Improvement of the cytotoxic T lymphocyte response against hepatocellular carcinoma by transduction of cancer cells with an adeno-associated virus carrying the interferon-γ gene

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Abstract. Dendritic cell (DC)-based antigen-targeted immunotherapy may offer effective adjuvant therapy for hepatocellular carcinoma (HCC), in which cytotoxic T lymphocytes (CTLs) are key. However, in a number of cases, the activity of CTLs is completely inhibited due to the downregulated expression of major human leukocyte antigen (HLA) class I molecules by HCC cells. The aim of the present study was to overcome this issue. Hep3B cells were transduced by HCC-specific recombinant adeno-associated virus (rAAV) carrying human α-fetoprotein promoter (AFPp) and the interferon-γ (IFN-γ) gene (rAAV/AFPp-IFN-γ). rAAV carrying the cytomegalovirus promoter (CMVp) and human α-fetoprotein (AFP) gene (rAAV/CMVp-AFP) was used to transduce professional antigen-presenting DCs for the purpose of stimulating a CTL response. It was observed that transduction of DCs with rAAV/CMVp-AFP resulted in: (i) AFP and interleukin-12 expression; (ii) high expression levels of cluster of differentiation (CD)80, CD83, CD86, CD40, HLA-death receptor and CD1a; (iii) T cell populations with marked IFN-γ expression; (iv) a high percentage of $CD69^+/CD8^+$ T cells; and (v) the activity of CTLs against HLA-A2-expressing Hep3B cells. The transduction of Hep3B cells with rAAV/AFPp-IFN-γ resulted in: (i) IFN-γ expression; (ii) upregulated expression of HLA-A2; and (iii) an improved CTL response against HLA-A2-deficient Hep3B cells. rAAV/CMVp-AFP-transduced DCs elicited an AFP-specific and HLA-class I-restricted CTL response against Hep3B cells. In conclusion, it was shown that the transduction of Hep3B with rAAV/AFPp-IFN-γ upregulated the expression of HLA-A2 and improved the sensitivity to CTL response.

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Introduction

According to data from the National Center for Health Statistics, primary hepatocellular carcinoma (HCC) is one of the most common types of cancer in the world. α-fetoprotein (AFP) is a glycoprotein that is produced in the endodermal cells of the yolk sac and fetal liver. The synthesis of AFP decreases markedly following birth. However, expression of the AFP gene is reactivated in adults during hepatocarcinogenesis (1). Human T cell repertoires recognize AFP-derived peptide epitopes in the context of major human leukocyte antigen (HLA) class I molecules and induce AFP-specific protection (1,2). Thus, AFP, as a tumor-associated antigen, may be a suitable target for dendritic cell (DC)-based cytotoxic T lymphocyte (CTL)-mediated antigen-targeted immunotherapy. CTLs are the predominant effector cells against cancers (3). DCs are the most effective antigen-presenting cells, and stimulate naive T lymphocytes to initiate an antigen-specific, HLA class I-restricted CTL response (4). However, in a number of cases, HCC cells express few or no HLA class I molecules due to genetic, transcriptional and post-transcriptional regulation, which results in escape of recognition of tumor cells by CTLs (5-8).

Various protocols for generating DCs *in vitro* from peripheral blood mononuclear cells have been developed (9). Recombinant adeno-associated virus (rAAV) is one of the safest virus vectors in gene therapy (10,11). AAV type 2 vectors have been demonstrated to be effective vectors for delivery of antigen genes into human DCs, and to generate a marked CTL response against the antigen-positive target cells (10,11). It has been reported that interferon-γ (IFN-γ) upregulates the expression of HLA class I molecules (12,13). The present study demonstrated that rAAV carrying the cytomegalovirus promoter (CMVp) and AFP gene (rAAV/CMVp-AFP)-transduced DCs elicited an AFP-targeted, HLA-A2-restricted CTL response against Hep3B cells. In order to improve the activity of CTLs

against HLA-A2-deficient Hep3B cells, HCC-specific rAAV carrying human α-fetoprotein promoter (AFPp) and the IFN-γ gene (rAAV/AFPp-IFN-γ) were used to transduce Hep3B cells in order to recover the expression of HLA-A2.

Materials and methods

Cells. Hep3B (hepatocellular carcinoma), NCI-H2126 (lung cancer), HEK293 (embryonic kidney cells), HeLa (cervical cancer), Hs578T (breast cancer), LNCaP-FGC (prostate cancer), LoVo (colorectal adenocarcinoma), PANC-1 (pancreatic cancer), SNU-1 (gastric carcinoma), THP-1 (monocytic leukemia) and K562 (myeloid leukemia) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). All of these cells were cultured in complete Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) or RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 5 or 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.). The primary human hepatocytes, hNHeps, were obtained from Lonza Group AG (Basel, Switzerland) and cultured in HCM hepatocyte culture medium (Lonza Group AG). Peripheral blood mononuclear cells (PBMCs) from healthy donors were separated using a routine Ficoll (Sigma-Aldrich, St. Louis, MO, USA) gradient method and cultured in AIM-V medium (Invitrogen; Thermo Fisher Scientific, Inc.). All blood donors were enrolled by the Provincial Hospital Affiliated to Shandong University (Jinan, China) and provided written informed consent. The study protocol received *a priori* approval by the Human Research Internal Review Board of the Provincial Hospital Affiliated to Shandong University. The HLA haplotype of all donors was HLA-A2.

Construction of rAAV vectors. The wild-type AAV type 2 genome, pSM620, was digested in order to delete the internal AAV sequences from map units 3-97, including the p5 promoter, and a specially designed polylinker was ligated in place, resulting in the AAV vector plasmid, dl3-97 (14). The cytomegalovirus enhancer and the SV40 early mRNA polyadenylation signal DNA were derived from the pEGFP-N1 plasmid (Clontech Laboratories, Inc., Mountain View, CA, USA) and inserted into the dl3-97 vector (provided by Professor Yong Liu, Gene and Biotherapy Center, Cancer Institute, University of Arkansas for Medical Sciences, Little Rock, AR, USA). The plasmid, pDRIVE-hAFP (Invivogen, San Diego, CA, USA) was digested with *Pac*I and *Nco*I to obtain 275 bp AFP promoter DNA. Subsequently, the DNA was inserted into the downstream of CMV enhancer gene (dl3-97/AFPp). CMV immediate early promoter was inserted into the dl3-97 vector using the identical method (dl3-97/CMVp), which was derived from the pEGFP-N1 plasmid.

Human AFP and IFN-γ cDNA were amplified by reverse transcription-polymerase chain reaction (RT-PCR). The total mRNA was isolated from the Hep3B cells and phytohemagglutinin-stimulated human T lymphocytes using an Oligotex mRNA isolation Mini kit (Qiagen, Inc., Valencia, CA, USA). The RNA was reverse transcribed using the SuperScript™ first-strand synthesis system, according to the manufacturer's protocols (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequent to the generation of the first-strand of cDNA, PCR amplification was conducted using the following primer pairs: Sense, 5'-CTTCCACCACTGCCAATAAC-3' and antisense, 5'-TTGTCTTCTCTTCCCCTG-3' for AFP, which amplifies the sequence from nucleotides 12 to 1,902 (15), and sense, 5'-TTTCTCTCGGAAACGATG-3' and antisense, 5'-GGC AGGACAACCATTAC-3' for IFN-γ, which amplifies the sequence from nucleotides 95 to 622 (16). The PCR conditions for AFP were 30 cycles of denaturation at 94˚C for 50 sec, annealing at 58˚C for 55 sec and elongation at 72˚C for 50 sec. The PCR conditions for IFN-γ were 30 cycles of denaturation at 94˚C for 50 sec, annealing at 58˚C for 55 sec and elongation at 72˚C for 40 sec. According to the routine method of molecular cloning, AFP and IFN-γ cDNA were inserted into the dl3-97 vectors, respectively. The dl3-97 vectors and AFP and IFN-γ cDNA were digested with a restriction enzyme (New England BioLabs, Inc., Ipswich, MA, USA). The cDNA was inserted downstream of the promoter using ligase (New England BioLabs, Inc.). The AAV vectors were generated, including rAAV/CMVp-AFP and rAAV/AFPp-IFN-γ. The enhanced green fluorescent protein (eGFP) was derived from pEGFP-N1, and also inserted into the AAV vectors to generate rAAV/AFPp-eGFP.

Generation of rAAV virus. The pSH3 plasmid expresses the AAV type 2 *rep* and *cap* genes and adenovirus type 5 E2A, VA1 and E4 genes, to allow rAAV DNA replication and packaging into viral particles without contaminating the wild-type AAV and adenovirus (17). The rAAV vectors were co-lipofected into HEK293 cells with the pSH3 plasmid using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), and the rAAVs were harvested after 4 days. A one-step column purification technique, which used gravity flow based on its affinity to heparin, without ultracentrifugation, was performed in order to generate the purified rAAV (18). The rAAVs were titered as described previously by dot blot hybridization using a DIG DNA Labeling and Detection kit (Roche Diagnostics, Basel, Switzerland) (10).

Analysis of cell-specific expression of rAAV with AFP promoter. To observe cell-specific expression of the rAAV with an AFP promoter, Hep3B and a series of AFP-negative control cells were infected with rAAV/AFPp-eGFP virus. After 5 days, the cells were observed under a BX61 inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Analysis of IFN-γ expression in the rAAV/AFPp-IFN-γ-transduced Hep3B cells. At day 5 of transduction, the Hep3B cells were harvested. Intracellular staining was performed to analyze the expression of IFN-γ in rAAV/AFPp-IFN-γ-transduced Hep3B cells. The cells were fixed with 4% parafomaldehyde (Sigma-Aldrich) at 4˚C for 30 min and permeabilized with 1 ml 0.5% saponin (Sigma-Aldrich) for 20 min at room temperature. The cells were subsequently incubated with phycoerythrin (PE)-conjugated mouse anti-human IFN-γ monoclonal antibody (1:100; cat. no. 559326; BD Pharmingen, San Diego, CA, USA). A FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) was used for data acquisitions, and 10,000 cells were counted for each sample. rAAV/AFPp-eGFP-transduced Hep3B cells served as a control.

The mRNA expression of IFN-γ was analyzed by RT-PCR under conditions of 30 cycles of denaturation at 94˚C for 50 sec, annealing at 58˚C for 55 sec and elongation at 72˚C for 40 sec. At day 5 of transduction, the total mRNA was isolated from rAAV/AFPp-IFN-γ-transduced Hep3B cells using an Oligotex mRNA isolation Mini kit. Subsequent to the generation of the first strand of the cDNA, PCR amplification was conducted using the primer pairs, 5'-TTTCTCTCGGAAACG ATG-3' and 5'-GGCAGGACAACCATTAC-3', which amplify the sequence from nucleotides 95 to 622 (16). Transcription factor IIB was also amplified by PCR as a control, the resulting products were analyzed by 2% gel electrophoresis.

The expression of IFN-γ was also measured in the Hep3B cell medium by enzyme-linked immunosorbent assay (ELISA) using the Human IFN-γ Direct ELISA kit (Invitrogen; Thermo Fisher Scientific, Inc.). From day 6 to day 60, the secretion of IFN-γ was measured in duplicate, according to the manufacturer's protocol.

Observation of HLA-A2 expression in rAAV/AFPp-IFN-γ-transduced Hep3B cells. At day 5 of rAAV/AFPp-IFN-γ transduction, the Hep3B cells were harvested. To observe HLA-A2 expression, the cells were fixed with 4% paraformaldehyde at 4˚C for 30 min and stained with PE-conjugated mouse anti-human HLA-A2 monoclonal antibody (1:100; cat. no. 558570; BD Pharmingen). rAAV/AFPp-eGFP-transduced Hep3B cells served as a control.

The mRNA expression of HLA-A2 was also analyzed by RT-PCR. At day 5 of transduction, the total mRNA was isolated from rAAV/AFPp-IFN-γ-transduced Hep3B cells using an Oligotex mRNA isolation Mini kit. Subsequent to the generation of the first strand of the cDNA, PCR amplification was conducted using the primer pairs, 5'-ATGGCCGTCATG GCGCCCCGAAC-3' and 5'-GGCAGCTGTCTCACACTT-3'. The PCR conditions were 30 cycles of denaturation at 94˚C for 50 sec, followed by annealing at 58˚C for 55 sec, and elongation at 72˚C for 60 sec. The resulting PCR products were analyzed by 2% gel electrophoresis.

Generation of AFP-expressing DCs. PBMCs were obtained from the peripheral blood of HLA-A2-positive healthy volunteers, separated by Ficoll density-gradient centrifugation at 400 x g for 20 min, and incubated in six-well culture plates at 37˚C for 2 h in AIM-V medium. Following incubation, the non-adherent cells were removed, and the adherent PBMCs were cultured in AIM-V medium with 800 units/ml human granulocyte macrophage colony-stimulating factor (GM-CSF; R&D Systems, Inc., Minneapolis, MN, USA) and infected with 10^{10} encapsidated genomes (eg)/ml of rAAV/CMVp-AFP. Uninfected PBMCs served as controls. After 8 h, the medium/virus solution was removed, and replaced with fresh AIM-V medium containing 800 units/ml GM-CSF and 1,000 IU/ml human interleukin-4 (IL-4; R&D Systems, Inc.). Every 2 days, the culture was replaced with the fresh medium, including the cytokines. At day 4, 50 IU/ml human tumor necrosis factor-α (R&D Systems, Inc.) was added into the medium. At day 6, the DCs were harvested. To analyze AFP expression, the intracellular staining was conducted as described above. The DCs were stained with PE-conjugated mouse anti-human AFP monoclonal antibody (1:100; cat. no. 563002; BD Pharmingen) and analyzed by fluorescence-activated cell sorting (FACS).

The mRNA expression of AFP was also analyzed by RT-PCR. At day 6 of transduction, the total mRNA was isolated from rAAV/CMVp-AFP-transduced DCs using an Oligotex mRNA isolation Mini kit. Subsequent to the generation of the first strand of the cDNA, PCR amplification was conducted using the primer pairs, 5'-AGTTTGAGGAGAATATTTG-3' and 5'-GGTTGCTAGTTATTTTGTT-3'. The PCR conditions were 30 cycles of denaturation at 94˚C for 50 sec, annealing 58˚C for 55 sec and elongation at 72˚C for 50 sec. The resulting PCR products were analyzed by 2% gel electrophoresis.

Characterization of DCs. Following 6 days of culture, the transduced and control DCs were harvested. A panel of fluorescein isothiocyanate (FITC)-conjugated mouse anti-human monoclonal antibodies was used, including HLA-death receptor (DR; cat. no. 555811), cluster of differentiation (CD)1a (cat. no. 555806), CD40 (cat. no. 555588), CD80 (cat. no. 555683), CD83 (cat. no. 555910) and CD86 (cat. no. 555657). They were all diluted at 1:100 and obtained from BD Pharmingen. For FACS analysis, all the samples were stained with the antibodies.

Analysis of the level of expressed cytokines in AFP-expressed DCs. Following 6 days of culture, the transduced and control DCs were harvested. The intracellular staining assay was performed to analyze the expression of IL-10 and IL-12 using PE-conjugated rat anti-human IL-10 (1:100; cat. no. 559330; BD Pharmingen) and FITC-conjugated mouse anti-human IL-12 (1:100; cat. no. 554574; BD Pharmingen) monoclonal antibodies.

Analysis of activated T cell populations. The rAAV/CMVp-AFP-transduced DCs were mixed with $CD3^+$ T cells subsequent to the DCs being harvested at day 6 (ratio, T:DC, 20:1). $CD3^+$ T cells were isolated from the PBMCs using a Pan T Cell Isolation kit II, according to the manufacturer's protocol (Miltenyi Biotec, Inc., Auburn, CA, USA). The DC-T cell mixtures were cultured in AIM-V medium in the presence of 20 units/ml recombinant human IL-2 (R&D Systems, Inc.) and 20 ng/ml recombinant human IL-7 (R&D Systems, Inc.). The medium in the DC-T cell culture was replaced with the fresh medium, including the cytokines, every 2 days. At day 8, the primed T cell populations were analyzed for their surface markers with immunofluorescence staining by FACS. A panel of FITC- or PE-conjugated mouse anti-human monoclonal antibodies recognizing the following antigens was used: CD4 (cat. no. 555347), CD8 (cat. no. 555634), CD25 (cat. no. 555431) and CD69 (cat. no. 557050). They were all diluted 1:100 and obtained from BD Pharmingen.

Analysis of the expression levels of cytokines in primed T cells. At day 8 post-priming, the T cells were harvested. The intracellular staining assay was performed to analyze the expression of IFN-γ and IL-4. The T cells were incubated at 37˚C for 6 h in AIM-V medium containing 50 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 500 ng/ml ionomicin (Sigma-Aldrich). Brefeldin A (10 ng/ml;

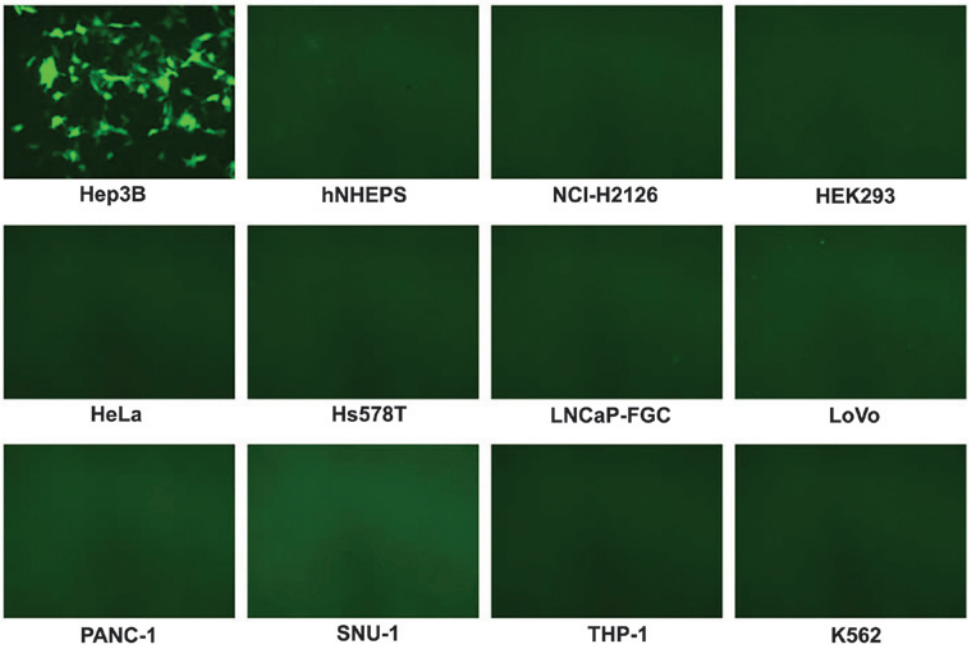

Figure 1. Cell-specific expression of rAAV with the AFP promoter. At day 5 of rAAV/AFPp-eGFP transduction, the eGFP signals were only observed in the infected AFP-positive Hep3B cells, and not in other AFP-negative cell lines, including hNHEPS. rAAV, recombinant adeno-associated virus; AFP, α-fetoprotein, eGFP, enhanced green fluorescent protein; hNHEPS, primary human hepatocytes.

Sigma-Aldrich) was added for the final 5 h of the incubation to completely inhibit cytokine secretion. The cells were washed and fixed with 4% paraformaldehyde and permeabilized with phosphate-buffered saline/0.5% saponin (Sigma-Aldrich) for 10 min. Following incubation, the cells were labeled with FITC-conjugated mouse anti-human IFN-γ (1:100; cat. no. 552882; BD Pharmingen) and PE-conjugated mouse anti-human IL-4 (1:100; cat. no. 559333; BD Pharmingen) monoclonal antibodies for 30 min on ice, prior to flow cytometric analysis.

Cytotoxicity assays. After 8 days of the DC-T cell culture, 6-hour chromium-51 (^{51}Cr) release assays were used to analyze the activity of the CTLs elicited by the rAAV/CMVp-AFP-transduced and control DCs against the target cells (11). The target cells included untreated, rAAV/AFPp-IFN-γ- and rAAV/AFPp-eGFP-transduced Hep3B cells. The ^{51}Cr-labeled target cells were mixed with the CTLs (1:20) and incubated for 6 h at 37˚C with 5% CO_2. To determine the structures on the target cells, the anti-human HLA-A2 monoclonal antibody was used to completely inhibit the cytotoxicity. The ^{51}Cr-labeled targets were preincubated with mouse anti-human HLA-A2 antibody (1:100; cat. no. 551230; BD Pharmingen) for 2 h prior to the ^{51}Cr release assay being performed. To demonstrate the AFP-specific activity of the CTLs, a series of AFP-negative cells were also assessed. K562 cells were used as targets to observe natural killer (NK) cell activity.

Statistics. All data are expressed as the mean ± standard deviation and differences between groups were analyzed using the Student's t-test with SPSS, version 15.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

A series of rAAV vectors were constructed. To construct the rAAV vectors for the present study, the AFP promoter and CMV immediate early promoter were successfully inserted into the dl3-97 vectors. AFP and IFN-γ mRNA was isolated from the human cells and amplified by RT-PCR (data not shown). This cDNA and eGFP cDNA were also successfully cloned into the rAAV vectors. All the cDNA was sequenced and determined to be identical to the published sequence (15,16). The virus stocks of rAAV were generated and titered (data not shown). The viral titers were from 10^{11} to 10^{12} eg/ml.

rAAV with an AFP promoter was specifically expressed in AFP-positive Hep3B cells. To verify that rAAV with the AFP promoter only expressed protein in AFP-positive cells, the AFP-positive Hep3B, and a series of AFP-negative, cells were transduced using rAAV/AFPp-eGFP. After 5 days, only the transduced Hep3B cells expressed eGFP protein (Fig. 1).

IFN-γ was expressed in rAAV/AFPp-IFN-γ-transduced Hep3B cells. To observe whether IFN-γ was expressed in the rAAV/AFPp-IFN-γ-transduced Hep3B cells, intracellular staining was performed. As shown in Fig. 2A, at day 5 post-transduction, the percentage of the IFN-γ-positive cells present was 68.57%. The rAAV/AFPp-eGFP-transduced and untransduced Hep3B cells were IFN-γ-negative. The mRNA expression of IFN-γ analyzed by RT-PCR is presented in Fig. 2B. Furthermore, the secretion of IFN-γ was also observed at various times post-transduction using ELISA. As presented in Fig. 2C, the secretion remained comparably stable for at least 60 days.

HLA-A2 expression was elevated in rAAV/AFPp-IFN-γ-transduced Hep3B cells. To observe whether the expression

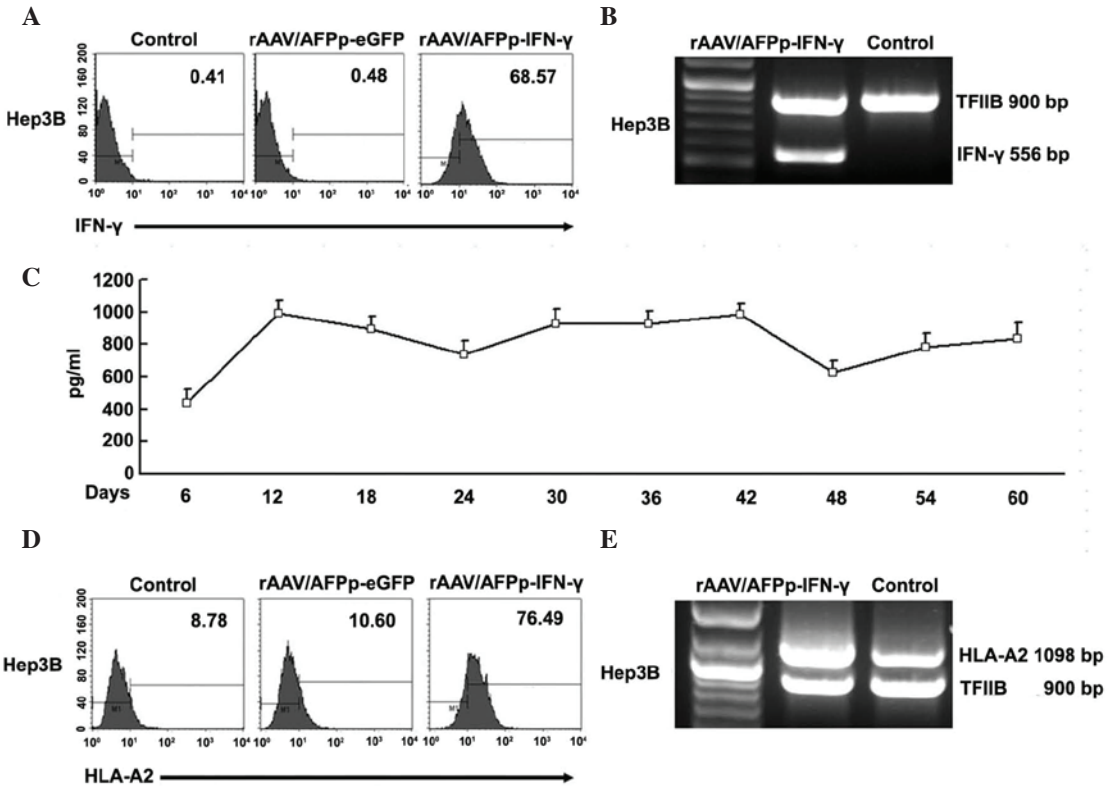

Figure 2. IFN-γ and HLA-A2 expression in the rAAV/AFPp-IFN-γ-transduced Hep3B cells. (A) The expression of IFN-γ was observed in rAAV/AFPp-IFN-γ-transduced Hep3B cells. (B) mRNA expression of IFN-γ was analyzed by RT-PCR. (C) The secretion of IFN-γ by rAAV/AFPp-IFN-γ-transduced Hep3B cells remained comparably stable for at least 60 days. (D) Upregulated expression of HLA-A2 was observed in rAAV/AFPp-IFN-γ-transduced Hep3B cells. P<0.05 vs. untransduced Hep3B cells. (E) Upregulated expression of HLA-A2 mRNA was analyzed by RT-PCR. IFN-γ, interferon-γ; HLA, human leukocyte antigen; rAAV, recombinant adeno-associated virus; RT-PCR, reverse transcription-polymerase chain reaction; AFPp, α-fetoprotein promoter; eGFP, enhanced green fluorescent protein; TFIIB, transcription factor II B.

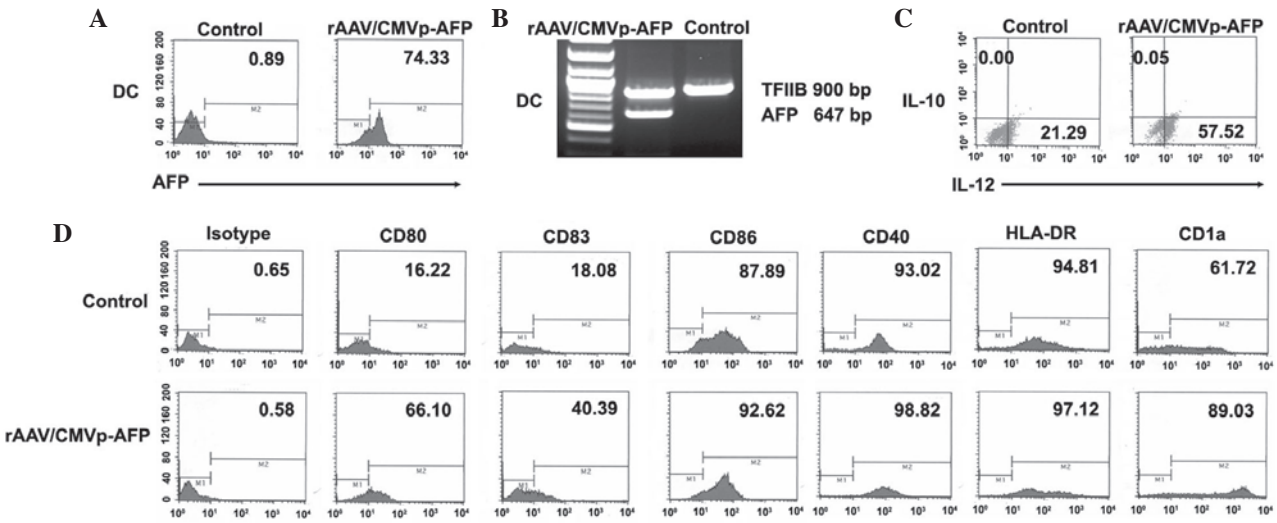

Figure 3. Characterization of DCs by FACS. (A) Percentage of AFP-positive DCs was 74.33% at day 6 following rAAV/CMVp-AFP transduction. (B) mRNA expression of AFP was assessed in rAAV/CMVp-AFP-transduced DCs by reverse transcription-polymerase chain reaction. (C) Higher levels of IL-12 production were detected in rAAV/CMVp-AFP-transduced DCs than in untreated DCs. Almost no IL-10 was detected in the two groups of the DCs. (D) Phenotype of the rAAV/CMVp-AFP-transduced DCs was determined by FACS analysis. The expression levels of CD1a, CD80 and CD83 of the rAAV/CMVp-AFP-transduced DCs were higher. P<0.05 vs. untreated DCs. CD, cluster of differentiation; rAAV, recombinant adeno-associated virus; DCs, dendritic cells; AFP, α-fetoprotein, FACS, fluorescence-activated cell sorting; CMVp, cytomegalovirus promoter; TFIIB, transcription factor II B; IL-10, interleukin-10; IL-12, interleukin-12.

of HLA-A2 was elevated in rAAV/AFPp-IFN-γ-transduced Hep3B cells, the level of expression of HLA-A2 was observed at day 5 post-transduction. As presented in Fig. 2D, the expressed IFN-γ effectively promoted the expression of HLA-A2. The percentage of HLA-A2-positive cells was higher compared with that of the untransduced cells (P<0.05). In addition,

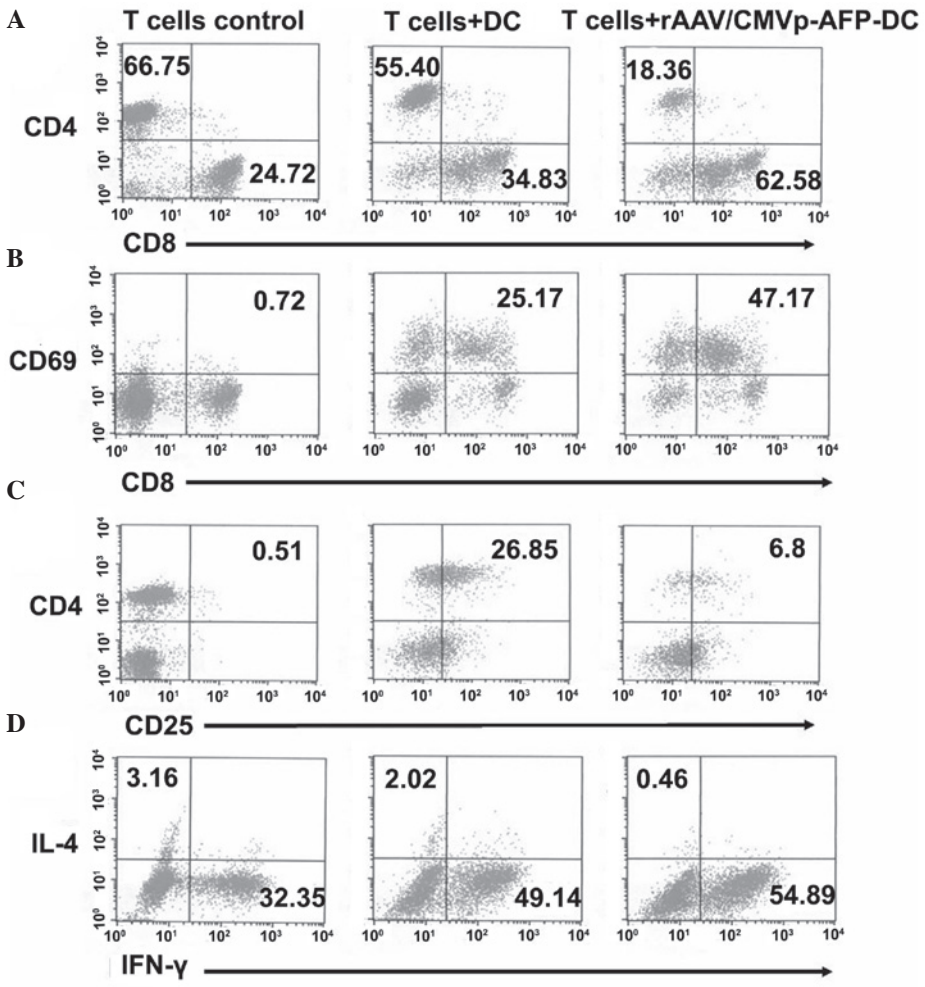

Figure 4. Two-color flow cytometric characterization of surface markers and cytokine secretion in primed T cell populations. (A) The CD8 and CD4 prevalence within the activated T cell populations resulting from the indicated treatments. (B) The CD69 marker prevalence in $CD8^+$ T cells resulting from the indicated treatments. (C) The CD25 marker prevalence in $CD4^+$ T cells resulting from the indicated treatments. The percentages of $CD25^+/CD4^+$ T cells in the T cells primed by rAAV/CMVp-AFP-transduced DCs was lower compared with those primed by untreated DCs ($P<0.05$). (D) The secretion of IFN-γ and IL-4 in the activated T cell population resulting from the indicated treatments The ratio of the expressed IFN-γ/IL-4 molecules in the T cells elicited by rAAV/CMVp-AFP-transduced DCs was higher compared with those primed by untreated DCs ($P<0.05$). CD, cluster of differentiation; IFN-γ, interferon-γ; IL-10, interleukin-10; IL-12, interleukin-12; DCs, dendritic cells; rAAV, recombinant adeno-associated virus; CMVp, cytomegalovirus promoter; AFP, α-fetoprotein.

rAAV/AFPp-eGFP transduction did not result in the elevation of level of HLA-A2 expression ($P>0.05$). The upregulated mRNA expression of HLA-A2 is presented in Fig. 2E.

AFP-pulsed monocyte-derived DCs were generated. To generate AFP-pulsed DCs, the PBMCs were transduced by rAAV/CMVp-AFP at day 0, and cultured in AIM-V medium containing the cytokines. At day 6, the monocyte-derived DCs were harvested and analyzed for AFP protein expression by FACS. The percentage of AFP-positive DCs at day 6 following rAAV/CMVp-AFP transduction was 74.33%, as presented in Fig. 3A. The mRNA expression of AFP analyzed by RT-PCR is presented in Fig. 3B.

DCs were activated by rAAv/CMVp-AFP. The intracellular staining assay was performed to analyze the expression of IL-10 and IL-12 in rAAV/CMVp-AFP-transduced DCs after 6 days of culture. A notably higher level of IL-12 production was detected in rAAV/CMVp-AFP-transduced DCs than in untreated DCs ($P<0.05$). Almost no IL-10 was detected in the rAAV/CMVp-AFP-transduced DCs and untreated DCs in the present study (Fig. 3C).

The phenotype of the rAAV/CMVp-AFP-transduced DCs was determined by FACS analysis to observe whether significant differences were present between untreated and rAAV/CMVp-AFP-transduced DCs. The results demonstrated that the expression levels of HLA-DR, CD40 and CD86 were not significantly different in the two groups of DCs ($P>0.05$). However, the expression levels of CD1a, CD80 and CD83 of the rAAV/CMVp-AFP-transduced DCs were higher ($P<0.05$), as presented in Fig. 3D.

T cells primed by rAAV/CMVp-AFP-transduced DCs were activated. The DC-T cell mixtures were cultured for 8 days. The primed T cell populations were analyzed for their surface CD markers by FACS analysis. As presented in Fig. 4A, the T cells primed by rAAV/CMVp-AFP-transduced DCs had a greater ratio of CD8/CD4 (3.4:1) than the T cells stimulated by untreated DCs (0.63:1; $P<0.05$). Furthermore, the expression level of the CD69 molecules, an early activation marker of T cells, was

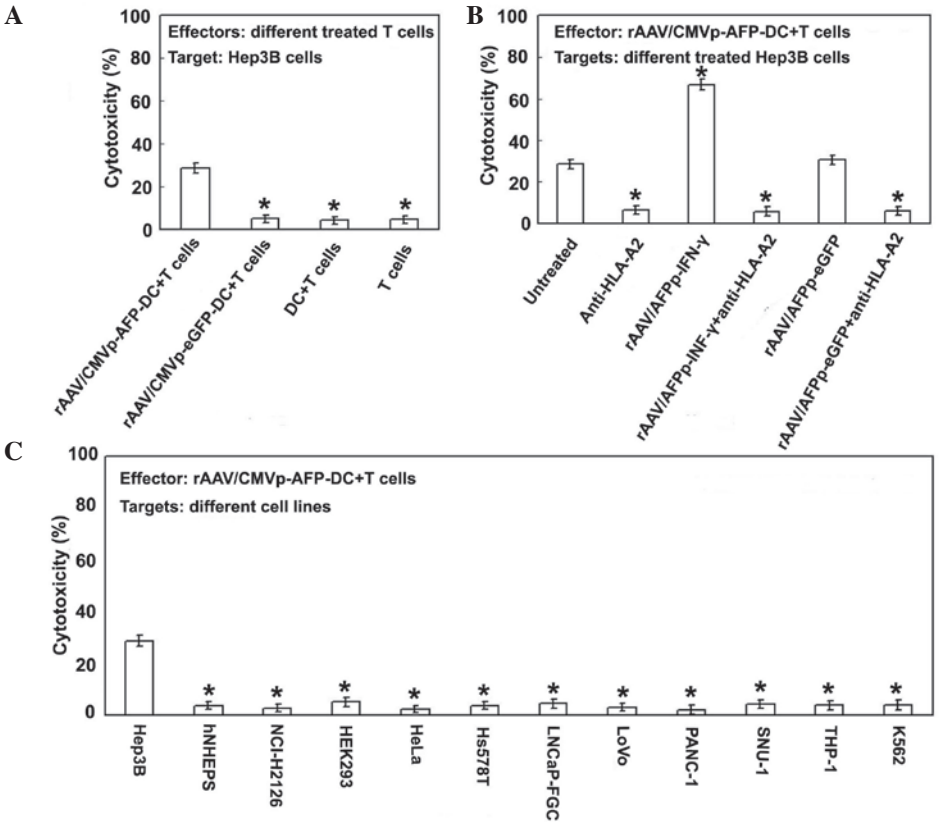

Figure 5. Cytotoxic response of CTLs resulting from rAAV/CMVp-AFP-transduced DCs against the target cells. (A) The activity of CTLs primed by rAAV/CMVp-AFP-transduced DCs against Hep3B cells. *P<0.05 vs. rAAV/CMVp-AFP-DC+T cells. (B) Activity of CTLs primed by rAAV/CMVp-AFP-transduced DCs against the rAAV/AFPp-IFN-γ-transduced Hep3B cells was higher than in untreated and rAAV/AFPp-eGFP-transduced Hep3B cells. The activity declined when the target cells were completely inhibited by an anti-HLA-A2 antibody. *P<0.05 vs. untreated cells. (C) CTLs primed by the rAAV/CMVp-AFP-transduced DCs did not function against AFP-negative cancer cells and primary hepatic cells. NK cell activity was observed using K562 cells as the target. *P<0.05 vs. Hep3B cells. CTLs, cytotoxic T lymphocytes; rAAV, recombinant adeno-associated virus; CMVp, cytomegalovirus promoter; AFPp, α-fetoprotein promoter; DCs, dendritic cells; HLA, human leukocyte antigen; IFN-γ, interferon-γ; eGFP, enhanced green fluorescent protein; NK, natural killer.

observed in the primed $CD8^+$ T cells (19). The percentage of the $CD69^+$ T cells elicited by the rAAV/CMVp-AFP-transduced DCs was higher than that primed by the untreated DCs (47.17 vs. 25.17%; P<0.05; Fig. 4B). $CD25^+/CD4^+$ T regulator (Treg) cells are critical, as they are involved in the suppression of the Th1 response (20,21). As presented in Fig. 4C, the percentages of $CD25^+/CD4^+$ T cells in the T cells primed by the untreated and rAAV/CMVp-AFP-transduced DCs were 26.85 and 6.8%, respectively (P<0.05).

IFN-γ expression was higher in the T cells primed by rAAV/CMVp-AFP-transduced DCs. The intracellular staining assay was performed to analyze the expression of IFN-γ and IL-4 in the T cells on day 8 post-priming. The ratio of the expressed IFN-γ/IL-4 molecules in the T cells elicited by rAAV/CMVp-AFP-transduced DCs (119.3:1) was higher compared with those primed by untreated DCs (24.3:1; P<0.05; Fig. 4D).

rAAV/CMVp-AFP-transduced DCs elicited an AFP-specific and HLA class I-restricted CTL response. ^{51}Cr release assays were conducted over 6 h to analyze the activity of the CTLs elicited by rAAV/CMVp-AFP-transduced DCs against a series of the cells. The activity of CTLs primed by rAAV/CMVp-AFP-transduced DCs observed against Hep3B cells was 28.6±2.3%. The CTLs primed by the untreated or rAAV/CMVp-eGFP-transduced DCs did not lyse or induce cell death in Hep3B cells (Fig. 5A). As presented in Fig. 5B, the level of CTLs against the rAAV/AFPp-IFN-γ-transduced Hep3B cells (66.7±2.8%) was markedly higher than untreated (28.6±2.3%) and rAAV/AFPp-eGFP-transduced cells (30.6±2.4%; P<0.05). To determine whether the CTL response was HLA class I (HLA-A2)-restricted, the target cells were completely inhibited with an anti-HLA-A2 antibody prior to performing the ^{51}Cr release assays. As presented in Fig. 5B, the activity was significantly decreased (P<0.05). To verify that the activity was AFP-specific, a series of AFP-negative cancer cells and primary hepatic cells were also assessed. The results of the ^{51}Cr release assays demonstrated that the CTLs primed by the rAAV/CMVp-AFP-transduced DCs did not function against these cells (Fig. 5C). In addition, NK cell activity was observed using K562 cells as the target. As presented in Fig. 5C, in K562 cells cell death did not occur at notable levels (3.86±1.9%).

Discussion

A number of previous studies have reported that AFP serves as a target for immunotherapy (1,2). The CTLs are activated by antigen-presenting DCs, and HLA class I genes are key

in cellular immune responses mediated by CTLs. To elicit a CTL immune response, the tumor antigenic peptides must form complexes with HLA class I heavy chain and β2-microglobulin (22). However, in certain cases of HCC, few or no HLA class I molecules are expressed by the tumor cells (6-8). A deficiency of HLA class I results in failure of the CTL response.

In the present study, transduction of rAAV/CMVp-AFP resulted in a total of 74.33% of AFP-positive DCs, and high levels of expression of HLA-DR, CD1a, CD40, CD80, CD83 and CD86. In addition, in the rAAV/CMVp-AFP-transduced DCs, the expression level of IL-10 was very low, and the expression level of IL-12 was markedly increased. Mature DCs release large quantities of IL-12, which stimulate a Th1 immune response. The release of IL-10, however, completely inhibits the DC maturation process by interfering with the upregulation of co-stimulatory molecules and production of IL-12, subsequently limiting the ability of the DCs to initiate a Th1 response (23,24). The data from the present study indicated that mature AFP-pulsed DCs were generated. After 8 days of DC-$CD3^+$ T cell culture, activation of the CTLs was achieved by rAAV/CMVp-AFP-transduced DCs. The ratio of the $CD8^+/CD4^+$ T cells (3.4:1) was notably higher compared with the T cells stimulated by untreated DCs (0.63; $P<0.05$). These data suggest that the predominant T cell population was the CTLs (62.58%); 73.7% of the $CD8^+$ T cells were CD69-positive, which is an early activation marker of T cells. Thus, large quantities of activated $CD8^+$ T cells were generated. Furthermore, the Treg cells ($CD25^+/CD4^+$) were present only at as level of 6.8% in the T cell population. In addition, in the primed T cells, the expression level of IL-4 was particularly low, and the expression level of IFN-γ was markedly increased. IFN-γ is a representative Th1 cytokine, and IL-4 is a representative Th2 response cytokine. These data suggest that the use of rAAV/CMVp-AFP-transfected DCs was effective in generating a marked Th1 response. However, the cytotoxicity assays demonstrated that the level of the CTL activity against the AFP-positive Hep3B cells was 28.6±2.3% due to a low level of HLA-A2 expression in the Hep3B cells (8.78%). This suggests that a deficiency of the HLA-A2 expression in the Hep3B cells completely inhibits the CTL response.

HLA class I genes may be induced by type 1 interferon (IFN-α/β) and IFN-γ (12,13). To recover the expression of HLA-A2 in the Hep3B cells, in the present study the human IFN-γ gene was transduced into Hep3B cells by rAAV/AFPp. It is well known that AAV infects a variety of human cells, and rAAVs have a similar ability to infect the cells (25). To provide valuable data for anti-HCC immunotherapy, the transduced genes were expressed in the AFP-positive target cells, but not in the other cells. The AAV main promoter, p5, was replaced with the human AFP promoter in an AAV type 2 vector. All the cells were infected by rAAV/AFPp-eGFP. As presented in Fig. 1, the eGFP was expressed in the AFP-positive cells, Hep3B, but not in AFP-negative cells, including hNHeps. These results indicate that the expression of rAAV with the AFP promoter is AFP-positive and target-specific.

Subsequently, the present study investigated the ability of human IFN-γ gene transduction by rAAV to promote the AFP-specific, HLA-A2-restricted CTL response against the Hep3B cells. IFN-γ proteins were continuously expressed in the rAAV/AFPp-IFN-γ-transfected Hep3B cells for at least 60 days. The expression of HLA class I genes was upregulated at the transcriptional level by IFN-γ. It has been demonstrated that IFN-γ resulted in an increased expression of HLA class I molecules in normal cells (26,27). In the current study, the expression levels of HLA-A2 were upregulated by IFN-γ subsequently to the HLA-A2-deficient HCC cells, Hep3B, being transduced with rAAV/AFPp-IFN-γ. This may be one mechanism underlying the higher percentages of induced cell death in rAAV/AFPp-IFN-γ-transduced Hep3B cells compared with untreated and rAAV/AFPp-eGFP transduced cells.

IFN-γ is the characteristic cytokine of Th1 cells, and is produced by NK cells and CTLs. The level of IFN-γ has been shown to be consistent with the CTL activity (27). The IFN-γ concentration increased when the cytokine was secreted from the rAAV/AFPp-IFN-γ-transduced Hep3B cells into the culture medium of the CTL and Hep3B cell mixtures, thus, the anti-AFP CTL activity was further enhanced. This may be another mechanism that underlies the advantageous transduction of the IFN-γ gene into Hep3B cells.

It was observed that blocking HLA-A2 inhibited cytotoxity, consistent with HLA-A2 restriction. The anti-AFP CTLs were only able to induce cell death in the AFP-expressed Hep3B cells, and exerted little toxicity on the AFP-negative primary hepatocytes and other tumor cells, all of which were AFP-negative. It was demonstrated that the CTLs are HLA class I (HLA-A2)-restricted and AFP antigen-specific.

In conclusion, the data from the present study indicated that rAAV/CMVp-AFP-transduced DCs elicit an AFP-specific and HLA-class I-restricted CTL response against Hep3B cells. The transduction of Hep3B cells with rAAV/AFPp-IFN-γ upregulated the expression of HLA-A2 and improved the sensitivity to the CTL response. The present study also provides a foundation for future studies. There are various possibilities for improving this basic protocol. First, the HLA-A2 gene may be transduced directly into Hep3B cells using rAAV/AFPp in order to compare its ability to improve the CTL response with rAAV/AFPp-IFN-γ. Secondly, the DC vaccine may be further pretreated with additional important cytokines (for example, IL-12) prior to mixing with T cells to enhance DC phenotype and function. Thirdly, a burgeoning body of evidence has demonstrated that rAAV6 is a more efficient serotype for transducing human DCs (28,29), and rAAV3 serotype may specifically and efficiently transduce human HCC cells *in vitro* and *in vivo* (30-32). These approaches may markedly improve the transduction techniques. Furthermore, naturally occurring chemical compounds may also be used to facilitate rAAV vector-mediated transgene expression (33-36).

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