HCV NS3 or core proteins. From the immature DCs, HCV core expression induced IFN-γ, TNF-α, IL-6, IL-2 and IL-12 p40, but not IL-10. On the other hand, expression of HCV NS3 induced very low levels of IFN-γ, TNF-α and IL-2. IL-10 expression was most prominent in uninfected immature DCs, whereas DCs expressing HCV NS3, core or control vector had much-reduced IL-10 mRNA expression. From the immature DCs, core expression led to a profound increase in Th1 cytokine mRNA induction compared with NS3. Upon maturation with LPS for 4 h, the induction of IFN-γ, TNF-α, IL-6 and IL-12 p40 was further increased in core-expressing DCs, whereas IL-2 induction was not increased. In the NS3-expressing DCs, however, IFN-γ and IL-10 mRNA induction was increased. Interestingly, the copy number of both of these cytokine messages was much higher than that induced in core-expressing DCs. The cytokine mRNA levels were significantly reduced 24 h after stimulation with LPS (data not shown). The uninfected or control vector-infected DCs produced significantly lower amounts of all of the cytokine mRNAs tested, i.e. IFN-γ, TNF-α, IL-6, IL-2, IL-12 p40 and IL-10. Therefore, expression of the HCV-derived proteins NS3 or core in DCs led to induction of various cytokines.

The cytokines IFN-γ, TNF-α, IL-10 and IL-12, secreted into the supernatants of immature and mature DCs expressing...
HCV NS3 or core antigens, were determined by ELISA assays (Table 1). From the mature DCs expressing HCV core, significantly high amounts of IL-12 and TNF-α were produced in the supernatant, whereas, from immature DCs, lower amounts of these cytokines were produced. Immature uninfected DCs produced higher levels of IL-10 than control vector-, core- or NS3-expressing DCs. However, from the mature DCs, NS3-expressing DCs induced higher levels of IL-10 than control vector- or core-expressing DCs. Except for IFN-γ, cytokines tested in the supernatant correlated qualitatively with mRNA expression. Core-expressing mature DCs produced more IFN-γ in the supernatant than NS3-expressing mature DCs, in contrast to mRNA expression.

Phenotype analysis of immature and mature DCs expressing HCV-derived core or NS3 proteins

In order to investigate whether HCV core or NS3 protein expression leads to a modulation of DC phenotype that could affect T-cell stimulation, we determined the expression of various lineage, maturation and costimulation markers expressed by DCs (Fig. 5). In each of the histograms, the percentage of positive cells is indicated at the top and the mean fluorescence channel (MFC) is indicated below the percentage of positive cells. Among the immature DCs, CD11c is expressed on >96% of uninfected DCs, indicating the myeloid DC lineage of these cells. Compared with the uninfected DCs, the control vector-infected DCs had lower MFC in CD11c expression. However, upon infection with control vector, NS3 or core recombinant adenoviral vectors, CD11c was not altered. CD83 is a maturation marker and only 6–9% of the immature DCs expressed CD83. However, upon maturation with LPS, >50% of the DCs expressed CD83. There was no significant difference in CD83 expression between DCs uninfected or infected with control, NS3 or core vectors. Other activation markers for DCs, such as CD86, CD1a, CD40, HLA-DR and HLA class I, were upregulated significantly on DCs matured with LPS. However, in both the immature and mature DCs, expression of these markers was not altered upon infection with control adenoviral vector or the recombinant adenoviral vectors containing HCV NS3 or core proteins. The expression of HLA class I molecules, as detected by a pan anti-class I antibody (HLA-A, B, C) was also not altered upon expression of NS3 or core in both immature and mature DCs. The culture incubation time for the phenotype analysis was the same as that for the Western blotting and immunofluorescence experiments. Therefore, at the time of phenotype analysis,
the HCV proteins were being expressed by almost all of the DCs. These experiments suggest that the DCs are normal with respect to their phenotypic differentiation upon expression of HCV NS3 or core proteins.

**Stimulation of allogeneic T cells by immature and mature DCs expressing HCV-derived core or NS3 proteins**

Allogeneic T-cell stimulation by limiting numbers of DCs is used as a hallmark of the function of DCs as professional APCs. The ability of immature and mature DCs expressing HCV NS3 or core to stimulate allogeneic T-cell proliferation was determined. Varying DC : T-cell ratios (1 : 200, 1 : 100, 1 : 40 and 1 : 20) were used in these studies. Immature DCs expressing NS3, core or control vector stimulated allogeneic T cells to proliferate more than the uninfected DCs (Fig. 6). Expression of core or NS3 in DCs did not have a significant effect on allo-T-cell proliferation (Fig. 6a, c). Similarly, the mature DCs expressing NS3 or core (Fig. 6b, d) stimulated the proliferation of allo-T cells to the same degree as the control vector-expressing or uninfected mature DCs. Compared with the immature DCs, however, the mature DCs stimulated allo-T-cell proliferation to a much higher degree (Fig. 6b, d). Therefore, the DCs expressing HCV core or NS3 proteins have normal capability for stimulating allogeneic T-cell proliferation.

**Stimulation of TT-specific memory T cells by DCs expressing HCV-derived core or NS3 proteins**

The capability of HCV core- or NS3-expressing DCs to process and present a protein antigen to memory T cells was examined in the next series of experiments. For these studies, donors were pre-selected whose PBMCs gave a specific TT memory T-cell response. In this experiment, 24 h after infection with recombinant adenoviral vectors, TT was added followed by maturation with LPS. The TT-loaded DCs at various concentrations were added to autologous T cells. The DCs expressing HCV core or NS3 proteins stimulated autologous T cells to respond to TT antigen as efficiently as T cells stimulated with DCs not expressing HCV antigens (Fig. 7). At all of the doses of TT-loaded DCs tested, there did not seem to be any defect in TT-specific T-cell proliferative response upon stimulation with DCs expressing HCV NS3 or core. Therefore, the DCs expressing HCV NS3 or core are able to efficiently process and present another foreign antigen to stimulate T cells.

**DISCUSSION**

DCs play a central role in initiating and directing the nature of adaptive antigen-specific T-cell responses against a pathogen. The majority of HCV-infected individuals become chronic carriers; however, the mechanism of pathogenesis and progression to chronicity remains unclear. Inefficient T-cell responses against HCV-derived antigens have been suggested to be one of the major causes of the progression to chronicity in HCV-infected patients (Battegay et al., 1995; Cerny et al., 1995; Koziel et al., 1992; Lauer et al., 2002; Lechner et al., 2000; Rehermann et al., 1996; Shirai et al., 1994; Shoukry et al., 2003; Thimme et al., 2001; Urbani et al., 2002). It has been suggested that patients with chronic HCV are unable to prime and maintain vigorous T-cell responses that are initiated during the acute phase of HCV infection (Battegay et al., 1995; Cerny et al., 1995; Koziel et al., 1992; Lauer et al., 2002; Lechner et al., 2000; Rehermann et al., 1996; Shirai et al., 1994; Shoukry et al., 2003; Thimme et al., 2001; Urbani et al., 2002). In several reports, it has been suggested that, in chronically HCV-infected patients, the functions of DCs are impaired (Auffermann-Gretzinger et al., 2001; Bain et al., 2001; Kakumu et al., 2000; Kanto et al., 1995; Koziel et al., 2000; Kanto et al., 2000; Lechner et al., 2000; Rehermann et al., 1996; Shirai et al., 1994; Shoukry et al., 2003; Thimme et al., 2001; Urbani et al., 2002). In several reports, it has been suggested that, in chronically HCV-infected patients, the functions of DCs are impaired (Auffermann-Gretzinger et al., 2001; Bain et al., 2001; Kakumu et al., 2000; Kanto et al., 1999). Direct HCV infection of blood DCs has also been implicated in DC dysfunction (Tsubouchi et al., 2004). However, these studies are difficult to reconcile because patients with chronic HCV are immunocompetent and do not have a general immune deficiency (Neumann-Haefelin et al., 2005). Also, there have been several reports providing evidence that the functions of DCs in chronic HCV patients or chronically HCV-infected chimpanzees are normal (Larsson et al., 2004; Longman et al., 2004).
Among various HCV-derived antigens, NS3 antigen has been shown to be immunodominant and T cells reactive against NS3 have been shown to be important in viral clearance (Pape et al., 1999). In contrast, HCV-derived core antigen has been suggested to be immunosuppressive (Sarobe et al., 2003). Treatment of immature DCs with core or NS3 proteins has been shown to inhibit DC differentiation (Dolganiuc et al., 2003). In another report, it was shown that DCs transduced to express HCV core and E1 were poor stimulators of allogeneic T cells (Sarobe et al., 2002).

The objective of our study was to determine whether human DCs endogenously expressing HCV core or NS3 antigens are...
functionally and/or phenotypically normal and can stimulate T cells efficiently. By using recombinant adenoviral vectors, 100% of the immature and mature DCs were infected and expressed HCV core or NS3 proteins (Fig. 3). In most of the experiments, we used recombinant vectors at 100–200 m.o.i. and obtained 100% infection. Also, at this dose of the adenoviral vectors, we did not see any reduction in the cell number or loss of viability compared with uninfected DCs (data not shown). These initial experiments not only verified our ability to express HCV core or NS3 proteins efficiently in human DCs, but also suggested that the expression of these HCV proteins does not lead to cell death or toxicity to cells.

DCs infected with recombinant viruses, including retroviruses, *Vaccinia virus*, adenovirus, adeno-associated virus and *Canarypox virus*, have been shown to be effective in the induction of tumour or viral antigen-specific T-cell responses (Bronte et al., 1997; Brossart et al., 1997; Butterfield et al., 1998; Chiriva-Internati et al., 2002; Gahn et al., 2001; Kaplan et al., 1999; Motta et al., 2001; Song et al., 1997; Specht et al., 1997). The choice of adenovirus vector for our study was based on the following rationale. Adenovirus vector, unlike retroviruses, can express the transgene in non-dividing primary differentiated cells, such as DCs. Adenovirus does not inhibit the maturation and function of DCs, unlike *Vaccinia virus*, which induces apoptosis of immature DCs, inhibits maturation of DCs and diminishes

**Fig. 6.** Allogeneic T-cell proliferation against DCs expressing HCV core or NS3. For the assay, $2 \times 10^5$ allogeneic T cells were cultured with $10^3$–$10^4$ DCs infected with rAd/CMV5 vector containing core or NS3 or a control vector in 96-well flat-bottomed plates in AIM-V (serum-free) medium for 4 days at 37°C with 5% CO2 and humidity. (a, c) Unstimulated DCs; (b, d) DCs stimulated with LPS (100 ng ml$^{-1}$). Tritiated thymidine ($^{3}$H]Tdr, 0.5 μCi per well) was added to each well for 18 h incubation. [H]Tdr incorporation into the DNA of proliferating cells was measured after harvesting the plates and counting in a MicroBeta Trilux liquid scintillation counter (Perkin-Elmer). Each group was set up in at least three replicate wells and mean ± SEM values are shown. The experiment shown here is representative of more than three repeated experiments.

**Fig. 7.** DCs expressing HCV antigens core or NS3 efficiently present TT antigen to autologous T cells. DCs ($10^3$–$3 \times 10^4$), uninfected or infected with control vector, rAd/core or rAd/NS3 were incubated with TT (0.1 μg ml$^{-1}$) for 12 h followed by maturation with LPS (100 ng ml$^{-1}$) for 4 h. These DCs were then added to autologous T cells ($2 \times 10^5$ cells per well) in 96-well flat-bottom plates and incubated for 5 days. Tritiated thymidine ($^{3}$H]Tdr, 0.5 μCi per well) was added to each well for 18 h incubation. [H]Tdr incorporation into the DNA of proliferating cells was measured after harvesting the plates and counting in a MicroBeta Trilux liquid scintillation counter (Perkin-Elmer). Each group was set up in at least three replicate wells and mean ± SEM c.p.m. values are shown. The experiment shown here is representative of more than three repeated experiments.
their T-cell stimulatory activity. *Canarypox virus* also leads to apoptosis of immature human DCs.

DCs initiate T-cell responses mainly by three mechanisms: production of cytokines, provision of costimulatory molecules and stimulation of T cells. In our studies, we examined all of these three functions of DCs expressing HCV core or NS3 proteins. Expression of HCV core in immature DCs induced the expression of IFN-γ, TNF-α, IL-6, IL-2 and IL-12 p40 (Fig. 4). On the other hand, expression of HCV NS3 in immature DCs did not induce the expression of any of these cytokines. These results are somewhat in contrast to the suggestion of HCV core being immunosuppressive. Uninfected immature DCs produced IL-10, which was reduced upon infection with control, core- or NS3-containing adenovirus vectors. IL-10 has previously been shown to be produced by immature unstimulated DCs (Levings et al., 2005). It has been suggested that, as the role of DCs is not only to sense danger, but also to tolerate the immune system to antigens encountered in the absence of maturation/inflammatory stimuli, immature DCs produce IL-10 to achieve this function. Our results in fact suggest that HCV-derived core is able to induce inflammatory cytokines from human immature DCs. Further, this initial induction of cytokines by the DCs would be very important in priming and initiating T-cell responses against core antigen. From the mature DCs, it was interesting that NS3 induced the expression of IFN-γ and IL-10, whereas core induced the expression of TNF-α, IL-6, IL-2 and IL-12 p40. In all of these experiments, control adenoviral vector-induced cytokine expression was significantly lower than that induced by HCV core or NS3, suggesting HCV antigen NS3- or core-dependent induction of these cytokines. The exact mechanism of how these HCV antigens induce the expression of these cytokines from DCs is not clear. The reciprocal induction of IFN-γ and IL-10 versus TNF-α, IL-6, IL-2 and IL-12 p40 by NS3 and core, respectively, by mature DCs suggests important regulation of priming and stimulation of T cells by these HCV antigens. When comparing the amounts of cytokine message being induced by immature DCs and mature DCs, all cytokines tested, with the exception of IL-2, were induced at 2–4 log higher copy numbers by mature DCs than by immature DCs. This observation is consistent with literature reports of cytokine induction by DCs upon maturation (Radstake et al., 2004; Wan & Bramson, 2001). It is, however, possible that, as core induces high amounts of pro-inflammatory cytokines from DCs, they may get exhausted and, therefore, the DCs examined from chronic HCV patients may show impaired function due to longer exposure to HCV-derived core antigen.

Upon examining the phenotype of immature and mature DCs expressing HCV NS3 or core antigens, we did not observe significant differences in HLA class I, class II, CD40, CD83, CD86, CD1a or CD11c expression (Fig. 5). Taken together, these results suggest that expression of these HCV-derived antigens in DCs does not alter their phenotype, their ability to provide costimulation to T cells or their antigen-presenting capability. Upon stimulation with LPS, all of these markers were upregulated to approximately the same extent in DCs expressing or not expressing HCV antigens. These results are in accordance with the DCs obtained from chronically HCV-infected chimpanzees (Larsson et al., 2004). However, it is possible that maturation of DCs by stimuli such as double-stranded RNA or poly-I:C may be affected by these HCV antigens, as the virus may have evolved to evade these signalling pathways.

In agreement with the results obtained from the DC phenotype analysis and cytokine induction, allogeneic T-cell stimulation by DCs expressing HCV core and NS3 antigens was not reduced compared with the control vector-infected or uninfected DCs (Fig. 6). All of the DCs were able to stimulate allo-T cells. Upon maturing the DCs with LPS, we observed a twofold increase in T-cell proliferation. Interestingly, the HCV core-expressing DCs stimulated allo-T cells to a higher degree than the non-transduced, control vector-infected DCs or NS3-expressing DCs, further supporting our observation of induction of inflammatory cytokines by HCV core from DCs. In addition to allo-T-cell stimulation, we examined the processing and presentation of another foreign antigen (TT) by DCs expressing HCV core or NS3 antigens, which was normal (Fig. 7). These results show that the HCV antigens NS3 and core do not impair the ability of DCs to process and present foreign antigens to T cells.

In conclusion, our results have shown that expression of HCV proteins NS3 or core by human DCs does not affect their ability to mature phenotypically and function as APCs. Our results are in apparent contradiction to other reports suggesting that DCs are impaired in chronic HCV patients (Auffermann-Gretzinger et al., 2001; Bain et al., 2001; Kakumu et al., 2000; Kanto et al., 1999). However, it is possible that, in chronic HCV infections, a multitude of host and viral factors together may contribute to apparent impairment of DCs. Nevertheless, as these reports are contradictory (Auffermann-Gretzinger et al., 2001; Bain et al., 2001; Kakumu et al., 2000; Kanto et al., 1999; Larsson et al., 2004; Longman et al., 2004), our studies using individual HCV antigen-expressing DCs present a rather unique prospect to clarify the role of DC function in relation to individual HCV proteins in HCV infection. Further studies with other structural and non-structural proteins of HCV may clarify whether other HCV proteins impair DC function. These studies are important for rational design of cellular-vaccine approaches for the immunotherapy of chronic HCV.

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