Development of a dendritic cell vaccine encoding multiple cytotoxic T lymphocyte epitopes targeting hepatitis C virus

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Abstract. The aim of the present study was to develop a dendritic cell (DC) vaccine encoding hepatitis C virus (HCV) multiple cytotoxic T lymphocyte (CTL) epitopes that can stimulate T cell responses *in vitro*, and can be used for immunization *in vivo*. DCs were infected with recombinant replication-defective adenoviruses (Ads) expressing 2 HCV sequences fused with green fluorescent protein (GFP) and FLAG tags. One sequence (sequence 1) contained the HCV CTL epitopes, NS4B 1793-1801 and P7 774-782, as well as the HCV Th epitope, NS3 1248-1261. A second sequence (sequence 2) was the positive epitope control which contained HCV core 35-44, core 132-140 and NS3 1248-1261. The efficiency of infection was detected by flow cytometry and the expression of HCV epitopes in the DCs was confirmed by RT-PCR and western blot analysis. Ad infection significantly enhanced DC maturation and interleukin (IL)-12p70 production, resulting in T cell proliferation and increased interferon-γ secretion. The CTLs stimulated by Ad-infected DCs specifically killed Huh7.5 human hepatoma cells. The recombinant Ad-expressing multiple CTL HCV epitopes effectively infected the DCs *in vitro* and promoted T cell antiviral immune responses, thereby laying the foundation for the development of anti-HCV DC vaccines.

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Introduction

Infection with hepatitis C virus (HCV) has become a global health issue with a prevalence of 170 million individuals infected worldwide (1). The use of pegylated interferon together with ribavirin remains the standard of care treatment for non-genotype 1-infected patients. For genotype 1-infected patients, who represent the majority of cases, current standard treatment consists of triple therapy: pegylated interferon, ribavirin and a first generation protease inhibitor (telaprevir or boceprevir). Although triple therapy represents a major advance by increasing the possibility of viral eradication, it also presents new challenges which include the need for strict compliance, risk of additional side-effects, the development of resistant variants and drug-drug interactions (2,3). No vaccine is currently available to prevent hepatitis C. Therefore, the development of effective preventive and therapeutic vaccines for HCV is mandatory (4).

HCV contains a single-stranded, positive-sense RNA genome of approximately 9,600 nucleotides, encoding structural (core, E1, E2 and p7) and non-structural (NS2, NS3, NS4a, NS4b, NS5a and NS5b) proteins (5). The outcome of HCV infection appears to be determined by a complex interplay between immunological factors, host genetics and viral escape mutations. Thus, a vaccine must be effective against different HCV genotypes crossing multiple viral quasispecies, elicit broad T cell responses and sustain long-lived memory $CD4^+$ and $CD8^+$ T cell responses (6). Major histocompatibility complex (MHC) class I is directly associated with antigen presentation to $CD8^+$ T cells and the induction of effective cross-genotype $CD8^+$ T cells has the potential to limit the escape of HCV from immune responses (7). Therefore, a robust multi-specific and cross-genotype $CD8^+$ T cell response to different viral epitopes is required for a successful response against HCV infection.

Peripheral blood dendritic cells (DCs) from HCV-infected patients have been shown to exhibit reduced function compared with DCs from healthy subjects (8,9). These defects in DCs correlate with an impairment of the effector function of HCV-specific $CD8^+$ T cells. HCV-specific tetramer-positive T cells are found in peripheral blood mononuclear cells

(PBMCs) from chronically infected patients, but they usually display an impaired proliferative capacity (10). This phenomenon may be the consequence of the 'helpless' stimulation of $CD8^+$ T cells due to the inefficient presentation of HCV antigens by DCs. Thus, the impaired $CD8^+$ T cells may contribute to viral persistence, whereas approaches to avoid or restore $CD8^+$ T cell dysfunction may facilitate the development of prophylactic and therapeutic vaccines. As DCs are the most potent antigen-presenting cells, the use of DC-based vaccines has vast applications (11). Several studies have developed protocols using PBMCs for the generation of monocyte-derived DCs (mdDCs) (12). There are several methods for the introduction of foreign genes into DCs for antigen presentation (13). The use of adenovirus (Ad) as a gene transfer vector has been proven to be particularly effective in the infection of human mdDCs (14).

We have previously reported the use of multiple CTL HCV epitopes predicted by bioinformatics for designing HCV vaccines (15). We also observed the proliferation of PBMCs and detected the level of interferon-γ (IFN-γ) in the supernatant of cells stimulated with each predicted peptide. The HCV epitopes, NS4B 1793-1801 and P7 774-782 induced high frequencies of IFN-γ producing T cells, and the specific CTL for other epitopes was not detected in peripheral blood lymphocytes from patients with HCV. Moreover, NS4B 1793-1801 exhibited a high binding affinity for HLA-A2 molecules, and its affinity for peptide-MHC class I complexes was good, indicating that the high binding affinity for MHC class I molecules is an important factor for immunogenicity (16). Based on these results, the aim of the current study was to construct recombinant Ads expressing multiple CTL HCV epitopes and infected DCs, and to assess the stimulation of antiviral T cell responses.

Materials and methods

Construction and production of recombinant Ad encoding multiple CTL HCV epitopes. In the current study, DCs were infected with recombinant replication-defective Ads expressing 2 HCV sequences fused with green fluorescent protein (GFP) and FLAG tags. Sequence 1 contained the HCV CTL epitopes, NS4B 1793-1801 SMMAFSAAL and P7 774-782 AAWYIKGRL, as well as the HCV Th epitope, NS3 1248-1261 GYKVLVLNPSVAAT as previously described (17). The linker peptide between the epitopes was AAY, which has been shown to promote the presentation of epitopes (18). Sequence 2 was a positive control that included the HCV CTL epitopes, core 35-44 YLLPRRGPRL and core 131-140 ADLMGYIPLV which are epitopes confirmed by Cerny *et al* (19), as well as the HCV Th epitope, NS3 1248-1261. The GFP gene and the FLAG gene, engineered into both sequences, were used to verify the expression of peptides. The multiple CTL HCV epitope vector was prepared using the AdEasy system (K4930-00; Invitrogen, Carlsbad, CA, USA) (20). Briefly, the HCV gene was cloned into the pTrack plasmid containing the human cytomegalovirus (CMV) promoter. The *Pme*I-linearized pTrack-CMV-HCV vector and supercoiled pAdEasy-1 were co-transformed into *Escherichia coli*, strain BJ5183. The resultant AdHCV plasmid was characterized by restriction endonuclease digestion. To generate the Ad vector particles, *Pac*I-digested AdHCV plasmids were transfected into human embryonic kidney (HEK) 293T cells using the calcium phosphate co-precipitation method (21). The AdHCV particles were propagated and purified according to the AdEasy protocol (20). The Ad vector containing sequence 1 was termed Ad1, while the Ad vector containing sequence 2 was termed Ad2. The control AdGFP vector containing the GFP gene but without the FLAG tag under the control of the CMV promoter was plaque-purified and replicated into the 293T cells. Viral particles were harvested from freeze-thaw lysates. After 2 rounds of purification by CsCl ultracentrifugation, the Ad vector titers were determined by plaque assay on the 293T cells (22). Ad vectors were stored in small aliquots at -80˚C, and thawed immediately before use and kept on ice prior to dilution and addition to the cells.

Generation of immature DCs (imDCs). Informed consent was obtained from all donors prior to participation in the study. The study protocol was approved by the Human Ethics Committee of Tangdu Hospital, Xi'an, China and was carried out in conformity with the guidelines of the Helsinki declaration. PBMCs obtained from the peripheral blood of healthy adults were isolated by Ficoll-Hypaque (Sigma, St. Louis, MO, USA) density gradient separation (23). $CD14^+$ monocytes were isolated using CD14 isolation beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity was assessed by staining with a FITC anti-CD14 antibody (BD Biosciences, San Jose, CA, USA) and was routinely found to be >95%. To generate imDCs, the purified cells were adjusted to $1x10^6$ cells/ml, and cultured in serum-free X-VIVO™ 15 medium (04-744Q; Lonza, Basel, Switzerland) in the presence of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; 100 ng/ml) and interleukin-4 (IL-4; 100 ng/ml) (both from PeproTech, Rocky Hill, NJ, USA) in 6-well plates at 37˚C in a 5% CO_2 atmosphere for 5 days (24).

Ad infection of imDCs and induction of DC maturation. For Ad infection, $5x10^5$ imDCs/ml were resuspended in fresh X-VIVO™ 15 medium and seeded into 24-well plates. Recombinant Ads (Ad1, Ad2 and AdGFP) were added to the imDCs at various multiplicities of infection (MOIs) ranging from 50 to 1,000. Following incubation for 4 h, the cells were washed with PBS and further cultured in X-VIVO 15 medium as previously described (25). In some experiments, the control mature DCs (mDCs) were generated by the addition of a maturation cocktail to the imDC for the final 2 days. The maturation cocktail consisted of 1,000 U/ml IL-1β, IL-6 and tumor necrosis factor (TNF)-α, as well as 1 μg/ml prostaglandin E2 (PGE2) (all from PeproTech). The Ad-infected cells were evaluated by fluorescence microscopy and flow cytometry.

Flow cytometryic analysis. DCs on day 8 (mDCs) and after infection with Ads for 48 h (Ad1-DCs, Ad2-DCs and GFP-DCs) were assessed for cell surface phenotypes by flow cytometry. Briefly, the DCs were washed and resuspended in PBS and incubated with various fluorochrome-conjugated monoclonal antibodies in 5 ml FACS tubes at 4˚C for 30 min in the dark. Fluorescein isothiocyanate (FITC)-conjugated antibodies against CD80, phycoerythrin (PE)-conjugated antibodies against CD83, peridinin chlorophyll protein complex (PerCP)-conjugated antibodies against CD86 and allophy-

cocyanin (APC)-conjugated antibodies against HLA-DR (BD Biosciences) were used. The cells were then washed twice and fixed in PBS containing 1% formaldehyde. The phenotype of the cells was analyzed by flow cytometry using a BD FACSCalibur.

ELISA detection of IL-12p70. Following Ad infection, the DCs were cultured in 24-well plates for 48 h. Some wells with uninfected DCs were used as the control samples. For both infected and uninfected DCs, cytokine release into the supernatant was evaluated by enzyme-linked immunosorbent assay (ELISA) using an IL-12p70 ELISA detection kit (BD Biosciences).

RNA isolation, cDNA synthesis and reverse transcription. Twenty-four hours after infection, the DCs were harvested and total RNA was prepared from 1 or $2x10^6$ DCs using the RNeasy mini kit (74104; Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesized using the cDNA synthesis kit (Fermentas/Thermo Scientific Molecular Biology, Pittsburgh, PA, USA) according to the manufacturer's instructions. The upstream primers for sequences 1 and 2 were 5'-ATGTCAATG ATGGCTTTCAGCG-3' and 5'-ATGTCATTGTTGCCGC GCAGG-3', respectively, and the downstream primer (5'-CTAC TTATCGTCGTCATCCTTGT-3') was the same for both sequences. The primers were specific for the NS4B and P7 peptides. The conditions used for PCR amplification were as follows: 95˚C for 5 min, 35 cycles at 95˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 45 sec followed by a final extension at 72˚C for 10 min. Polymerase chain reaction products were visualized on 2% agarose gels.

Western blot analysis. Target protein expression was detected by western blot analysis. Total protein was extracted using RIPA reagent (PL005-PL008 PL035; Sangon Biotech, Shanghai, China). Following the removal of cell debris by centrifugation (13,200 x g at 10 min), 50 μg from each lysate sample were boiled for 5 min in sample buffer, separated using 15% SDS-PAGE and transferred onto PVDF membranes (Millipore). Non-specific reactivity was blocked in 5% non-fat milk in TBST for 1 h at room temperature. The membranes were then incubated with polyclonal mouse anti-FLAG antibody (f1804; Sigma) followed by goat anti-mouse IgG-HRP (sc-2031; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Reactive protein was detected by enhanced chemiluminescence (ECL) (Millipore, Billerica, MA, USA).

T cell proliferation assay. T cell proliferation was assessed using the cell counting kit-8 (CCK-8). 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) produces a water-soluble formazan dye upon reduction in the presence of an electron carrier. The amount of formazan dye generated by the activity of dehydrogenases in the cells is directly proportional to the number of living cells (26). Proliferative responses were measured in triplicate in flat-bottom 96-well microtiter plates. Autologous non-adherent T cells ($1x10^5$/well, obtained after removal of adherent T cells) were co-cultured with various concentrations of infected or uninfected DCs (10^3-10^4) in 200 μl of X-VIVO™ 15 medium at 37˚C for 4 days. The assay included a blank control (medium only) and a negative control (T cells alone). After the addition of 20 μl of WST-8/well for 4 h, the optical density (OD) was recorded at a wavelength of 450 nm according to the manufacturer's instructions. The stimulating index of proliferation was calculated as follows: stimulating index of proliferation = (mean experimental OD - mean blank control OD)/(mean negative control OD - mean blank control OD).

Cytotoxicity assay and IFN-γ ELISA. The release of lactate dehydrogenase (LDH) was measured using a CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI, USA). Effector T cells were incubated with Ad-infected DCs at a ratio of 10:1 for 7 days at 37˚C with 5% CO_2. T cells alone, T cells incubated with uninfected DCs and T cells incubated with GFP-DCs were used as the negative controls. Huh7.5 cells electrotransfected with FL-J6/JFH transcripts were used as target cells (27,28). Huh7.5 cells were electroporated at 230 V, 950 μF using 4 mm electroporation cuvettes, and then cultured in opti-MEM for 2 days. A total of $1x10^3$ target cells in a volume of 100 μl of phenol red-free DMEM were plated into individual wells of a 96-well U-bottom plate in triplicate. Effector cells were added at an effector:target ratio (E:T) of 25:1, 50:1 and 100:1. The cell mixture was incubated for 4 h at 37˚C. The spontaneous release by target and effector cells was determined by incubation of the respective populations alone. Following incubation, the conditioned medium was collected and centrifuged at 250 x g for 4 min. A sample (50 μl) of each supernatant was transferred to separate wells of a 96-well plate and substrate solution (50 μl) was added. After 30 min of incubation at room temperature, the absorbance was measured at 490 nm using a microplate reader. Maximum release was determined by cell lysis with 1% Triton X-100. The results were calculated as the means of triplicate assays, and the percentage of specific lysis was calculated according to the following formula: cytotoxicity (%) = [(experimental - effector spontaneous - target spontaneous)/(target maximum - target spontaneous)] x100%. To quantify cytokine production, 100 μl of each supernatant from the cytotoxicity assay were collected and evaluated using an IFN-γ ELISA detection kit (BD Biosciences) according to the manufacturer's instructions.

Statistical analysis. All data were analyzed using SPSS 13.0 software. One-way analysis of variance (ANOVA) was used for multiple group comparison. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Production of high titer recombinant Ads expressing multiple CTL HCV epitopes. Recombinant Ads encoding multiple CTL epitopes from HCV were constructed. GFP was used as a positive marker for epitope expression. The titers of the viral stocks were determined by a plaque assay using 293T cells, and were $1.68x10^{10}$, $1.74x10^{10}$ and $1.56x10^{10}$ pfu/ml for AdGFP, Ad1 and Ad2, respectively.

Analysis of Ad transfection efficiency by flow cytometric assessment of GFP. $CD14^+$ monocytes were cultured in the presence of GM-CSF and IL-4 for 5 days and then incubated with a maturation mix or transfected with Ad at different

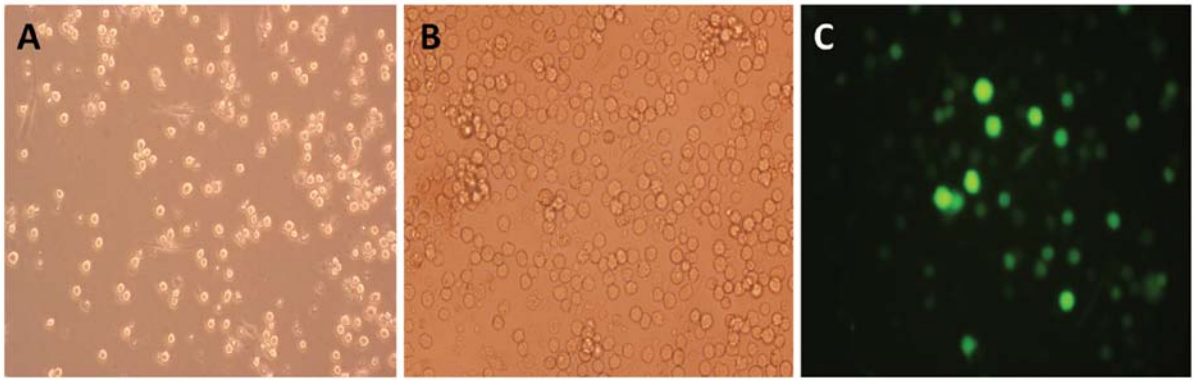

Figure 1. Morphology of mature dendritic cells (mDCs) on day 7. (A) mDCs at a magnification of x200. (B) Ad1-DCs at a magnification of x400. (C) Ad1-DCs expressing green fluorescent protein (GFP) at a magnification of x400.

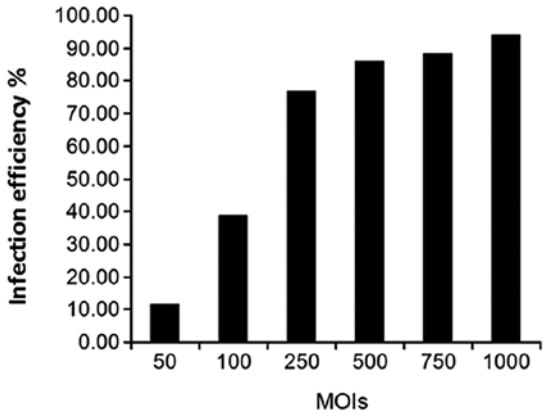

Figure 2. Transfection efficiency of adenoviruses (Ads) using various multiplicities of infections (MOIs). Immature dendritic cells (DCs) were infected with adenovirus green fluorescent protein (AdGFP) at various MOIs, then cultured for 48 h. Flow cytometry was used to determine the efficiency of Ad infection by GFP expression. The infection efficiencies were 11.4, 38.8, 76.8, 85.7, 88.3 and 93.8%, at an MOI of 50, 100, 250, 500, 750 and 1,000, respectively.

MOIs, and the cultures were further incubated until day 7. Upon examination by fluorescence microscopy, cells initially showed identical morphological changes from $CD14^+$ monocytes (day 1) to imDCs (day 5) as well as mDCs (day 7) (Fig. 1). The formation of veils and clusters was evident during the differentiation of monocytes into highly mobile imDCs. After maturation, there were no morphological differences between the mDCs and Ad1-DCs, as shown by microscopic examination. Flow cytometry was used to determine the efficiency of Ad infection by GFP expression (Fig. 2). The results revealed that with the increase in MOI, the number of GFP-positive cells increased, and the infection efficiency ranged from 11.4 to 93.8%. Achieving higher levels of gene transfer in DCs using Ad requires a high MOI over a prolonged culture period, which is frequently associated with DC apoptosis (29). We, therefore, used an MOI of 250 in the subsequent experiments, which had an infection efficiency of approximately 75%, but was less cytotoxic in our experiments.

Identification of multiple CTL HCV epitopes in DCs. The expression of multiple CTL HCV epitopes in DCs was measured by RT-PCR and western blot analysis. Twenty-four hours after infection with Ad1 and Ad2, the DCs were harvested and total RNA was extracted for RT-PCR analysis to detect HCV epitopes. A 150-bp fragment corresponding to the expected size is shown in Fig. 3A. Forty-eight hours after the Ad infection of DCs, western blot analysis was performed to assess recombinant protein expression. As CMCE was fused with FLAG, anti-FLAG was used as the primary antibody. A 10-kDa protein was detected in the Ad1- and Ad2-infected DCs, but not in the uninfected mDCs, indicating that sequence 1 and 2 target proteins were successfully expressed in DCs (Fig. 3B).

Assessment of DC phenotype by examining cell surface activation markers. The activation of DCs by Ad treatment was determined by comparing the expression level of DC activation markers (normally expressed at low levels in imDCs) on cytokine-treated control DCs and Ad-treated DCs using fluorescence activated cell sorter (FACS) analysis. The following markers were upregulated upon DC activation: MHC class II (HLA-DR molecules), co-receptor molecules, such as CD80 and CD86, and the maturation marker, CD83 (Fig. 4). The expression of CD80, CD83, CD86 and HLA-DR in imDCs was very low (data not shown). Following infection with Ad1, the DCs had higher expression rates of CD80, CD83, CD86 and HLA-DR (76.87, 87.75, 97.51 and 97.85%, respectively) compared with the uninfected mDCs (48.29, 60.89, 91.23 and 92.30%, respectively). Ad2-DC and AdGFP showed a similar expression of these cell surface markers. The upregulation of activation markers indicates that imDCs have undergone functional maturation and can effectively present antigen to T cells and secrete cytokines, such as IL-12p70.

IL-12p70 secretion by Ad-infected DCs and IFN-γ production by T cells co-cultured with Ad-infected DCs. IL-12p70 production by DCs was evaluated by ELISA before and after Ad infection. At an Ad MOI of 250, the infection efficiency was approximately 75%. The results revealed that the levels of IL-12p70 were significantly higher after 24 h of infection compared with the uninfected DCs ($P<0.05$) (Fig. 5A). Thus, after Ad infection, IL-12p70 secretion increased slightly in the DCs infected with Ad1 compared with the DCs infected with Ad2, although the difference was not statistically significant. IL-12p70 secretion by Ad1-DCs and Ad2-DCs was more

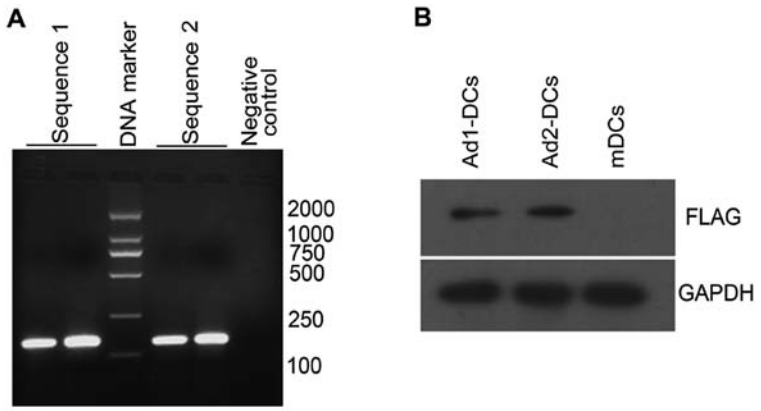

Figure 3. Expression of multiple cytotoxic T lymphocyte (CTL) hepatitis C virus (HCV) epitopes in dendritic cells (DCs). (A) PCR amplification of specific sequences. Lanes 1 and 2, sequence 1; lane 3, DNA marker; lanes 4 and 5, sequence 2; lane 6, negative control. (B) Western blot analysis of HCV epitope expression. Expression of HCV epitopes fused with FLAG in Ad1-DCs and Ad2-DCs using anti-FLAG antibody. Lane 1, Ad1-DCs; lane 2, Ad2-DCs; lane 3, mDCs.

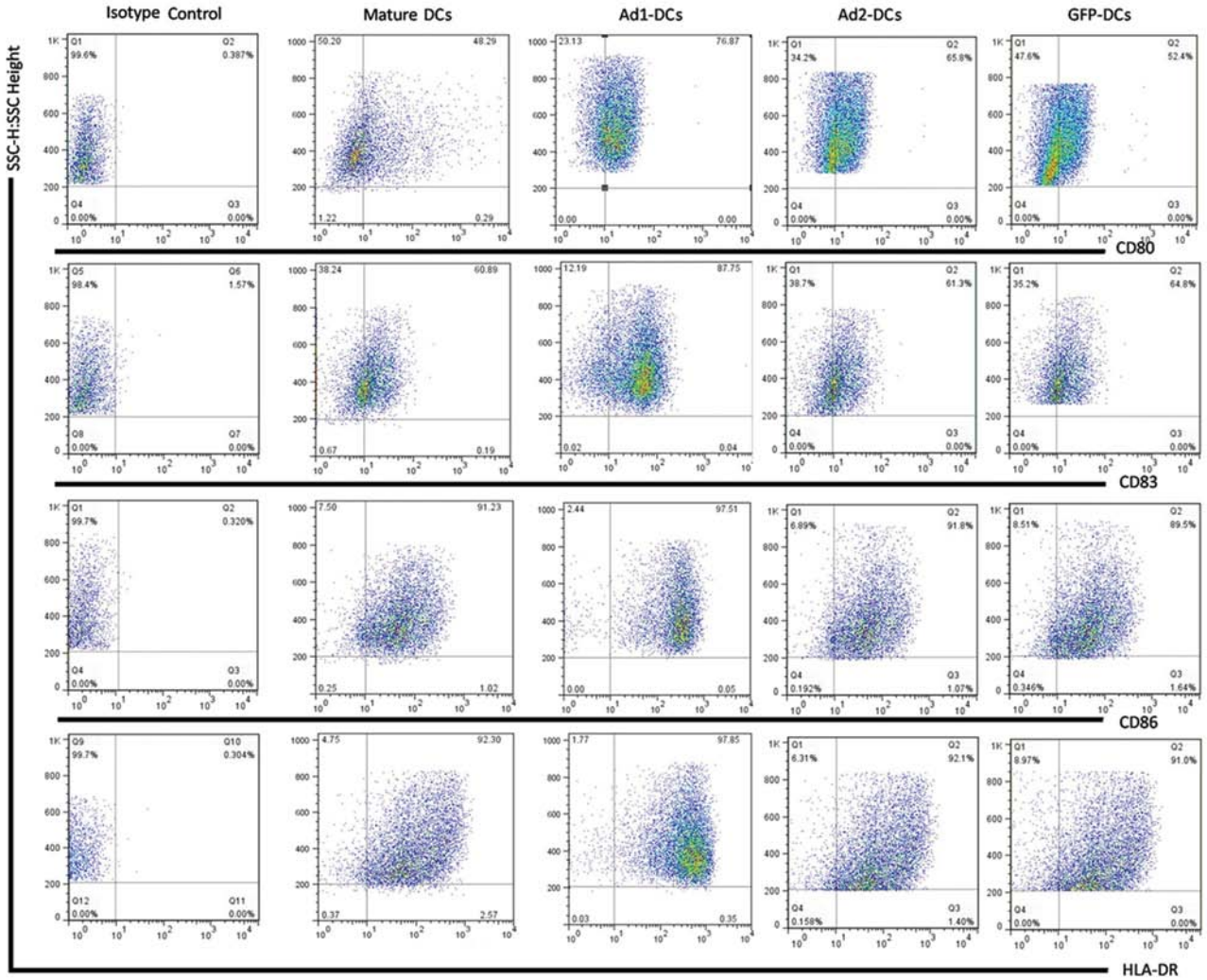

Figure 4. Cell surface markers of dendritic cells (DCs) measured by flow cytometry. This figure illustrates that the DCs infected with adenovirus (Ad) (Ad1-DC, Ad2-DC and AdGFP) express similar levels of CD80, CD83, CD86 and HLA-DR, compared with $CD14^+$ monocyte-induced mature DCs. The results indicated that infected DCs exhibited a mature phenotypic change toward antigen presentation.

than that of GFP-DCs and mDCs. The cells activated by DCs infected with Ad1 or Ad2 vectors were incubated with Huh7.5 cells that had been electrotransfected with FL-J6/JFH transcripts. Supernatants were harvested to assess IFN-γ production. IFN-γ secretion by Ad1-DC-T was higher than that by other groups (Fig. 5B).

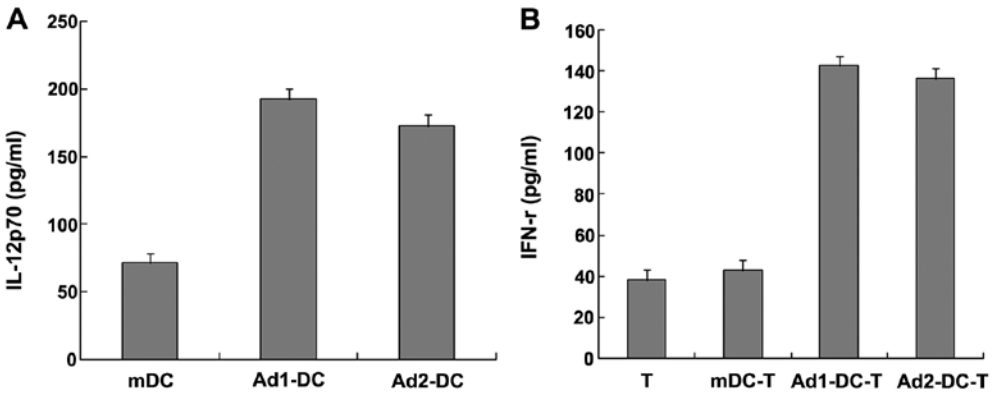

Figure 5. Interleukin (IL)-12p70 and interferon (IFN)-γ release into the supernatant of cultured cells as evaluated by ELISA. (A) Twenty-four hours after infection, IL-12p70 levels were measured in the supernatants of Ad1-DC, Ad2-DC cultures, mature dendritic cells (DCs) without infection and green fluorescent protein (GFP)-DC cultures. The results revealed that after adenovirus (Ad) infection, the level of IL-12p70 significantly increased. When the DCs were infected with Ad1 or Ad2, the level of IL-12p70 released was higher than that of the other DC groups (P<0.05). (B) IFN-γ release in the supernatants of cytotoxic T lymphocytes (CTLs) co-cultured with Huh7.5 cells. The levels of IFN-γ released by Ad1-DC-T and Ad2-DC-T were higher than those released by the GFP-DC-T, DC-T and T cells alone (P<0.05). Data represent the means ± SD from 3 independent samples.

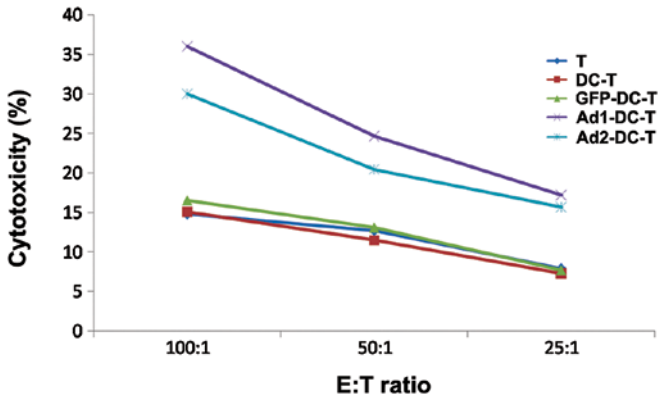

Figure 6. Cytotoxicity measured by lactate dehydrogenase (LDH) release assay. Cytotoxic activity of effector T cells was measured by LDH release from FL-J6/JFH-transfected Huh7.5 hepatocytes co-cultured with T cells pulsed with or without dendritic cells (DCs) at 3 different effector-to-target ratios. Data are representative of 3 independent experiments.

Table I. Stimulation of auto-T cell proliferation by imDCs and mDCs as detected by WTS-8 assay.

DC:T	1:10	1:20	1:100
mDCs	3.2±0.3	1.9±0.6	0.6±0.3
GFP-DCs	3.4±0.2	2.0±0.2	0.6±0.2
Ad1-DCs	6.8±0.2[a]	3.9±0.3[a]	2.7±0.3[a]
Ad2-DCs	6.7±0.3[a]	3.8±0.2[a]	2.5±0.2[a]

Proliferation of T cells activated by DCs infected with or without Ads (Ad1-DCs and Ad2-DCs) compared with those activated by uninfected mDCs and GFP-DCs ([a]P<0.05). Data represent the means ± SD from 3 independent samples. DCs, dendritic cells; imDCs, immature DCs; mDCs, mature DCs; GFP, green fluorescent protein; Ad, adenovirus.

Stimulation of autologous T cells by Ad-infected DCs. Autologous T cell stimulation using increasingly limited numbers of DCs is considered to be a hallmark to determine the function of DCs as professional APCs. The ability of Ad1-DCs, Ad2-DCs, GFP-DCs and mDCs to stimulate autologous T cell proliferation was determined by WTS-8 assay using different DC:T cell ratios (1:100, 1:20 and 1:10). Both Ad1-DCs and Ad2-DCs stimulated autologous T cell proliferation to a much higher degree than the GFP-DCs and uninfected mDCs, although there was no statistically significant difference between Ad1-DCs and Ad2-DCs in the stimulation of T cell proliferation (Table I).

Cytotoxicity assays. The functional capability of the CTLs responding to Ad-infected DCs was tested by determining whether they could specifically lyse target cells. The effector T cells were plated in 96-well plates in medium containing IL-2 (20 ng/ml). The DCs were added at a 1:10 ratio, and the cells were co-cultured at 37˚C in 5% CO_2 for 7 days. The cytotoxic activity was assayed using Huh7.5 cells electrotransfected with FL-J6/JFH transcripts as the targets. The results indicated that Ad1-DCs and Ad2-DCs specifically induced high CTL activity against Huh7.5 cells electrotransfected with FL-J6/JFH transcripts, whereas the GFP-DC-T cells, the uninfected mDC-T cells and the unstimulated T cells alone had little or no cytotoxicity against the target cells (Fig. 6). The percentages of lysed Huh7.5 cells mediated by Ad1-DC-T (36%) and Ad2-DC-T (30%) were much higher than those of GFP-DC-T (16%), DC-T (15%) and T (15%) at an E:T ratio of 100:1 (P<0.001). No significant differences were detected between Ad1-DC-T and Ad2-DC-T in terms of target cell lysis (P>0.05).

Discussion

DCs are professional antigen-presenting cells with potent immunostimulatory capabilities. Due to their pivotal role in antigen processing and antiviral immunity, the use of *ex vivo* genetically manipulated DCs to augment the immune response is an attractive approach for immunotherapy (30). Previous studies have demonstrated that DC-based vaccines stimulate T cell immunity against viral infection and tumor growth (10). However, the efficacy of the DC vaccine correlates with the

level of antigen expressed by the DCs, which greatly depends on the efficiency of gene transfer. The use of viral vectors is a more promising strategy for antigen loading, as viral vectors efficiently express full-length endogenous antigen in DCs. Expression of full-length antigen allows the DCs to present a wide variety of both MHC class I and II epitopes. Consistent with the data presented in this study, other studies have shown that DCs infected with adenovirus effectively elicit a strong and specific CTL response (30), and the safety and efficiency of Ad-based DC vaccines have been documented in clinical trials (31). The modification by adenoviruses is only transient, thereby reducing the risk of insertional mutagenesis exerted by retroviral infection. Although Ad vectors have seen their share of setbacks in recent years, they remain viable tools for the prevention or treatment of a multitude of diseases (32).

In our study, Ad1-DCs and Ad2-DCs stimulated higher T lymphocyte proliferation than GFP-DCs and uninfected mDCs. However, there was no significant difference between Ad1-DCs and Ad2-DCs in promoting auto-T cell proliferation (Table I). As a positive control, sequence 2 included the HCV CTL epitopes, core 35-44, core 131-140, as well as the HCV Th epitope, NS3 1248-1261. HCV core 35-44 and core 131-141 have been demonstrated to induce an HCV-specific CTL response (19). The Th epitope, NS3 1248-1261 has been associated with viral clearance in acute hepatitis C infection, and has found to be present in patients with a diverse HLA background (17). Our study demonstrates that HCV epitopes are expressed in DCs and can be presented to T cells, promoting T cell proliferation.

IL-12 is produced primarily by antigen-presenting cells and plays a critical role in host defense through its ability to stimulate both innate and adaptive immune effector cells. IL-12 has been found to stimulate natural killer (NK) cells to proliferate, produce IFN-γ and exhibit potent cytotoxicity. $CD4^+$ T cells, upon IL-12 stimulation, undergo differentiation to become Th1 effectors at the expense of Th2 differentiation. IL-12 can also directly activate $CD8^+$ T cells and enhance their cytolytic potential (33). The production of IL-12 is critical for the induction of Thl immunity directed towards the elimination of intracellular pathogens and viruses. The core protein of HCV seems to have a suppressive action on IL-12 production at the transcriptional level. A specific Thl cell defect correlates with insufficient Th and CTL responses, and lower production of type 1 cytokine (IL-2, IFN-γ, lymphokine-activated killer cells). Taken together, these factors may be responsible for the observed failure to resolve HCV infections (34). The enhanced IL-12 release by infected DCs compared with uninfected DCs and GFP-DCs demonstrates that HCV epitopes promote the maturation and function of DCs in an antigen-specific manner. IL-12 stimulates the proliferation of activated CD8 T cells.

The T cell functional assays further proved that DCs correctly presented specific HCV epitopes to T cells following Ad infection, leading to the T cell production of IFN-γ, inhibiting HCV replication and effectively killing Huh7.5 cells transfected with FL-J6/JFH transcripts. In another study, FL-J6/JFH (35) plasmids encoding the full-length HCV chimeric genome were transcribed into HCV RNA *in vitro*, and the resulting RNA was transfected into Huh7.5 cells by electroporation. In our previous study (27), we showed that high levels of virus were present in the supernatant of Huh7.5 transfected cells, and the elevated expression of HCV NS5A proteins was observed in the transfected cells by indirect immunofluorescence staining. Large numbers of enveloped or non-enveloped virus-like particles (VLPs) were also observed in the transfected Huh7.5 cells by transmission electron microscopy (27). Therefore, in the current study, Huh7.5 cells transfected with FL-J6/JFH transcripts were employed as target cells to simulate the natural HCV infection of human hepatocytes.

Upon T cell receptor engagement with peptide/MHC class I complexes, $CD8^+$ T cells vigorously expand and exhibit multiple effector functions, including cytotoxicity mediated by perforin-granzyme and Fas/FasL pathways and the production of cytokines (e.g., IFN-γ). HCV-specific $CD8^+$ T cells exert strong antiviral effects through 2 distinct mechanisms. The first requires direct cell-cell contact resulting in cytolysis, and the second is a non-cytolytic inhibition mediated by IFN-γ. Of note, studies on acute HCV infection in humans and chimpanzees have indicated the presence of both effector functions in HCV-specific $CD8^+$ T cells. For example, several studies have shown a correlation between the appearance of virus-specific $CD8^+$ T cell responses in the peripheral blood and liver, and the onset of liver disease, indicating cytolytic effector functions (36,37). In addition, certain studies have demonstrated that activated HCV-specific $CD8^+$ T cells inhibit viral replication by both cytokine-mediated and direct cytolytic effects (38,39). A number of studies on chimpanzees and acutely infected humans have supported the idea that an effective HCV vaccine must be able to induce strong HCV-specific cytotoxic IFN-γ + T cells which are able to target the virus (40). IFN-γ inhibits protein synthesis and RNA replication of subgenomic and genomic HCV replicons (41). IFN-γ may enhance the immune lysis of HCV-infected cells and inhibit hepatic fibrosis by an effect on TGF-β and on HCV-induced carcinogenesis (34). Thus, a successful prophylactic or therapeutic dendritic cell vaccine must elicit HCV-specific $CD8^+$ T cells with a strong ability to produce IFN-γ.

Several studies have been published indicating progress with therapeutic HCV vaccines (42-44). For example, IC41 is a synthetic peptide vaccine containing 7 relevant HCV T cell epitopes and Th1/Tc1 adjuvant poly-L-arginines (42), and CIGB-230 is a mixture of pIDKE2, a plasmid expressing HCV structural antigens, and a recombinant HCV core protein (43); both of these vaccine candidates have recently been shown to be safe, and are currently being used in a clinical trial. A phase I clinical trial has recently been completed which assessed dendritic cell immunotherapy in HCV-infected individuals using autologous mdDCs presenting HCV-specific HLA A2.1-restricted CTL epitopes pulsed with lipopeptides. The vaccine was found to be safe, but was unable to generate sustained responses or alter the outcome of HCV infection (44). These vaccines, although still in their early developmental stages, show a bright future for HCV vaccine development.

In conclusion, in this study, we successfully constructed recombinant Ads encoding multiple CTL HCV epitopes, although more epitopes may be required to eliminate the infection. The recombinant Ads effectively infected DCs and induced DC maturation and the production of IL-12. Ad-infected DCs stimulated T lymphocyte proliferation, IFN-γ production and induced specific T cell cytotoxicity against HCV-expressing hepatocytes. This preliminary *in vitro* study

lays the foundations for further development of anti-HCV DC vaccines. We believe that a DC-based vaccine against HCV infection combined with adjuvants may block negative and enhance positive regulatory signals, which may improve the vaccine-induced immune response against HCV infection. This may lead to a reduction in the HCV viral load, and may thus attenuate the progression of chronic liver disease (45).

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