Uptake and Presentation of Hepatitis C Virus-like Particles by Human Dendritic Cells

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Abstract

Hepatitis C virus (HCV) is a major cause of chronic hepatitis worldwide. Interaction of dendritic cells (DC) with viral particles may play an important role in the immunopathogenesis of HCV infection. Since the synthesis or purification of infectious virions is limited, we used HCV-like particles (HCV-LPs) to study the interaction of HCV with human DCs. Immature DCs exhibited an envelope-specific and saturable binding of HCV-LPs indicating receptor-mediated DC-HCV-LP interaction. Confocal microscopy revealed that HCV-LPs were rapidly taken up by DCs in a temperature-dependent manner. Competition experiments demonstrated that C-type lectins such as mannose receptor or DC-SIGN were not sufficient for mediating HCV-LP binding. HCV-LP uptake was followed by DC activation. DCs pulsed with HCV-LPs stimulated HCV core-specific CD4+ T cells, indicating that uptake of HCV-LPs by DCs leads to antigen processing and presentation on MHC class II molecules. Finally, HCV-LP-derived antigens were efficiently cross-presented to HCV core-specific CD8+ T cells. These findings demonstrate that HCV-LPs represent a novel model system to study HCV-DC interaction allowing to define the molecular mechanisms of HCV uptake, DC activation and antigen presentation to T cells. Furthermore, HCV-LP-mediated DC activation and efficient antigen presentation may explain the marked immunogenicity of HCV-LPs in vivo.
Hepatitis C virus (HCV), a positive strand RNA virus that belongs to the Flaviviridae family, is one of the major causes of chronic hepatitis worldwide. Current therapy based on pegylated interferon and ribavirin is characterized by limited efficacy, high cost, and major adverse effects. Therefore, the development of a preventive and potentially also therapeutic vaccine against HCV infection has a very high priority.

Clearance of acute HCV infection is associated with a strong HCV-specific CD4+ and CD8+ T cell response directed against epitopes of the structural and nonstructural proteins. Dendritic cells (DCs) play a key role in the induction and maintenance of antiviral T cell immune responses. As antigen presenting cells, DCs capture antigens, process them into peptides, and present them on molecules of the MHC to T cells.

A pathway by which DCs internalize antigens for presentation of T cells is receptor-mediated endocytosis, in which C-type lectins play an important role. A growing number of C-type lectins with specificity for mannosylated antigens have been found to be expressed by DCs such as Langerin (CD207), mannose receptor (CD206), DEC-205 (CD205) and DC-SIGN (CD209). Interestingly, DC-SIGN has been shown to play a role in infection of DCs by Ebola virus and dengue virus, another member of the Flaviviridae family. Furthermore, two recent studies using recombinant HCV envelope E2 protein and HCV pseudotype particles (HCVpp) have provided evidence that DC-SIGN or L-SIGN can bind HCV E2 on DCs or liver sinusoidal endothelial cells.
Several studies have suggested that DCs can be infected with HCV \(^{19-21}\). Furthermore, defects in DC function such as impaired maturation or allostimulation have been described in HCV-infected individuals by some investigators \(^{20,22,23}\) but not by others \(^{24,25}\). These contradictory findings indicate that the issue of HCV-induced defects in DC function requires further investigation. Due to the difficulties to synthesize or purify sufficient quantities of infectious virions for the study of virus-host interaction, the study of HCV-DC interaction has been limited to individually expressed recombinant viral proteins as surrogate viral antigens \(^{26}\). We and others have previously shown that cellular binding and entry of insect cell-derived HCV-like particles (HCV-LPs) represent a convenient and powerful surrogate system for the study of virus-host cell interaction allowing the definition of the cell surface proteins and viral epitopes required for viral binding and entry into target cells \(^{27-31}\). HCV-LPs are generated by self-assembly of HCV structural proteins in insect cells and are characterized by morphological, biophysical, and antigenic properties similar to putative virions from infected humans \(^{27-29,32-34}\). In vivo, HCV-LPs induce a strong humoral and cellular immune response including HCV-specific helper T cells and cytotoxic T lymphocytes \(^{35-37}\). The marked immunogenicity of HCV-LPs in animal models strongly suggests HCV-LP uptake and antigen presentation by DCs \textit{in vivo}. 

Virus-like particles (VLP) of several viruses including human immunodeficiency virus (HIV)\(^{38}\), hepatitis B virus (HBV)\(^{39}\), papillomavirus\(^{40}\) and parvovirus\(^{41}\) have been successfully used to study viral particle uptake, virion-mediated activation and antigen presentation by DCs. In this study, we used HCV-LPs as a model system to study the molecular interaction of HCV virions with human DCs.
Material and Methods

Recombinant proteins

HCV-LPs were synthesized as described\textsuperscript{27,32}. HCV-LP core concentration was determined as previously described\textsuperscript{27} using purified recombinant core protein C\textsubscript{1-120}\textsuperscript{42} as a standard. HCV-LP E2 concentration was determined by an E2-specific ELISA as described\textsuperscript{33}. Recombinant core protein C22-3 was kindly provided by M. Houghton (Chiron, Emeryville, CA). IL-2, IL-3, IL-4, IL-6, and TNF-\(\alpha\) were obtained from CellGenix (Freiburg, Germany), IL-1\(\beta\) from R&D Systems (Minneapolis, MN), GM-CSF from Novartis (Basel, Switzerland), CD40 ligand from Leinco Technologies (St. Louis, MO), prostaglandin E2, mannan, lipopolysaccharide (LPS, \textit{Escherichia coli} 026:B6), and poly-L-lysine from Sigma (St. Louis, MO), endoglycosidase H (EndoH), and peptide N-glycosidase F (PNGaseF) from New England Biolabs (Beverly, MA).

Antibodies

Mouse monoclonal anti-E2 (16A6, AP33, and 2F10), anti-E1 (11B7) and anti-galactosidase antibodies (mAb) have been described\textsuperscript{27}. Chimpanzee anti-E2 mAb (49F3) was generated by immunization of a chimpanzee with recombinant E2 protein\textsuperscript{43}. Anti-E2 antibodies 16A6 and 49F3 bind to an E2 epitope located between HCV amino acid 516 and 530 (E. Depla, unpublished observations 2004). Anti-human DC-SIGN (Clone 120507), CCR5 (clone 45531.111), CCR3 (clone 61828) and CXCR4 mAbs (clone 12G5, from Dr. J. A. Hoxie\textsuperscript{44}) were generous gifts from the NIH AIDS Research and Reference Reagent Program (Germantown, MD). Anti-human DC-SIGN mAb (AZN-D1) was a kind gift from Dr. T. B. H. Geijtenbeek (Department of Molecular Cell Biology and Immunology, Vrije Universiteit, Amsterdam, The Netherlands). Anti-human DC-SIGN/L-SIGN mAb (Clone 120612) was purchased from R&D Systems, anti-human CD205 (DEC-205) and anti-human Toll-like receptor (TLR) 2 mAb (clone TL2.1) from eBioscience (San Diego, CA), anti-human
DCGM4 mAb (anti-Langerin) from Beckman Coulter (Fullerton, CA). Anti-human lysosomal-associated membrane protein mAb (LAMP-2), biotinylated anti-human TLR4 (clone HTA125), PE-conjugated anti-human CCR7, R-PE-conjugated anti-human mannose receptor mAb and streptavidin-PE were purchased from Becton Dickinson (San Jose, CA). R-PE-conjugated anti-mouse and R-PE-conjugated or Cy3-conjugated anti-human IgG antibodies were obtained from Dako (Glostrup, Denmark). Fluorescein-conjugated anti-mouse IgG was purchased from ICN Biomedicals (Irvine, CA).

**Generation of DCs from human peripheral blood monocytes (PBMCs)**

DCs were generated from PBMCs of healthy, anti-HCV negative blood donors as described by Sallusto and Lanzavecchia. To obtain monocyte-derived DCs, CD14+ cells were cultured in the presence of IL-4 (1000 U/ml) and GM-CSF (800 U/ml). Immature DCs (imDCs) were collected on day 5 before exposure to the maturation stimuli. For DC maturation, IL-6 (1000 U/ml), IL-1β (10 ng/ml), TNF-α (10 ng/ml), and prostaglandin E2 (1 µg/ml) or LPS (10 µg/ml) were added for the last two days (day 5 to 7) of culture.

**Isolation of human blood DCs**

Blood pre-DCs were isolated from PBMCs of healthy, anti-HCV negative blood donors using DC isolation kit BDCA-1 for myeloid pre-DCs 1 and kit BDCA-4 for plasmacytoid pre-DCs 2, respectively, according to the manufacturer’s protocol (Miltenyi Biotech, Bergisch Gladbach, Germany). ImDCs 1 were generated by short-term culture (48 h) of pre-DCs 1 in DC medium supplemented with IL-4 (1000 U/ml) and GM-CSF (800 U/ml). ImDCs 2 were induced by incubation of pre-DCs 2 with IL-3 (40 ng/ml).
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Cytofluorimetric cell surface phenotyping

Cell staining was performed using FITC- and PE-conjugated mAb directed against epitopes of CD80, CD83, CD86, CD123, HLA-DR, CCR7, and mannose receptor. Lineage cocktail (Lin) comprises a combination of anti-CD3, -CD14, -CD16, -CD19, -CD20, and -CD56 antibodies (Becton Dickinson). For staining by flow cytometry, DCs were incubated with the respective antibodies or IgG isotype control antibodies and cellular fluorescence was monitored using a FACSCalibur flow cytometer with CellQuest 3.11 software (Becton Dickinson). For the detection of C-type lectin, CCR and TLR cell surface expression, DCs were incubated with the respective antibodies or IgG isotype control and stained with R-PE conjugated anti-mouse IgG.

Analysis of cellular HCV-LP binding

Cells (0.5-1 x 10^5 cells/100μl) were incubated with HCV-LPs or insect cell control preparations (derived from insect cells infected with a recombinant baculovirus containing the cDNA for β-glucuronidase (GUS)) and cell-bound HCV-LPs were detected using anti-E2 antibody (16A6) and flow cytometry as described. To assess whether binding of HCV-LPs was envelope-dependent, HCV-LPs were pre-incubated with mouse anti-envelope antibodies as described. The HCV-LP-antibody mixture was added to imDCs and incubated for 1 h at 4°C in PBS containing 0.5% BSA. Cellular binding of HCV-LPs was quantified by flow cytometry using chimpanzee anti-E2 mAb (49F3) and R-PE-conjugated anti-human IgG antibody. Inhibition of HCV-LP binding to imDCs by a human polyclonal anti-HCV antiserum was performed as described. To assess the role of HCV-LP envelope protein glycosylation for HCV-LP binding, HCV-LPs and insect cell control preparations were treated with glycosidases, followed by immunoblotting with anti-E1 and E2 monoclonal
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antibodies as described\textsuperscript{35}. To minimize denaturation of HCV-LPs during glycosidase treatment, digestions with glycosidases were performed in the absence of denaturation buffer according to the manufacturer’s protocol. Binding of glycosylated (mock-treated) and de-glycosylated (EndoH and PNGaseF treated) HCV-LPs to imDCs was analyzed side by side as described above.

To characterize the role of cell surface proteins for HCV-LP-DC interaction, DCs were pre-incubated with antibodies directed against C-type lectins, CCRs, TLRs or control IgG (anti-galactosidase mAb) (50 µg/ml) in PBS-0.5% BSA. Following an 1 h incubation, HCV-LPs (1 µg/ml HCV-LP E2) were added to the cells and cell-bound HCV-LPs were detected as described above using chimpanzee anti-E2 mAb (49F3) and flow cytometry. For inhibition with mannan, DCs were incubated with mannan (3 mg/ml) prior to the addition of HCV-LPs. As a positive control, FITC-dextran uptake was measured in the presence or absence of mannan as described\textsuperscript{20}.

\textit{Analysis of HCV-LP entry by immunofluorescence}

HCV-LP entry was analyzed by immunofluorescence and confocal laser scanning microscopy as described\textsuperscript{30}. Briefly, imDCs were incubated with HCV-LPs or insect cell control preparation for 1 h at 4°C (HCV-LP binding) or 37°C (HCV-LP entry). After removal of non-bound HCV-LPs by washing with ice-cold PBS, imDCs were bound to poly-L-lysine treated cover slides and fixed with PBS/3.5% paraformaldehyde. DCs were permeabilized in PBS-0.1% Triton X-100 and stained for HCV-LP binding and entry using chimpanzee anti-E2 antibody 49F3 (dilution 1:200 in PBS, 1.5 h incubation) and Cy3-conjugated anti-human IgG (dilution 1:500 in PBS, 45 min). DCs were counterstained with mouse anti-human LAMP-2.
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Antibody (dilution 1:700 in PBS, 1.5 h incubation) and FITC-conjugated anti-mouse IgG (dilution 1:700, 45 min). Prior to analysis by confocal microscopy, cover slides were mounted in antifade reagent with 4’-6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR). Stained cells were analyzed using a Leica TCS SP2 AOBS laser scanning confocal microscope (Carl Zeiss Corp., Jena, Germany) with Argon (488 nm) and Helium (543 nm) lasers. For staining of cell nuclei, DAPI was excited with a diode laser (405 nm). Digitalized images were analyzed using Zeiss-LSM Image Browser version 2.8 (Carl Zeiss Corp.).

Analysis of HCV-LP-induced activation

To investigate DC activation, imDCs were replated on day 6 at a density of 1 x 10^5 cells/well in 96-well plates. DCs were then exposed to insect cell control preparations (10 μg/ml), native or denatured HCV-LPs (total protein concentration 10 μg/ml), or LPS (10 μg/ml). Denaturation of HCV-LPs was performed by heat treatment (95°C for 15 min) as described. DCs were then incubated for 16 h in DC medium containing IL-4 (1000 U/ml) and GM-CSF (800 U/ml). Finally, DCs activation was analyzed by flow cytometry using HLA-DR-, CD86-, CD80-, and CD83-specific antibodies as described above.

Mixed lymphocyte reaction

Monocyte-derived imDCs from healthy, anti-HCV negative individuals were incubated with HCV-LPs or insect cell control preparations (total protein concentration 10 μg/ml) for 16 h. DCs were then exposed for 24 h to maturation stimuli as described above. Following maturation, DCs were collected, washed, irradiated (30 Gy), and used as stimulators for allogeneic PBMCs. Irradiated mDCs (10^1-10^4 cells) were then co-incubated with PBMCs (2 x 10^5 cells) for 6 days. During the last 16 h of incubation, cells were pulsed with [3H]thymidine.
(1 μCi/well; Amersham Buckinghamshire, UK), harvested, and the amount of radiolabel incorporated into the responder cell DNA was determined using a beta-counter (LKB, Pharmacia, Uppsala, Sweden). For cytokine analysis, mDCs were co-incubated with allogeneic CD4+ T cells at a DC/T cell ratio of 1:10. Cytokine secretion was quantified by ELISA on days 1 (for IL-4) and 5 (for IFN-γ) as described.

**Analysis of HCV core-specific CD4+ T cell clone stimulation by HCV-LPs**

A core-specific CD4+ T cell clone (recognizing an epitope in the HCV core protein comprising aa 21-44) was generated from a patient with acute hepatitis C as described. HLA restriction for the clone was defined as HLA-DQ3 using a panel of homozygous lymphoblastoid cell lines. For expansion, the T cell clone was restimulated with irradiated allogeneic PBMC, phytohemagglutinin (2 μg/ml), and IL-2 (50 U/ml). DCs generated from allogeneic HLA-DQ3 positive PBMC were incubated with different concentrations of recombinant core protein, HCV-LPs, or insect cell control preparation for 3 hours at 37°C. After washing, irradiated DCs (5 x 10^4 cells/well) were incubated with the core-specific CD4+ T cell clone (5 x 10^4 cells/well) for 3 days. On day 3, CD4 cells were labeled by incubation for 16 h with [3H]thymidine (2 μCi/well) and the amount of radiolabel incorporated into DNA was measured using a beta-counter.

**Cross-presentation of HCV-LPs to HCV core-specific CD8+ T cells**

HCV-specific CD8+ T cell responses were studied in a 75-year-old chronically HCV-infected patient (HCV genotype 1) by a comprehensive ELISPOT assay using 441 overlapping peptides (18-mers, overlapping by 11aa) spanning the whole HCV genotype 1 polyprotein (NIH AIDS Research & References Reagent Program) similarly as described. This method
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led to the identification of three intrahepatic HCV-specific CD8+ T cell responses in this patient, with the strongest response against a core peptide comprising aa 36-53 (LLPRRGPRLGVRATRKTS) – peptide 6. An HCV peptide-specific cytotoxic T lymphocyte (CTL) line was established from PBMCs by stimulation with this peptide as described previously 50. Intrahepatic CD8+ T cells were expanded polyclonally and antigen-non-specifically as described recently (purity confirmed by FACS analysis >95%) 50. To study HCV-LP antigen presentation on MHC class I molecules, autologous imDCs were harvested and incubated with HCV-LPs, insect cell control preparation, or without antigen for 40 h. After 24 h, medium was replaced and fresh antigen added. During the final 16 hours, CD40 ligand (1 µg/ml) was added to the cultures as a maturation stimulus. DCs were then extensively washed and co-cultured with core-specific intrahepatic CD8+ T cells or peripheral core-specific CD8+ T cell lines at a ratio of 1:2. After 5 h of incubation intracellular IFN-γ staining of core-specific CD8+ T cells was performed as described recently 6. As a positive control, unpulsed mature DCs were incubated for 1 h at 37°C with peptide 6 (10 µg/ml) and added to the CD8+ T cells following extensive washing. Approval was obtained from the University Hospital Freiburg institutional review board for these studies. Informed consent was provided according to the Declaration of Helsinki.
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Results

Binding of HCV-LPs to human DCs

The induction of a broad humoral and cellular immune response against HCV structural proteins by HCV-LPs in mice in vivo\textsuperscript{35-37,51} has suggested that HCV-LPs are efficiently presented by antigen-presenting cells (APCs). In this study we aimed to analyze the interaction of HCV-LPs with human professional APC. For that purpose, human imDCs were generated \textit{in vitro} from monocytes of healthy donor PBMCs. ImDCs were characterized by moderate expression of HLA-DR and CD86, and low or absent expression of CD80 and CD83 (Fig. 1, \textit{A}). In contrast, mDCs generated by addition of pro-inflammatory cytokines were characterized by expression of high levels of HLA-DR, CD80, CD83, and CD86 (Fig. 1, \textit{A}). DC maturation was accompanied by down-regulation of CCR3 and CCR5 and up-regulation of CXCR4 expression (Table 1). CCR7 was exclusively detected on the cell surface of mDCs (Table 1). Surface expression of TLR2 and 4 on immature and mDCs was very low or non-detectable (Table 1).

HCV-LP binding to imDCs was dose-dependent and achieved saturation (Fig. 1, \textit{B} and \textit{C}). By contrast, mDCs demonstrated a significantly reduced HCV-LP binding that was not saturable at the HCV-LP concentration tested. CD3+ T- and CD19+ B-cells did not demonstrate any measurable HCV-LP binding. Monocytes (CD14+) showed very low binding at high HCV-LP E2 concentrations (Fig. 1, \textit{B} and \textit{C}). To study whether HCV-LPs interact with human DCs circulating in blood, freshly isolated myeloid (pre-DCs 1) and plasmacytoid pre-DCs (pre-DCs 2) from peripheral blood were analyzed for HCV-LP binding. Pre-DCs 1 strongly expressed CD1c while pre-DCs 2 were characterized by expression of CD123 (Fig. 2, \textit{A}). HCV-LPs did not bind to pre-DCs 1 or pre-DCs 2 (Fig. 2, \textit{B}). However, following
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maturation of pre-DCs into imDCs using medium containing defined cytokines, binding of HCV-LPs to blood DCs was induced (Fig. 2, C).

**HCV-LP-DC interaction is mediated by epitopes in the E1 and E2 N-terminal regions**

To study whether HCV-LP binding was mediated by the HCV structural proteins, we analyzed cellular HCV-LP binding in the presence of anti-envelope antibodies and human polyclonal anti-HCV antiserum. As shown in Fig. 3A, HCV-LP binding to DCs was markedly inhibited by pre-incubation with a well-characterized anti-HCV antiserum containing high-titer anti-E2 antibodies but not by a control serum. To map viral envelope protein epitopes mediating HCV-LP-DC cell surface interaction, we used a panel of well-characterized anti-E1/E2 mAbs for inhibition of HCV-LP binding. As shown in Fig. 3A, HCV-LP binding to imDCs was markedly inhibited by pre-incubation of HCV-LPs with anti-E2 (AP33 and 2F10) and anti-E1 mAb (11B7). Anti-envelope mediated inhibition of binding was concentration-dependent (data not shown) and absent for control mouse monoclonal IgG (Fig. 3A). These data suggest that the E2 hypervariable region-1 (aa 398-403; targeted by mAb 2F10) as well as the E1 and E2 N-terminal regions (aa 212-224 targeted by anti-E1 mAb 11B7 and aa 412-423 targeted by anti-E2 mAb AP33) play an important role for docking of HCV-LPs to the cell surface of human DCs. To assess whether cellular binding of HCV-LPs is dependent on native envelope protein conformation, we compared the binding profiles for native and heat-denatured HCV-LPs. Compared to native HCV-LPs, heat-denatured HCV-LPs exhibited a strongly reduced binding to imDCs (Fig. 3A). These data suggest that proper conformation of the HCV-LP envelope is crucial for DC-binding.
Next, we assessed the impact of envelope glycosylation for HCV-LP binding. Using glycosidase digestion of HCV-LPs we previously demonstrated that the HCV-LP envelope proteins are extensively N-glycosylated \(^{35}\). Similar to expression of full-length envelope proteins in mammalian cells \(^{52}\), the envelope proteins of HCV-LPs do not acquire the complex carbohydrate forms associated with trafficking through the Golgi apparatus \(^{35}\). Digestion of HCV-LP envelope proteins with glycosidases (Fig. 3, B) revealed that envelope proteins of HCV-LPs used in this study (H77C strain) are extensively N-glycosylated as shown previously for HCV-LPs derived from the HCV-J strain \(^{35}\). HCV-LPs digested with EndoH or PNGaseF exhibited a partial decrease in DC binding compared to mock-digested HCV-LPs (Fig. 3, C). Since we can not exclude that the reduction of HCV-LP binding was related to changes in HCV-LP envelope conformation following glycosidase treatment, the partial reduction in HCV-LP binding does not support a major role of envelope glycosylation for HCV-LP-DC cell surface interaction.

\textit{HCV-LP binding to imDCs requires molecules other than C-type lectins}

HCV-LP binding was sensitive to pre-treatment of DCs with trypsin, indicating that binding of HCV-LPs is mediated by a cellular protein (Fig. 4, C). To further characterize the protein interacting with HCV-LPs, we assessed the role of several DC cell surface molecules implicated in the interaction of DCs with microbial pathogens.

For efficient capture of a variety of antigens, DCs use C-type lectins. ImDCs demonstrated high level expression of mannose receptor, DC-SIGN, and DEC-205 (Fig. 4, A). Langerin was not expressed on DCs (Fig. 4, A). Upon maturation of imDCs expression of mannose receptor and DC-SIGN decreased to lower levels (Fig. 4, A). In contrast, DEC-205 expression increased during maturation of DCs. To study whether C-type lectins play a crucial role in mediating binding of HCV-LPs to DCs, binding of HCV-LPs was analyzed in the
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presence or absence of mannan. Incubation of imDCs with mannan abolished binding and uptake of FITC-dextran by C-type lectins (Fig. 4, B). In contrast, binding of HCV-LPs to imDCs was only marginally inhibited by mannan (Fig. 4, B). Pre-incubation of imDCs with anti-human DC-SIGN (AZN-D1) or DC-SIGN/L-SIGN (Clone 120612) antibodies that had been previously shown to inhibit binding of recombinant C-terminally truncated E2 to DC-SIGN on imDCs \(^{16,17}\) did not inhibit HCV-LP binding. Similar results were obtained for DEC-205 (Fig. 4, C), another C-type lectin recognizing mannose-like carbohydrates on microbial pathogens \(^{53}\). These results suggest that C-type lectins are not sufficient to mediate HCV-LP-DC interaction and that other or additional cell surface molecules are required for HCV-LP binding and entry.

Next, we studied whether defined TLRs or CCRs play an important role for HCV-LP binding. Recent studies have indicated that innate immune responses induced by several viruses is mediated by binding of viral envelope protein to TLR2 or TLR4 \(^{54}\). Pre-incubation of TLR2 and 4 on imDCs by anti-TLR specific antibodies did not result in inhibition of HCV-LP binding (Fig. 5, C). Furthermore, overexpression of either human TLR2 or TLR4 in CHO cells (Fig. 5, A) was not able to induce cellular binding of HCV-LPs (Fig. 5, B). These data demonstrate that TLR2 and 4 do not represent cell surface molecules mediating binding of HCV-LPs.

CCR3, CCR5 and CXCR4 have been shown to act as cofactors for HIV entry \(^{55,56}\). CCR7 and CXCR4 expression did not correlate with HCV-LP binding (Table 1). Although CCR3 and CCR5 expression partially correlated with HCV-LP binding to immature and mDCs, anti-CCR3, and anti-CCR5 antibodies -recently shown to inhibit HIVgp120-CCR interaction \(^{57}\)- were not able to inhibit HCV-LP binding (data not shown). These data suggest that these CCRs do not play a major role for HCV-LP binding.

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Internalization of HCV-LPs into imDCs

After particle binding, the next step is uptake or entry of particles into the cell. Therefore, temperature-dependent HCV-LP entry into monocyte-derived imDCs was analyzed using HCV-LP-specific immunofluorescence and confocal microscopy. As shown in Fig. 6, incubation of imDCs with HCV-LPs at 4°C resulted in the exclusive detection of HCV-LPs on the cell surface, consistent with HCV-LP binding to the cell membrane. By contrast, incubation at 37°C for 1 h resulted in the translocation of E2 immunoreactivity into the cell consistent with HCV-LP entry. Furthermore, staining of cells with anti-LAMP antibody revealed that HCV-LPs partially co-localized with LAMP-2. In contrast, imDCs incubated with insect cell control preparation did not show any measurable E2-specific immunofluorescence (Fig. 6) and PBMC-derived T-cells incubated with HCV-LPs did not show any evidence for HCV-LP-cell binding and entry (Fig. 1, C and data not shown). Thus, imDCs specifically bind and rapidly internalize HCV-LPs in a temperature-dependent manner.

Activation of DCs by native but not by denatured HCV-LPs

DC activation and maturation may be mediated by endogenous cytokines or the antigen itself. To determine whether HCV-LPs can induce DC activation, imDCs were exposed to HCV-LPs or insect cell control preparations. DC activation was then measured by flow cytometry of defined cell surface markers. After exposure to HCV-LPs for 16 h, CD80 and CD83 were markedly and significantly up-regulated indicating HCV-LP mediated DC activation (Fig. 7, Table 2). Although this up-regulation was less pronounced than for DCs incubated with LPS it was highly reproducible for different HCV-LP and DC preparations (Table 2). By contrast, incubation of DCs with insect cell control preparations did not activate DCs. Denatured HCV-LPs (generated by heat treatment of native HCV-LPs) also failed to elicit DC activation (Fig.
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7, Table 2). This finding suggests that conformation of structural proteins of HCV-LPs is important for DC activation.

**Immunostimulatory properties of DCs pulsed with HCV-LPs in mixed lymphocyte reaction**

DCs in individuals with chronic HCV infection have been shown to display an impaired allostimulatory function. To study whether HCV-LP can modify the allostimulatory function of human DCs, the allostimulatory capacity of DCs pulsed with HCV-LPs was studied in a mixed lymphocyte reaction. As shown in Fig. 8, DCs pulsed with HCV-LPs exhibited no quantitative or qualitative difference in their allostimulatory function as assessed by T-cell proliferation (Fig. 8, A) and cytokine secretion (Fig. 8, B) compared to DCs pulsed with insect cell control preparations or without antigen. These data indicate that HCV-LPs do not alter the allostimulatory function of human DCs.

**DCs pulsed with HCV-LPs induce HCV core-specific CD4+ T cell responses**

Since HCV-LPs can activate imDC, we also examined whether HCV-LPs are processed by imDCs and whether HCV-LP-derived antigens are presented to CD4+ T cells. DCs pulsed with HCV-LPs, but not with insect cell control preparations, induced a marked and dose-dependent CD4+ T cell clone proliferation indicating efficient HCV-LP processing and presentation of HCV-LP derived antigens on MHC class II molecules (Fig. 9).

**Cross-presentation of HCV-LPs to intrahepatic HCV core-specific CD8+ T cells**

To study whether HCV-LPs are cross-presented to CD8+ T cells, we assessed presentation of HCV-LP derived antigens to core-specific intrahepatic CD8+ T cells derived from an HCV-
infected individual. Autologous DCs pulsed with HCV-LPs markedly stimulated IFN-γ production by intrahepatic CD8+ T cells (Fig. 10). In contrast, DCs pulsed with an insect cell control preparation (GUS Ctrl; Fig. 10) or intrahepatic CD8+ T cells incubated with HCV-LPs in the absence of DCs (data not shown) did not result in IFN-γ production of intrahepatic CD8+ T cells. These control experiments rule out that IFN-γ production of CD8+ T cells was due to non-specific effects related to contaminating insect cell proteins or a direct activation of intrahepatic CD8+ T cells by non-processed HCV-LP-derived antigens. Similar results were obtained using peripheral core-specific CD8+ T cell lines established from the same donor (data not shown). These data demonstrate that human DCs can efficiently cross-present HCV-LPs to HCV-specific CD8+ T cells.

**Discussion**

In this study, we demonstrate for the first time that HCV-LPs interact specifically with human monocyte-derived DCs and defined subsets of blood DCs. DC-HCV-LP interaction is characterized by the following four key findings: (i) human DCs bind and rapidly internalize HCV-LPs in a dose-dependent manner; (ii) HCV-LP binding and entry requires cell surface molecules other than C-type lectins; (iii) incubation of DCs with HCV-LPs results in the up-regulation of costimulatory molecules indicating HCV-LP-mediated DC activation; (iv) DCs pulsed with HCV-LPs elicit a strong HCV-specific CD4+ and CD8+ T cell response demonstrating efficient HCV-LP antigen processing and presentation.

HCV-LPs demonstrated a dose-dependent binding to monocyte-derived imDCs and purified blood imDCs. Maturation of imDCs by pro-inflammatory cytokines was accompanied by a marked reduction of HCV-LP binding, indicating down-regulation of DC
cell surface molecules that bind HCV-LPs during maturation. These observations suggest that
imDCs are most likely to be the key antigen-presenting cells interacting with HCV-LPs in
humans. Since recent studies have shown that HCV is internalized by imDCs\textsuperscript{19,20}, our results
indicate that HCV-LPs demonstrate a similar tropism for DCs as virions.

ImDCs express several receptors including C-type lectins that mediate recognition and
internalisation of pathogens \textsuperscript{58}. Previous studies have demonstrated that recombinant C-
terminally truncated HCV envelope glycoprotein E2 and HCVpp can bind to DC-SIGN and
L-SIGN \textsuperscript{59,60}. In contrast to observations using the above mentioned ligands, blocking of C-
type lectins by mannan, as well as pre-incubation of imDCs with specific DC-SIGN/L-SIGN
antibodies, did not result in marked inhibition of HCV-LP binding. Although our results do
not exclude binding of HCV-LPs to DC-SIGN, our observations indicate that DC-SIGN is not
sufficient to mediate HCV-LP binding to DCs and other cell surface receptors are required for
HCV-LP binding.

Since glycosylation of HCV-LPs did not appear to play a major role for HCV-LP
binding to DCs and HCV-LP binding could be markedly inhibited by anti-envelope antibodies
directed against non-glycosylated envelope epitopes, it is unlikely that differences in envelope
glycosylation play a key role for the observed differences in ligand-cell interaction
(recombinant envelope proteins versus HCV-LPs). This conclusion is supported by the
observation that both C-terminally truncated E2 purified from the intracellular fraction
\textsuperscript{17} (containing carbohydrate forms sensitive to digestion with EndoH) as well as secreted C-
terminally truncated E2 \textsuperscript{16,59} (containing EndoH resistant complex carbohydrate forms
associated with trafficking through the Golgi apparatus) similarly interact with DC-SIGN\textsuperscript{16,59}.

Alternatively, differences in envelope protein conformation may play an important
role for the interaction of model ligands with DC-SIGN. In contrast to C-terminally truncated
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recombinant E2 used for interaction studies with DC-SIGN \(^{16,17,59,61}\), E2 of HCV-LP is expressed in its full length together with glycoprotein E1 as part of an E1/E2 heterodimer \(^{33,35}\). Several investigators have shown that HCV-LPs contain E2 in a native conformation, which may resemble properly folded E2 in the virion \(^{27,28,33}\). Thus, it is conceivable that the observed interaction of E2 with cell surface DC-SIGN is predominantly a feature of C-terminally truncated envelope proteins, whereas properly folded E1/E2 heterodimers containing full-length E2 in its native conformation may exhibit a different interaction profile. This hypothesis is supported by two recent studies characterizing the cellular entry of HCV E1 and E2 glycoproteins complexes as part of retroviral HCVpp. Although HCVpp can bind to DC-SIGN\(^{18}\), DC-SIGN is not able to mediate entry of HCVpp into DCs\(^{18}\) or hepatoma cells\(^{62}\). Recent evidence suggests that C-type lectins may rather play a role for facilitating transmission of HCV from DCs or L-SIGN-positive liver sinusoidal endothelial cells to neighboring hepatocytes \(^{18,59,60}\).

Since the detailed structural and functional properties of infectious virions are still poorly characterized, it is unknown whether the surface properties of HCV-LPs or other surrogate systems such as HCVpp are identical with the infectious virion. The ongoing development of recombinant infectious virions may ultimately allow to answer the role of DC-SIGN for binding and entry of the native virion in the future.

Several cell surface molecules including CD81\(^{63}\), scavenger receptor class B type I (SR-BI)\(^{64}\) and heparan sulfate\(^{30}\) have been suggested as mediators of HCV envelope protein binding. Interestingly, preliminary studies of our laboratory indicate that HCV-LP binding to DCs can be partially inhibited by anti-CD81 mAb (H. Barth and T. Baumert, unpublished observations 2004), that was recently reported to block HCV E2-CD81 interaction\(^{65}\). Further
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studies analyzing the role of CD81, SR-BI and heparan sulfate in mediating HCV-LP binding to DCs are in progress.

Following cell surface binding, antigen-receptor complexes are internalized into lysosomal compartments of DCs where proteinases mediate antigen processing\(^6^6\). Confocal microscopy demonstrated that HCV-LPs partially co-localized with LAMP-2 following HCV-LP uptake, suggesting that HCV-LPs may be targeted into the endosomal-lysosomal pathway. Trafficking and processing of HCV-LPs into the endosomal-lysosomal pathway is corroborated by the finding of efficient MHC class II restricted HCV-LP antigen presentation to CD4+ T cells. DCs are also capable of transporting antigens from the endocytic compartment to the cytosol, leading to “cross-presentation” on MHC class I molecules to CD8+ T cells. This pathway can be used by particular antigens such as VLPs but not by recombinant soluble proteins (for review see\(^6^7\)). In this study, we show for the first time that human DCs cross-present HCV-LPs to HCV-specific CD8+ T cells.

Incubation of imDCs with HCV-LPs also led to a small but reproducible and significant up-regulation of the costimulatory molecules CD80 and CD83 indicating HCV-LP-induced DC activation. These data are in line with two previous reports on activation of mouse and human monocyte-derived imDCs by papillomavirus VLPs\(^4^0,6^8\). Similar to the results using denatured HCV-LPs, non-assembled papillomavirus structural protein L1 failed to activate DCs\(^4^0\). In contrast to a marked DC activation induced by lipopolysaccharide (LPS), HCV-LP-induced activation was of substantially lower magnitude. This finding is not surprising since neither HCV\(^1\) nor HCV-LPs (T. F. Baumert and T. J. Liang, unpublished observations) generally induce a systemic inflammatory reaction in humans (HCV) or mice (HCV-LPs) in vivo.

A growing number of studies has characterized the function of DCs in chronic hepatitis C. Similar to findings in other chronic viral infections\(^6^9\), two observations have been
made: (i) an impaired DC maturation has been described in patients with chronic but not in resolved HCV infection\textsuperscript{23}; (ii) DCs in chronic HCV infection appear to display an impaired allostimulatory function\textsuperscript{20,22,23}. In contrast to these findings, two recent studies did not show evidence for an impaired DC function in HCV-infected chimpanzees or humans\textsuperscript{24,25}. Since incubation of DCs with HCV-LPs resulted in an increased activation of DCs and did not modify the allostimulatory function of DCs, our findings suggest that uptake and processing of the virion do not alter DC function. Therefore, it is conceivable that the observed defects in patients with chronic HCV infection in some\textsuperscript{20,22,23} but not in other\textsuperscript{24,25} studies may either require ongoing HCV viral replication or are possible consequences of chronic HCV infection associated with disease progression.

In conclusion, the interaction of HCV-LPs with DCs represents a powerful model system to study HCV-DC interaction allowing the characterization of HCV uptake, activation and presentation of HCV structural proteins on the molecular level. This includes the isolation of DC receptor(s) required for HCV binding and uptake, the exploration of signaling pathways including TLRs in mediating DC activation, the mapping of viral epitopes presented to T cells and finally the detailed exploration of HCV cross-presentation. Furthermore, our model system may allow to evaluate HCV-specific defects in dendritic cells postulated in patients with chronic HCV infection\textsuperscript{70}.

Finally, our findings of HCV-LP-mediated DC activation and efficient antigen presentation may explain the marked immunogenicity of HCV-LPs \textit{in vivo} \textsuperscript{35-37,51}. Together with similar findings of papillomavirus VLP-DC interaction \textsuperscript{68} and the successful development of a human papillomavirus VLP-based vaccine for the prevention of chronic
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papillomavirus infection\textsuperscript{71}, our findings suggest that HCV-LPs may be a potent vaccine candidate for the induction of antiviral cellular immune responses in humans.

Acknowledgments

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The abbreviations use are: CCR, chemokine receptor; CMV, cytomegalovirus; DC, dendritic cell; E1 and E2, envelope glycoproteins 1 and 2; GM-CSF, granulocyte-macrophage colony-stimulating factor; GUS, β-glucuronidase; HCV, hepatitis C virus; HCV-LP, hepatitis C virus-like particle; HIV, human immunodeficiency virus; HCVpp, HCV pseudotype particles; IFN-γ, interferon-γ; IL, interleukin; imDC, immature DC; LAMP, lysosomal-associated membrane protein; LPS, lipopolysaccharide; mDC, mature DC; MHC, major histocompatibility complex; MFI, mean fluorescence intensity; mAb, monoclonal antibody; TLR, Toll-like receptor; VLP, virus-like particle.
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Figure Legends

FIG. 1. Binding of HCV-LPs to monocyte-derived DCs. (A) Immunophenotyping of immature (imDC) and mature DCs (mDCs) used for HCV-LP binding. DC phenotypes were characterized by flow cytometry of HLA-DR, CD86, CD80, and CD83 surface expression (LIN - combination of anti-CD3-, CD14-, CD16-, CD19-, CD20-, and CD56 antibodies). Histograms corresponding to cell surface expression of the respective cell surface molecules (shadowed graphs) are overlaid with histograms of cells incubated with the appropriate isotype control antibody (unshadowed graphs). The absence of CD3, CD14, CD16, CD19, and CD56 expression excluded the presence of B cells, T cells, NK cells, or macrophage/monocytes in the DC preparation. (B) Binding of HCV-LPs to monocytes, immature and mature DCs. Flow cytometry histograms of HCV-LP binding to target cells (unshadowed graphs) are shown. Background fluorescence (grey shadowed graph) was measured using cells incubated with insect cell control preparations. Cellular binding of HCV-LPs was analyzed as described in Material and Methods. (C) Dose-dependent binding of HCV-LPs to target cells. Immature (closed squares) and mature DCs (open squares) (left panel), monocytes (triangles), T- (open circles), and B-cells (open diamonds) (right panel) were incubated with increasing concentrations of HCV-LP or insect cell control preparations. On the y-axis, net mean fluorescence intensity (Δ MFI) values for each HCV-LP E2 concentration were calculated by subtracting the MFI of insect cell control preparations from that obtained with the respective HCV-LP E2 concentration (x-axis). Concentration of HCV-LP E2 was quantified by an ELISA as described earlier 33.

FIG. 2. Interaction of HCV-LPs with blood DCs. (A) Immunophenotyping of freshly isolated myeloid (pre-DCs 1) and plasmacytoid (pre-DCs 2) pre-DCs from peripheral blood used for HCV-LP binding. Phenotypes of pre-DCs were characterized by flow cytometric
analysis of CD1c expression for pre-DCs 1 (left panel) and CD123 expression for pre-DCs 2 (right panel). Histograms corresponding to cell surface expression of the indicated molecules (unshadowed graphs) are overlaid with histograms corresponding to cells incubated with the appropriate isotype control antibody (grey shadowed graphs). (B and C) HCV-LP binding to pre-DCs and imDCs. Freshly isolated pre-DCs as well as myeloid and plasmacytoid imDCs (imDCs 1 and 2) were incubated with HCV-LPs (HCV-LP E2 concentration ≈ 2 µg/ml) and binding of HCV-LPs was analyzed as described in Fig. 1. Flow cytometry histograms of HCV-LP binding to pre-DCs (B) and imDCs (C) are shown (unshadowed graphs). Background fluorescence (grey shadowed graph) was measured as described above using cells incubated with insect cell control preparations.

**FIG. 3. Viral envelope glycoproteins and HCV-LP binding to DCs.** (A) Inhibition of HCV-LP binding to imDCs by anti-HCV antibodies. HCV-LPs (1 µg HCV-LP E2/ml) were pre-incubated with anti-E2 (AP33, 2F10; 50 µg/ml), anti-E1 antibody (11B7; 50 µg/ml), or an anti-HCV antiserum (dilution 1:50) containing high-titer antibodies against the HCV envelope glycoprotein E2 for 1 h at 37°C. Incubation of HCV-LPs with control IgG (50 µg/ml) or anti-HCV negative serum (dilution 1:50) served as negative controls. HCV-LP binding was analyzed by flow cytometry using chimpanzee anti-E2 mAb (49F3) and R-PE conjugated anti-human IgG. Denaturation of HCV-LPs was performed by heat-treatment as described²⁷,³⁵. Data are shown as percent binding relative to the binding of HCV-LPs without antibody (100%). (B) Analysis of envelope glycoprotein glycosylation. HCV-LPs were digested with EndoH or PNGaseF for 2 h at 37°C as described in Materials and Methods. Glycosidase (+) and mock-digested HCV-LP envelope proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting with anti-E1 and anti-E2 mAbs as described previously²⁷,³⁵. Sizes of molecular weight (MW) markers in kilodaltons...
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are indicated on the left (E1↑ and E2↑ – deglycosylated forms of E1 and E2). (C) Impact of envelope glycoprotein glycosylation for HCV-LP binding. ImDCs were incubated with increasing concentrations of glycosidase- (↓) and mock-digested HCV-LPs and particle binding was analyzed by flow cytometry as described in Fig. 1.

FIG. 4. HCV-LP binding to DCs and C-type lectins. (A) Expression of mannose receptor, DC-SIGN, DEC-205 and Langerin on monocyte-derived DCs. C-type lectin expression on imDCs and mDCs was analyzed by FACS using cell surface protein-specific antibodies (NC, negative control corresponding to cells incubated with the respective isotype control antibody). (B, left panel) Inhibition of FITC-dextran binding and uptake into imDCs by mannan. FITC-dextran binding and uptake at 37°C was measured by FACS in the absence or presence of mannan (3 mg/ml). (B, right panel) Mannan does not inhibit cellular HCV-LP binding to imDCs. HCV-LPs in subsaturating concentrations (1 μg HCV-LP E2/ml) were added to imDCs in the absence or presence of mannan (3 mg/ml) and HCV-LP binding was analyzed as described in Fig. 1. (C) Quantitative analysis of cellular HCV-LP binding in the presence of anti-DC-SIGN, anti-DC-SIGN/L-SIGN, anti-DEC-205, anti-Langerin antibodies or control IgG. DCs were preincubated with mannan (3 mg/ml) or antibody (50 μg/ml) for 1 h at 25°C. HCV-LPs (1 μg HCV-LP E2/ml) were added to the cells and HCV-LP binding was analyzed by flow cytometry as described in Fig. 3, A. To study whether trypsin pretreatment of DCs modifies HCV-LP binding, imDCs were exposed to 0.5% trypsin prior to the addition of HCV-LPs as described recently. Mean ± SD of three experiments is shown (HCV-LP binding in the absence of antibody = 100%).

FIG. 5. Analysis of HCV-LP binding to cell lines stably expressing TLR2 and 4. (A) Cell surface expression of TLR2 and TLR4 in CHO-CD14, CHO-CD14/TLR2, and CHO-
CD14/TLR4 cells. CHO cell lines \(^{72}\) were incubated with anti-TLR2, anti-TLR4, or isotype matched control mAbs, and analyzed for cell surface expression of TLR2 and TLR4 by flow cytometry. Black unshadowed graphs represent TLR-specific staining, grey shadowed graphs show staining with isotype-matched control Abs. (B) HCV-LP binding to the human hepatoma cell line Huh-7 \(^{31}\) and TLR-transfected CHO-cell lines. Cells were incubated with HCV-LPs for 1 h at 4°C and HCV-LP binding was quantified by flow cytometry. Data are shown as Δ MFI (mean ± SD) of a representative experiment performed in triplicate. (C) Binding of HCV-LPs to imDCs in the presence of anti-TLR antibodies. ImDCs were pre-incubated with anti-TLR2, anti-TLR4 or control mAb (50 μg/ml) prior to the addition of HCV-LPs and cellular HCV-LP binding was analyzed as described in Fig. 3, A. Data are shown as percent binding relative to the binding of HCV-LPs without antibody pre-incubation (100%). Mean ± SD of three experiments is shown (HCV-LP binding in the absence of antibody = 100%).

**FIG. 6. Temperature-dependent HCV-LP entry into imDCs.** Monocyte-derived imDCs were incubated with HCV-LPs or insect cell control preparation (GUS Ctrl) for 1 h at 4°C (for HCV-LP binding) or 37°C (for HCV-LP entry). Then, DCs were washed in ice-cold PBS, fixed, and permeabilized. HCV-LPs were detected with chimpanzee anti-E2 mAb (49F3) and Cy3-conjugated anti-human IgG antibody (red fluorescence). For co-staining of cytosolic lysosomal protein, cells were incubated with a mouse anti-LAMP-2 mAb and FITC-conjugated anti-mouse IgG antibody (green fluorescence). Nuclear staining was performed using 4′, 6-diamidino-2-phenylindole (DAPI; blue fluorescence) as described in Materials and Methods. Comparative analysis of stained imDCs by confocal laser scanning microscopy revealed temperature-dependent HCV-LP internalization.
**FIG. 7. DC activation by native HCV-LPs.** ImDCs were exposed to insect cell control preparations (GUS Ctrl), native HCV-LPs, denatured HCV-LPs or LPS at a final concentration of 10 μg/ml. After 16 h, DC activation was assessed by flow cytometric analysis of HLA-DR, CD80, CD86, and CD83 cell surface expression (dark line). Histograms corresponding to background expression of the respective cell surface molecules in unexposed DCs are shown as a grey line. A representative result of three independent experiments is shown.

**FIG. 8. Allostimulatory function of DCs pulsed with HCV-LPs.** (A) Responder PBMCs (2 x 10^5 cells/well) were cultured for 6 days in the presence of increasing concentrations of irradiated allogenic stimulator DCs pulsed with HCV-LPs (circle), insect cell control preparation (square) or without antigen (open triangle). T cell proliferation was measured by [3H]thymidine incorporation during the last 16 h of culture as described in Materials and Methods. The results are expressed as counts per minute (cpm, mean ± SD of an experiment performed in triplicate). (B) IL-4/IFN-γ production by allogeneic CD4+ T cells. CD4+ T cells were cocultured with DCs (T/DCs ratio: 10:1) as described above. For IL-4 and IFN-γ determination cell culture supernatants were collected on days 1 and 5, respectively. Data are shown as pg/ml (mean ± SD of an experiment performed in triplicate). The thresholds for cytokine detection are indicated (---).

**FIG. 9. Core-specific CD4+ T cell clone stimulation by imDCs pulsed with HCV-LPs.** Monocyte-derived imDCs were pulsed with corresponding concentrations of recombinant core protein, HCV-LPs (1-100 μg/ml; equivalent to approximately 0.1-10 μg HCV-LP core/ml) and insect cell control preparations (GUS Ctrl). DCs were then incubated with core-
specific CD4+ T cells for 3 days and T cell proliferation was measured by [3H]thymidine incorporation. Results are shown as counts per minute (cpm; mean ± SD of a representative experiment performed in triplicate).

**Fig. 10. Cross-presentation of HCV-LPs to intrahepatic core-specific CD8+ T cells.** Autologous imDCs were loaded with HCV-LPs (50 μg/ml; equivalent to approximately 5 μg HCV-LP core/ml), insect cell control preparation (GUS Ctrl; 50 μg/ml) or core peptide 6 (comprising core aa 36-53; 10 μg/ml) as described in Material and Methods. The pulsed DCs were washed and subsequently cocultured with intrahepatic core-specific CD8+ T cells at a ratio 1:2. After 5 h of incubation the cells were stained with antibodies to CD8 and IFN-γ and analyzed by flow cytometry. Percentages of CD8+ T cells that produced IFN-γ in the respective quadrants are indicated on the dot-plots. Controls include isotype-control stained CD8+ T cells to determine the quadrant boundaries (not shown).
**Table 1.** Cell surface expression levels of chemokine receptors and TLRs on monocyte-derived imDCs and mDCs. Expression of cell surface receptors was determined by flow cytometry as described in Materials and Methods. Mean ± SD of a representative experiment performed in triplicate is shown.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>imDC</th>
<th>mDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR3</td>
<td>172 ± 2</td>
<td>84 ± 1.5</td>
</tr>
<tr>
<td>CCR5</td>
<td>314 ± 1.5</td>
<td>117 ± 1.5</td>
</tr>
<tr>
<td>CXCR4</td>
<td>108 ± 0.5</td>
<td>258 ± 2</td>
</tr>
<tr>
<td>CCR7</td>
<td>9 ± 1</td>
<td>89 ± 1.5</td>
</tr>
<tr>
<td>TLR2</td>
<td>23 ± 0.5</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>TLR4</td>
<td>20 ± 0.5</td>
<td>0 ± 0</td>
</tr>
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</table>

Δ MFI: net mean fluorescence intensity
Table 2. Upregulation of CD80 and CD83 expression during HCV-LP induced DC activation. ImDCs were incubated with insect cell control preparation (GUS Ctrl), native HCV-LPs, denatured HCV-LPs, and lipopolysaccharide (LPS) as described in Fig. 7. Cell-surface expression of CD80 and CD83 was determined by flow cytometry as described in Materials and Methods. Δ MFI (net mean fluorescence intensity) was calculated by subtracting the MFI of DCs incubated without antigen (background) from the MFI of DCs incubated with the respective antigen. Mean ± SD of three independent experiments using imDCs from three different individuals is shown. Differences in expression of DC activation markers between native HCV-LPs versus GUS Ctrl treated DCs (*) as well as DCs incubated with native HCV-LPs versus denatured HCV-LPs (**) were statistical significant (p < 0.05; two-sided student’s t-test).

<table>
<thead>
<tr>
<th></th>
<th>Δ MFI</th>
<th>CD80</th>
<th>CD83</th>
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<tr>
<td>GUS Ctrl</td>
<td>10 ± 8</td>
<td>7 ± 6</td>
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</tr>
<tr>
<td>Native HCV-LPs</td>
<td>41 ± 9</td>
<td>48 ± 19</td>
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<tr>
<td>Denatured HCV-LPs</td>
<td>5.6 ± 4</td>
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<td>LPS</td>
<td>140 ± 6</td>
<td>157 ± 21</td>
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</tbody>
</table>

* p < 0.05; ** p < 0.01
Fig. 1

A

imDC

mDC

B

Monocytes

imDC

mDC

C

Δ MFI

HCV-LP E2 (µg/ml)

Δ MFI

HCV-LP E2 (µg/ml)
Fig. 2

A  

CD1c

CD123

Counts

Counts

FL2-H

FL2-H

B  

pre-DCs 1

pre-DCs 2

Counts

Counts

FL2-H

FL2-H

C  

imDCs 1

imDCs 2

Counts

Counts

FL2-H

FL2-H
Fig. 3

A

% HCV-LP binding

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Binding</th>
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<tr>
<td>HCV-LPs + PBS</td>
<td>100</td>
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<tr>
<td>Denatured HCV-LPs</td>
<td></td>
</tr>
<tr>
<td>HCV-LPs + control serum</td>
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</tr>
<tr>
<td>HCV-LPs + anti-HCV serum</td>
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<tr>
<td>HCV-LPs + Control IgG</td>
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<tr>
<td>HCV-LPs + anti-E2 AP33</td>
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B

C

EndoH

- EndoH E1

MW 108 90

50.7

35.5

E2

E2+

E1

E1+

HCV-LPs

PNGaseF

- PNGaseF E1

MW 108 90

50.7

35.5

E2

E2+

E1

E1+

HCV-LPs

HCV-LPs +

Δ MFI

HCV-LP E2 (µg/ml)

0 1 10

500 400 300 200 100

0 1 10

600 500 400 300 200 100

0 1 10

PNGaseF

HCV-LPs

HCV-LPs +

Δ MFI

HCV-LP E2 (µg/ml)

0 1 10

600 500 400 300 200 100

0 1 10

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Fig. 4

A  
Mannose Receptor

DC-SIGN

DEC-205

Langerin

B  
FITC-Dextran Uptake

HCV-LP Binding

C

% HCV-LP binding

DCs + PBS  
DCs + Trypsin  
DCs + Mannan  
DCs + Control IgG  
DCs + anti-DC-SIGN  
DCs + anti-L-SIGN  
DCs + anti-DC-SIGN (AZN-D1)  
DCs + anti-DEC-205  
DCs + anti-Langerin
Fig. 5

A

anti-TLR2

anti-TLR4

B

C

Δ MFI

% HCV-LP binding

HUH-7 + HCV-LPs
CHO-CD14 + HCV-LPs
CHO-CD14/TLR2 + HCV-LPs
CHO-CD14/TLR4 + HCV-LPs

DCs + PBS
DCs + Control IgG
DCs + anti-TLR2
DCs + anti-TLR4
Fig. 6

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</table>
Fig. 7

**HLA-DR**

**CD 86**

**CD 80**

**CD 83**

**GUS Ctrl**

**Native HCV-LPs**

**Denatured HCV-LPs**

**LPS**
Fig. 8

A

[\text{[^3]H} \text{thymidine incorporation (x 10^3 cpm)}]

Number of DCs

B

IL-4 (pg/ml)

IFN-\gamma (pg/ml)

CD4+

CD4 + DCs

CD4 + DCs + GUS

CD4 + DCs + HCV-LPs

CD4 +

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CD4 + DCs + GUS

CD4 + DCs + HCV-LPs
**Fig. 9**

![Graph showing [³H]thymidine incorporation (x 10³ cpm)](image)

**Fig. 10**

![Histograms showing IFN-γ expression in CD8-positive cells for different DC preparations](image)
Uptake and presentation of hepatitis C virus-like particles by human dendritic cells

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