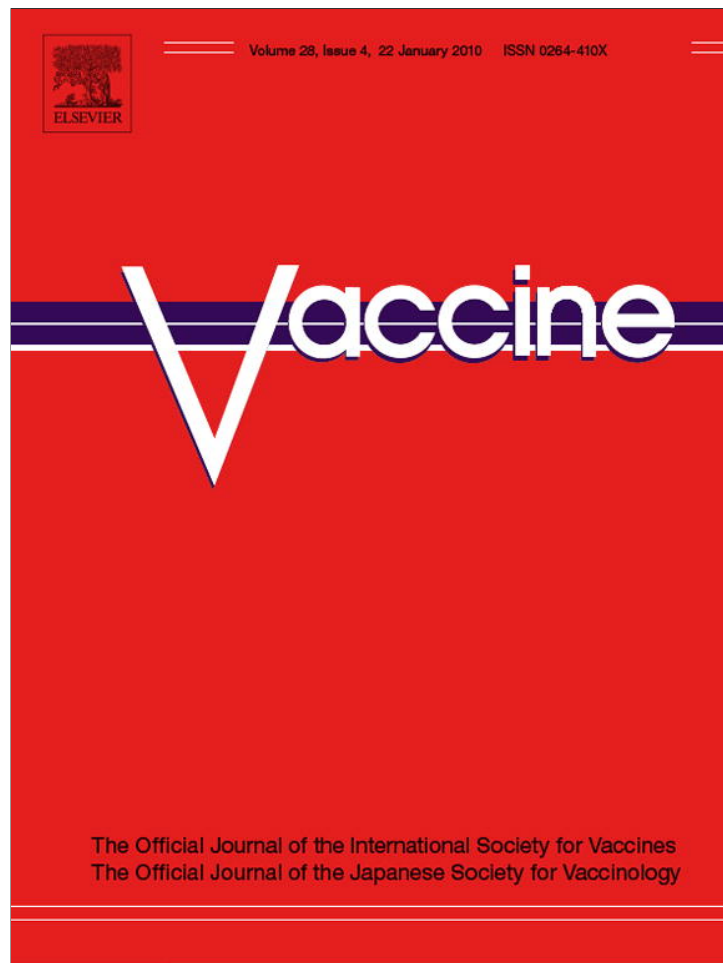


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## Monocytes transduced with lentiviral vectors expressing hepatitis C virus non-structural proteins and differentiated into dendritic cells stimulate multi-antigenic CD8<sup>+</sup> T cell responses

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### ABSTRACT

Halting the spread of hepatitis C virus (HCV) and also eradicating HCV in subjects with chronic infection are major goals for global health. To this end, several years of research on HCV vaccine development have led to the conclusion that multi-antigenic and multi-functional vaccine types are necessary for effectiveness against HCV infection. In this study, we evaluated lentiviral vectors (LV) expressing clusters of HCV structural (LV-HCV-S) and non-structural (LV-HCV-NS) genes for future vaccine development. Batches of high titer LV were used to transduce differentiated dendritic cells (DC) and monocytes. We report successful delivery of HCV gene clusters, particularly into monocytes, leading to >80% LV-HCV-NS and >70% LV-HCV-S and transduced cells, respectively. Intracellular expression of HCV proteins in monocyte-derived DC resulted in immunophenotypic changes, such as downregulation of CD83 and CD86. Monocytes expressing NS proteins and differentiated into DC stimulated allogeneic and autologous CD8<sup>+</sup> and CD4<sup>+</sup> T cells *in vitro* and resulted in antigen-specific CD8<sup>+</sup> T cell responses against NS3, NS4a and NS5b. Hence, lentiviral-mediated expression of the multi-antigenic HCV-NS cluster in monocytes subsequently differentiated into DC is a novel potential anti-HCV vaccine modality.

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### 1. Introduction

Approximately 130 million chronic hepatitis C virus (HCV) infections have been estimated worldwide. The standard treatment consisting of recombinant IFN- $\alpha$  alone or in combination with ribavirin, has proven efficacious in approximately half of HCV-infected patients [1]. However, many patients cannot be treated using this standard regimen as IFN- $\alpha$  is associated with frequent and sometimes severe side effects. Moreover, between 10% and 50% of individuals infected with HCV are unable to clear the virus following an acute infection and, as a result, become persistently

infected leading to chronic infection, which can lead to liver cirrhosis and development of hepatocellular carcinoma. Therefore, prophylactic vaccines (to hinder HCV epidemics) and therapeutic adjuvant immunotherapy approaches (to prevent disease progression or to ultimately cure chronic HCV patients) have been actively explored in past years but have not yet ultimately succeeded in clinical trials [2–4].

HCV contains a single-stranded, positive-sense RNA genome of approximately 9500 nucleotides. The genome consists of a single open reading frame (ORF), which encodes a large polyprotein of approximately 3000 amino acid residues. The polyprotein is cleaved by cellular and viral proteases into at least ten different products consisting in structural (the core, the E1 and E2 envelope proteins and p7) and non-structural proteins (NS2, NS3, NS4a, NS4b, NS5a and NS5b) [5,6]. Although some structural proteins can stimulate antibody and T cell responses, a major drawback in their use for vaccination strategies is their high mutability, which may be involved in evasion of the innate and adaptive host immune

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response and seroconversion [6,7]. In contrast, studies in acute or persistently HCV-infected humans have demonstrated that immunity against conserved domains of HCV-NS proteins are generally correlated with viral clearance [4,8–10].

It is not clearly known how an individual develops into a chronic hepatitis virus carrier state; however, a defective immune response of the host is thought to play a critical role in the underlying pathogenic mechanism. Peripheral blood DC from HCV-infected patients compared to normal controls have shown decreased expression of CD86, decreased production of IL-12 and lower allostimulatory capacity [11–13]. The apparent defects in DC correlate with an impairment of the effector function of HCV-specific CD8<sup>+</sup> T cells in chronic HCV infection. HCV-specific tetramer positive T cells are frequently found in PBMCs from chronically infected patients, but they display an impaired proliferative capacity [14]. This phenomenon may be the consequence of “helpless” stimulation of CD8<sup>+</sup> T cells during the inefficient presentation of HCV antigens by DC, leading to anergy or ultimately tolerance. Thus, the defective functions of HCV-specific CD8<sup>+</sup> T cells might contribute to viral persistence in chronically infected patients, and approaches to avoid or revert their dysfunction may facilitate the development of prophylactic and immunotherapeutic vaccines.

DC provide the most potent pathway for initiating T and B cell immune responses [15]. Thus, the use of DC-based vaccines in the treatment of patients with acute and chronic infections is a field with vast applications. Since blood is the most accessible tissue for clinical studies, various groups have developed protocols using peripheral blood mononuclear cells (PBMC) for the *in vitro* production of DC. GM-CSF and IL-4 added to peripheral blood monocytes in culture promote the generation of “immature DC”, the most prevalent form of DC in tissues [16,17]. DC differentiated *in vitro* and genetically manipulated with cDNA, RNA or engineered viral vectors have been evaluated in several clinical trials. These strategies have proven to be feasible, safe and effective to activate both CD8<sup>+</sup> CTL and CD4<sup>+</sup> T-helper cells [18]. In addition, genetic manipulations of DC in order to ectopically express immune modulators may potentially help to overcome immune dysfunctions that occurred *in vivo*, as is the case for chronic HCV infections.

LV are a subtype of retroviral vectors that were intensively developed during the last decade. Unlike non-integrating viral vectors such as adenovirus and vaccinia, LV integrate in the genome and offer an approach by which efficient, long lasting, non-toxic and non-immunogenic gene delivery into monocytes and DC may be obtained. In contrast to previously developed onco-retroviral vectors (such as Moloney Murine Leukemia Virus), lentiviruses are able to infect non-proliferating cells, due to the karyophilic properties of the lentiviral pre-integration complex, which allows recognition by the cell nuclear import machinery. Therefore, LV can transduce primary quiescent cells, cells that are growth-arrested in culture, as well as terminally differentiated cells. The lentiviral packaging system was originally developed as a tripartite transient transfection procedure [19] and later evolved into further generations where the four accessory genes of HIV (*vif*, *vpr*, *vpu* and *nef*) were deleted from the viral packaging system [20] and a 400-nucleotide deletion in the 3' long terminal repeat resulted into self-inactivating (SIN) LV. The risk of vector mobilization and production of replication competent LV is drastically reduced for the SIN vectors [20]. In most cases, LV are pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G), which is a rhabdovirus envelope protein that is reported to bind to ubiquitous cell surface phospholipids, thereby achieving a wide host range. Previous observations from other groups and from our own research demonstrate that lentiviral vector transduction is a suitable methodology for efficient gene delivery into DC or monocytes [21–24]. Due to their robust infectivity and persistent transgene delivery capabilities into APCs, LV have emerged as a novel potent approach for genetically engineered DC [24].

Of considerable interest for vaccine development, intravenous or subcutaneous LV administration has resulted consistently into potent CTL responses specific against several cancer antigens such as Melan-A [25,26], NY-ESO 1 [27,28] and TRP2 [29,30]. LV vaccines are also currently in preclinical testing for protection or treatment of human immunodeficiency virus (HIV). Several routes of LV administration have been explored in mice, leading to consistent and persistent anti-HIV/SIV immune responses [31,32].

In this report, we demonstrate the high capability of LV to transfer whole sets of HCV structural or non-structural gene clusters *in vitro* into monocytes prior to their differentiation into DC. Notably, gene delivery of the HCV-NS cluster into monocytes resulted in its persistent expression in differentiated DC leading to potent stimulation of CD4<sup>+</sup> and CD8<sup>+</sup> allogeneic and autologous responses.

## 2. Materials and methods

### 2.1. Cell culture

Human embryonic kidney 293T cells were cultured in DMEM with 10% FBS and penicillin (100 U/ml) and streptomycin (100 mg/ml).

### 2.2. Lentiviral vector construction and production

The self-inactivating (SIN) lentiviral vectors used in this study are derived from the RRL-sin-cPPT-hCMV-GFP vector (described previously [33]). The control lentiviral vector contains a truncated form of the human CD34 surface antigen [34] kindly provided by Prof. Christopher Baum, Department of Experimental Hematology, Hannover Medical School. For construction of the LV-HCV vectors, RRL-sin-cPPT-hCMV-GFP was cut with XbaI and Sall restriction enzymes (to remove GFP). For construction of the LV-HCV-S vector, a PCR was performed with primers XbaI-5'HCV (GGGGTCTAGACCACCACATGAGCAGC-AATCCTAAACCTCAAAGA) and P7-Sall (GTCGACTCAGGCGTAAGCTCCTGGTGG), and the 2.4 kb insert was subcloned into the LV corresponding to nucleotides 342–2762 of HCV-NS sequence (NCBI AF139594). For construction of the LV-HCV-NS vector, a PCR was performed with primers XbaI-NS2 (TCTAGACCACCACATGGACCGGGAGATGGCT) and 3'HCV-Sall (GGGGGAATTCTCATCGATTGGGGAGCAAGTAGATGCC), and the 6.6 kb insert was subcloned into the LV corresponding to nucleotides 2763–9389 of HCV-NS sequence (NCBI AF139594). The structural integrity of all constructs was reconfirmed by restriction digestion and sequencing analysis of the promoters and transgenes. Large-scale production of lentiviral constructs was performed by transient co-transfection of 293T cells exactly as described [33]. Lentiviral titer was determined by assessing viral p24 antigen concentration by ELISA (Cell Biolabs, Inc., San Diego, CA, USA) and hereafter expressed as  $\mu\text{g}$  of p24 equivalent units per milliliter (1  $\mu\text{g}$  p24 equals approximately  $1.5 \times 10^7$  infective particles).

### 2.3. Analyses of protein expression by Western blot

Cells were harvested at determined time-points, washed with PBS, and lysed with Mammalian Cell Lysis Kit (Sigma–Aldrich, St. Louis, MO, USA). Equal amounts of protein (Bradford method) were boiled in SDS sample buffer (40 mM Tris–HCl, pH 7.4, 5% glycerol, 5% mercaptoethanol, 2% SDS, 0.05% bromphenol blue). The samples were fractionated on 10% SDS-acrylamide gels (Biorad, Hercules, CA) and subjected to immunoblot analysis and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using rabbit anti-HCV core (provided by Dr. M. Lai), monoclonal mouse anti-NS3 (provided by Dr. M. Lai), monoclonal mouse anti-NS5a (Biodesign, Saco, ME, USA), or monoclonal mouse anti-E2

(Bioscience) primary antibodies followed by incubation with corresponding secondary antibodies conjugated to HRPO (Santa Cruz Labs, CA, USA). Membrane detection was performed using the ECL system (Amersham, Piscataway, NJ, USA). Documentation of the chemiluminescent immunodetected signal was performed by Quantity One 4.40 software (Biorad).

#### 2.4. Transduction of monocytes and monocyte-derived DC

Peripheral blood mononuclear cells were obtained from HLA-A2.1 positive adult healthy volunteers and studies performed in accordance with protocols approved by the Hannover Medical School Ethics Review Board. CD14<sup>+</sup> cells were isolated using CD14 isolation beads (Miltenyi Biotec, Bergisch-Gladbach, Germany) from the peripheral blood donated by healthy individuals and further cultured in X-Vivo 15 medium (Lonza, Basel, Switzerland) in the presence of recombinant granulocyte macrophage colony-stimulating factor and interleukin-4 (80 ng/ml each; Cellgenix, Freiburg, Germany) for 8 h before transduction.  $5 \times 10^6$  CD14<sup>+</sup> monocytes or monocyte-derived DC were transduced on six-well plates (Omnilab, Bremen, Germany) in the presence of 5  $\mu$ g p24 equivalent/ml of virus plus 5  $\mu$ g/ml Protamine Sulfate (American Pharmaceutical Partners, Inc., Schaumburg, IL). For subsequent DC differentiation, granulocyte macrophage colony-stimulating factor and interleukin-4 were added on day 1 of culture, and subsequently every 3 days. 24 h post-transduction, cells were washed twice with phosphate-buffered saline, and further cultured in X-Vivo 15 medium supplemented with the appropriate cytokines. In some experiments, on day 6, 500 ng/ml of soluble CD40L (R&D Systems, Wiesbaden, Germany) or 500 U/ml of IFN- $\alpha$  (R&D Systems) was added to the monocyte-DC 24 h before phenotypic analysis or co-culture with PBMCs.

#### 2.5. Intracellular staining for expression of HCV proteins

Intracellular expression of HCV proteins by transduced cells was detected by flow cytometry. Briefly,  $1 \times 10^5$  to  $1 \times 10^6$  293T cells, monocytes or monocyte-derived DC were transduced with LV vectors expressing either the structural or non-structural HCV proteins. At the appropriate time-points, cells were washed with PBS and fixed with 3% paraformaldehyde for 10 min at room temperature before permeabilization with 0.5% Triton X-100 for 5 min at room temperature. Permeabilized cells were washed with cold PBS and blocking was performed with 50  $\mu$ g/ml of mouse IgGs for 15 min at 4 °C before staining with either anti-core (Hepatitis C core Dianova GmbH, Hamburg, Germany) or anti-NS5a (designated 9E10 and kindly provided by Dr. Charles Rice, New York, USA) and incubated for further 30 min at 4 °C. Cells were washed three times with cold PBS before staining with FITC labeled goat anti-mouse secondary antibody (Dianova GmbH) for another 30 min at 4 °C. Cells were washed and analyzed using a BD FACSCalibur (Becton Dickinson, Heidelberg, Germany) and analysis done using either BD CellQuest or Dako Cytomation, Summit (Dako, Glostrup, Denmark) software.

#### 2.6. Flow cytometry analyses of immunophenotypic markers

The phenotype of monocytes or monocyte-derived DC was analyzed as previously described [35]. Briefly, 2 or 7 days post-transduction with the lentiviruses, the cells were detached from the wells by incubation in PBS (without Mg<sup>2+</sup> and Ca<sup>2+</sup>) for 30 min at 37 °C in 5% CO<sub>2</sub> incubator. After incubation cells were resuspended and washed once with PBS and then incubated with mouse IgG (50  $\mu$ g/ml) on ice for 15 min before further staining with the corresponding monoclonal antibodies. Monoclonal antibodies reactive against CD209, CD83 and CD86 conjugated with PerCP, APC and

PE, respectively or their respective isotypes conjugated with similar colors were used (Becton Dickinson, Heidelberg, Germany). 30 min after incubation with the monoclonal antibodies, cells were resuspended in 100  $\mu$ L of 1% paraformaldehyde for fixation. Cells were further analyzed using a FACSCalibur cytometer and acquisition and analyses were done using CellQuest software (Becton Dickinson) or Dako Cytomation Summit software (Dako).

#### 2.7. RT-Q-PCR analysis of genomic DNA

Genomic DNA from control and transduced monocytes was purified using a QIAmp Blood Minikit (QIAGEN GmbH, Hilden, Germany). Quantification of the vector copy number in the samples was performed in 25  $\mu$ l reaction containing 300 ng of genomic DNA (equivalent to  $5 \times 10^4$  genomes) according to the instructions provided with the UltraRapid Lentiviral Titer Kit (System Biosciences, Mountain View, CA, USA). For internal control, G3PDH primers were used: 5'-ACCACAGTCCATGCCATCAC-3', and 5'-TCCACCACCCTGTGCTGTA-3' Amplifications were carried out in an StepOnePlus™ real-time PCR System (Applied Biosystems Inc. Foster City, CA, USA); after the initial denaturation (10 min at 95 °C), amplification was performed with 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The numbers of integrated lentiviral copies were calculated based on analyses of DNA standards provided with the kit. Ct values were plotted against the input DNAs, and a standard reference curve was obtained.

#### 2.8. Analyses of activated T cells by flow cytometry

To assess influence of HCV proteins on the ability of either monocytes or monocyte-derived DC to stimulate T cells autologously or allogeneically,  $1 \times 10^5$  transduced monocytes (48 h post-transduction) or monocyte-DC (7 day old DC) were co-cultured with  $1 \times 10^6$  (1:10 ratio) of either allogeneic or autologous PBMCs derived from healthy donors in a round bottom 96 well plate (Sarstedt, Nümbrecht, Germany) for 48–72 h in RPMI medium supplemented with 10%FBS, penicillin, streptomycin and L-glutamine. FastImmune kit (Becton Dickinson) which consisted of a mixture of anti-CD3 PerCP/CD4 APC anti-CD69 PE was used to analyze expression of CD69 as described in the manufacturer's protocol. A gate on the CD3<sup>+</sup> lymphocyte fraction was set for quantitative analysis of CD4<sup>+</sup>/CD69<sup>+</sup>, CD8<sup>+</sup>/CD69<sup>+</sup>-expressing subpopulations. A mixture of respective PerCP, APC, and PE-conjugated isotypes (Becton Dickinson, Heidelberg, Germany) were used to set up the appropriate background fluorescences. Acquisition and analysis was done using the CellQuest software and a FACSCalibur apparatus (Becton Dickinson).

#### 2.9. Analyses of antigen-specific T cell responses by tetramer staining

CD3<sup>+</sup> T cells were positively selected from PBMCs using CD3-magnetic particles (Becton Dickinson) according to manufacturer's protocol. Two separate stimulation assays were performed: in the presence of low and high concentrations of rhlL-2 (Invitrogen, Karlsruhe, Germany). For the first protocol (Fig. 8A), T cells were washed with PBS and resuspended in RPMI medium plus 10% FCS and 5 U/ml rhlL-2.  $1 \times 10^5$  CD3<sup>+</sup> cells were then co-cultured with autologous  $1 \times 10^4$  transduced monocytes that were differentiated into DC. After 5 days of co-culture, cells were resuspended in PBS plus 2% FCS, co-stained with PE-conjugated MHC Class-I tetramers (NS3 (1073 tetramer (CINGVCWTV)), NS4 (1798 tetramer (SLMAF-TAAV)) and NS5b (2594 tetramer (ALYDVVTKL)); Beckman Coulter, Krefeld, Germany) and an APC conjugated anti-CD8 antibody and analyzed by flow cytometry. An irrelevant tetramer provided by the manufacturer was used as a negative control. For the second

protocol (Fig. 8B), co-cultures were supplemented with fresh rhIL-2 (25 U/ml) on day 3 and cells were harvested for flow cytometry analyses 3 days after.

### 2.10. CFSE proliferation assay

Enriched CD3<sup>+</sup> T cells were resuspended in 15 ml of PBS supplemented with 0.2% BSA. The cells were centrifuged at 600 g for 7 min and the cell pellets were resuspended in 1 ml of 4 μM CFSE solution (Invitrogen Karlsruhe, Germany) and incubated at 37 °C for 10 min. CFSE staining was stopped by adding 10 ml of cold FCS followed by further incubation on ice for 5 min. 1 × 10<sup>5</sup> CD3<sup>+</sup> T cells were resuspended in RPMI medium plus 10% FCS and 5 U/ml rhIL-2 (R&D Systems) and co-cultured with 1 × 10<sup>4</sup> day 7 Mock or with HCV-NS transduced cells for 5 days. On day 5 post-priming, T cells were re-stimulated with 1 μg/ml of HCV-NS3 peptides (NS3-1073 (CINGVCWTV) or NS3-1406 (KLSGLGINAV)) in U bottom 96 well plates. 5 days after, assessment of proliferation of the CD8<sup>+</sup> fraction (stained with an APC conjugated anti-CD8 antibody) was performed by flow cytometry. Loss of CFSE staining was used to determine the level of proliferation, relative to unstimulated T cells.

## 3. Results

### 3.1. Production of high titer lentiviral vectors expressing HCV gene clusters

Self-inactivating third-generation lentiviral vector backbones containing the HCV structural protein cluster (E1, E2, core, p7) or the non-structural protein cluster (NS2, NS3, NS4a, NS4b, NS5a and NS5b) were constructed (Fig. 1A). LV expressing the green fluorescent protein (GFP) or the truncated human CD34 surface antigen (thCD34) were used as experimental marking controls. Lentiviral vectors were produced by transient co-transfection of 293T cells and concentrated by ultracentrifugation. Batches of virus expressing the HCV proteins showed titers consistently within the expected normal range of 5–10 p24 μg/ml, approximately equivalent to 2.5–5.0 × 10<sup>8</sup> viral particles/ml (Fig. 1B).

### 3.2. Efficient infectivity of LV-HCV vectors and co-expression of HCV proteins in 293T cells

293T cells transduced with LV-HCV-S or LV-HCV-NS were harvested 3 days after transduction and expression of structural or

non-structural proteins was analyzed by immunoblot. Core and E2 were detectable in 293T cells transduced with LV-HCV-S, whereas NS3 and NS5a were detectable after transduction with LV-HCV-NS (Fig. 2A). The frequency of transduced cells in the population was performed by intracellular staining of HCV-core and HCV-NS5a proteins. Cells transduced with LV-HCV-S and LV-HCV-NS showed 100% positivity for core and NS5a, respectively (Fig. 2B). 293T cells (mock or transduced) stained with the fluorescence-conjugated secondary antibody alone showed no background signal.

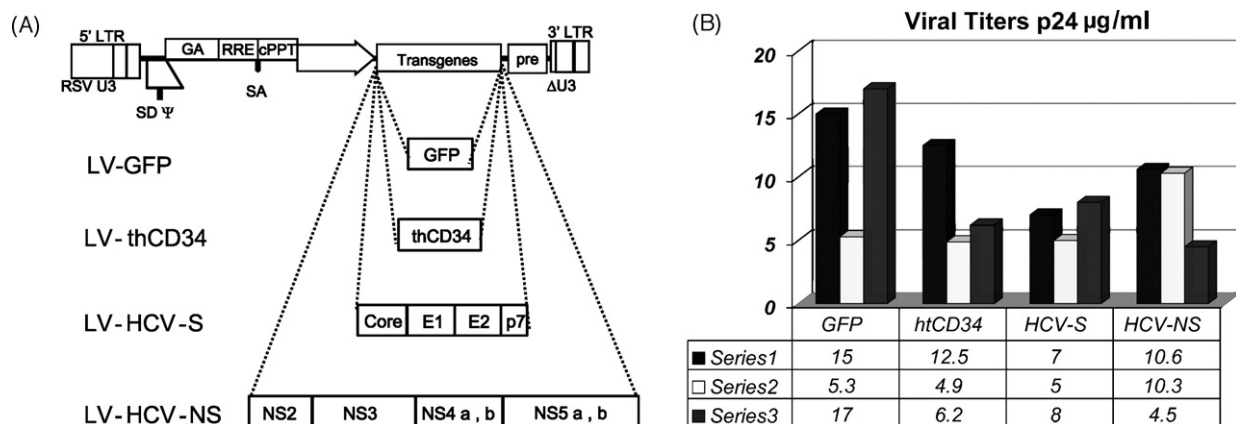
### 3.3. Transduction of monocyte-derived DC with LV-HCV: transient expression of HCV proteins

Transduction of *ex vivo* generated monocyte-derived DC was carried out with LV-HCV-S and LV-HCV-NS. Monocytes were maintained in the presence of standard differentiation cytokines (GM-CSF and IL-4) for 7 days and transduced with LV at 5 μg/ml p24 equivalent. Cells were subsequently maintained in the presence of GM-CSF and IL-4 for 2 or 7 days and harvested for flow cytometry analyses (Fig. 3A). Under these conditions, we observed high DC transduction frequency, as monitored in parallel transduction with the LV-thCD34 marking vector (Fig. 3B). Transduction of DC with LV-HCV-S and LV-HCV-NS resulted in >70% of the cells expressing core or NS5a 2 days after. Nevertheless, expression of core and NS5a in DC was transient, decreasing drastically 7 days post-transduction (Fig. 3C). These results were reproducible in triplicate experiments.

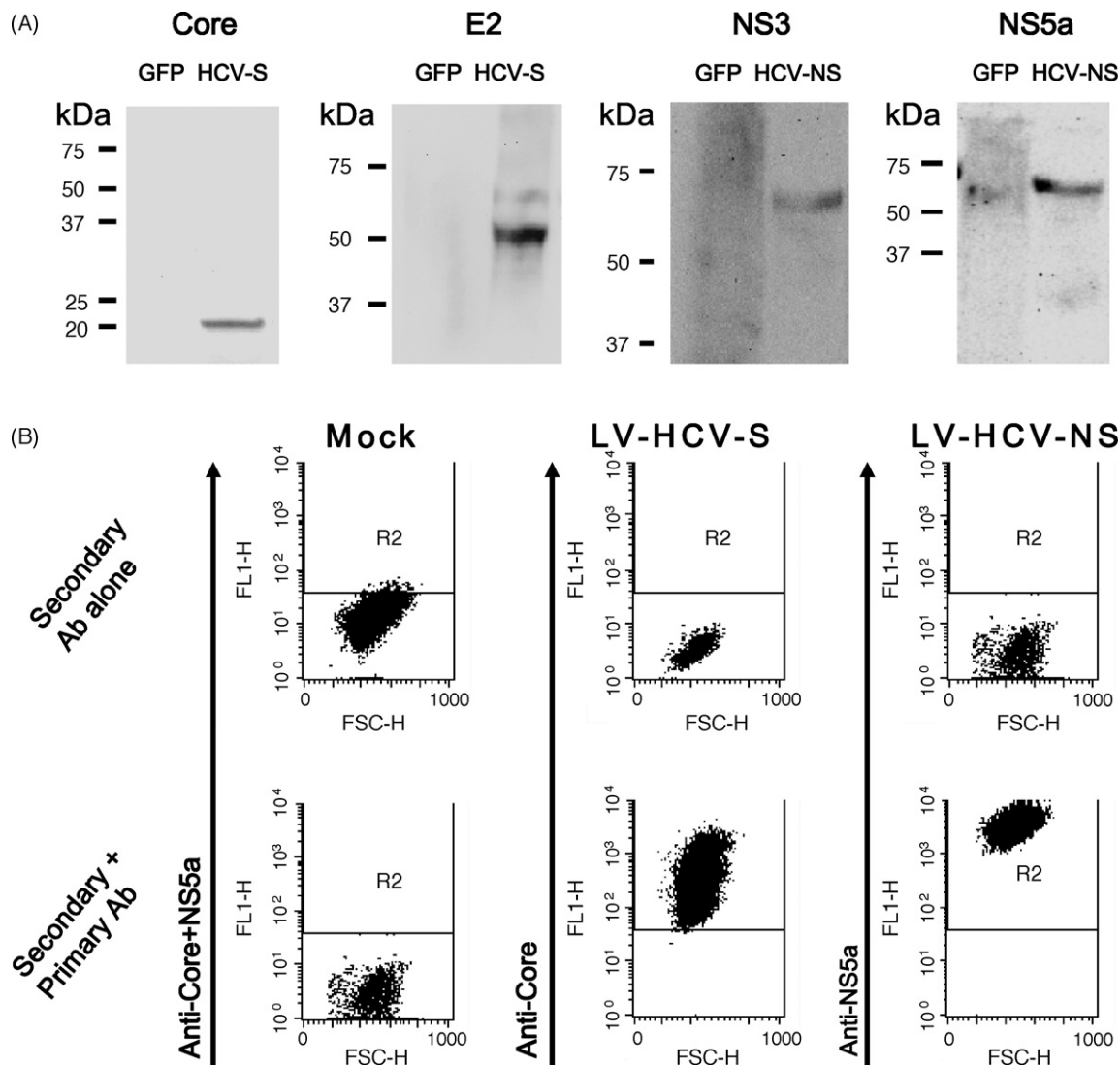
DC phenotype after transduction was analyzed by immunostaining of CD209 (DC-SIGN, a DC-specific marker), CD86 (a co-stimulatory molecule expressed in DC) and CD83 (a DC maturation marker). CD209 expression remained unchanged for transduction controls and for the LV-HCV-S and LV-HCV-NS transduction groups throughout the 2–7 day culture period (Fig. 3D and E). On day 2 of analyses, CD86 and CD83 expression were also not much altered due to transduction (Fig. 3D). On day 7, however, a noticeable decrease of CD86 and CD83 expression was observed for DC transduced with LV-HCV-S or LV-HCV-NS in comparison with the LV-thCD34 transduction control (Figs. 3E and 5).

### 3.4. Transduction of monocytes with LV-HCV vectors: robust expression of HCV proteins in subsequently differentiated DC

We have previously reported that transduction of monocytes with LV and subsequent differentiation into DCs provided an alternative efficient methodology for expression of transgenes [36].



**Fig. 1.** Lentiviral vectors constructs and titers. (A) Diagram representing the lentiviral backbones (expressing GFP or thCD34 marking genes or expressing HCV-S or HCV-NS cluster genes). The long terminal repeats (LTR), the splice donor site (SD), the splice acceptor site (SA), the packaging signal ψ CMV enhancer/promoter element, the truncated and out of frame gag gene (Ga) upstream to the Rev responsive element (RRE), the central polypurine and termination sequence (cPPT), the woodchuck hepatitis pre-element (Wpre) and the self-inactivating mutation in the 3'LTR (Δu3) are indicated (not in scale). (B) Titer (p24 μg/ml) of 3 independent lentiviral vector high titer productions for each vector type.



**Fig. 2.** Expression of HCV gene products in 293T cells. (A) Immunodetection by Western blot of structural (core, E2) and non-structural (NS3, NS5a) gene products in 293T cells after transduction with LV-HCV-S or LV-HCV-NS. Protein extracts obtained from LV-GFP transduced cells were included as controls. (B) Immunodetection by flow cytometry of structural (Core) and non-structural (NS5a) gene products. 293T cells were transduced at a concentration of 0.1  $\mu$ g/ml p24 equivalent lentiviral vectors and expression of HCV proteins was analyzed 48 h post-transduction by intracellular staining. Anti-core or anti-NS5a monoclonal antibodies plus a secondary fluorochrome-conjugated antibody were used. Cells stained with secondary antibody alone were used as controls.

Therefore, we also evaluated the effects of LV-HCV-S and LV-HCV-NS transduction in monocytes. Monocytes were preconditioned with GM-CSF and IL-4 for 8 h, transduced with LV overnight and further cultured in the presence of GM-CSF and IL-4 (Fig. 4A). Using this protocol, high transduction for the LV-thCD34 marking vectors was observed (Fig. 4B). Intracellular staining for detection of HCV proteins 2 days after transduction with LV-HCV-S or LV-HCV-NS demonstrated >70% of the cells expressing Core or NS5a, respectively (Fig. 4C). Lentiviral transduction mediating expression of HCV proteins in monocytes was performed in triplicate independent experiments analyzed by intracellular staining analyses and was highly consistent. In addition, quantitative PCR analyses of DNA collected on day 3 post-transduction confirmed 4–9 copies of LV integration per cell (Table 1). On day 7 post-transduction, NS5a expression persisted in LV-HCV-NS transduced cells, whereas expression of Core in LV-HCV-S transduced cells dropped (Fig. 4C).

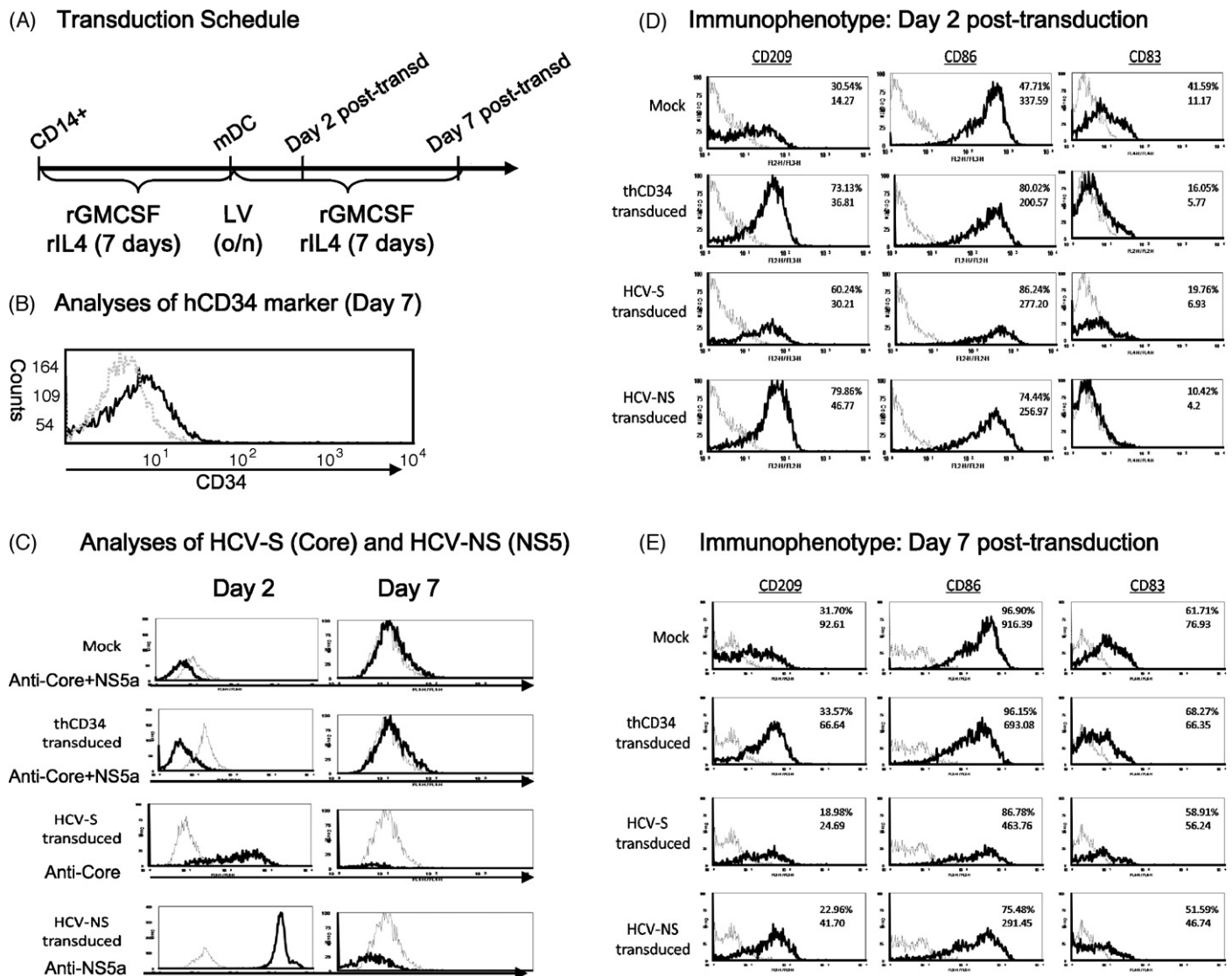
Immunophenotypic analyses of differentiating DC demonstrated that, from day 2 to day 7 post-transduction, frequency of cells expressing CD209 and CD86 increased (Figs. 4D,E and 5A). On the other hand, lower frequency of cells expressing the maturation marker CD83 was observed in cells transduced with LV-HCV-S

and LV-HCV-NS vectors compared with the control LV-thCD34 (Fig. 4E and 5B). Thus, transduction with LV-HCV-S and LV-HCV-NS did not inhibit expression of DC differentiation markers but down regulated expression of the maturation marker CD83.

In order to access the persistency of LV expression within the longevity of *ex vivo* grown, monocyte-derived DCs (which is approximately 2 weeks), we extended the analyses of NS5a and Core expression to 14 days post-transduction (Fig. 6). We included intermediate time-points (days 3, 5, 7, and 10 post-transduction) in order to obtain a kinetic perspective. The frequency of NS5a-expressing cells corresponded to 80–100% of the cells throughout the period of analyses, whereas the frequency of Core-expressing

**Table 1**  
Relative numbers of lentiviral copies in transduced monocytes subsequently differentiated into DCs, assessed by real-time quantitative PCR.

	Relative numbers of lentiviral (LV) integrations
Mock	0.303677
HCV-NS	9.552092
HCV-S	4.506394



**Fig. 3.** Transduction of monocyte-derived DC. (A) Experimental scheme:  $5 \times 10^6$  DC were transduced with  $5 \mu\text{g/ml}$  p24 equivalent of LV vectors expressing either the thCD34 marking gene, structural or non-structural HCV proteins. (B) Analyses of thCD34 marking gene expression 7 days post-transduction. (C) Intracellular detection of core and NS5a in cells at 2 days or 7 days post-transduction. (D and E) Immunophenotypic analyses of DC on days 2 and 7 post-transduction, respectively. On the upper left corner are the frequencies and the mean fluorescence intensities for each staining.

cells dropped from 70% on day 3 to 40% on day 14 (Fig. 6A). The expression level of the transgenes was followed as mean fluorescence analyses (Fig. 6B). For both NS5a and Core, we observed a peak of expression between day 5 and day 7 post-transduction.

### 3.5. Stimulation of allogeneic and autologous CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses by DC expressing HCV-S and HCV-NS clusters

In order to evaluate whether HCV proteins could lead to stimulatory effects on T cells, monocytes transduced with LV-HCV-S or LV-HCV-NS were co-incubated with allogeneic and autologous PBMCs and the immune effects on T cells were analyzed by flow cytometry (Fig. 7A).

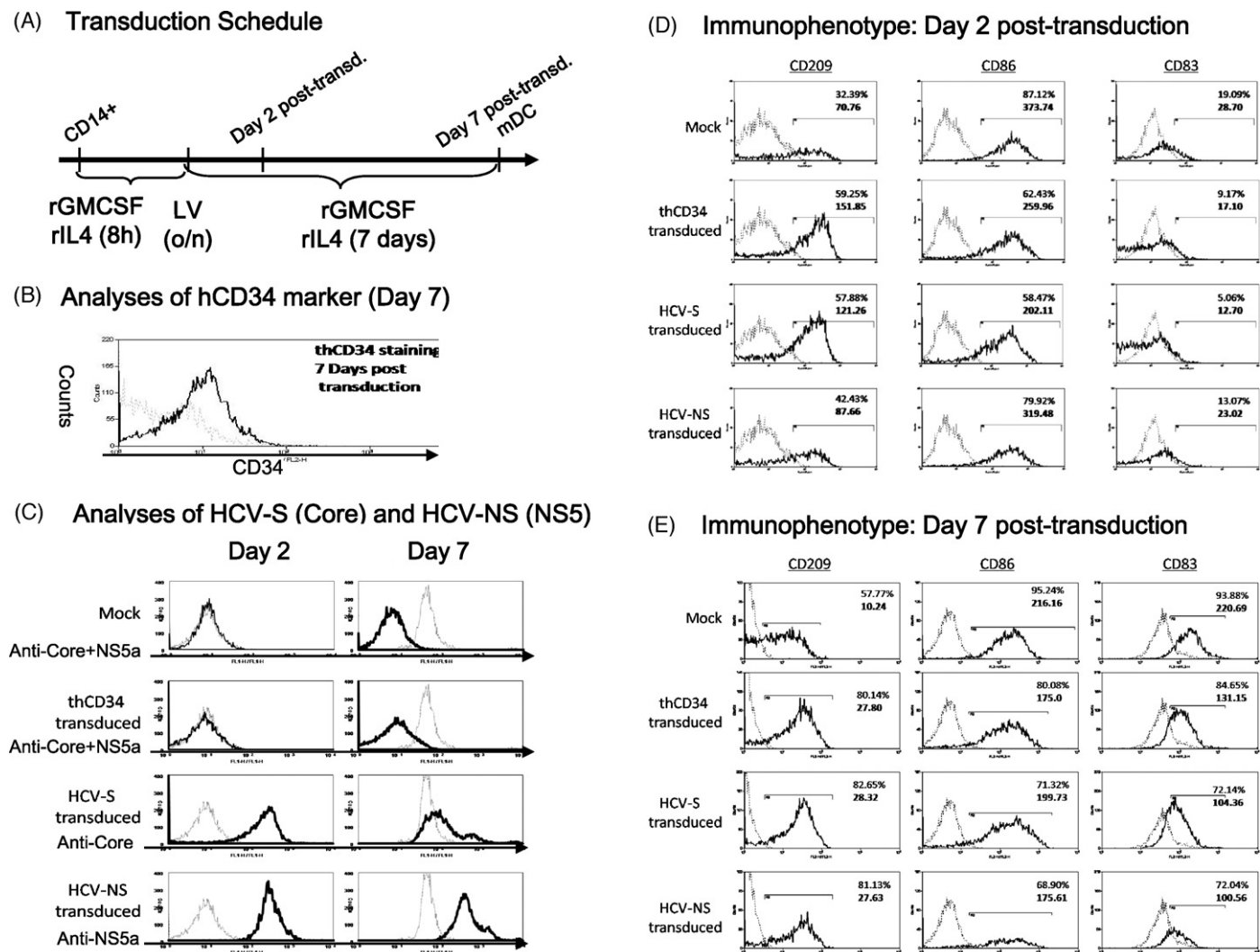
Stimulation of CD8<sup>+</sup> and CD4<sup>+</sup> T cells was evaluated by upregulation of the activation marker CD69. The frequency of CD69<sup>+</sup> cells was calculated by subtracting the baseline levels of unstimulated T cells (PBMCs maintained in the absence of DC). Whereas the effects of LV-HCV-S transduction seemed to lead to variable effects on the different systems evaluated, LV-HCV-NS transduced DC were consistently more stimulatory than untransduced Mock DC (Fig. 7B–E), for both allogeneic and autologous responses.

We further evaluated whether immunomodulatory factors that promote DC maturation such as CD40 ligand (CD40L) and interferon-alpha (IFN- $\alpha$ ) could influence T cell activation. For the allogeneic system, treatment of DC/LV-HCV-S with CD40L and IFN- $\alpha$  promoted variable results on T cell stimulation. Activation of allogeneic CD4<sup>+</sup> T cells was slightly enhanced upon CD40L treatment whereas the effect was negligible for CD8<sup>+</sup> T cell activation.

Notably, the stimulatory effects observed on CD8<sup>+</sup> and CD4<sup>+</sup> T cells caused by co-culture with LV-HCV-NS transduced DC were dramatically increased upon treatment with CD40L. IFN- $\alpha$  did not produce consistent effects on CD69 upregulation of autologous T cells. In all, these results indicated that expression of HCV-NS in matured monocyte-derived DC augmented the allogeneic T cell stimulation and promoted autologous T cell activation.

### 3.6. Antigen-specific CD8<sup>+</sup> T cell responses against DC expressing HCV-NS cluster

Ultimately, we sought to confirm that DC expressing HCV proteins could stimulate immune responses in an antigen-specific manner. We focused our analyses on monocytes transduced with



**Fig. 4.** Transduction of monocytes followed by ex vivo differentiation into DC. (A) Experimental scheme:  $5 \times 10^6$  CD14<sup>+</sup> monocytes were transduced with 5  $\mu$ g/ml p24 equivalent of LV vectors expressing either the thCD34 marking gene, structural or non-structural HCV proteins. (B) Analyses of thCD34 marking gene expression 7 days post-transduction. (C) Intracellular detection of Core and NS5a in cells at 2 days or 7 days post-transduction. (D and E) Immunophenotypic analyses of differentiating DC on days 2 and 7 post-transduction, respectively. On the upper left corner are the frequencies and the mean fluorescence intensities for each staining.

LV-HCV-NS and differentiated in DC, as we had seen in previous experiments that these DC had maintained the longest expression of HCV proteins and stimulated the highest levels of T cell responses.

Autologous CD3<sup>+</sup> T cells were stimulated once with DC in the presence of low or high levels of rhIL-2 and reactivity was assessed by tetramer analyses of CD8<sup>+</sup> T cells. T cells stimulated with Mock DC resulted in 0.7–1.5% of the CD8<sup>+</sup> T cells being reactive against NS3-1073 or NS3-1406 tetramers (baseline with negative control was 0.4%) (Fig. 8A). For the T cells co-cultured with DC expressing HCV-NS, the reactivity increased to 2.0–2.4% of the cells becoming NS3 reactive (baseline with negative control being 1.4%). The T cell reactivity against NS3 increased drastically upon co-culture of T cells and DC in high concentration of IL-2. T cells stimulated with Mock DC resulted in 3–5% of CD8<sup>+</sup> T cells reactive against NS3 (1073 tetramer (CINGVCWTV)), NS4 (1798 tetramer (SLMAF-TAAV)) and NS5b (2594 tetramer (ALYDVVTKL)). In contrast, DC transduced with LV-HCV-NS and used to stimulate T cells resulted into approximately 20% of the CD8<sup>+</sup> T cells above baseline showing reactivity against the different tetramers (Fig. 8B). The increased reactivity of T cells stimulated with DC expressing HCV-NS was confirmed by CD8<sup>+</sup> T cell proliferation assay based on CFSE staining. Loss of CFSE stain (represented as CSFE Low CD8<sup>+</sup> T cells) was sig-

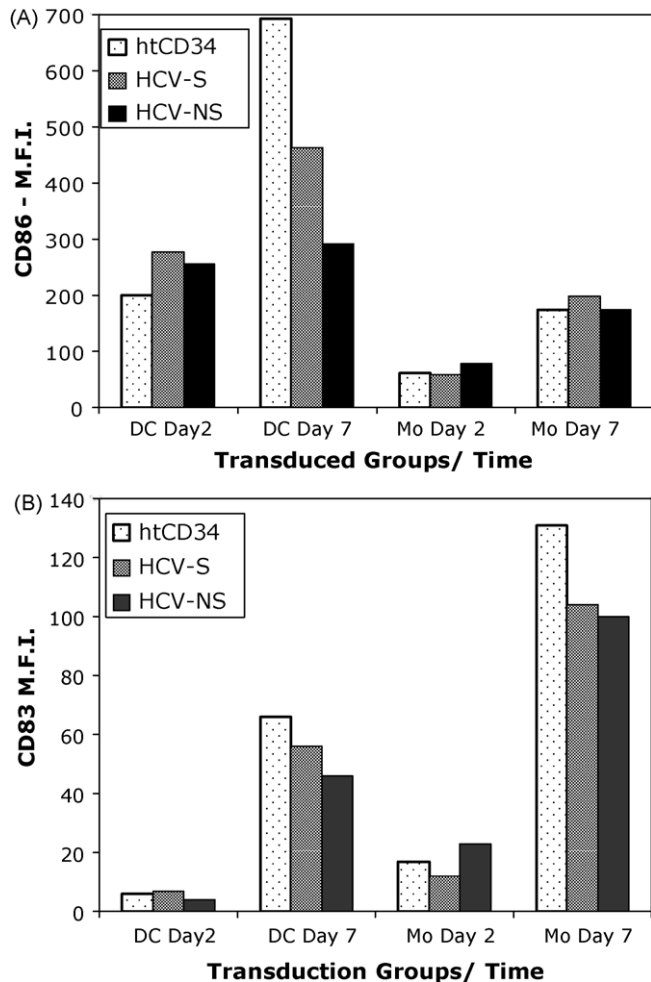
nificantly higher for T cells stimulated with DC expressing HCV-NS and re-primed with NS3 peptides than Mock DC (Fig. 9).

In summary, these results demonstrate that DC expressing HCV-NS proteins are capable of ex vivo priming or re-stimulation of antigen-specific CD8<sup>+</sup> T cell responses.

#### 4. Discussion

Over the past decade, lentiviral vectors have been actively pursued in the field of gene therapy for their robust, consistent and persistent gene delivery capabilities in the absence of overt cytotoxic or antigenic side effects. Advances in lentiviral vector biosafety enhancements were accompanied by recent development of clinical trials [37]. In addition, a number of experimental studies in mice have demonstrated their potent applicability in the vaccination field, both directly or as gene delivery vehicles to antigen presenting cells [24,38]. To our knowledge, this is the first report regarding lentiviral vectors for gene delivery into dendritic cells for stimulation of anti-HCV responses.

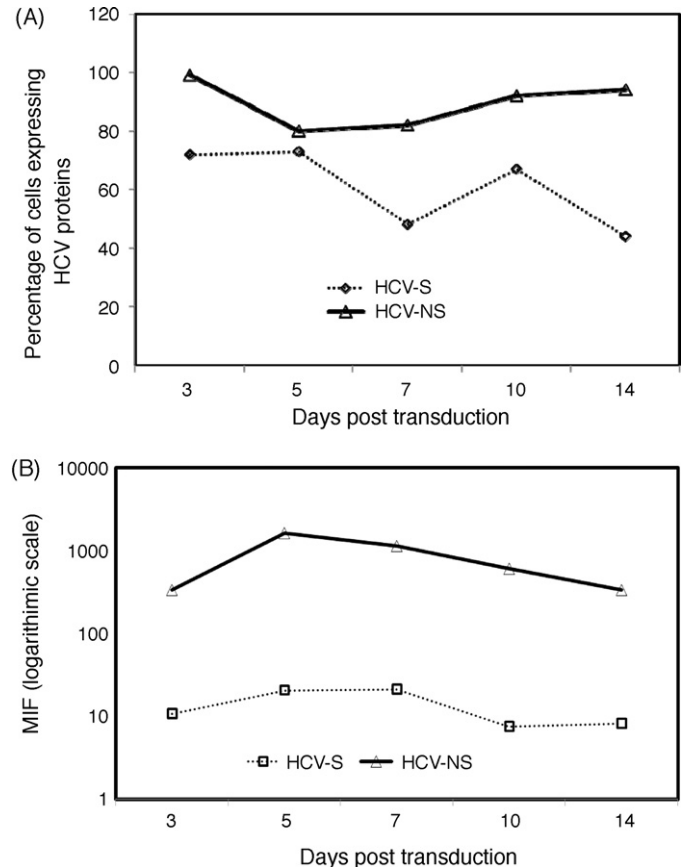
The results presented here demonstrate the feasibility of inserting the HCV genome into two sets of LV for expression of structural and non-structural gene clusters. The gene-cargo capacity of the lentiviral vector backbone described here is approximately 7–8



**Fig. 5.** CD86 and CD83 expression levels after lentiviral transduction of differentiated DC (DC) or monocytes subsequently differentiated into DC (Mo). Dendritic cells or monocytes were transduced with a control vector (expressing htCD34) or LV-HCV vectors (expressing the structural or non-structural cluster). On days 2 and 7 post-transduction, the differentiated DCs were analyzed for expression of CD86 (A) and CD83 (B). The mean fluorescence intensity (MFI) obtained for immune staining against CD86 and CD83 is shown.

kilobases, which was enough to accommodate the 2.4 kb HCV-S and 6.6 kb HCV-NS gene clusters, as viral titers were in the normal range and infectivity comparable to LV expressing marking genes. Of note, in contrast to recombinant “non-gutless” adenoviral vectors, lentiviral vectors express only the transgenic products in target cells, avoiding co-expression of potentially antigenic gene-vector proteins. In addition, the lentiviral vectors are pseudotyped with the vesicular stomatitis viral protein G, resulting into receptor-independent efficient infection of a broad-range of cell types containing heparan sulphate on the cell surface. This is in contrast to the frequently used Adenovirus serotype 5 (Ad5)-based vectors, known to be hindered on infectivity due to the paucity of the coxsackie and adenovirus receptor (CAR) on hematopoietic cells.

Although the LV-HCV vectors could readily transduce pre-differentiated DC, we observed a higher transducibility of monocytes during DC differentiation, possibly due to the fact that monocytes at this stage are more metabolically active, facilitating viral infection. Overall, DC differentiation, morphology and viability were comparable to DC transduced with control marking genes. A downregulation of CD86 and CD83 immunophenotypic markers was more evident in transduced DCs than transduced monocytes subsequently differentiated in DCs. In addition, mono-



**Fig. 6.** Kinetics of HCV proteins expression in monocytes transduced with LV-HCV. Monocytes were transduced with LV vectors expressing either the structural protein (HCV-S) or the non-structural protein (HCV-NS). Intracellular detection of core and NS5a in cells at different time-points after transduction was performed by flow cytometry. (A) Frequency of cells expressing Core or NS5a. (B) Expression level of core and NS5a is indicated as mean fluorescence intensities (MFI).

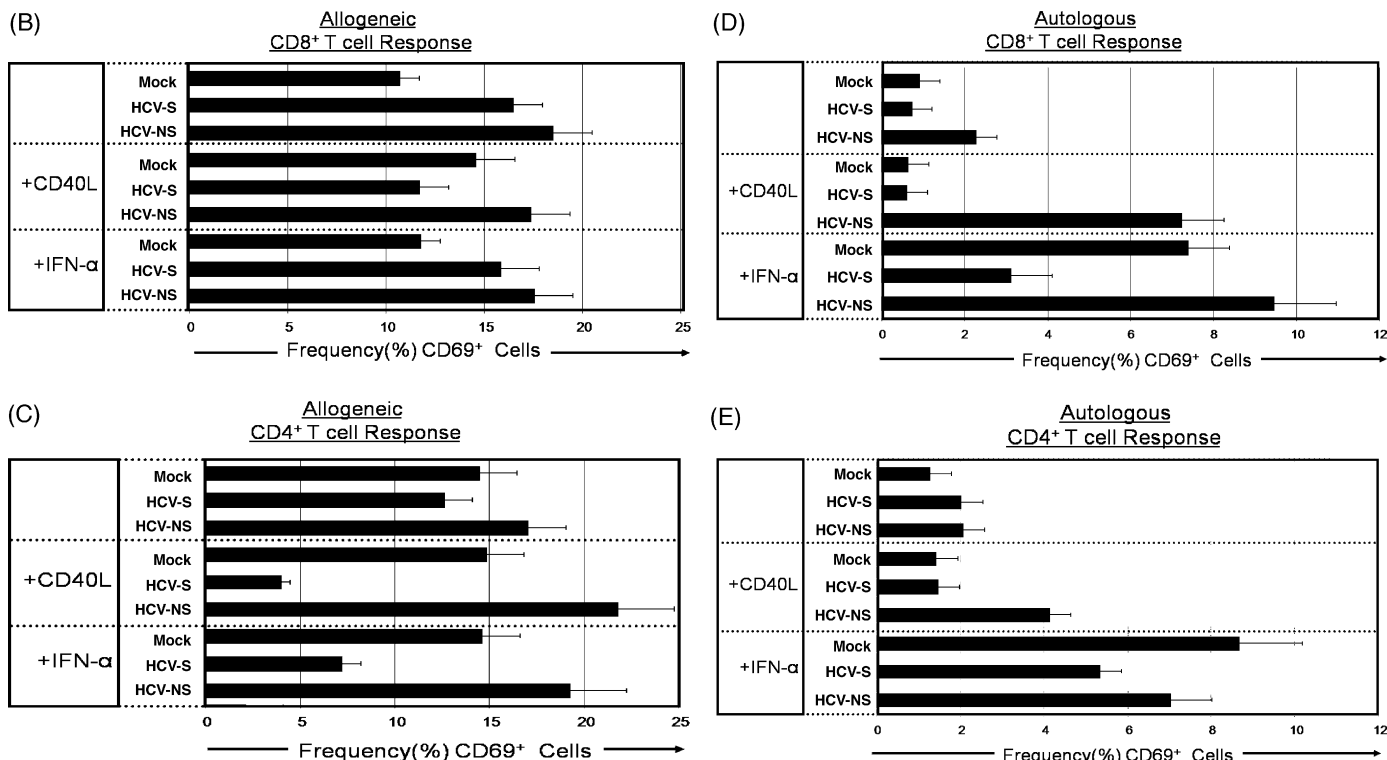
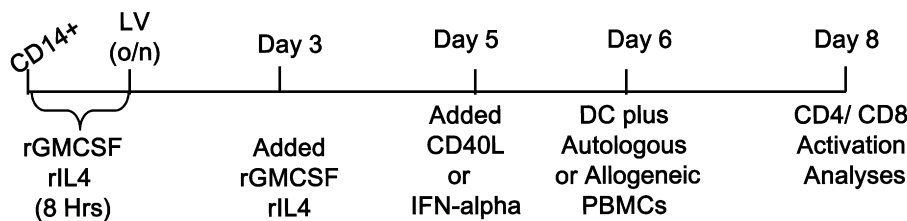
cytes transduced with LV-HCV-NS that differentiated into DCs activated autologous and allogeneic CD8<sup>+</sup> T cells at high levels. This may reflect the fact that several immunogenic NS antigens expressed by in LV transduced monocytes may be presented through the major histocompatibility complex (MHC) class-I pathway.

Therefore, a relevant observation obtained from our studies is that monocytes are not only better transduced with the LV vectors expressing HCV proteins, but also seem to be less susceptible to immune suppression effects. This may be due to experimental set up, i.e. the supra-physiological amounts of cytokines added to the cells for their differentiation may counterbalance immune-escape mechanisms of HCV. Since monocytes are readily available in much higher frequencies (20–30%) than DC (1%), their exploitation as cell targets for gene delivery would greatly facilitate the development of genetically programmed cellular vaccines.

Thus, the lentiviral vector system recapitulates the competence of “natural” HCV to infect monocytes as reported by independent groups [39,40] and could be explored in the future as an experimental model to elucidate basic mechanism(s) of the proposed immune defects caused by persistent HCV occult infection of the lymphatic system [40].

We demonstrate the proof-of-principle that delivery of HCV genes into monocytes and subsequent differentiation into DC leads to T cell immune-stimulation: our results showed that the HCV-NS cluster is more suitable for immune-stimulation than the structural cluster.

(A) Transduction and Co-culture Schedule



**Fig. 7.** Stimulation of autologous and allogeneic PBMCs by transduced monocyte-derived DC. (A) Experimental scheme for lentiviral transductions, CD40L or IFN-alpha treatment and co-culture (1:10 DC:PBMCs). (B and C) Activation of allogeneic CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes assessed by measuring upregulation of CD69 expression. (D and E) Activation of autologous CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes assessed by measuring upregulation of CD69 expression. The error bars represent deviation in the mean values of experimental duplicates.

Overall, experiments performed herein with DC genetically modified with multi-antigenic LV strongly correlated with previous findings using recombinant adenoviral vectors as gene delivery vehicles. Priming and stimulation of CD4<sup>+</sup> and CD8<sup>+</sup> cells with DC transduced with adenoviral vectors expressing single non-structural proteins such as NS3 NS4, NS5a and NS5b have been demonstrated by several groups [41–44]. These experimental models are consistent with the clinical finding that T-helper responses against non-structural proteins are associated with successful hepatitis C viral clearance, reinforcing the opinion that they may be ideal antigen candidates for vaccination or therapy [45]. This is actually an important factor for the preferred use of more genetically stable non-structural proteins as suitable vaccination antigens, in detriment to the highly mutable structural proteins.

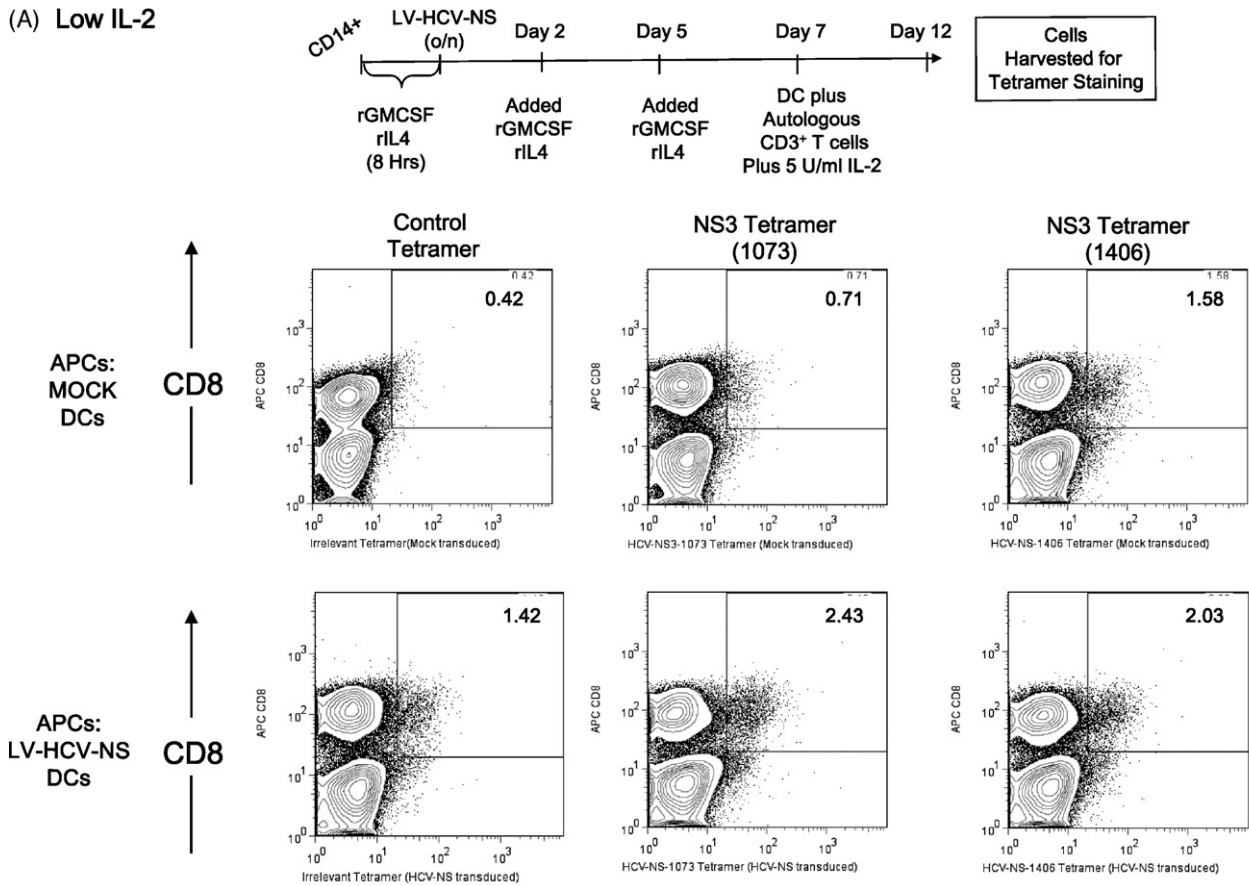
In addition to this fact, our results also correlated with previous findings demonstrating the inherent propensity of structural proteins to deregulate the function of DC in the activation of autologous our allogeneic T cells (particularly CD4<sup>+</sup> T cells). Work by Sarobe and co-workers convincingly showed that dendritic cells transduced with adenoviral vectors for core and E1 expression were inhibited in their capacity to stimulate T cells in vitro and in vivo [41,46]. Notably, corroborating to our results, they specifically

observed that the expression of Core/E1 inhibited DC maturation (by CD40L) and the DC ability to activate CD4<sup>+</sup>- and CD8<sup>+</sup>-T cell responses [41]. Another study describing HCV causing immunosuppression in DCs during maturation was based on bone marrow obtained from mice transgenic for HCV structural proteins and used for production of DCs *ex vivo*. There, the capacity of DCs expressing HCV structural proteins to stimulate T cells was significantly impaired, mostly through the MHC class-I pathway [47].

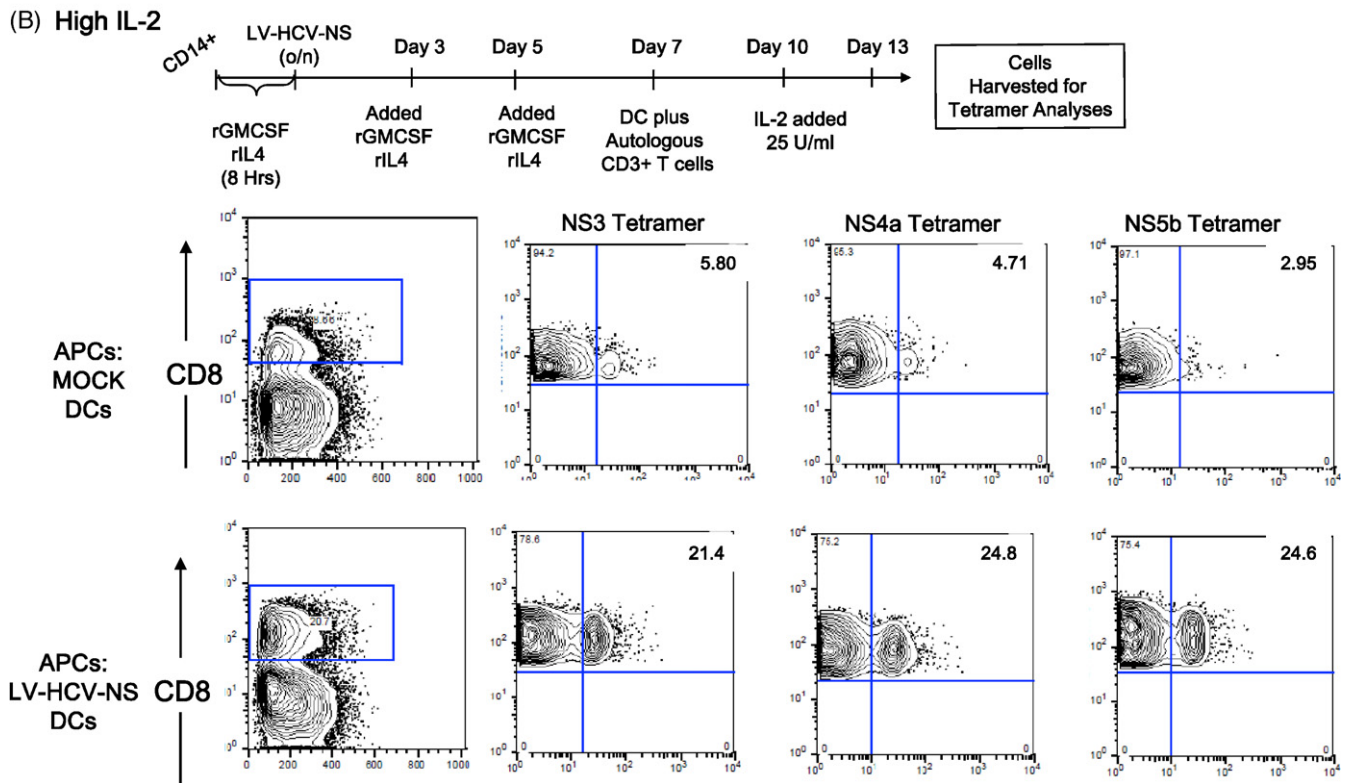
In summary, we demonstrate a novel application of lentiviral vectors to efficiently deliver HCV-NS gene cluster into monocytes, prior to their *ex vivo* differentiation into DC which in the future could be adapted with co-expression of co-stimulatory molecules such as CD40L [23] for optimal rational programming of DC vaccines inducing potent CD8<sup>+</sup> and CD4<sup>+</sup> T cell stimulation.

In addition to the clinical applications of DC genetically modified with LV as vaccines for HCV, this system also offers a practical solution for pinpointing the function of single or combination genes in HCV infection and drug resistance, in analogy to the “replicon” approach [48]. Ultimately, lentiviral vectors expressing HCV clusters can lead to understanding the effects of the occult and persistent HCV infection in immunostimulatory functions of DC, a topic of still much dispute in the field.

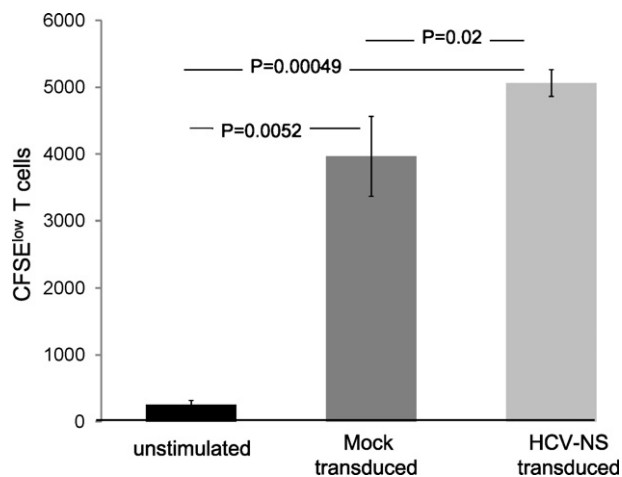
(A) Low IL-2



(B) High IL-2



**Fig. 8.** Analyses of antigen-specific autologous responses by tetramer staining. (A) Experimental scheme for lentiviral transductions, co-culture of DC and CD3<sup>+</sup> T cells and IL-2 treatment at low concentration and flow cytometry analyses of tetramer staining against two different NS3 epitopes. (B) Experimental scheme for lentiviral transductions, co-culture of DC and CD3<sup>+</sup> T cells and IL-2 treatment at high concentration and flow cytometry analyses of tetramer staining against NS3, NS4a and NS5b epitopes.



**Fig. 9.** Analyses of antigen-specific T cell proliferation by CFSE staining. T cells maintained alone ("unstimulated") or co-cultured with Mock DC or LV-HCV-NS transduced DC were compared. *P*-values were calculated from triplicate samples.

### Conflict of interest

None declared.

### Acknowledgments

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### References

- [1] Manns MP, Wedemeyer H, Cornberg M. Treating viral hepatitis C: efficacy, side effects, and complications. *Gut* 2006;55(9):1350–9.
- [2] Klade CS, Wedemeyer H, Berg T, Hinrichsen H, Cholewinska G, Zeuzem S, et al. Therapeutic vaccination of chronic hepatitis C nonresponder patients with the peptide vaccine IC41. *Gastroenterology* 2008;134(5):1385–95.
- [3] Stoll-Keller F, Barth H, Fafi-Kremer S, Zeisel MB, Baumert TF. Development of hepatitis C virus vaccines: challenges and progress. *Exp Rev Vac* 2009;8(3):333–45.
- [4] Wedemeyer H, Schuller E, Schlaphoff V, Stauber RE, Wiegand J, Schiefke I, et al. Therapeutic vaccine IC41 as late add-on to standard treatment in patients with chronic hepatitis C. *Vaccine* 2009;27(37):5142–51.
- [5] Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989;244(4902):359–62.
- [6] Dustin LB, Rice CM. Flying under the radar: the immunobiology of hepatitis C. *Annu Rev Immunol* 2007;25:71–99.
- [7] Farci P, Shimoda A, Coiana A, Diaz G, Peddis G, Melpolder JC, et al. The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. *Science* 2000;288(5464):339–44.
- [8] Lamonaca V, Missale G, Urbani S, Pilli M, Boni C, Mori C, et al. Conserved hepatitis C virus sequences are highly immunogenic for CD4(+) T cells: implications for vaccine development. *Hepatology* 1999;30(4):1088–98.
- [9] Rehmann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat Rev Immunol* 2005;5(3):215–29.
- [10] Klade CS, Kubitschke A, Stauber RE, Meyer MF, Zinke S, Wiegand J, et al. Hepatitis C virus-specific T cell responses against conserved regions in recovered patients. *Vaccine* 2009;27(23):3099–108.
- [11] Kanto T, Hayashi N, Takehara T, Tatsumi T, Kuzushita N, Ito A, et al. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. *J Immunol* 1999;162(9):5584–91.
- [12] Bain C, Fatmi A, Zoulim F, Zarski JP, Trepo C, Inchauspe G. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology* 2001;120(2):512–24.

- [13] Ciesek S, Liermann H, Hadem J, Greten T, Tillmann HL, Cornberg M, et al. Impaired TRAIL-dependent cytotoxicity of CD1c-positive dendritic cells in chronic hepatitis C virus infection. *J Viral Hepat* 2008;15(3):200–11.
- [14] Wedemeyer H, He XS, Nascimbeni M, Davis AR, Greenberg HB, Hoofnagle JH, et al. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol* 2002;169(6):3447–58.
- [15] Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998;392(6673):245–52.
- [16] Romani N, Gruner S, Brang D, Kampgen E, Lenz A, Trockenbacher B, et al. Proliferating dendritic cell progenitors in human blood. *J Exp Med* 1994;180(1):83–93.
- [17] Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 1994;179(4):1109–18.
- [18] Ribas A. Clinical trials with tumor antigen genetically modified dendritic cells. *Semin Oncol* 2005;32(6):556–62.
- [19] Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 1996;272(5259):263–7.
- [20] Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, et al. A third-generation lentivirus vector with a conditional packaging system. *J Virol* 1998;72(11):8463–71.
- [21] Dyall J, Latouche JB, Schnell S, Sadelain M. Lentivirus-transduced human monocyte-derived dendritic cells efficiently stimulate antigen-specific cytotoxic T lymphocytes. *Blood* 2001;97(1):114–21.
- [22] Gruber A, Kan-Mitchell J, Kuhlen KL, Mukai T, Wong-Staal F. Dendritic cells transduced by multiply deleted HIV-1 vectors exhibit normal phenotypes and functions and elicit an HIV-specific cytotoxic T-lymphocyte response in vitro. *Blood* 2000;96(4):1327–33.
- [23] Koya RC, Kasahara N, Favaro PM, Lau R, Ta HQ, Weber JS, et al. Potent maturation of monocyte-derived dendritic cells after CD40L lentiviral gene delivery. *J Immunother* 2003;26(5):451–60.
- [24] Koya RC, Kimura T, Ribas A, Rozengurt N, Lawson GW, Faure-Kumar E, et al. Lentiviral vector-mediated autonomous differentiation of mouse bone marrow cells into immunologically potent dendritic cell vaccines. *Mol Ther* 2007;15(5):971–80.
- [25] Esslinger C, Chapatte L, Finke D, Miconnet I, Guillaume P, Levy F, et al. In vivo administration of a lentiviral vaccine targets DCs and induces efficient CD8(+) T cell responses. *J Clin Invest* 2003;111(11):1673–81.
- [26] Chapatte L, Colombetti S, Cerottini JC, Levy F. Efficient induction of tumor antigen-specific CD8+ memory T cells by recombinant lentivectors. *Cancer Res* 2006;66(2):1155–60.
- [27] Palmowski MJ, Lopes L, Ikeda Y, Salio M, Cerundolo V, Collins MK. Intravenous injection of a lentiviral vector encoding NY-ESO-1 induces an effective CTL response. *J Immunol* 2004;172(3):1582–7.
- [28] Lopes L, Fletcher K, Ikeda Y, Collins M. Lentiviral vector expression of tumour antigens in dendritic cells as an immunotherapeutic strategy. *Cancer Immunol Immunother* 2006;55(8):1011–6.
- [29] Kim JH, Majumder N, Lin H, Watkins S, Falo Jr LD, You Z. Induction of therapeutic antitumor immunity by in vivo administration of a lentiviral vaccine. *Hum Gene Ther* 2005;16(11):1255–66.
- [30] Kimura T, Koya RC, Anselmi L, Sternini C, Wang HJ, Comin-Anduix B, et al. Lentiviral vectors with CMV or MHCII promoters administered in vivo: immune reactivity versus persistence of expression. *Mol Ther* 2007;15(7):1390–9.
- [31] Buffa V, Negri DR, Leone P, Borghi M, Bona R, Michelini Z, et al. Evaluation of a self-inactivating lentiviral vector expressing simian immunodeficiency virus gag for induction of specific immune responses in vitro and in vivo. *Viral Immunol* 2006;19(4):690–701.
- [32] Iglesias MC, Mollier K, Beignon AS, Souque P, Adotevi O, Lemonnier F, et al. Lentiviral vectors encoding HIV-1 polyepitopes induce broad CTL responses in vivo. *Mol Ther* 2007;15(6):1203–10.
- [33] Stripecke R, Koya RC, Ta HQ, Kasahara N, Levine AM. The use of lentiviral vectors in gene therapy of leukemia: combinatorial gene delivery of immunomodulators into leukemia cells by state-of-the-art vectors. *Blood Cells Mol Dis* 2003;31(1):28–37.
- [34] Fehse B, Richters A, Putimtseva-Scharf K, Klump H, Li Z, Osterstag W, et al. CD34 splice variant: an attractive marker for selection of gene-modified cells. *Mol Ther* 2000;1(5 Pt 1):448–56.
- [35] Koya RC, Weber JS, Kasahara N, Lau R, Villacres MC, Levine AM, et al. Making dendritic cells from the inside out: lentiviral vector-mediated gene delivery of granulocyte-macrophage colony-stimulating factor and interleukin 4 into CD14+ monocytes generates dendritic cells in vitro. *Hum Gene Ther* 2004;15(8):733–48.
- [36] Stripecke R. Lentiviral vector-mediated genetic programming of mouse and human dendritic cells. *Methods Mol Biol* 2009;506:139–58.
- [37] Manilla P, Rebello T, Afable C, Lu X, Slepshkin V, Humeau LM, et al. Regulatory considerations for novel gene therapy products: a review of the process leading to the first clinical lentiviral vector. *Hum Gene Ther* 2005;16(1):17–25.
- [38] Karwacz K, Mukherjee S, Apollonia L, Blundell MP, Bouma G, Escors D, et al. Nonintegrating lentivector vaccines stimulate prolonged T-cell and antibody responses and are effective in tumor therapy. *J Virol* 2009;83(7):3094–103.
- [39] el-Awady MK, Tabli AA, Redwan el RM, Youssef S, Omran MH, Thakeb F, et al. Flow cytometric detection of hepatitis C virus antigens in infected peripheral blood leukocytes: binding and entry. *World J Gastroenterol* 2005;11(33):5203–8.

- [40] Pham TN, King D, Macparland SA, McGrath JS, Reddy SB, Bursey FR, et al. Hepatitis C virus replicates in the same immune cell subsets in chronic hepatitis C and occult infection. *Gastroenterology* 2008;134(3):812–22.
- [41] Sarobe P, Lasarte JJ, Zabaleta A, Arribillaga L, Arina A, Melero I, et al. Hepatitis C virus structural proteins impair dendritic cell maturation and inhibit in vivo induction of cellular immune responses. *J Virol* 2003;77(20):10862–71.
- [42] Li W, Krishnadas DK, Li J, Tyrrell DL, Agrawal B. Induction of primary human T cell responses against hepatitis C virus-derived antigens NS3 or core by autologous dendritic cells expressing hepatitis C virus antigens: potential for vaccine and immunotherapy. *J Immunol* 2006;176(10):6065–75.
- [43] Li W, Krishnadas DK, Kumar R, Tyrrell DL, Agrawal B. Priming and stimulation of hepatitis C virus-specific CD4+ and CD8+ T cells against HCV antigens NS4, NS5a or NS5b from HCV-naïve individuals: implications for prophylactic vaccine. *Int Immunol* 2008;20(1):89–104.
- [44] Echeverria I, Zabaleta A, Silva L, Diaz-Valdes N, Riezu-Boj JI, Lasarte JJ, et al. Monocyte-derived dendritic cells from HCV-infected patients transduced with an adenovirus expressing NS3 are functional when stimulated with the TLR3 ligand poly(I:C). *J Viral Hepat* 2008;15(11):782–9.
- [45] Gerlach JT, Ulsenheimer A, Gruner NH, Jung MC, Schraut W, Schirren CA, et al. Minimal T-cell-stimulatory sequences and spectrum of HLA restriction of immunodominant CD4+ T-cell epitopes within hepatitis C virus NS3 and NS4 proteins. *J Virol* 2005;79(19):12425–33.
- [46] Sarobe P, Lasarte JJ, Casares N, Lopez-Diaz de Cerio A, Baixeras E, Labarga P, et al. Abnormal priming of CD4(+) T cells by dendritic cells expressing hepatitis C virus core and E1 proteins. *J Virol* 2002;76(10):5062–70.
- [47] Hiasa Y, Takahashi H, Shimizu M, Nuriya H, Tsukiyama-Kohara K, Tanaka T, et al. Major histocompatibility complex class-I presentation impaired in transgenic mice expressing hepatitis C virus structural proteins during dendritic cell maturation. *J Med Virol* 2004;74(2):253–61.
- [48] Bartenschlager R, Kaul A, Sparacio S. Replication of the hepatitis C virus in cell culture. *Antiviral Res* 2003;60(2):91–102.