Immunotherapy for Lewis lung carcinoma utilizing dendritic cells infected with CK19 gene recombinant adenoviral vectors

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Abstract. Dendritic cells (DCs) as 'professional' antigen-presenting cells (APCs) initiate and regulate immune responses to various antigens. DC-based vaccines have become a promising modality in cancer immunotherapy. Cytokeratin 19 (CK19) protein is expressed at high levels in lung cancer and many other tumor cells, suggesting CK19 as a potential tumor-specific target for cancer immune therapy. We constructed a recombinant adenoviral vector containing the CK19 gene (rAd-CK19). DCs transfected with rAd-CK19 were used to vaccinate C57BL/6 mice bearing xenografts derived from Lewis lung carcinoma (LLC) cells. The transfected DCs gave rise to potent CK19-specific cytotoxic T lymphocytes (CTLs) capable of lysing LLC cells. Mice immunized with the rAd-CK19-DCs exhibited significantly attenuated tumor growth (including tumor volume and weight) when compared to the tumor growth of mice immunized with rAd-c DCs or DCs during the 24-day observation period ($P<0.05$). The results revealed that the mice vaccinated with the rAd-CK19-DCs exhibited a potent protective and therapeutic antitumor immunity to LLC cells in the subcutaneous model along with an inhibitive effect on tumor growth compared to the mice vaccinated with the rAd-c DCs or DCs alone. The present study proposes a meaningful mode of action utilizing rAd-CK19 DCs in lung cancer immunotherapy.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide (1). Despite recent advances in treatment, the overall survival of patients with lung cancer remains only 15% at 5 years, and this declines to less than 2% in patients with metastatic disease (2). Traditional chemotherapy predominantly kills drug-sensitive cells, leaving behind a heterogeneous population of resistant cells, including those that have the potential to re-populate the tumor or seed new metastatic sites. Immunotherapy has been considered as a valuable treatment for lung carcinoma. It has many advantages including: its specificity to tumor cells and its capability of eliciting a long-lasting immune memory response to kill tumor cells.

Many antitumor vaccines have utilized cell-surface proteins as target molecules, such as livin protein (3-5). Cytokeratin 19 (CK19), a type I cytokeratin, belongs to a group of intermediate filament proteins forming the cytoskeleton. CK19 is the smallest known acidic cytokeratin and is not paired with a basic cytokeratin, unlike the other proteins of this group. *In vitro* studies of rat lung alveolar epithelial cells have shown high expression of CK19 in intensively proliferating type II pneumocytes (6). Increased expression of this protein was found by immunohistochemical staining in lung cancer cells in humans, and a particularly strong overexpression was observed in cases of squamous cell carcinoma and adenocarcinoma (7). Since CK19 is overexpressed in a variety of human tumors, but poorly or unexpressed in normal differentiated cells, it may be a suitable target for tumor immunotherapy (8). In the present study, we explored an immunization strategy using H-2^b positive mouse DC2.4s transduced with rAd-CK19 dendritic cells (DCs) to induce the resistance of C57BL/6 mice to Lewis lung carcinoma (LLC) cells. The present study should be valuable for DC-based lung cancer immunotherapy.

Materials and methods

Mice and cell lines. C57BL/6 mice [SCXK (SU) 2011-0003] (21-35 days of age and 18-22 g in weight) were purchased from the Cavens Experimental Animal Center (Changzhou, China). Lewis carcinoma cell line (H-2^b) was cultivated in RPMI-1640 medium containing antibiotics and 10% fetal calf serum (BioWit Technologies, Shenzhen, China).

Generally, DCs were extracted from bone marrow (BM) (9,10). Firstly, BM cells were drawn from femurs and tibias of C57BL/6 mice. BM cells were flushed into RPMI-1640

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medium (non-serum) and cultured on plates (6-wells) at $5x10^6$ cells/well in normal RPMI-1640 medium, which was cultured with 10% heat-inactivated fetal bovine serum, murine interleukin (IL)-4 and recombinant murine colony-stimulating factor (GM-CSF). The next day, the non-adherent cells were gently removed, and fresh medium was added with IL-4 and GM-CSF. Half of the culture medium was replaced with fresh medium containing IL-4 and GM-CSF, every 2 days. Finally, after 7 days, the non-adherent cells were harvested. The present study was approved by the Ethics Committee of the Second Hospital of Shandong University.

Construction of the adenoviral vector encoding CK19. Firstly, we amplified CK19 cDNA from the plasmid pIRES2-EGFP-CK19 by polymerase chain reaction (PCR). Then, the products were digested using *Hind*III and *Not*I. The shuttle plasmid pDC316-EGFP-cmv (BioWit Technologies) was inserted to construct pDC316-EGFP-cmv-CK19 at the same site, and then it was identified by PCR. HEK-293 cells were transfected with the pDC316-EGFP-cmv-CK19 plasmid. A recombinant adenovirus encoding human CK19 (rAd-CK19) was constructed using AdMax Kit D (BioWit Technologies) (Fig. 1). The transfected cells were harvested among the cells with normal CPE. The cells were repeatedly washed with phosphate-buffered saline (PBS), and the cells were frozen and thawed 3 times at 70˚C/37˚C. The supernatant was collected after the lysate was subjected to centrifugation (3,000 rpm) at 4˚C for 10 min. This was the preliminary crude lysate containing rAd-CK19, and then the rAd-CK19 went through 3 rounds of amplification *in vitro*. The titer of the rAd-CK19 passaged was calculated using endpoint dilution assay for 4 times (11,12). The rAd-CK19 was evaluated by the PCR method after being generated.

Assay of the expression of CK19 mRNA in Lewis lung carcinoma (LLC) cells, DCs and CK19-DCs by RT-PCR. The RNA drawn from CK19-DCs was used to evaluated the expression of CK19 by RT-PCR on the basis of the sequences of the multiple clone site of the pcDNA3.1 vector and CK19. The primers for CK19 used for amplification were: upper, 5'-GGT GTCGACCTAGCCAAGAT-3' and lower, 5'-TCTGGATCT GCTCAGAGTGG-3'. The primers for β-actin were: upper, 5'-TTCTTGGGTATGGAATCCTGTG-3' and lower, 5'-GAG GAGCAATGATCTTGATCTT-3'.

Transduction of DCs with rAd-CK19. The DCs were infected with either rAd-c or rAd-CK19 at a multiplicity of infection (MOI) of 200 and were incubated in medium containing GM-CSF (20 ng/ml) and IL-4 (10 ng/ml) for 2 h. The infected cells were cultured in complete medium for 48 h. Then, the DCs were used for subsequent experiments after being washed with PBS. Cell viability was assessed by trypan blue dye exclusion. We used flow cytometry to evaluate the transduction efficiency of the rAd-CK19-DCs.

Assay of the expression of CK19 in LLC cells, DCs and CK19-DCs by western blotting. rAd-CK19 DCs, LLC cells and rAd-c DCs were used for immunofluorescence technique and western blotting assays. Assays were carried out with an anti-CK19 polyclonal antibody (Shandong Medical College Jinan, China) via chemiluminscence utilizing an Amersham ECL Advance system with semi-dry transfer.

Analysis using flow cytometry for the phenotype of the DCs. rAd-CK19-DCs, non-infected DCs and rAd-cDCs were harvested and suspended in FACS buffer (PBS with 0.2% BSA and 0.09% NS) on day 9. LLC cells were immunostained with anti-mouse MHC II, CD80 or CD86 antibodies (PE-labeled) with an isotype-matched control antibody. The antibodies and LLC cells were cultured at 4˚C for 30 min, and washed with PBS twice. Finally, the DC cells were analyzed using FACScan (BioWit Technologies).

Assay of T cell proliferation. CK19-DCs were treated with mitomycin C (25 μg/ml) for 30 min at 37˚C while the DC and mock-DC groups were used as controls. After washing with PBS for several times, the cells ($4x10^5$ cells/ml) were harvested in RPMI-1640 medium (10% FCS). The cell suspension (100 μl/well) was added into plates (96-well) which were filled with $CD4^+$ T cells ($3x10^5$ cells/100 μl/well) isolated from the C57BL/6 mice; PBS was used as the control. Finally, the T cell proliferation was determined by MTT assay after 24, 48, 72 and 96 h.

Tumor immunization and prophylactic therapy. To evaluate the immunotherapeutic effect of the rAd-CK19-DCs, we used C57BL/6 mice (21-35 days of age; the mice were randomly divided into 3 groups, 6 mice/group) which were injected with tumor cells (LLC cells at $5x10^5$/mouse) into the right flanks. When the subcutaneous tumors grew to 4-6 mm, the mice were treated by subcutaneously injection with rAd-CK19-DCs, rAd-cDCs or DCs ($5x10^5$/mouse) into the left flanks every 3 days, for a total of 3 times. Approximately 14 days after the last injection, the mice were sacrificed (cervical dislocation), and this was recorded as the date of death. To evaluate the antitumor effect, the tumor was measured with digital calipers every 2 days. Tumor volumes were calculated using the formula: $D \times d^2 \times 0.52$ (D=length, d=width). After sacrifice of the mice, the tumors were removed and weighed (g).

Cytotoxic T lymphocyte (CTL) assay. In order to evaluate the ability of rAd-CK19-DCs to induce tumor-specific CTLs, C57BL/6 mice were subcutaneously injected with DCs, rAd-CK19-DCs or rAd-c DCs for 2 times every 7 days. Splenocytes were collected and cultured in complete medium after the final injection. Non-adherent cells were harvested and cultured in plates (24-well) with mitomycin-C-treated LLCs (10^6 cells/ml). Then the splenocytes were cultured in medium containing 10 U/ml of IL-2 at 37˚C for 5 days. The effector cells (E) treated with LLCs, and target cells (T) were put into 96-well plates at different E:T ratios (60:1, 40:1 and 20:1). The plates were centrifuged at 250 x g for 4 min, and then the cells were collected. The negative controls were the cells without effector cells. The cytotoxicity ratio was calculated using the following formula: Experimental release - spontaneous release/maximum release - spontaneous release (%).

Statistical analysis. All statistical analyses were performed using the SPSS 13.0 software package. The Student's t-test and χ^2 test were used to compare data from two groups. Differences

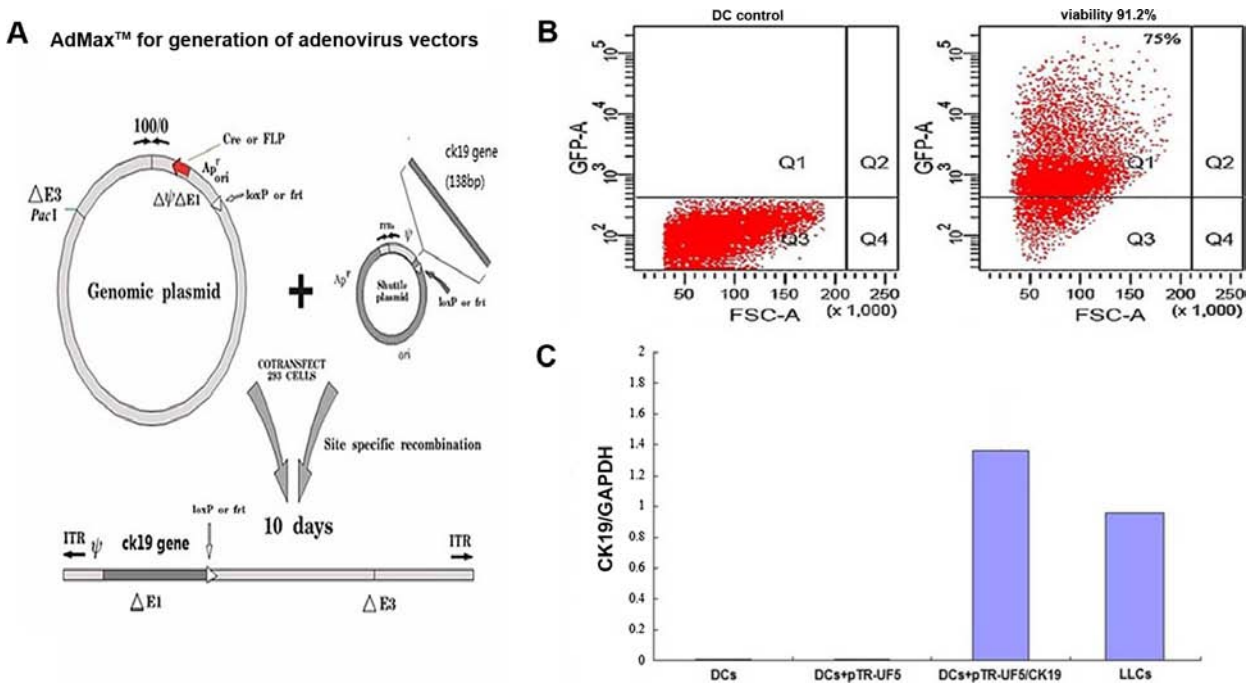

Figure 1. Construction of rAd-CK19 and transduction of DCs with rAd-CK19 and cell viability. (A) Demonstrative figure of the construction of the recombinant adenovirus and CK19 gene, using AdMax system. The shuttle plasmid pDC316-EGFP-cmv-CK19 was transfected into HEK-293 cells with the plasmid pBH-Glox E1, 3 Cre containing 5 type adenovirus genome. Then the recombinant, replication-deficient adenovirus rAd-CK19 gene was generated. (B) Transduction of DCs with rAd-CK19 and cell viability. After infection for 48 h, GFP expression was evaluated and the percentage of viable cells was evaluated using trypan blue staining; Q1, % of GFP-positive cells by flow cytometry. (C) Assay of CK19 mRNA expression in LLC cells, rAd-CK19-DCs and DCs by RT-PCR.

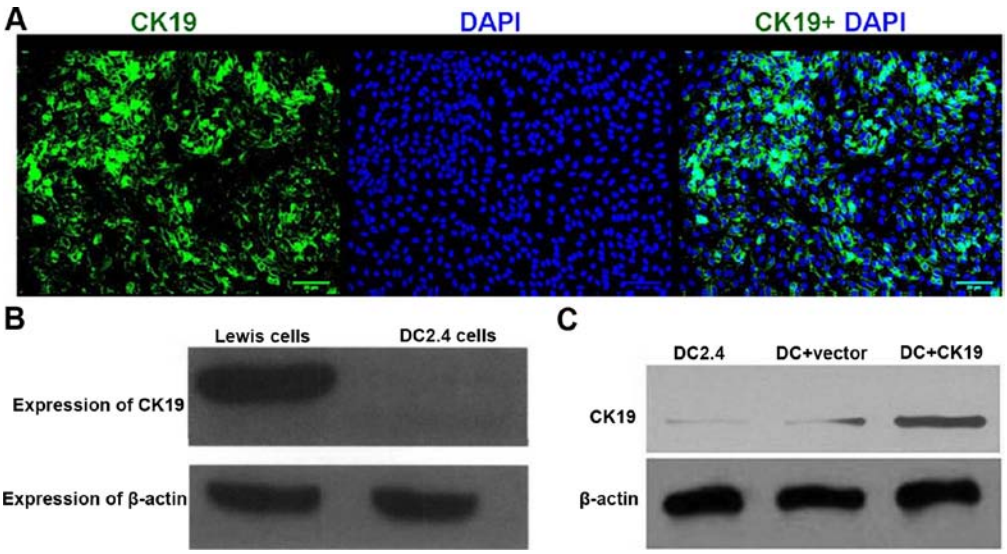

Figure 2. (A) Identification of CK19 expression in LLC cells by immunofluorescence (magnification, x100). (B) Expression of CK19 protein in LLC cells and DC2.4s by western blotting. (C) CK19 expression in DC2.4 and rAd-CK19-DCs. DC+vector was used as a negative control.

associated with a P-value of 0.05 were considered statistically significant.

Results

Preparation of the rAd-CK19. Following successful construction of the plasmid pDC316-EGFP-cmv-CK19 which was identified by PCR, HEK-293 cells were transfected with this plasmid as shown in Fig. 1A. HEK-293 cells were infected with rAd-CK19, and 3 days later the expression of GFP was also monitored by flow cytometry (Fig. 1B).

Transduction of the DCs with the adenoviral vector encoding CK19. DCs transfected with rAd-CK19 were analyzed for transduction efficiency after 7 days. The results of flow cytometry showed that ~80% of DCs were positive for GFP. When the MOI of gene transduction was 200, its cell viability was >80% (Fig. 1B).

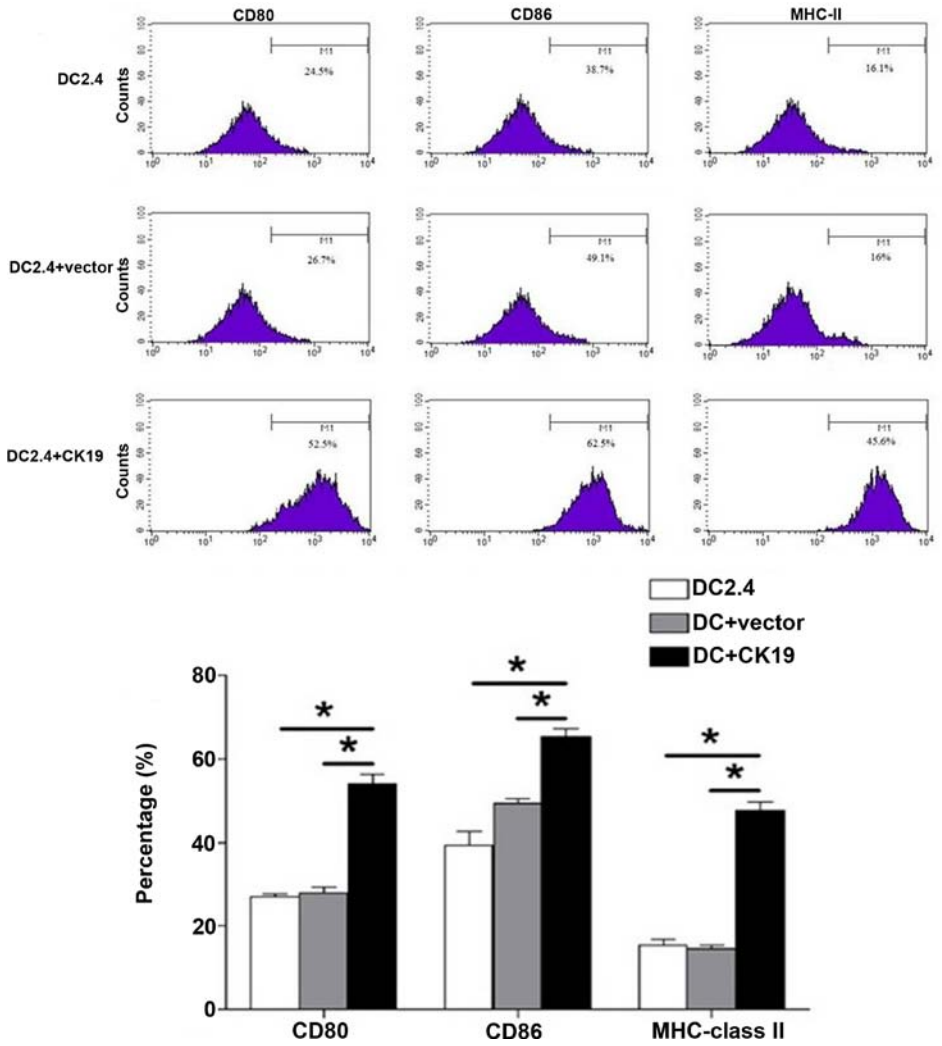

Figure 3. Variation of surface markers on CK19-DCs. The expression levels of MHC class II, CD86 or CD80 on CK19-DCs, DCs or vector-DCs were analyzed by FCM, and CK19-DCs exhibited markedly increased expression of MHC class II, CD80 and CD86.

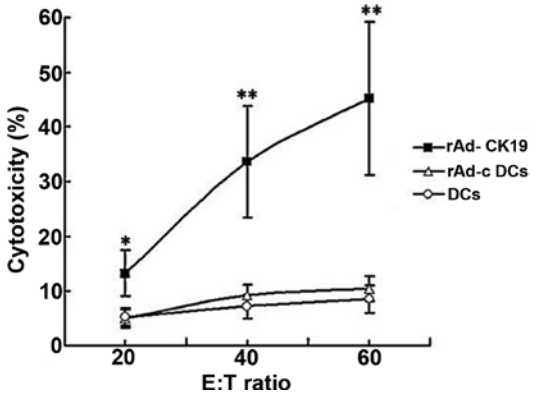

Figure 4. Induction of specific T cells by mice vaccinated with rAd-c DCs. Mice were subcutaneously vaccinated 3 times during a 3-day interval with DCs (○), rAd-CK19-DCs (■) or rAd-c DCs (△). After the last vaccination, spleen cells were isolated and stimulated with mitomycin C-treated LLC cells for 5 days. The stimulated cells were then assayed for cytolytic function using LLC cells as target cells. Results are shown as means ± SD, *P<0.01 compared with rAd-c DCs or DCs; **P<0.001 compared with rAd-c DCs or DCs.

Expression of CK19 in the rAd-CK19 DCs and LLC cells. As shown in Figs. 1 and 2, CK19 mRNA and protein were monitored in LLC cells. CK19 mRNA and protein were also detected in the rAd-CK19-DCs, while poor expression was observed in the DCs and rAd-c DCs.

Effect on the DCs after infection with rAd-CK19. To assess the effect on DC activity after infection with rAd-CK19, we analyzed three phenotypes of the rAd-c DCs, DCs or rAd-CK19-DCs by flow cytometry. The results showed (Fig. 3) that expression levels of MHC class II, CD80 and CD86 on rAd-CK19-DCs were significantly higher than levels on the rAd-c-DCs, which proved that rAd-infected DCs had enhanced MHC class II, CD80 and CD86 expression levels compared to the expression levels in the normal DCs. Thus, we concluded that infection with the recombinant adenovirus led to the maturation of the DCs. In contrast, there was no apparent effect on DC differentiation and activation by CK19 gene infection.

Activity of tumor-specific CTLs. As shown in Fig. 4, the E cells vaccinated with rAd-CK19-DCs induced significantly killing responses by CTLs against lung cancer cells. On the contrary, the control groups vaccinated with DCs or rAd-c DCs expressed less lysis. The results proved that E cells vaccinated with the rAd-CK19-DCs induced tumor-specific CTL responses.

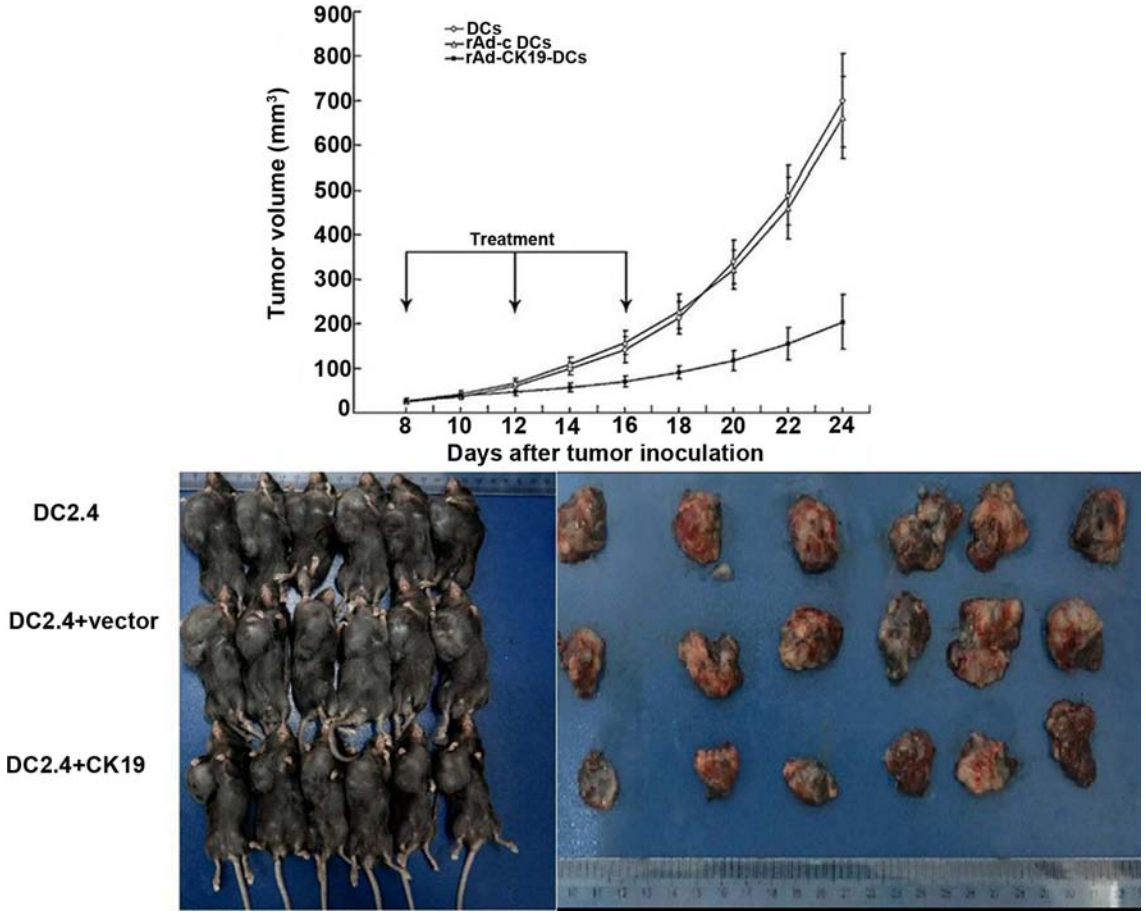

Figure 5. Effect of rAd-CK19-DCs on tumor growth in mice. LLC cells were subcutaneously injected into mice into the right flanks on day 1. When tumors grew to 4-6 mm, the mice were divided into groups with similar tumor size (6 mice/group). Then the mice were injected with rAd-c DCs (△), rAd-CK19 DCs (■) or DCs (○). Tumor volume was measured every 2 days. Mean ± SD of tumor volume is shown. The CK19-DC vaccine significantly attenuated the tumor growth as compared to that in the control groups.

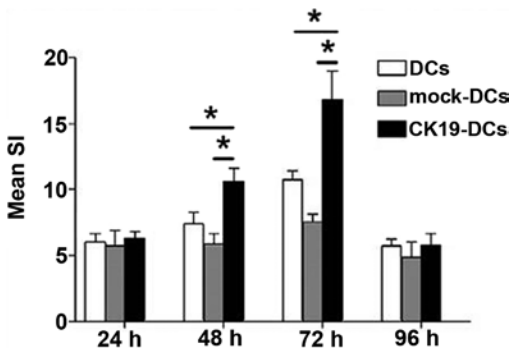

Figure 6. Capacity of CK19-DCs to sitimulate T cell proliferation. Proliferation of T cells was evaluated by MTT assay. The SI yielded 10.30±0.93 and 15.78±1.95 in the CK19-DCs compared with 5.67±0.58 and 6.99±1.51 in the rAd-c DCs (P<0.01), and 7.12±0.66 and 9.63±0.89 in the DCs at 24 and 48 h. The results are presented as the mean ± SD (n=5), *P<0.01.

Antitumor effects of immunization with rAd-CK19 DCs. We used a mouse LLC cell tumor model to evaluate the immunotherapeutic effect of rAd-CK19-DCs. Mice vaccinated with rAd-CK19-DCs exhibited markedly induced antitumor effects compared to the control group during the 24-day observation period. As shown in Fig. 5, immunization with rAd-CK19-DCs significantly attenuated the growth of tumors as compared to the tumor growth in the control groups immunized with rAd-c DCs or DCs (P<0.01). Tumor volume in the control groups was ~1-fold larger than that in the treated groups. There was no difference in tumor volume between the two control groups (P>0.05). The results of the tumor weights were the same as the tumor sizes. The average weights of the tumors (immunized with rAd-c DCs and DCs) were 2.800 and 2.788 g, while the average weight of the tumors (immunized with rAd-CK19 DCs) was 1.035 (P<0.01). These results suggested that the rAd-CK19-DC vaccine evoked a cytotoxic antitumor immune response to the target cells.

Proliferation of T cells driven by rAd-CK19 DCs. CK19-DCs had a higher capability to evoke special T cell proliferation than that noted in the control groups (rAdc-DCs and DCs). The proliferation of T cells, stimulated with CK19-DCs began at 48 h as compared with T cell proliferation of the control group which reached the highest peak after 72 h. The study suggested that CK19-DCs have the capability to stimulate T cell proliferation (Fig. 6).

Discussion

Host antigen-presenting cells (APCs) are critical for the presentation of tumor antigens (13). DCs are the most effective APCs, which have the capability to stimulate special immune

response dependent on the T cells. Antigen-loaded DCs migrate to lymphoid tissues and relate to naive T cells, where the labeled DCs stimulate immune responses dependent on co-stimulatory and adhesive molecules on the cell surface (9).

The ability to induce original $CD8^+$ T and $CD4^+$ cells, and to expand those cells at sites of tumor antigen expression provides an efficient method for priming T cells. Injecting DC vaccines to secondary lymphoid organs is a critical step to stimulate the initiation of a primary immune response, where DCs encounter naive T cells. Almost at the same time, the $CD4^+$ T and $CD8^+$ T cells infiltrate into the tumor site (14).

The use of a DC-based tumor vaccine is important for advanced stage tumors with poor responsiveness to chemotherapy, such as lung cancer. DCs can contain different proteins or peptides of tumors. As the source of antigens for immunotherapy, the lysate of tumor cells offers potential target antigens, which should induce a greater T-cell response against multiple known or unknown tumor-associated antigens of individual cancer cells (15). The immunotherapy of DCs loaded with tumor antigens has been proven to be an effective antitumor strategy in preclinical tumor therapy and some have been used in clinical therapy research, such as in melanoma and pancreatic cancer therapy (16-19).

Vaccination of DCs infected with adenovirus vectors encoding special or broad tumor antigens can stimulate protective and antigen-specific CTL responses and immunity to kill target cells (20-22). Transduction with replication-defective and recombinant adenovirus vectors encoding target genes is an efficient method for gene transfer into DCs (23,24). Xie *et al* and Zhou *et al* have shown that DCs pulsed with tumor antigens livin-α and XAGE-1b induced significant immune responses by CTLs against LLC cells (25,26).

In the present study, we generated rAd-CK19-DCs which expressed the CK19 gene at high levels through adenovirus transduction at an MOI of 200 (Fig. 1), and transduction efficiency of cell viability was >80% (Fig. 1). In addition, maturation, enhancement and activation of T cell stimulatory capacity have also been achieved for DC adenovirus-mediated transduction (27,28).

According to previous studies (12,29), adenovirus-mediated transduction induced maturation and upregulated surface antigen expression of DCs through a nuclear B-dependent mechanism. On the other hand, adenovirus vectors infected with target tumor antigens delivered immunogenic adenovirus genes and proteins, which induced special cellular responses (30). In the present study, the viral vector had no significant effect on the expression of the DC phenotype at an MOI of 200 and the upregulation of MHC class II, CD80 and CD86 by adenoviral vector was also detected in the rAdc-DCs (Fig. 3).

Cytokeratins, which belong to a family of 20 individual polypeptides, form an intracellular network of filaments that participates in maintaining the structural integrity of the cell. The expression pattern of cytokeratin polypeptides varies with the cell type and changes during transformation of normal epithelial cells into malignant cells in many types of cancer (31). Moreover, cytokeratins were applied in evaluation of the degree to which a tissue is abnormally differentiated (32). CK19 is the smallest member of the cytokeratin family, and its expression is highly tissue-specific. Many studies have shown that CK19 expression is correlated with LNM in various solid malignancies (33-36), including the liver, colon, stomach, pancreas, biliary tract and breast (37,38).

CK19, a type I cytokeratin, belongs to a group of intermediate filament proteins forming the cytoskeleton. CK19 is the smallest known acidic cytokeratin and is not paired with a basic cytokeratin, unlike the other proteins of this group. *In vitro* studies of rat lung alveolar epithelial cells have shown high expression of CK19 in intensively proliferating type II pneumocytes (6). Meanwhile, CK19 mRNA was assessed to investigate the prognostic significance of circulating cancer cells in peripheral blood of patients with non-small cell lung cancer (36).

Increased expression of this protein was found using immunohistochemical methods in lung cancer, particularly overexpression in squamous cell carcinoma and adenocarcinoma (7). Thus, according to these findings, we used an immunization strategy using the CK19 gene as the target. The immune system is tolerant to many self antigens, which is a potential obstacle for any immunotherapeutic strategy that relies on a vaccination against specific antigens. The tumor vaccination protocol with the addition of cytokines or other immune modulators may help to halt the immune escape of tumor cells. Steitz *et al* (39) reported that, in C57BL/6 mice, immunization with recombinant adenovirus vector encoding human xenogeneic tyrosinase-related protein 2 induced protection against the growth of B16 melanoma cells. Another study showed that, in syngeneic Fischer rats, a human xenogeneic DNA vaccine stopped the anti-apoptotic protein survivin to inhibit the growth of F98 glioblastoma (40). In the same way, the homologous epitopes encoded by the xenogeneic DNA act as molecular mimics recognized as non-self, resulting in an immune response against host self gene products. Downregulating specific tumor antigens may help in preventing tumor escape.

In conclusion, in the present study, we used the human xenogeneic CK19 antigen as a target to stop the immune tolerance of mice to the CK19 antigen. According to our results (Fig. 4), the mice vaccinated with the rAd-CK19 DCs generated special CTLs, which could recognize CK19-derived antigens stimulating significant killing responses. To test the antitumor effect of the rAd-CK19 DC vaccine *in vivo*, we used C57BL/6 mice with LLC as a tumor model with which to evaluate the antitumor effect of the rAd-CK19-DC vaccine. Vaccination with rAd-CK19 DCs prevented the growth of the LLC tumors in the mice. Thus, we considered that it provided significant protection against tumor growth (Fig. 5). In contrast, the control groups vaccinated with DCs or rAd-c DCs did not exhibit effective tumor protection. According to the above, we conclude that rAd-CK19-DC vaccine could generate an effective specific CK19 antitumor effect. In summary, rAd-CK19-DC-based vaccine may offer a promising approach to cancer immunotherapy in clinical trials, and may play an important role in fighting lung cancer.

Acknowledgements

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