Published in final edited form as:

Vaccine. 2006 April 24; 24(17): 3477–3489.

An effective cancer vaccine modality: Lentiviral modification of dendritic cells expressing multiple cancer-specific antigens

Bei Wang^a, Jin He^a, Chen Liu^b, and Lung-Ji Chang^{a,*}

a Department of Molecular Genetics and Microbiology Powell Gene Therapy Center and McKnight Brain Institute, University of Florida, College of Medicine, Gainesville, FL 32610-0266, USA

b Department of Pathology, Immunology and Laboratory Medicine, University of Florida, College of Medicine, Gainesville, FL 32610-0266, USA

Abstract

Viral modification of dendritic cells (DCs) may deliver a “danger signal” critical to the hyporeactive DCs in cancer patients. Using three highly differentially expressed hepatoma tumor-associated antigens (TAAs): stem cell antigen-2 (Sca-2), glycoprotein 38 (GP38) and cellular retinoic acid binding protein 1 (RABP1), we explored the therapeutic potential of the DCs modified with lentiviral vectors (LVs). Preventive and therapeutic injection of the LV-TAA-DC vaccine into tumor-bearing mice elicited a strong anti-tumor response and extended survival, which was associated with tumor-specific interferon- γ and cytotoxic T cell responses. In vivo elimination of the LV-TAA-DCs by a co-expressed thymidine kinase suicide gene abrogated the therapeutic effect. The modification of DCs with LVs encoding multiple TAAs offers a great opportunity in cancer immunotherapy.

Keywords

Dendritic cells; Lentiviral vectors; Hepatoma; Tumor-associated antigen

1. Introduction

Despite major advances in the development of cancer immunotherapeutics, cancer vaccines in the form of peptides, proteins, and DNA, either alone or with adjuvants have illustrated limited success. Recent insight into the role of dendritic cells (DCs) as the pivotal antigen-presenting cells (APCs) has provided the basis for developing more effective immunotherapy regimens [1–5]. There is known immunological defect in cancer patients involving either cancer cells or patients' own immune cells such as tolerogenic DCs and T cells. Cancer patients may have established immune tolerance to the cancer cells [6]. In addition, a growing body of literature points to restrictions in activation and/or maturation of DCs from the tumor bed of hosts [7, 8]. The rationale for using ex vivo-generated DCs as cancer vaccines is that DC vaccines may break the tolerance to the overburdened cancer antigens in the patients and induce a strong anti-cancer immunity.

DCs process and present antigens through different pathways which leads to a balanced host immune response. Direct loading of antigenic peptides to DCs is an effective way to induce specific immunity [9,10], but is major histocompatibility complex (MHC) type-restricted and the peptide–MHC complexes only engage T cells for short term [11]. A recent clinical trial using bone marrow-derived dendritic cells pulsed with multiple peptides specific to malignant

* Corresponding author. Tel.: +1 352 392 3315; fax: +1 352 392 3133. E-mail address: lchang@mgm.ufl.edu (L.-J. Chang)..

melanomas has failed to show benefit to the stage IV cancer patients. This result suggests that further immune modulation of the cancer patients' DCs is required [12].

An alternative approach to present cancer antigens is to pulse DCs with cancer cells or recombinant proteins [13,14]. However, the latter is limited by the availability of large amount of cancer antigens that can be loaded to DCs. To overcome these limitations, many non-viral and viral gene transfer technologies for modifying DCs have been developed [2,13,15,16]. Recently, HIV-1 derived lentiviral vectors (LVs) have emerged as powerful tools for gene delivery to a variety of dividing and non-dividing cells including DCs [17–19]. LVs have been shown to target DCs with high efficiency with little to no cytotoxicity, and the transduced DCs still retain their immunostimulatory potential [17,20]. We have reported that human monocyte-derived DCs can be efficiently transduced and modified by LVs and the altered DCs can induce a strong T helper type 1 (Th1) response [21].

Hepatocellular carcinoma (HCC) is a major health problem worldwide, causing half a million deaths per year [22]. Limited treatment options for this cancer results in a poor prognosis. Therefore, novel therapeutic strategies and a better understanding of HCC immunobiology are urgently required. Using murine hepatoma 1MEA7R cell line and its parental hepatocyte cell line BNL.CL2 as a model system, we have applied a modified suppression subtractive hybridization (SSH) cDNA screening method and identified a few highly differentially expressed murine hepatoma antigens; among the highest expressed tumor-associated antigens (TAAs) include stem cell antigen-2 (Sca-2), glycoprotein 38 (GP38) and cellular retinoic acid binding protein 1 (RABP1) [23]. These three TAAs are unaltered “self” antigens and, therefore, preexisting immunological tolerance to these antigens may exist in vivo. Self-antigens have been used as cancer antigens to induce anti-cancer immune responses as reported in several clinical trials [24–27]. The main goal of cancer immunotherapy is to enhance immunogenicity of cancer antigens or to break host's cancer tolerance. One way to achieve this is to modify DCs with LVs to induce a strong anti-cancer immune response in vivo.

Modification of the antigen presentation process and the immune modulatory functions of DCs using efficient gene transfer technologies may help induce a strong anti-cancer response. In this study, we have adopted a “multi-antigenic” approach by modifying DCs with LVs expressing multiple TAAs as cancer vaccines, which may overcome possible strong tolerance toward any single TAA [28]. We present evidence that an effective cancer vaccine can be developed based on the multiple LV-TAA-DCs. This LV-TAA-DC vaccine approach induced a strong anti-cancer immune response that may serve as a model system for future clinical applications.

2. Materials and methods

2.1. Mice and cell lines

Female Balb/c mice, 6–8 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in the animal facility at the University of Florida with institutional regulatory approval (Institutional Animal Care and Use Committee). The 1MEA7R mouse liver hepatoma cell line and its normal counterpart BNL.CL2 cell line, derived from Balb/c mice, were obtained from ATCC (Manassas, VA) and maintained in tissue culture at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Gaithersburg, MD) containing 10% heat-inactivated fetal bovine serum and 100 U/ml of penicillin-streptomycin (Gibco BRL).

2.2. Cloning of TAA cDNAs into lentiviral vectors

Full-length cDNA of TAAs were amplified by RT-PCR. Briefly, total RNA was harvested with Tri-reagent (Molecular Research Center Inc., Cincinnati, OH). RNA was reverse transcribed with M-MLV reverse transcriptase and oligo-dT₍₁₅₎ (Promega, Madison, WI). Gene-specific PCR primers used to amplify the three TAA cDNAs were designed according to NCBI published sequences with enhanced translational initiation motif (Sca-2 forward primer: 5'-AAT CTA GAC CAC CAT GTC TGC CAC TTC CAA CAT-3'; Sca-2 reverse primer: 5'-AAG AAT TCT GGT CAG GGG CTC AGC TGC AG-3'; GP38 forward primer: 5'-AAT CTA GAC CAC CAT GTG GAC CGT GCC AGT G-3'; GP38 reverse primer: 5'-AAG AAT TCT TAG GGC GAG AAC CTT CCA G-3'; RABP1 forward primer: 5'-AAT CTA GAC CAC CAT GCC CAA CTT CAC CGG TAC CC-3'; RABP1 reverse primer: 5'-AAG AAT TCT TAC TCC CGG ACA TAA ATT C-3'). RT-PCR was performed with the PCR Master Mix (Promega) for 35 cycles in a Mastercycler gradient (Eppendorf, Hamburg, Germany) at an annealing temperature of 58 °C. The PCR products were purified and cut with *Xba*I and *Eco*RI. The cDNA was then ligated into the lentiviral vector pTYF-EF at *Spe*I and *Eco*RI sites.

2.3. Production of high-titer lentiviral vectors

The lentiviral vector used for TAA gene expression is an improved self-inactivating construct, pTYF [29,30]. pTYF plasmid DNA was used for DNA cotransfection with the lentiviral helper plasmid (pNHP) and a VSV-G envelope plasmid (pHEF-VSVG) into 293T cells. The virus titer was determined by transducing TE671 cell line. This protocol routinely generates 10^{7-8} ml⁻¹ transducing units of LVs and 10^{9-11} ml⁻¹ after concentration.

2.4. Generation and transduction of DCs from mouse bone marrow cells

Mouse DCs were generated from bone marrow suspensions harvested from 6- to 10-week-old Balb/c mice, as previously described but with slight modifications [31]. Briefly, mouse bone marrow was flushed from femurs, passed through a 200 µm mesh to remove fibrous tissue, and red blood cells were lysed using ammonium chloride lysis buffer. Cells were cultured at 10^6 cells/ml in AIM-V medium (Invitrogen, San Diego, CA) containing 2% FBS, 20 ng/ml recombinant murine GM-CSF, and 20 ng/ml murine IL-4. On day 3 and 5 of culture, floating cells were gently removed, and fresh mGM-CSF/IL-4-containing medium was added. On day 7, non-adherent cells and loosely adherent proliferating BMDC aggregates were collected. Flow cytometry analysis was used to determine the purity and surface phenotype.

For LV transduction, day 6 BMDCs were plated in a 24-well plate containing 200 µl of medium supplemented with mGM-CSF/IL-4 and polybrene (8 µg/ml); concentrated LVs were then added to the cells at a multiplicity of infection (MOI) of 20–40, as previously described [29]. The infected cells were incubated at 37 °C for 3 h with gentle shaking every 30 min, 1 ml of DC medium was then added, and the culture was incubated with the viral vector for an additional 12 h. The cells were then treated with AIM-V medium containing 2 mM EDTA at 37 °C for 20 min and washed three times with PBS in order to collect the infected DCs. Transduced DCs for the vaccine were collected 2 days after infection and injected into mice immediately.

2.5. Analysis of migration of the injected LV-DC in vivo

mBMDCs were transduced with LV-eGFP and 5×10^5 GFP-DC were injected s.c. at the end of the tail. Draining lymph nodes (LNs) and the spleen were harvested 24 or 48 h after injection. Red blood cells were lysed using ammonium chloride and followed by flow cytometry analysis of GFP-positive cells.

2.6. LV transduction of BNL.CL2

BNL.CL2 cells were split into 24-well plate and were transduced 17 h later. The cells were infected with LV-Sca-2, LV-GP38, or LV-RABP1 at MOI 20 in 500 ml DMEM complete medium for 3 h. The cells were washed and continuously cultured in DMEM complete medium at 37 °C and 5% CO₂. Two weeks after transduction, total RNA was harvested and RT-PCR was performed, as described above. The quality of the cDNA was confirmed by PCR for GAPDH. Gene-specific PCR products were analyzed on 1% agarose gels containing 0.01 mg/ml ethidium-bromide.

2.7. Flow cytometry

The mouse bone marrow-derived DCs were incubated with optimal concentrations of APC-conjugated mAb against murine CD11c, FITC-conjugated mAb against murine H-2Kd or CD86, and PE-conjugated mAb against murine I-Ad or CD80 (BD Pharmingen, San Diego, CA) for 45 min at 4 °C in PBS buffer containing 2% FBS and 0.1% sodium azide (FCS buffer). After three washes, cells were resuspended in FCS buffer. Analysis was performed on a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA).

2.8. DC immunization and tumor challenge

To test the immunogenicity of the TAAs, five mice were vaccinated with 2×10^5 TAA-DCs s.c. weekly for 2 weeks. Spleens were harvested 10 days after last vaccination. For prophylaxis, the groups of mice were immunized with a subcutaneous injection in the tail containing either 5×10^5 TAA-DCs or nLacZ-DCs. After 7 days, they were challenged with a subcutaneous injection in the front leg flank of 5×10^5 viable 1MEA7R hepatoma cells. For therapeutic evaluation, groups of mice received 5×10^5 DC-nLacZ, DC-TAA or DC-TAA-TK (as indicated) 7 days after a 2×10^5 tumor challenge. To induce DC-TAA-TK apoptosis, mice injected with DC-TAA-TK were treated with GCV (10 mg/kg per day, i.p.) for 3 days. Mice were observed every other day, and when tumors became evident, their perpendicular diameters were measured using a set of calipers. Tumor size was expressed as the average of the diameters. Mice were euthanized when the tumor became ulcerated or surpassed 1 cm³ in size. Statistical analysis for evaluating the survival advantage of the vaccines was performed using SPSS software.

2.9. IFN- γ ELISPOT

Mice were immunized as described above. At the endpoint of the vaccine experiment, splenocytes were collected from each group of mice. Multiscreen 96-well assay plates (Biosource) were precoated overnight at 4 °C with anti-IFN- γ antibody according to vender's instruction. After washing with PBS/0.05% Tween-20 (PBST), plates were blocked for 1 h at 37 °C with PBS/1% BSA. Splenocytes were plated in triplicate wells at a density of 2×10^5 per 100 μ l in AIM V medium. For restimulation, 100 μ l of 4×10^4 1MEA7R, BNL.CL2 cells, or medium alone were added. Plates were cultured overnight at 37 °C with 5% CO₂, washed extensively with PBST and incubated with anti-IFN- γ mAb for 1 h at 37 °C. After washing, goat anti-biotin antibodies (Biosource) were added and incubated for 1 h at 37 °C. Thirty microliters of activator solution (Biosource) was added to develop spots and after 10–30 min the plates were washed with distilled water to stop the reaction. After being air-dried, the number of spots in each well is counted by computerized video-imaging as described before [32], using free ImageJ 1.32 software (National Institutes of Health, USA).

2.10. Intracellular cytokine staining (ICCS) and flow cytometry analysis

Before intracellular cytokine staining, pooled splenocytes were stimulated as indicated. GolgiPlug (BD Pharmingen, San Diego, CA) was added to the cell culture at 1 μ l/ml based on the vender's manual. At the end of the stimulation, cells were harvested and the murine Fc

receptors were blocked using 1 μ g Fc Block/ 10^6 cells (BD Pharmingen) in 100 μ l FCS buffer ($1 \times$ PBS with 2.5% FBS and 0.09% sodium azide) for 15 min at 4 °C. The cells were then washed and stained. Surface antigens of splenocytes were stained using PE-conjugated monoclonal antibody against murine CD8 α , and/or APC-conjugated monoclonal antibody against murine CD4 (BD Pharmingen) for 30 min at 4 °C. Following three washings, cells were fixed and permeabilized by adding 250 μ l BD Cytofix/Cytoperm solution (BD Pharmingen) for 20 min at 4 °C. After two washes with $1 \times$ BD Perm/Wash solution (BD, Pharmingen), intracellular IFN- γ and/or TNF- α were stained with FITC-conjugated monoclonal antibody against murine IFN- γ and APC-conjugated monoclonal antibody against murine TNF- α , respectively. After staining, flow cytometry analysis was performed using FACSCalibur with CellQuest software (Becton Dickinson).

2.11. In vitro antibody blocking assay

Pooled splenocytes (2×10^6) were pre-incubated with 10 μ g/ml functional grade purified monoclonal antibody at 37 °C for 1 h and kept throughout the assay. The antibodies used for blocking were: mouse IgG1 isotype antibody, rat anti-mouse CD4 monoclonal antibody, rat anti-mouse CD8 α monoclonal antibody, mouse anti-mouse H-2D^d monoclonal antibody, mouse anti-mouse H-2K^d monoclonal antibody (BD, Pharmingen), rat anti-mouse MHC class II (I-A/I-E) (eBioscience, San Diego, CA), or rat anti-mouse NKG2D monoclonal antibody (R&D system, Inc. Minneapolis, MN). Antibody blocked splenocytes were then stimulated with 1MEA7R for 16 h with GolgiPlug (BD Pharmingen) and intracellular staining for IFN- γ was performed as described above.

2.12. CTL stimulation and FATAL assay

Splenocytes were obtained from nLacZ-DC or TAA-DCs immunized mice and the red blood cells were lysed using ammonium chloride. Splenocytes were co-cultured with irradiated (10,000 rad) 1MEA7R tumor cells at 37 °C with 5% CO₂ for 5 days in AIM V medium containing 5% FBS, antibiotics, and 10 U/ml murine rIL-2. After 5 days of culture, viable cells were harvested and tested for cytolytic activity by the fluorometric assessment of T lymphocyte antigen specific lysis (FATAL) assay [33]. Target cells (BNL.CL2 or 1MEA7R cells) were first labeled with PKH-26 according to the vendor's instruction (final concentration of 2.5×10^{-6} M, Sigma, St. Louis, MO), at room temperature for 3–5 min. To stop the reaction, a volume of heat-inactivated FBS, equal to that of the cells and dye, was added to the cell suspension and was then incubated for 1 min at room temperature before centrifugation for 5 min at $400 \times g$. After a single wash with 10 ml PBS, the target cells were centrifuged for 5 min and the supernatant was discarded. PKH-26 labeled target cells were then labeled with 5- (and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, Oregon). CFSE was diluted to a final concentration of 2.5×10^{-6} M and added to the target cell suspension. Immediately following the addition of the CFSE, an equal volume of FCS was added to stop the reaction and cells were centrifuged for 5 min at room temperature. Finally, the target cells were washed twice with PBS, resuspended in AIM V medium, and dispensed in duplicate at 5×10^4 cells/well into 96-well U-bottom plates (Becton Dickinson). Effector cells were added at various E:T ratios and mixed with the target cells. The FATAL assay was incubated overnight (16 h) at 37 °C with 5% CO₂. Following incubation, the total contents of the U-bottom plate were transferred to 1.5 ml flow tubes and centrifuged for 5 min at room temperature. The supernatant was discarded and the cell pellet was resuspended in 150 μ l FCS buffer and immediately analyzed by flow cytometry. Flow cytometry was performed with FAC-SCalibur (Becton Dickinson). PKH-26 positive cells were gated and same cell numbers were acquired for each sample. The percentage of specific cell lysis in the target cell population was determined by the disappearance of the antigen labeled targets from the CFSE^{Hi} population compared to the fluorescence of the control targets in the CFSE^{Hi} population. To calculate the percentage of the specific lysis using the FATAL assay the following equations were used:

Percent survival = (mean CFSE^{Hi} percent of test well/mean CFSE^{Hi} percent of spontaneous release) × 100; percent specific lysis = 100 – % survival.

2.13. Immunohistochemistry staining

The paraffin-embedded tissue sections were deparaffinized in xylene and re-hydrated in water. Sections were incubated with 10% goat serum for 15 min, followed by the incubation with primary biotin-conjugated anti-mouse-CD3 monoclonal antibody (BD PharMingen) for 45 min at room temperature. Enzyme-labeled streptavidin was linked to the primary monoclonal antibody for 30 min. The substrate of color development, DAB (DAKO Corp., Carpinteria CA), was added to develop the color. Then, the sections were counterstained by hematoxylin.

2.14. Statistical analysis

All the in vitro experiments were run in triplicate and the results are the mean ± S.D. of triplicate determinations (or representative data from one or two independent experiments). Statistical analysis was performed using the Student's *t*-test and Kaplan–Meier analysis (for the mouse survival analysis). The difference was considered statistically significant when the *P*-value was less than 0.05. All statistical analyses were carried out with the SPSS 11.5 software (Lead Technologies Inc., Chicago, IL).

3. Results

3.1. The mouse bone marrow-derived dendritic cells maintain their surface phenotypes after lentiviral gene transfer

To explore a novel dendritic cell (DC) cancer vaccine strategy, we cloned into lentiviral vectors the full-length cDNAs of three highly differentially expressed tumor-associated antigens that have been previously identified using an improved suppression subtractive hybridization cDNA cloning method [23]. Mouse bone marrow-derived dendritic cells (BMDCs) were cultured and transduced with the three LVs as illustrated in Fig. 1. The DCs were generated from mouse bone marrow using a previously described method with minor modifications [31].

DCs can be efficiently transduced with LVs as we have recently described [21,34]. To verify the LV gene transfer, the mock-transduced and the LV-transduced DCs were analyzed by flow cytometry. The untransduced BMDCs expressed DC surface markers and T cell costimulatory molecules, including CD11c, MHC class I (H-2K^d) and II (I-A^k), CD40, CD80 and CD86 (Fig. 2A). After LV transduction, the DCs exhibited slightly increased expression of MHC class I and II molecules, CD80 and CD40 (Fig. 2B).

To verify the LV transgene expression, we examined Sca-2 expression after LV-Sca-2 gene transfer by flow cytometry. The surface Sca-2 expression in BMDCs was undetectable before LV transduction but increased expression was detected 4 days after LV infection (Fig. 2C). To determine the dose effect of LV transduction of mouse BMDCs, we infected the BMDCs with LV-Sca-2 at four different MOIs (1, 5, 20, and 40), and examined Sca-2 expression by flow cytometry 4 days later. At 40 MOI, more than 25% of the DCs expressed high levels of Sca-2 without obvious cytotoxicity (Fig. 2D and data not shown). The actual transduction efficiency is presumably higher than that detected by flow cytometry based on our previous assay using a sensitive reporter gene, placenta alkaline phosphatase [21]. Sca-2 expression was the highest of the three TAAs in the 1MEA7R hepatoma [23]. To compare the expression levels in BMDCs, we transduced immature DCs with LV-Sca-2, LV-GP38, LV-RABP1 and control LV-nLacZ, and RNA was harvested 48 h later and analyzed by semi-quantitative RT-PCR. The results show that Sca-2 expression was consistently higher than GP38 and RABP1 (Fig. 2E).

3.2. Analysis of in vivo immunogenicity of the three hepatoma TAAs

The target cancer antigens in our study are three highly expressed, non-altered self-antigens, Sca-2, GP38 and RAPB1. The immunogenicity of self-antigens might be low and the antigen-specific T cell clones could potentially have been deleted or tolerized. To examine the immunogenicity of these three hepatoma TAAs in vivo, we vaccinated mice with BMDCs transduced with all three LV-TAAs (LV-TAA-DCs) weekly for 2 weeks and harvested the splenocytes 10 days after the second vaccination. Specific T cell response to the individual antigen was analyzed using the normal hepatocytes (BNL.CL2), which were transduced with the LVs encoding the individual TAAs as target cells. We verified the expression of the TAA genes by RT-PCR using specific primers. Fig. 3A shows the detection of Sca-2, GP38 and RAPB1 RNA in both the LV-transduced BNL.CL2 (P) and 1MEA7R hepatoma cells (T). No TAA RNA was detected in untransduced BNL.CL2 cells (N).

To examine TAA-specific response in vivo, we pooled splenocytes from the same group of vaccinated mice and co-cultured them with BNL.CL2, TAA-BNL.CL2, or 1MEA7R for 16 h and measured specific immune response by intracellular cytokine staining for IFN- γ or TNF- α , co-stained with surface CD8 and CD4 antibodies (Fig. 3B and C). The result illustrated different CD4 and CD8 responses to the individual TAAs. RAPB1 induced a strong CD8 and CD4 T cell response, and GP38 elicited a strong CD8 T cell response and an intermediate CD4 T cell response. Interestingly, Sca-2 appeared to be the least immunogenic among the three TAAs, which elicited a weak CD4 and CD8 response. Nevertheless, compared with unmodified BNL.CL2 cells, all three TAAs were immunogenic in the transduced BNL.CL2 cells and elicited antigen-specific T cell responses in vivo.

3.3. Prophylactic LV-TAA-DC vaccination protects the mice from the hepatoma cell challenge

To determine the potential of the LV-TAA-DC vaccine, BMDCs transduced with the three hepatoma-specific LV-TAAs were tested in a syngeneic Balb/c hepatoma model. Two groups of mice, 10 per group, were immunized weekly for 3 weeks with 5×10^5 DCs transduced with either LV-nLacZ (control) or the three TAA LVs. The mice were then subcutaneously challenged with a lethal dose of 5×10^5 1MEA7R hepatoma cells 7 days after the last DC vaccination, as illustrated in Fig. 4A. After palpable, the tumor growth was monitored every 2 days using a caliper (Fig. 4B). Significant difference in tumor growth was noted between the control and the TAA vaccine groups after 2 months. The control DCs (DC-nLacZ) failed to protect the mice from the hepatoma cell challenge, while the LV-TAA-DC injected mice (DC-TAA) exhibited decreased rate of tumor growth (Fig. 4C), associated with prolonged survival (Fig. 4D).

3.4. Therapeutic LV-TAA-DC vaccination effectively suppresses tumor growth and prolonged survival of the tumor-bearing mice

The prophylactic LV-TAA-DC vaccination, although effective, did not reflect the cancer patient's clinical situations. To test the LV-TAA-DC vaccine in a preexisting cancer model, we injected Balb/c mice with 2×10^5 1MEA7R hepatoma cells, and 6 days later, immunized with the LV-TAA-DC vaccines weekly for 3 weeks, as illustrated in the diagram in Fig. 5A. Compared to the control mice receiving nLacZ-DC injection, the mice treated with LV-TAA-DCs exhibited a significant delay in tumor growth which coincided with prolonged survival (Fig. 5B and C). In repeated experiments, we observed significantly improved survival for mice receiving the LV-TAA-DC vaccine after 1×10^5 tumor cell implantation (data not shown).

3.5. In vivo migration and the validation of the efficacy of the LV-transduced DCs

To engage T cells for immune stimulation, DCs need to migrate to lymphoid organs, such as the draining lymph nodes and spleen. It has been reported that 10^2 – 10^3 antigen-presenting DCs

in the spleen are sufficient to achieve protective levels of CTL activation in mice [35]. To see if the LV-modified DCs could migrate to local LNs in vivo, we transduced BMDCs with a reporter LV-eGFP and followed by subcutaneous injection. At different time points, we collected lymphocytes from the draining LNs from the injection site and spleen and analyzed for GFP-positive cells by flow cytometry. As shown in Fig. 6A, 24 h after injection, GFP-positive cells were found both in the LNs (~1500 cells/2 × LNs/mouse) and spleen (~2000 cells/spleen), but became undetectable (<10 GFP-positive cells) after 48 h. This is consistent with the report that DCs capture epidermal antigens and migrate to peripheral LNs within 24 h [36].

It is not clear why the injected DCs could not be detected after 2 days although the in situ mouse CD8⁺ DCs are known to have 3 days lifespan in the spleen [37]. To see if there were functional LV-DCs in vivo 48 h after subcutaneous injection, we designed an in vivo LV-DC knockout experiment. One week after tumor challenge, three groups of mice were vaccinated with nlacZ-DCs, TAA-DCs, or TAA-TK-DCs. The TAA-TK-DCs were co-transduced with the LV-TAA and the LV-TK. LV-TK encodes the thymidine kinase gene of herpes virus which is cytotoxic in the presence of gancyclovir (GCV). Forty-eight hours after vaccination, the mice were given GCV (10 mg/kg per day, i.p.) for 3 days, to induce apoptosis of the injected TAA-TK-DCs. The result showed that like the control group of mice (nlacZ-DC), the TAA-TK-DC mice developed tumor and no protection effect was observed (Fig. 6B). This data suggest that the LV-DCs are functional and necessary for the protective effect 48 h after injection. Taken together, these data indicate that the LV-modified DCs retain the ability to migrate to LNs and spleen in vivo and can induce a protective immune response.

3.6. Immunohistological analysis of the LV-TAA-DC vaccinated mice

Tumor infiltrating lymphocytes (TILs) are often indicators of improved clinical prognosis in patients with hepatocellular carcinoma [38,39]. To see if the survival rate correlated with the existence of TILs in the mouse hepatoma model, we performed T cell-specific immunohistochemistry staining on tumor tissue sections. Consistent with the survival data, we found that the tumors from vaccinated mice were intensively infiltrated with T cells shown by anti-CD3 antibody staining (Fig. 7A, right panel), as opposed to the tumor tissues from two of the control mice (Fig. 7A, left panel).

One of the concerns with strong anti-TAA immune stimulation is the possibility of inducing an unwanted autoimmune response. We did not observe any adverse effect in the LV-TAA-DC vaccinated, tumor-survival mice after continued observation for over 2 years, the entire life span of the animal. Further examination of major organs (liver, lung, brain, heart, and kidney) revealed no infiltration of lymphocytes and no histological difference between the LV-TAA-DC vaccinated mice and the normal mice after 1 year (representative histological analysis, Fig. 7B, top panel, anti-CD3 Ab staining, and bottom panel, H&E staining).

3.7. LV-TAA-DCs induce tumor-specific cytotoxic T cell immune response

To confirm the existence of a systemic and specific anti-cancer immunity, we examined cell-mediated immune responses in mice treated with the LV-TAA-DCs. At the endpoint of each experiment, the splenocytes were harvested from the control and the LV-TAA-DC injected mice. We performed an IFN- γ ELISPOT assay to detect the anti-cancer specific immune response. The splenocytes were harvested and co-cultured with either the cancer cells (1MEA7R) or normal hepatocytes (BNL.CL2). After 24 h, the splenocytes were removed and IFN- γ production was determined. The results demonstrated that when restimulated with the cancer cells, splenocytes from the vaccinated mice produced a significant greater number of IFN- γ -secreting cells than that from the control mice, but not when they were stimulated with normal hepatocytes or medium (Fig. 8A).

To detect cancer cell-specific cytotoxic lymphocyte (CTL) activity, we conducted a target cell-specific FATAL assay [33]. Spleen T cells from the LV-TAA-DC-injected mice were restimulated with the hepatoma cells for 5 days and tested for CTL activity against different targets, as illustrated in Fig. 8B. The results demonstrate that the spleen cells from LV-TAA-DC primed (vaccine) mice displayed marked tumor-specific CTL activity, while no significant CTL lysis activity was detected in the control nLacZ-DC immunized mice. These results confirm that a cancer cell-specific immunity can be induced by the LV-TAA-DCs, which only target the hepatoma cells but not the normal hepatocytes.

To determine the contribution of different lineages of immune cells to the anti-tumor immunity, we blocked CD4, CD8, NKG2D, MHC class I (H-2K^d/D^d) or class II (I-A/I-E) molecules using specific Abs in co-cultures of splenocytes and stimulator cells, and performed ICCS for IFN- γ . As shown in Fig. 8C, CD4, CD8 and NK cells all contributed, although to different extents, to the IFN- γ production as compared with the control (unblocked) immune cells. The CD8 cells contributed the most (from 50% to 72%) to the hepatoma specific IFN- γ production, while CD4 T cells and NK cells only accounted for 19% and 10%, respectively, of the total IFN- γ production. Blocking H-2K^d/D^d partially eliminated the IFN- γ production by approximately 20–40%, suggesting that H-2K^d/D^d-independent mechanisms also exist, likely through the action of alternative MHC class I molecules, or other MHC class I-independent mechanisms. The I-A/I-E (MHC class II) did not appear to contribute to any of the IFN- γ -related anti-tumor response.

4. Discussion

The lack of specific anti-cancer immune response in cancer patients is often associated with immune tolerance to the cancer cells. Cancer vaccines composed of cancer cells alone, or cancer-specific genes, proteins and peptides, may not be sufficient to overcome the existing immune tolerance. To break the tolerance, it is necessary to introduce a “danger signal” in order to initiate a strong anti-cancer immunity. Such danger signals can be in the form of bacterial toxins or viral RNAs. In this study, we modified dendritic cells with LVs that encode multiple cancer antigens; these are highly differentially expressed hepatoma antigens identified previously by suppression subtractive hybridization cDNA cloning [23]. Combining the three hepatoma antigens, Sca-2, GP38, and RABP1, we demonstrated an effective anti-cancer immunity using modified dendritic cells.

The mBMDCs transduced with LVs express high levels of transgenes with little to no effect on DC surface phenotypes. However, further analysis will be necessary to evaluate possible “danger signal” effects from LV transduction. Antigen concentration is a crucial parameter for the induction of a potent immune response. Transduction of DCs with viral vectors that have been engineered to express TAAs can result in high levels of transgene expression necessary for a strong immune response [16]. Various viral vectors, including poxvirus [40], adenovirus [41], and conventional oncoretrovirus [42], have been used to genetically modify DCs. LVs offer several advantages over other vectors for targeting DCs. First, they can efficiently transduce non-dividing, monocyte-derived DCs, as well as bone marrow-derived DCs [19, 43]. Second, LVs establish stable, long-term infection with minimal cytotoxicity. Third, LVs express the transgene of interest (up to 9 kb) without viral gene expression and do not induce unwanted viral immune responses [44,45]. Finally, we have recently shown that DCs are amenable to genetic manipulations by LVs and can be modified to up-regulate its Th1 functions [21].

By evaluating both prophylactic and therapeutic properties of the LV-TAA-DCs in the Balb/c hepatoma model, we demonstrated a strong protection against the syngeneic hepatoma: 9 out of 15 mice challenged with cancer cells after vaccination remained tumor free for 140 days;

10 out of 15 mice with a pre-established tumor did not experience additional tumor growth for more than 120 days. However, not all of the mice were protected from the LV-TAA-DC vaccines. It is possible that cancer antigen epitopes are not efficiently recognized by host immune cells due to the immune escape functions of the cancer cells, such as the expression of immune suppressors, the up-regulation of apoptosis signals, and down-regulation of MHC, β 2-microglobulin or peptide transport proteins (TAP-1/2) [6,46,47]. Using all three TAAs for DC modification, our approach entails the co-delivery of multiple cancer-specific antigens to induce a broad range of anti-cancer immunity. DCs may be modified by LVs encoding additional immune modulatory molecules to overcome the intrinsic immune suppression from the tumor cells. Using human monocyte-derived DCs which are transduced with LVs encoding IFN- γ and IL-12, we have detected a strong Th1 response in a DC:T cell co-culture system [21].

It has been reported that the injected DCs do not migrate as efficiently as the resident DCs in situ from the skin to the draining lymphnodes [36,48]. However, the in vivo tracking experiment showed that the LV-TAA-DCs can migrate to the local draining lymphnodes within 48 h of vaccination. This could be due to the status of the DCs transduced with LVs, which may have activated the “danger signal” mechanism that promotes the DC migration. By using the suicide tk gene-modified DCs, we demonstrated a direct correlation of the viability of the DC vaccine with the ensuing animal survival; the DC vaccine needs to be viable for at least 48 h to be effective. This confirms that a prolonged engagement of the DC vaccine within the host immune system is necessary for the efficacy of the vaccine.

The TAAs are non-mutated self-antigens and could be expressed in normal tissues and cells. Targeting these antigens by modified DC vaccines may induce immune responses against normal organs and tissues leading to autoimmune diseases. However, we did not observe any abnormal autoimmune responses in the vaccinated mice. Tumor-free mice remained healthy for more than one and a half years. Possibilities of autoimmune responses were also studied by immunohistochemical staining of major organs of the vaccinated mice. We did not find any abnormal histological phenotypes or T cell infiltrations in the organs of the vaccinated mice. This does not exclude the possibility that mild autoimmune reactivity might have occurred early during vaccination. However, abnormal autoimmune diseases or related symptoms were not observed in any of the vaccinated mice. These results indicate that the LV-TAA-DC vaccination approach can induce a strong anti-tumor immune response while, at the same time, without provoking a harmful autoimmune reaction.

The contribution of different lineages of immune cells to the anti-cancer immunity of the DC vaccine is delineated using the antibody blocking assays. It appears that the protective immunity elicited by the LV-TAA-DC vaccine is primarily consisted of CD8 T cell response and less dependent on the MHC II-mediated immune reaction. If so, the MHC class I-restricted cancer-specific epitopes must serve as important anti-cancer immunogens. To identify potential MHC-I TAA peptides, we searched for H-2K^d binding nanomer peptides from each of the TAAs (Sca-2, CRABP1, and GP38) using the SYFPEITHI program (<http://syfpfeithi.bmi-heidelberg.com/>). The peptides were screened for proteasomal cleavage sites using the NetChop program (<http://www.cbs.dtu.dk/Services/NetChop>). The best-fit epitopes of the three TAAs that fulfill both criteria with the highest affinity score were synthesized and tested. To determine whether the selected peptides could stimulate CD8+ spleen T cells from the TAA-DC vaccinated mice, intracellular cytokine staining for IFN- γ was performed. Interestingly, all three predicted epitopes from the three TAAs failed to stimulate cancer-specific CD8 T cells from the LV-TAA-DC vaccinated mice (data not shown). This corroborates the recent report of the lack of anti-cancer efficacy with peptide-loaded DCs in treating melanoma patients [12]. It is possible that the predicted epitopes do not represent the best antigenic epitopes in vivo, or that the selected epitopes are not sufficient to induce a

strong anti-cancer response as compared with the whole antigens that are expressed by the LVs. Our studies strongly suggest that for the best vaccine efficacy, the presentation of the full-length antigen may be necessary.

Future studies should aim at optimization of the DC immune functions to overcome existing cancer tolerance. The combination of LV-TAA-DCs and the SSH method of identifying highly differentially expressed cancer antigens represents an important first step toward defeating the most immune resilient cancers.

Acknowledgements

We thank X. Chen and W. Chou for technical assistance and G. Eubanks for editorial assistance with the manuscript. Part of the study had been presented in 2005 Keystone meeting and European Society of Gene Therapy meeting. This work was supported by NIH grant HL59412. L.J. Chang is a consultant to Innova Science PTE with financial interest to the lentiviral vector system used in this study.

References

- Esche C, Shurin MR, Lotze MT. The use of dendritic cells for cancer vaccination. *Curr Opin Mol Ther* 1999;1(1):72–81. [PubMed: 11249687]
- Kirk CJ, Mule JJ. Gene-modified dendritic cells for use in tumor vaccines. *Hum Gene Ther* 2000;11(6):797–806. [PubMed: 10779157]
- Schuler G, Schuler-Thurner B, Steinman RM. The use of dendritic cells in cancer immunotherapy. *Curr Opin Immunol* 2003;15(2):138–47. [PubMed: 12633662]
- Ridgway D. The first 1000 dendritic cell vaccinees. *Cancer Invest* 2003;21(6):873–86. [PubMed: 14735692]
- Banchereau J, Palucka AK. Dendritic cells as therapeutic vaccines against cancer. *Nat Rev Immunol* 2005;5(4):296–306. [PubMed: 15803149]
- Smyth MJ, Godfrey DI, Trapani JA. A fresh look at tumor immuno-surveillance and immunotherapy. *Nat Immunol* 2001;2(4):293–9. [PubMed: 11276199]
- Gabrilovich DI, Nadaf S, Corak J, Berzofsky JA, Carbone DP. Dendritic cells in antitumor immune responses. II. Dendritic cells grown from bone marrow precursors, but not mature DC from tumor-bearing mice, are effective antigen carriers in the therapy of established tumors. *Cell Immunol* 1996;170(1):111–9. [PubMed: 8665591]
- Almand B, Resser JR, Lindman B, et al. Clinical significance of defective dendritic cell differentiation in cancer. *Clin Cancer Res* 2000;6(5):1755–66. [PubMed: 10815894]
- Mayordomo JI, Zorina T, Storkus WJ, et al. Bone marrow- derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumor immunity. *Nat Med* 1995;1:1297–302. [PubMed: 7489412]
- Brossart P, Wirths S, Stuhler G, Reichardt VL, Kanz L, Brugger W. Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells. *Blood* 2000;96(9):3102–8. [PubMed: 11049990]
- Thurner B, Roder C, Dieckmann D, et al. Generation of large numbers of fully mature and stable dendritic cells from leukapheresis products for clinical application. *J Immunol Methods* 1999;223(1):1–15. [PubMed: 10037230]
- Banchereau J, Ueno H, Dhodapkar M, et al. Immune and clinical outcomes in patients with stage IV melanoma vaccinated with peptide-pulsed dendritic cells derived from CD34+ progenitors and activated with type I interferon. *J Immunother* 2005;28(5):505–16. [PubMed: 16113607]
- Gilboa E, Nair SK, Lyster HK. Immunotherapy of cancer with dendritic-cell-based vaccines. *Cancer Immunol Immunother* 1998;46(2):82–7. [PubMed: 9558003]
- Santin AD, Hermonat PL, Ravaggi A, et al. Induction of human papillomavirus-specific CD4(+) and CD8(+) lymphocytes by E7-pulsed autologous dendritic cells in patients with human papillomavirus type 16- and 18-positive cervical cancer. *J Virol* 1999;73(7):5402–10. [PubMed: 10364287]
- Mitchell DA, Nair SK. RNA-transfected dendritic cells in cancer immunotherapy. *J Clin Invest* 2000;106(9):1065–9. [PubMed: 11067858]

16. Jenne L, Schuler G, Steinkasserer A. Viral vectors for dendritic cell-based immunotherapy. *Trends Immunol* 2001;22(2):102–7. [PubMed: 11286712]
17. Schroers R, Sinha I, Segall H, et al. Transduction of human PBMC-derived dendritic cells and macrophages by an HIV-1-based lentiviral vector system. *Mol Ther* 2000;1(2):171–9. [PubMed: 10933928]
18. 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. [PubMed: 11133750]
19. Chang L-J, Gay EE. The molecular genetics of lentiviral vectors—current and future perspectives. *Curr Gene Ther* 2001;1:237–51. [PubMed: 12109139]
20. Gruber A, Kan-Mitchell J, Kuhen 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. [PubMed: 10942374]
21. Chen X, He J, Chang L-J. Alteration of T cell immunity by lentiviral transduction of human monocyte-derived dendritic cells. *Retrovirology* 2004;1:37. [PubMed: 15518595]
22. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003;362(9399):1907–17. [PubMed: 14667750]
23. He J, Chang L-J. Functional characterization of hepatoma- specific stem cell antigen-2. *Mol Carcinog* 2004;40:90–103. [PubMed: 15170814]
24. Zeh HJ 3rd, Perry-Lalley D, Dudley ME, Rosenberg SA, Yang JC. High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy. *J Immunol* 1999;162(2):989–94. [PubMed: 9916724]
25. Dhodapkar MV, Steinman RM, Sapp M, et al. Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. *J Clin Invest* 1999;104(2):173–80. [PubMed: 10411546]
26. Fong L, Brockstedt D, Benike C, Wu L, Engleman EG. Dendritic cells injected via different routes induce immunity in cancer patients. *J Immunol* 2001;166(6):4254–9. [PubMed: 11238679]
27. Dudley ME, Rosenberg SA. Adoptive-cell-transfer therapy for the treatment of patients with cancer. *Nat Rev Cancer* 2003;3(9):666–75. [PubMed: 12951585]
28. Radvanyi L. Discovery and immunologic validation of new antigens for therapeutic cancer vaccines. *Int Arch Allergy Immunol* 2004;133(2):179–97. [PubMed: 14764946]
29. Chang L-J, Urlacher V, Iwakuma T, Cui Y, Zucali J. Efficacy and safety analyses of a recombinant human immunodeficiency virus type 1 derived vector system. *Gene Ther* 1999;6:715–28. [PubMed: 10505094]
30. Iwakuma T, Cui Y, Chang LJ. Self-inactivating lentiviral vectors with U3 and 5 modifications. *Virology* 1999;261(1):120–32. [PubMed: 10441560]
31. Inaba K, Inaba M, Romani N, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1992;176(6):1693–702. [PubMed: 1460426]
32. Cui Y, Chang L-J. Computer-assisted, quantitative cytokine ELISPOT analysis of human immune effector cell function. *BioTechniques* 1997;22:1146–9. [PubMed: 9187766]
33. Sheehy ME, McDermott AB, Furlan SN, Klenerman P, Nixon DF. A novel technique for the fluorometric assessment of T lymphocyte antigen specific lysis. *J Immunol Methods* 2001;249(1–2):99–110. [PubMed: 11226468]
34. Chen X, Chang L-J. Mycoplasma-mediated alterations of in vitro generation and functions of human dendritic cells. *J Biomed Sci* 2005;12:31–46. [PubMed: 15864737]
35. Ludewig B, Ehl S, Karrer U, Odermatt B, Hengartner H, Zinkernagel RM. Dendritic cells efficiently induce protective antiviral immunity. *J Virol* 1998;72(5):3812–8. [PubMed: 9557664]
36. Kissenpfennig A, Henri S, Dubois B, et al. Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. *Immunity* 2005;22(5):643–54. [PubMed: 15894281]
37. Kamath AT, Henri S, Battye F, Tough DF, Shortman K. Developmental kinetics and lifespan of dendritic cells in mouse lymphoid organs. *Blood* 2002;100(5):1734–41. [PubMed: 12176895]

38. Shirabe K, Matsumata T, Maeda T, Sadanaga N, Kuwano H, Sugimachi K. A long-term surviving patient with hepatocellular carcinoma including lymphocytes infiltration—a clinicopathological study. *Hepatology* 1995;42(6):996–1001. [PubMed: 8847059]
39. Wada Y, Nakashima O, Kutami R, Yamamoto O, Kojiro M. Clinicopathological study on hepatocellular carcinoma with lymphocytic infiltration. *Hepatology* 1998;27(2):407–14. [PubMed: 9462638]
40. Kim CJ, Prevette T, Cormier J, et al. Dendritic cells infected with poxviruses encoding MART-1/Melan A sensitize T lymphocytes in vitro. *J Immunother* 1997;20(4):276–86. [PubMed: 9220317]
41. Sloan JM, Kershaw MH, Touloukian CE, et al. MHC class I and class II presentation of tumor antigen in retrovirally and adenovirally transduced dendritic cells. *Cancer Gene Ther* 2002;9(11):946–50. [PubMed: 12386833]
42. Kondo E, Topp MS, Kiem HP, et al. Efficient generation of antigen-specific cytotoxic T cells using retrovirally transduced CD40- activated B cells. *J Immunol* 2002;169(4):2164–71. [PubMed: 12165546]
43. Chinnasamy N, Chinnasamy D, Toso JF, et al. Efficient gene transfer to human peripheral blood monocyte-derived dendritic cells using human immunodeficiency virus type 1-based lentiviral vectors. *Hum Gene Ther* 2000;11(13):1901–9. [PubMed: 10986562]
44. Kordower JH, Emborg ME, Bloch J, et al. Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science* 2000;290(5492):767–73. [PubMed: 11052933]
45. Lizee G, Gonzales MI, Topalian SL. Lentivirus vector-mediated expression of tumor-associated epitopes by human antigen presenting cells. *Hum Gene Ther* 2004;15(4):393–404. [PubMed: 15053864]
46. Hahne M, Rimoldi D, Schroter M, et al. Melanoma cell expression of Fas(Apo-1/CD95) ligand: implications for tumor immune escape. *Science* 1996;274:1363–6. [PubMed: 8910274]
47. Ellem KA, Schmidt CW, Li CL, et al. The labyrinthine ways of cancer immunotherapy—T cell, tumor cell encounter: “how do I lose thee? Let me count the ways”. *Adv Cancer Res* 1998;75:203–49. [PubMed: 9709811]
48. Adema GJ, de Vries IJ, Punt CJ, Figdor CG. Migration of dendritic cell based cancer vaccines: in vivo veritas? *Curr Opin Immunol* 2005;17(2):170–4. [PubMed: 15766677]

Fig. 1.

An overview of multi-antigenic DC vaccine strategy. Highly differentially expressed murine hepatoma antigens were identified by the SSH cDNA cloning method. The full-length cDNA was cloned into the lentiviral pTYF vector. Lentiviral vectors encoding different TAAs were produced and used to co-transduce mouse bone marrow-derived immature DCs. The transduced DCs were harvested and used as anti-cancer vaccines.

Fig. 2.

Analysis of BMDC surface phenotype before and after LV-TAA transduction. DCs were generated from the bone marrow of Balb/c mice as described in Section 2. Immature DCs (day 7) were harvested and transduced with LVs. (A) Mouse BMDC phenotype analysis. The d7 BMDCs were collected and incubated with fluorescence-conjugated monoclonal Abs against murine CD 11c, H-2K^d (MHC class I), I-A^k (MHC class II), CD80, CD86 and CD40 for 30 min at 4 °C as indicated and analyzed by flow cytometry; shadowed area depicts isotype Ig control. (B) Mouse BMDC phenotype analysis after LV transduction. Forty-eight hours after transduction, DCs were incubated with fluorescence-conjugated Abs as indicated; shadowed area, isotype Ig control; dotted line, mock-transduced DCs; solid line, LV-TAA-DCs. (C) Flow cytometry analysis of BMDCs transduced by LV-Sca-2 (MOI = 40). (D) Quantitative analysis of LV-Sca-2 expression in DCs. Day 7 DCs were transduced with different MOI of LV-Sca-2: 1, 5, 20 and 40. Four days after transduction, the DCs were harvested and stained with FITC-conjugated Ab against murine Sca-2 and the transduction efficiency was analyzed by flow cytometry. The graphs were representative of two repeated experiments. (E) Semi-quantitative analysis of LV-TAA expression in BMDCs. Day 5 mBMDCs were transduced with LV-Sca-2, LV-GP38, LV-RABP1 or LV-nLacZ. The total cellular RNA was harvested 48 h later, reverse transcribed and amplified after serial dilution (1, 1/10, 1/100, 1/1000 and 0) using gene-specific primers. RT-PCR of GAPDH RNA was included as internal control. No-RT control was done

using 500 ng RNA in stead of cDNA. Same amount of PCR products were loaded and run in 1% agarose gel.

Fig. 3.

Analysis of in vivo immunogenicity of LV-TAA-DCs. (A) Expression of TAAs in LV-transduced BNL.CL2 cells. Normal hepatocytes (BNL.CL2) were transduced with LV-Sca-2, LV-GP38 or LV-RABP1. The level of transgene expression was determined by RT-PCR using total cellular RNA and the TAA gene-specific primers as depicted. N, negative control (BNL.CL2); P, positive control (1MEA7R); T, transduced BNL.CL2. RT-PCR of GAPDH RNA was included as control. (B) and (C) Analysis of cell-mediated immune response by intracellular cytokine staining. Balb/c mice were injected with the LV-TAA-DCs twice in weekly intervals and the splenocytes (1×10^6) of five mice were pooled and stimulated for 16 h with 2×10^5 BNL.CL2, BNL.CL2 transduced with a LV-TAA or 1MEA7R cells. The intracellular IFN- γ or TNF- α was stained using specific Abs; left panel represents CD8 T cells and IFN- γ staining, and right panel CD4 T cells and TNF α staining. Error bars depict standard deviations (S.D.) of three independent experiments.

Fig. 4.

In vivo protective immunity against tumor challenge by prophylactic immunization with LV-TAA-DCs. (A) DC immunization and tumor injection scheme. Balb/c mice (10 per group) were immunized s.c. with nLacZ-DCs or LV-TAA-DCs weekly for 3 weeks at a dose of 5×10^5 DCs/injection. Seven days after immunization, the mice were injected s.c. with 5×10^5 of 1MEA7R hepatoma cells. (B) Representative tumor size in mice at endpoints; scale is shown in cm. (C) Tumor growth kinetics. The tumor size was measured over time using caliper and the mean tumor diameter (in mm) was determined. Error bars depict standard deviation (S.D.), $n = 10$. The LV-TAA-DC vaccines resulted in statistically significant protection compared to the control nLacZ-DCs after day 14 ($P < 0.01$). (D) Survival Kaplan-Meier curves. The curves were generated from the survival data ($n = 10$).

Fig. 5. Suppression of the tumor growth and extension of the survival of tumor-bearing mice after LV-TAA-DC immunization. (A) Tumor implantation and DC immunization scheme. The mice, 10 per group, were inoculated s.c. with 2×10^5 hepatoma cells on day 0, and on day 6 the mice received 5×10^5 LV-TAA-DCs or nLacZ-DCs weekly for 3 weeks. (B) Tumor growth kinetics. The measurement was made when the tumor was palpable; error bars depict SD. (C) Kaplan-Meier survival plots. The plots were generated from the survival data, $n = 10$ per group. Mice that became moribund were killed. Data are representative of two independent experiments.

Fig. 6.

In vivo migration and knockout kinetic study of the injected LV-DCs. (A) Detection of LV-GFP expression in the injected DCs after migration to the draining lymph nodes and the spleen. The draining lymph nodes and the spleen were harvested 24 or 48 h after injection and analyzed by flow cytometry for GFP-positive cells. (B) Kaplan–Meier survival plots of mice with or without the knockout the LV-TAA-DC in 48 h. The mice were inoculated with 1MEA7R tumor cells, and 1 week later, vaccinated with DC-nLacZ, DC-TAA or DC-TAA-TK. Forty-eight hours after vaccination, the DC-TAA-TK injected mice were treated with GCV i.p. for 3 days. The plots were generated from the survival data, $n = 5$ per group.

Fig. 7. Immunohistochemical staining of tumor infiltrating T cells and histological evaluation of organs of the vaccinated mice. (A) Immunohistochemical staining of tumor infiltrating T lymphocytes. The paraffin-embedded tissue sections were prepared and stained with monoclonal anti-CD3 antibody as described in Section 2. The left panel showed tumors from the control mice, and the right panel from the LV-TAA-DC vaccinated mice. (B) H&E and infiltrating T cell staining in liver and lung of the LV-TAA-DC vaccinated mice. The upper panel represents H&E staining and the lower panel anti-CD3 antibody staining.

Fig. 8.

Analyses of tumor-specific immune response in the LV-TAA-DC-immunized mice. (A) ELISPOT assays for immune cells releasing IFN- γ . Splenocytes harvested from the DC-injected mice were restimulated with medium alone, 1MEA7R hepatoma cells or BNL.CL2 normal hepatocytes. 1MEA7R or BNL.CL2 cells were cultured with splenocytes at 1:5 ratios for 24 h at 37 °C, and the IFN- γ -secreting cells (spots) were visualized and quantified. The column represents the average of counted spots per well which was normalized against background from three independent experiments with S.D. error bars. Representative spot-containing wells are shown below each corresponding column. (B) FATAL assay for anti-cancer cytotoxicity. The splenocytes were restimulated for 5 days with irradiated 1MEA7R tumor cells. The FATAL assays, as described in Section 2, were performed to evaluate the cytotoxic activity against 1MEA7R and BNL.CL2 cells. The representative of three experiments is shown. (C) In vitro antibody blocking experiments. The pooled splenocytes (1×10^6) were pre-incubated with blocking antibodies for 1 h at 37 °C as indicated at bottom of the graph, and then stimulated with 1MEA7R (2×10^5) for 16 h and treated with Brefeldin A. Intracellular IFN- γ was stained and analyzed by FACS. The experiments were repeated three times and presented with S.D. error bars. Student's *t*-test was performed with $P < 0.05$ for CD8 and MHC I, and $P < 0.08$ for CD4 and NK.