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Immunotherapy with Dendritic Cells Modified with Tumor-Associated Antigen Gene Demonstrates Enhanced Antitumor Effect Against Lung Cancer[1]

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Abstract

BACKGROUND: Immunotherapy using dendritic cell (DC) vaccine has the potential to overcome the bottleneck of cancer therapy. *METHODS:* We engineered Lewis lung cancer cells (LLCs) and bone marrow–derived DCs to express tumor-associated antigen (TAA) ovalbumin (OVA) via lentiviral vector plasmid encoding *OVA* gene. We then tested the antitumor effect of modified DCs both *in vitro* and *in vivo*. *RESULTS:* The results demonstrated that *in vitro* modified DCs could dramatically enhance T-cell proliferation ($P < .01$) and killing of LLCs than control groups ($P < .05$). Moreover, modified DCs could reduce tumor size and prolong the survival of LLC tumor-bearing mice than control groups ($P < .01$ and $P < .01$, respectively). Mechanistically, modified DCs demonstrated enhanced homing to T-cell–rich compartments and triggered more naive T cells to become cytotoxic T lymphocytes, which exhibited significant infiltration into the tumors. Interestingly, modified DCs also markedly reduced tumor cells harboring stem cell markers in mice ($P < .05$), suggesting the potential role on cancer stem-like cells. *CONCLUSION:* These findings suggested that DCs bioengineered with TAA could enhance antitumor effect and therefore represent a novel anticancer strategy that is worth further exploration.

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Introduction

Immunotherapy is a promising approach for the treatment of cancer. Emerging evidence from basic and clinical studies indicates that immunotherapy has the potential to overcome the bottleneck of cancer therapy [1,2]. One of the most attractive methods of immunotherapy is to use antigen presenting cells, such as dendritic cells (DCs), to load with tumor-associated antigens (TAAs) so that potentially specific and superior therapeutic effect can be achieved [3,4].

DCs play a pivotal role in the immune response. They bridge the innate and adaptive immunity through capturing antigens and presenting them to T cells [5]. DCs-based immunotherapy is therefore one of the promising approaches in cancer therapy. In a phase III randomized controlled trial, the DC-based therapy sipuleucel-T showed

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significantly better median overall survival in patients with metastatic hormone-resistant prostate cancer [6]. However, several other phase III trials did not yield clinical significance for lung cancer including the studies with tecemotide (START study using MUC1) and belagenpumatucel-L (STOP study targeting 4 TGF-β2), as well as MAGE-A3 (MAGRIT study using melanoma associated antigen A3) in the adjuvant setting for non–small cell lung cancer [7–10]. Potential explanations may involve that modified DCs *in vitro* could not effectively and sufficiently enter the T cell–rich regions *in vivo*, such that the DC-based vaccines could not activate sufficient T cells to generate adequate amount of cytotoxic T lymphocytes (CTLs). The main goal of DC-based vaccination is to stimulate tumor antigen-specific CTLs that can get into the tumor sites and recognize and eliminate cancer cells in an antigen-specific way [11]. However, whether TAA-modified DCs and activated CTLs can successfully reach the tumor sites remains to be elucidated.

Cancer stem-like cells (CSCs) are a subset of cells characterized by the capacity for self-renewal, differentiation, and tumorigenesis [12]. CSCs may be responsible for innate resistance to chemotherapy and radiation, as well as disease recurrence after definitive therapy [13,14]. Although CSC theory remains controversial, some studies have demonstrated promising results through targeting CSCs in cancer therapy [15]. Eradicating CSC by efficient targeting agents may have the potential to cure cancer [16,17]. One study used CSC lysate (using *ALDH* as the stem cell marker) as a source of antigen to pulse DCs and induced significantly higher antitumor immunity than DCs pulsed with the lysates of unsorted whole tumor cell lysates, with production of higher amount of interferon-γ and granulocyte macrophage-colony stimulating factor (GM-CSF) [18]. Another study presented seven patients with glioblastoma treated with DC-based vaccine targeting CSCs, and the reported progression-free survival was 2.9 times longer in vaccinated patients compared to the control group (median 694 vs 236 days, $P = .002$) [19]. These results indicated that DC-based immunotherapy especially targeting CSC has the potential to provide therapeutic benefit. However, there are very few studies regarding whether DC-based vaccines with TAAs could exert anti-CSC effect *in vivo*.

In this study, DCs from murine bone marrow were modified to express ovalbumin (OVA) via lentiviral infection. Modified DCs were tested for therapeutic effect against murine Lewis lung cancer cell (LLC) expressing OVA both *in vitro* and *in vivo*. In addition, experiments were carried out to investigate whether (1) modified DCs could have enhanced homing to T cell–rich regions *in vivo*, (2) activated CTLs could infiltrate tumors, and (3) DC-based vaccines could reduce tumor cells harboring CSC markers *in vivo*, which therefore could be suggestive of potential anti-CSC effect.

Materials and Methods

Reagents

QIANGEN Midi Plasmid Kit, QIANGEN Mini Purification Kit, EndoFree Plasmid Maxi Kit, and DH5α were purchased from QIANGEN (Beijing, China). Recombinant mouse GM-CSF and interleukin-4 (IL-4) were purchased from R&D Systems (Minneapolis, MN). Fluoroisothiocyanate-conjugated rat anti-mouse CD3 and CD8 antibody (Ab) and phycoerythrin-conjugated rat anti-mouse CD11c, CD80, CD86, and CD1a Abs were purchased from PharMingen (San Diego, CA). Rat anti-mouse CD3, CD11c, CD133, and nestin Abs and rat anti-chicken OVA Ab were purchased from BOSTER (Wuhan, China). BCA kit, PVDF membrane, and ECL Western blot substrate kit were purchased from Beyotime (Shanghai, China).

Identification of OVA Expression Vector

OVA expression vectors including pHR-CMV-EGFP-OVA, pHR-CMV-EGFP, pLTR-VSVG, and pHIV-pack were obtained from Cancer Institute of Tongji University. All plasmids were confirmed by either sequencing or restriction enzyme digestion prior to the experiments. The sequencing results were compared with the data published on GenBank.

Cell Lines and Animals

293T packaging cell line was maintained in Dulbecco's modified Eagle's medium (GIBCO BRL, Germany) supplemented with 10% fatal calf serum (FCS) (HyClone, Logan, UT), penicillin (50 U/ml), and streptomycin (50 μg/ml) in the 5% CO_2 thermostat incubator. LLCs were grown in RPMI-1640 (HyClone, Logan, UT) supplemented with 10% FCS, penicillin (50U/ml), and streptomycin (50 μg/ml) in 5% CO_2 thermostat incubator. All cell lines were obtained from Tongji University School of Medicine (Shanghai, China). All mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). Animals were housed and maintained under optimal conditions of light, temperature, and humidity with free access to food and water. All procedures involving animal treatment and care in this study were approved by the Animal Care Committee of Tongji University School of Medicine.

Isolation of DCs and T Cells

Bone marrow–derived immature DCs were generated from the femurs and tibiae of 5- to 6-week-old mice. Briefly, bone marrow was flushed from the femur and tibia of mice, and red blood cells were lysed with 0.84% ammonium chloride. Cells were cultured in RPMI-1640 complete medium for 2 hours to allow for adherence. Nonadherent cells were collected and incubated with culture medium supplemented with recombinant murine GM-CSF (10 ng/ml) and IL-4 (10 ng/ml). On day 6, nonadherent cells were harvested as DCs and used for the subsequent experiments. The purity of isolated DCs was tested through flow cytometry (FCM) analysis using CD80, CD86, and CD1a expression. Hematoxylin and eosin (H&E), Wright's, and immunohistochemical (IHC) staining of CD11c was performed for morphology observation of purified DCs.

T cells were isolated from the spleen of 6-week-old mice by using Nylon Wool Fiber Column (Hedebio, Beijing, China) according to the manufacturer's instructions. Briefly, cell suspension was prepared from mice spleen. The column was washed with 20 ml of minimum essential medium (MEM) and warmed at 37°C. Then, 15 ml of warm MEM containing 5% FCS passed through, and the stopcock valve was closed. Next, 2 ml of 2 to 4×10^8 cells suspended in MEM containing 5% FCS at 4°C were added, and the valve was opened slowly to allow the suspension get settled in the fiber bed. After suspension sank thoroughly, the stopcock was closed, and another 1 ml of MEM containing 5% FCS at 37°C was added, followed by incubation at 37°C for 60 minutes. After incubation, the suspension was collected, and 20 ml of MEM containing 5% FCS at 37°C was added with a flow rate of 3 to 4 ml/min. The purity of isolated T cells was tested by FCM analysis using CD3 expression.

Lentivirus-Mediated OVA Transduction

293T and LLC cells (293T: 1×10^3 cells per dish, LLC: 1×10^3 cells per dish) were cultured in 96-well plates prior to transduction. To generate lentivirus, 293T cells were transduced with pHR-CMV-EGFP-OVA or pHR-CMV-EGFP plasmid along with pLTR-VSVG and pHIV-packaging plasmids (pHR-CMV-EGFP-OVA/pHR-CMV-EGFP:

pLTR-VSVG: pHIV-packaging = 1:4:2) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). After 2 days, we collected the supernatant, which contained the lentivirus encoding either pHR-CMV-EGFP-OVA or pHR-CMV-EGFP. We then applied the lentiviral supernatant to infect LLCs and DCs after filtering through a 0.45-μm microfilter. After 3 to 5 days, green light in the cells expressing either EGFP-OVA or EGFP were observed under fluorescence microscope. All the cells in each well were collected and sorted by FACScalibur FCM (Becton Dickinson, San Jose, CA). The positive cells [LLC-EGFP-OVA, DC-EGFP-OVA (LLCs or DCs transduced with pHR-CMV-EGFP-OVA), and DC-EGFP (DCs transduced with pHR-CMV-EGFP)] were incubated in 6-cm dishes in 5% CO_2 thermostat incubator with medium change every 2 days.

Western Blot

LLC-EGFP-OVA, DC-EGFP-OVA, and DC-EGFP cells were homogenized on ice for 1 hour in a lysis buffer containing NaCl (100 mM), Na_4P_2O (20 mM), glycerol (1% v/v), Tris (10 mM; pH 7.4) (all from Gibco), Triton X-100 (2% v/v), EDTA (1 mM), NaF (1 mM), and SDS (0/1% w/v). Subsequently, the lysates were centrifuged. Then, the supernatants were collected and stored at −20°C. Total protein concentration was determined using a BCA kit according to the manufacturer's instructions. Lysates were subjected to SDS-PAGE followed by protein transfer onto the PVDF membrane. Membranes were then blocked (5% skim milk) and incubated with anti-OVA Ab at 4°C overnight. After washing, the membranes were incubated in horseradish peroxidase–conjugated sheep anti-rabbit IgG for 45 minutes. OVA protein expression was detected using an ECL Western blot substrate kit (Beyotime, Shanghai, China).

T-Cell Proliferation and Cytotoxic Assay In Vitro

Purified T cells and modified DCs were co-cultured at a ratio of 10:1 with IL-2 (1.45 ng/ml). After incubation for 3 days, cells were collected for proliferation assay using CCK-8 kit (Dojingdo Molecular Technology, Japan). For cytotoxic assay, LLC-EGFP-OVA cells were cultured in six-well plates (1×10^5 cells). After 3 days of co-culture with purified T cells and modified DCs, T cells were collected and added into 6-cm dishes. After another 6 hours of incubation, LLCs were collected and CCK-8 was performed.

Tumor-Bearing Mice and Immunization Protocols

Eight- to 10-week-old BALB/c mice were housed and maintained under optimal light, temperature, and humidity. LLC-EGFP-OVA cells were used for tumor induction in mice. LLC-EGFP-OVA cells were harvested and washed, and the density was adjusted to 1×10^7 cells/ml phosphate-buffered saline (PBS). Mice were given subcutaneous injection into the back next to the right forelimb (100 μl). The day of the inoculation was considered as day 0 in all groups. Purified DC, DC-EGFP, or DC-EGFP-OVA plus T cells (5×10^7 cells/ml PBS, DCs:T = 1:10) were injected directly into tumor site twice (day 12 and 16 after LLC injection) and through tail vein once (day 20 after LLC injection). As per the immunization protocols, three groups (DC + T, DC-EGFP + T, DC-EGFP-OVA + T; 10 mice per group) were included in the study.

Tumor Volume and Survival of Tumor-Bearing Mice

Tumor growth was evaluated by measuring two perpendicular diameters of each tumor using a caliper. Tumor volume was calculated as (length × width^2)/2 and measured every 4 days. On day 48, mice were sacrificed by cervical dislocation and tumors processed for IHC analysis. To determine whether DC immunization prolonged the survival of tumor-bearing mice, another three groups of mice that underwent the same tumor inoculation and DC immunization were used. The survival of these mice was monitored at 5-day intervals, and the distribution of survival percentages over time was estimated using the Kaplan-Meier curves.

Measurement of Cytokines

A sandwich ELISA was used for detecting IL-12 in mice serum. Assays of cytokines in mice serum were performed as recommended by the manufacturer (BD Biosciences). The serum levels of cytokines were determined by measuring the absorbance at 450-nm wavelength with a micro-plate reader. Cytokine concentrations in the samples were calculated using standard curves generated from recombinant cytokines, and the results were expressed in picograms per milliliter.

IHC of Mice Lymph Node and Tumor Tissue

Formalin-fixed, paraffin-embedded mice lymph node and tumor tissue were prepared for H&E staining. For IHC study, 4-μm–thick sections were prepared and placed at 60°C for 1 hour. For deparaffinization, the slides were placed in xylol solution (3 times), absolute ethanol, and ethanol 95°, respectively (twice, 5 minutes each time), and then washed and placed in running water. The slides were buffered in Tris-EDTA (pH = 9) and then placed in microwave at full power until the buffer reached the boiling point. After that, the microwave power was reduced to 40°C, and all tissues stayed there for 15 minutes. Then slides were removed and placed at room temperature for 15 minutes. After rinsing under running water and TBS buffer, slides were transferred to a damp chamber where monoclonal Ab diagnostic kits for CD3, CD8, CD11c, OVA, CD133, and nestin were applied on them, respectively. Antibodies were diluted by diluent and carefully covered all tissue sections. The control specimens were covered with just TBS buffer, and all samples were placed at room temperature for 30 minutes. After washing with TBS buffer, hematoxylin was used as background stain. All IHC-stained slides were read by two expert pathologists.

Statistical Analyses

All data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using the Student's *t* test or one-way analysis of variance. Differences were considered to be statistically significant when $P < .05$. Kaplan-Meier curves were used in the analysis of the time-to-event variables, and the 95% confidence interval for the median time to event was calculated. The log-rank test was used to compare cumulative survival in the two groups. All statistical analyses were performed using GraphPad Prism 5.0 software (La Jolla, CA).

Results

Confirmation of OVA Expression Plasmid Vector by Gene Sequencing

The correct sequence of *OVA* expression plasmid vector (pHR-CMV-EGFP-OVA) was verified by comparing to the sequence data on GenBank. The sequencing map and corresponding result were shown in Supplemental Figure S1.

Establishment of OVA-Expressing Stable Clones of LLCs and DCs

Bone marrow–derived immature DCs were generated from the femurs and tibiae of mice (male, 6 weeks old). Mature DCs were isolated by FCM using CD80, CD86, and CD1a as the markers. After 6 days of incubation, the percentage of CD80+, CD86+, and CD1a + mature

DCs was 40.3%, 48.6, and 45.7% respectively (Figure 1, *M–O*). Triple-positive DCs were used for the subsequent experiments. H&E, Wright's, and immunocytochemical (CD11c) staining showed that mature DCs had a large number of dendrites (Figure 1, *Q*, *S*, and *U*). As shown in Figure 2, the GFP positive rate was nearly 100% in 293T cells after 48 hours of transduction (Figure 2, *A–D*). Then the collected lentiviral supernatant was applied to infect LLCs and purified DCs. After 7 days of incubation, infected LLCs and DCs were sorted by FCM (Figure 2, *E–L*). The results indicated that positive rate of DC-EGFP-OVA (DCs modified by vector that carried *OVA* gene segment) and DC-EGFP (DCs modified by blank vector) cells was 58.6% and 55.4%, respectively (Figure 2). Then, isolated LLC-EGFP-OVA (LLC carried *OVA* gene segment), DC-EGFP, and DC-EGFP-OVA cells were cultured for 3 months to establish the stable clones. Immunocytochemical staining and Western blot assay had validated the expression of OVA protein in LLC-EGFP-OVA and DC-EGFP-OVA stable clones (Figure 2, *M–O*).

The OVA-Expressing Modified DCs Enriched T-Cell Proliferation and Enhanced the Killing of OVA-Expressing LLCs In Vitro

To test whether modified DCs were capable of inducing T-cell proliferation and achieve cytotoxic effect, either modified or unmodified

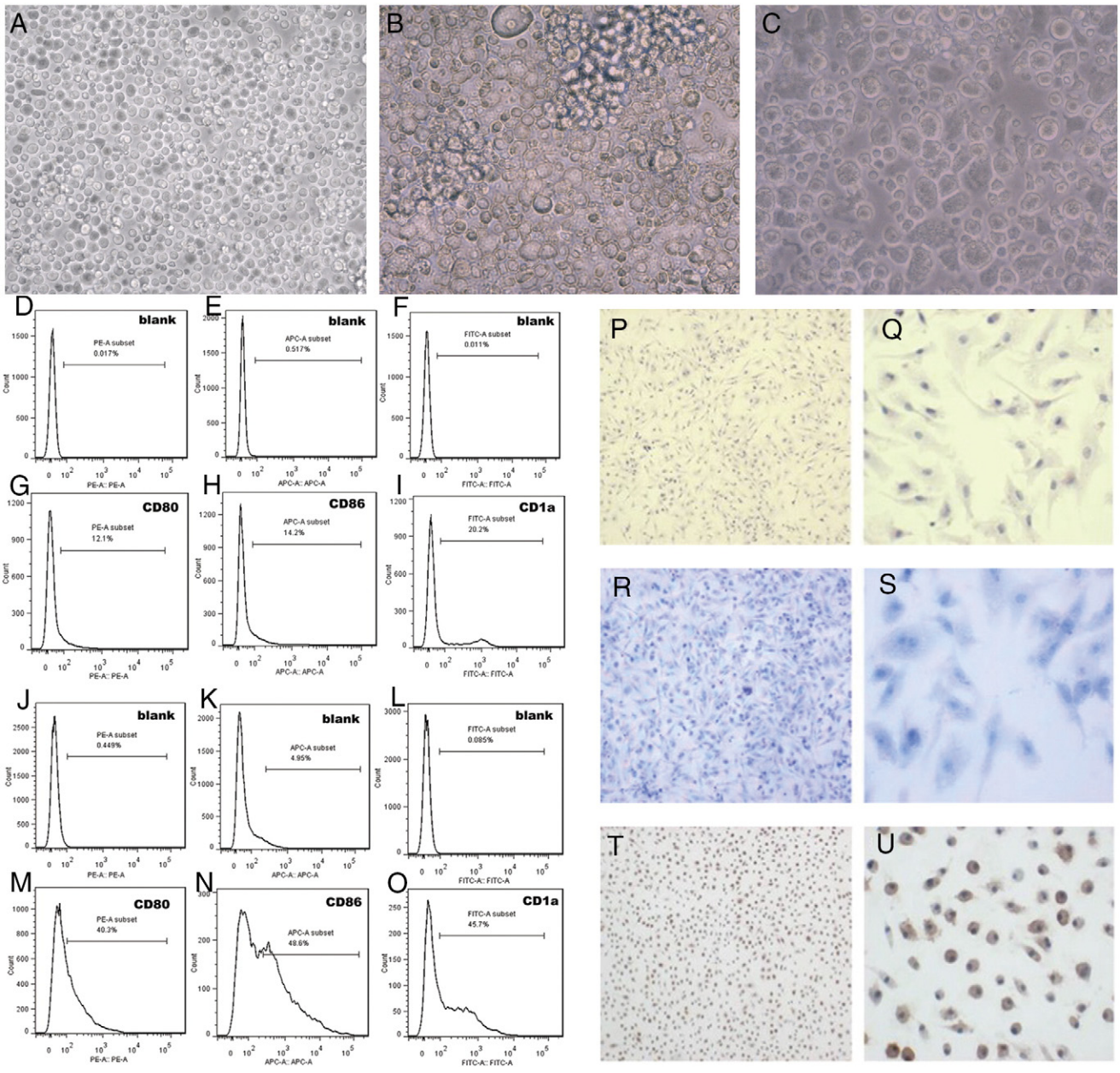

Figure 1. Isolation and morphological observation of bone marrow–derived immature DCs, as well as morphological staining of purified mature DCs. (A–C) Morphological observation of bone marrow–derived immature DCs after 1, 3, and 6 days of incubation with recombinant murine GM-CSF (10 ng/ml) and IL-4 (10 ng/ml) (×200). (D–O) FCM analysis of CD80, CD86, and CD1a expression in bone marrow–derived DCs after 3 (D–I) and 6 (J–O) days of incubation with recombinant murine GM-CSF (10 ng/ml) and IL-4 (10 ng/ml). (P and Q) H&E staining of purified mature DCs (P: ×100, Q: ×400). (R and S) Wright's staining of purified mature DCs (R: ×100, S: ×400). (T and U) Immunocytochemistry of CD11c staining of purified mature DCs (T: ×100, U: ×400).

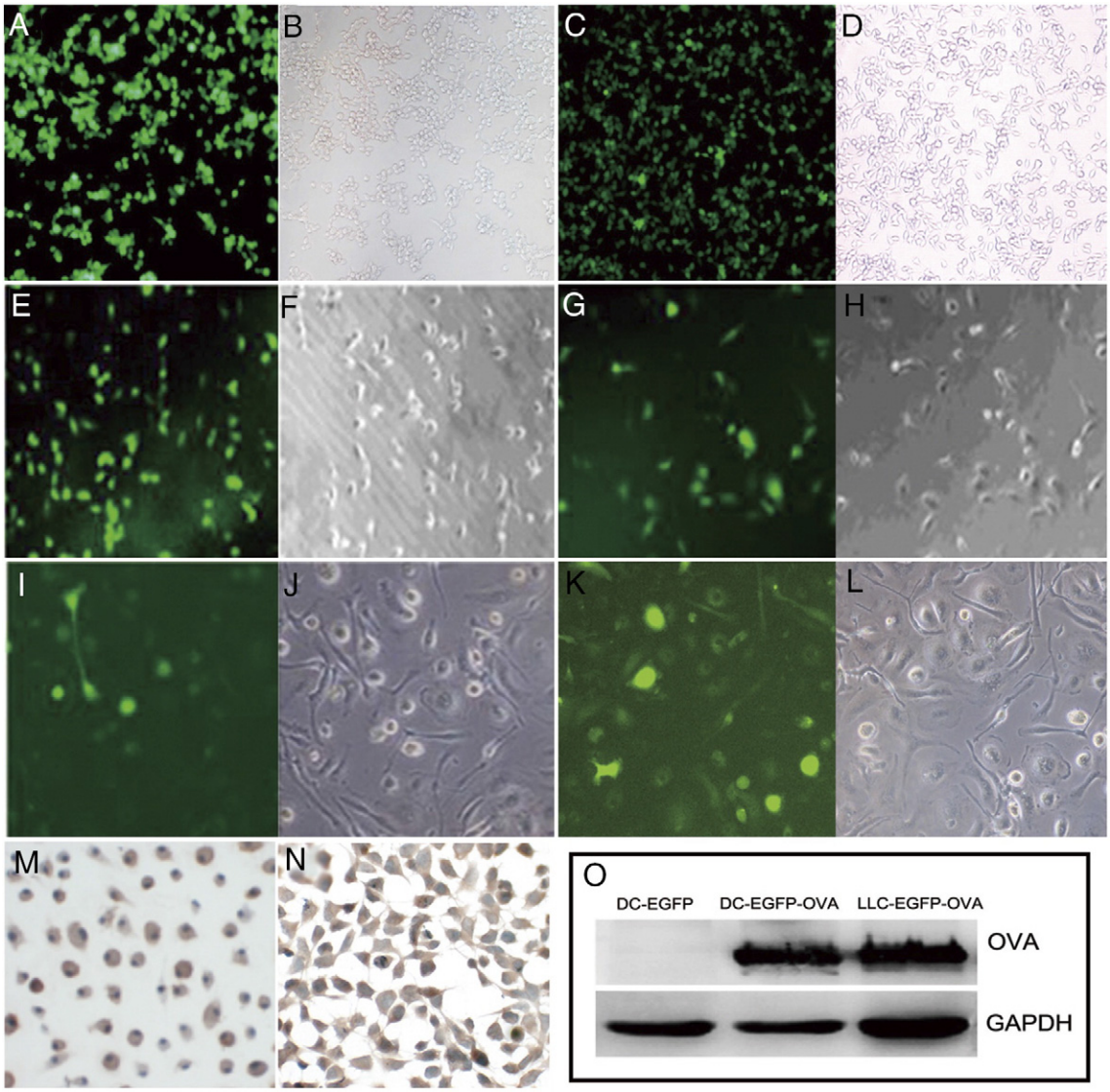

Figure 2. Establishment of OVA-expressing LLCs and DCs via lentiviral delivery system. (A–D) 293T cells were transduced with lentiviral vector encoding either EGFP alone (A and B, ×400), or EGFP and OVA (C and D, ×400). Positive cells were shown green due to EGFP expression. (E–H) LLCs were infected with lentiviral supernatant collected from above transduced 293T cells (E and F: expressing EGFP alone; G and H: expressing both EGFP and OVA, ×400). (I–L) DCs infected by lentivirus produced by above transduced 293T cells (I and J: expressing EGFP alone; G and H: expressing both EGFP and OVA, ×400). (M) Immunocytochemistry of OVA protein expression in DC-EGFP-OVA cells (×400). (N) Immunocytochemistry of OVA protein expression in LLC-EGFP-OVA cells (×400). (O) Western blot assay validated the expression of OVA protein in LLC-EGFP-OVA and DC-EGFP-OVA cells but not DC-EGFP cells.

DCs were co-cultured with T cells isolated from syngeneic mice spleen. The CCK-8 assay showed that T cells co-cultured with DC-EGFP-OVA cells had a higher proliferation rate than that T cells co-cultured with either DC-EGFP cells or unmodified DCs ($P < .01$, Figure 3*A*). For cytotoxic assay, T cells were first collected after co-cultured with either DC-EGFP-OVA or DC-EGFP cells. Then LLC-EGFP-OVA cells were co-cultured with either of the modified T cells in 6-cm dishes at a ratio of 50:1. Figure 3*B* demonstrated that DC-EGFP-OVA cells could induce T cells to kill LLC-EGFP-OVA cells much more effectively ($P < .05$). In addition, Hoechst 33258 staining showed that DC-EGFP-OVA plus T cells could induce significantly more apoptosis in OVA-expressing LLCs than unmodified DCs plus T cells ($P < .01$). However, the increased ratio of modified DCs over T cells showed no added apoptotic effect in this system (1:5 vs 1:10, $P > .05$) (Figure 3*C*). We therefore used the ratio of 1:10 in the subsequent experiments.

The OVA-Expressing Modified DCs Enhanced the Killing of OVA-Expressing LLCs In Vivo and Prolonged the Overall Survival of Tumor-Bearing Mice

To evaluate the immune-mediated protection generated by DC-EGFP-OVA vaccination *in vivo*, we injected mice of experimental group three times with DC-EGFP-OVA plus T cells in medium at 4-day intervals (days 12, 16, and 20). Mice in control groups received either DC-EGFP cells or DCs along with T cells in PBS at the same intervals. LLC-EGFP-OVA tumors in mice of both control groups grew progressively and developed into palpable tumors 10 days earlier than those in the group treated with DC-EGFP-OVA plus T cells (Figure 4*A*). The average tumor size in the treatment group was significantly smaller than those of both control groups ($P < .01$; Figure 4, *A* and *B*). Moreover, the tumor-bearing mice treated with DC-EGFP-OVA plus T cells had significantly longer

overall survival ($P < .01$) (Figure 4*C*). All of the mice in the control groups died by day 72. The median survival time was 44 vs 37 days after tumor inoculation for the DC-EGFP and DC group, respectively, and 67 days for the DC-EGFP-OVA group ($P < .0443$ and $P < .0082$, respectively). The experiment ended on day 72 after initial tumor inoculation.

Modified DCs Demonstrated Enhanced Homing to Lymph Nodes and Resulted in Significantly More Cytotoxic T Cells in Both the Lymph Nodes and Tumors

To assess whether DC-EGFP-OVA cells can enter the T cell–rich regions and activate T cells to generate CTL, IHC of OVA, CD11c, CD3, and CD8 expression was performed on mice axillary lymph nodes after immunization. As shown in Figure 5, *A–D*, DC-EGFP-OVA cells demonstrated significantly enhanced homing to lymph nodes than DC-EGFP cells (CD11c expression: 15.9% vs 1.6%, $P < .0001$; OVA expression: 9.6% vs 0.8%, $P < .0001$) (Figure 5*E*). Figure 5, *F–I* showed that DC-EGFP-OVA cells potently activated T cells to generate CTL, which accumulated in the relative DC-EGFP-OVA–rich regions of lymph nodes (CD3 positive rate: 9.7% vs 3.5%, $P < .0001$; CD8 positive rate: 5.9% vs 2.1%, $P = .0006$) (Figure 5*J*). Moreover, Figure 5, *K–N* showed that there were significantly more activated CTLs that infiltrated into the tumor sites than control groups (CD3 positive rate: 6.9% vs 2.4%, $P < .0001$; CD8 positive rate: 5.0% vs 1.7%, $P < .0001$) (Figure 5*O*).

Modified DCs Downregulated CSC Markers in Tumors

To test whether OVA-expressing modified DCs could have potential effect on CSCs of OVA-expressing LLCs *in vivo*, we performed IHC of putative CSC markers CD133 and nestin in the tumors before and after treatment. Figure 6 demonstrated that positive expression rate of both CD133 and nestin significantly reduced after treatment with DC-EGFP-OVA + T (20.7% vs 9.5% and 14.8% vs 7.6%; $P < .05$, respectively) but barely changed in tumors treated with either DC-EGFP + T or DC + T, suggesting potential effect on CSCs, although more studies are certainly needed. Due to the antitumor effect of IL-12 (Tugues et al., Cell Death and Differentiation, 2015), we also measured serum IL-12 level along the

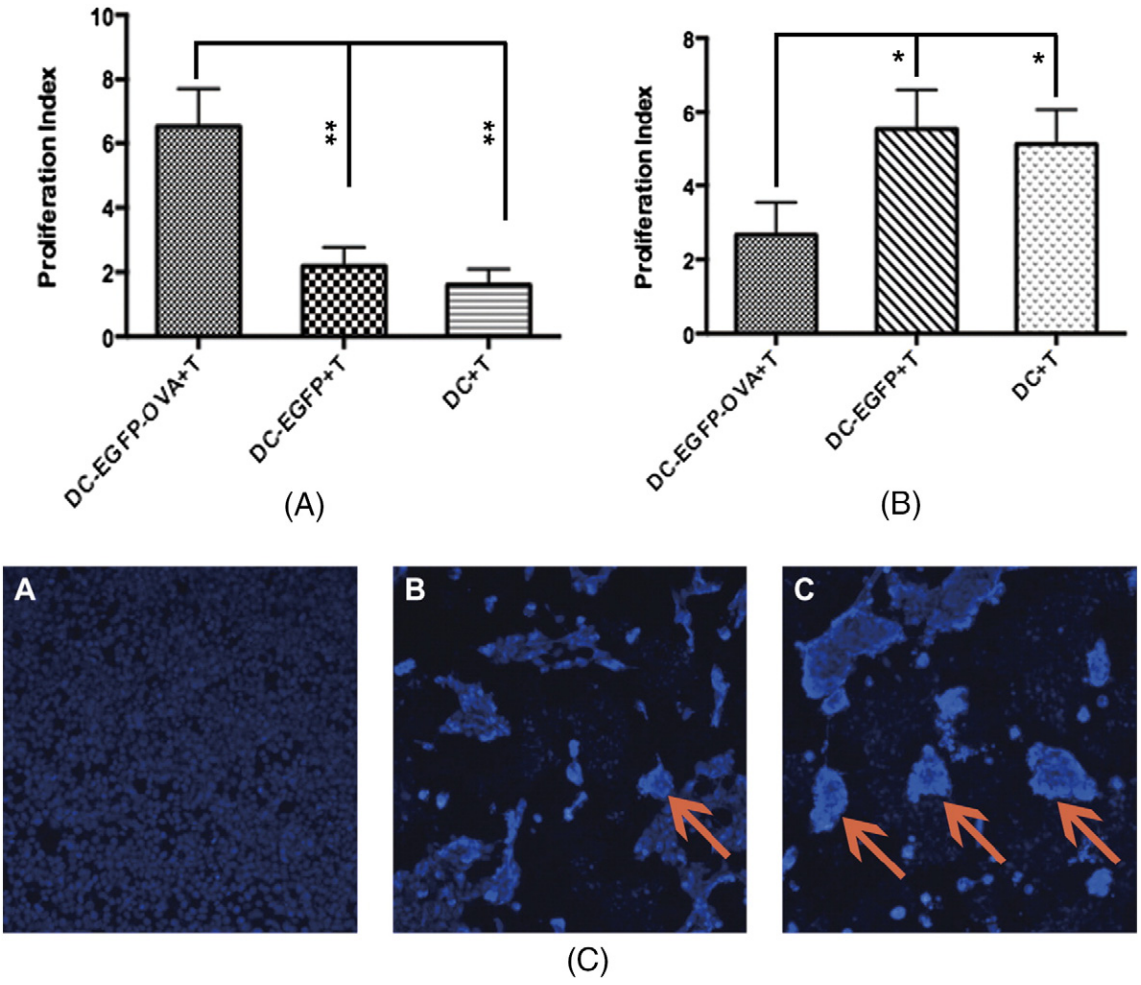

Figure 3. The OVA-expressing modified DCs enriched T-cell proliferation and enhanced the killing of OVA-expressing LLCs *in vitro*. (A) DC-EGFP-OVA cells enhanced more significantly the proliferation of T cells than DC-EGFP and unmodified DCs ($P < .01$ and $P < .01$, respectively) (×100). (B) DC-EGFP-OVA cells induced more effectively the T cells to kill LLC-EGFP-OVA cancer cells than DC-EGFP and unmodified DCs ($P < .05$) (×100). (C) Hoechst 33258 staining showed that DC-EGFP-OVA–activated T cells could remarkably induce apoptosis of LLC-EGFP-OVA cancer cells (×100) [(C) A: DC:T cells = 1:5; (C) B: DC-EGFP-OVA:T cells =1:5; (C) C: DC-EGFP-OVA:T cells =1:10; $*P < .05$; $**P < .01$]. However, further increasing the ratio of modified DCs to T cells did not yield more apoptosis [(C) B versus (C) C].

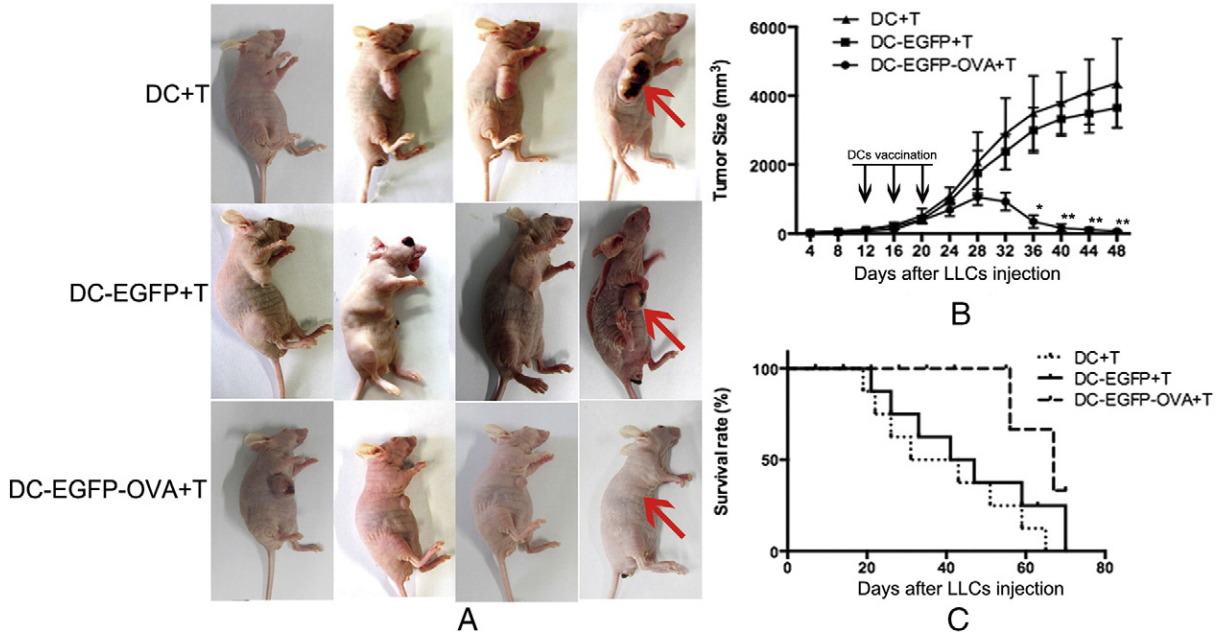

Figure 4. The OVA-expressing modified DCs enhanced the killing of OVA-expressing LLCs *in vivo* and prolonged the overall survival of tumor-bearing mice. (A) Representative tumor-bearing mice from each group showing that mice that received DC-EGFP-OVA plus T cells had much smaller tumors. (B) The tumor growth curve showing average tumor sizes from each group. After 20 days of tumor cell inoculation, mice in the DC-EGFP-OVA + T group grew significantly smaller tumors than those in the two control groups. (C) A comparison of overall survival. Whereas the median survival time was 44 and 37 days for the DC-EGFP + T and DC + T group, respectively ($P < .0443$ and $P < .0082$, respectively), it was 67 days for the DC-EGFP-OVA + T group, suggesting that DC-EGFP-OVA plus T cells treatment could significantly prolong the overall survival.

course of treatment which demonstrated that mice treated with DC-EGFP-OVA + T had persistently higher level of serum IL-12 ($P < .01$) (Supplemental Figure S2).

Discussion

DCs are critical regulators of innate and acquired immunity. They are uniquely potent in their ability to capture and process antigens, and induce efficient activation of T cells that play vital roles in cancer immunotherapy through expressing high levels of major histocompatibility complex (MHC)-peptide complexes and co-stimulatory molecules [8,20,21]. Previous studies have shown that DCs generated from murine bone marrow and loaded with *OVA* plus other antigens elicited strong allogeneic stimulatory activity in mixed lymphocyte reactions and efficiently activated CD4+ and CD8+ T cells [22,23]. Importantly, murine bone marrow–derived DCs stimulated with *OVA* plus bacterial OprI lipoprotein could delay tumor growth and prolong mice survival in syngeneic melanoma mouse model [23]. In our present study, we also demonstrated that murine bone marrow DCs modified with *OVA* induced proliferation of isolated T cells and immune response against lung cancer cells expressing *OVA in vitro*. Lung cancer mouse model vaccinated with *OVA* gene-modified DCs had a smaller tumor size and longer survival time than control groups. Thus, DCs modified with TAAs might be a valuable vaccination strategy for future cancer immunotherapy.

After vaccine administration, activated DCs must closely interact with *naïve* T cells, which then exert their cytotoxic, helper, or regulatory function. Maximizing the homing to T cell–rich areas through DC-based vaccines has been shown to enhance antitumor immune responses [24]. We therefore performed IHC of CD3, CD8, CD11c, and OVA expression in lymph node and tumor sites of tumor-bearing mice. The results showed that modified DCs had enhanced homing to T cell–rich areas in lymph node and enhanced the activation of *naïve* T cells to become CTLs, especially CD8+ CTLs. As the result, we observed that significantly more CD8+ T cells had infiltrated in the tumors. Indeed, multiple lines of evidence show that CD8+ T cells are key components of antitumor immunity [25]. They can exhibit their robust antitumor effect through direct cytolytic activity and their cytokine secretion [26]. Therefore, the increased tumor-infiltrating CD8+ T cells had likely contributed to the therapeutic effect in this study.

As is known, immune response to tumor cell can be divided into two steps: induction and effector phase [27]. In the induction phase, DCs handle the antigens from the tumor and present them to naïve T cells. Prior to this step, DCs must receive immunogenic stimuli to mature. Afterward, the mature DCs process the captured antigen and present it on MHC class II molecules, at which point they are transported to the draining lymph node and start to interact with T cells and induce corresponding immune response. After MHC and processed antigens binding together, T-cell receptor will interact with them, and a co-stimulatory signal is needed to activate T cells to eliminate tumor cells. In the present study, although we have demonstrated that effector CD8+ T cells had significantly infiltrated the tumor microenvironment after vaccination with modified DCs, their function could still be impaired to a certain extent by tumor cells as previously reported [26,28]. The major reasons to cause impaired function of CD8+ T cells include the increased expression of co-inhibitory receptors such as programmed death-1 (PD-1), CD244, CD160, T-cell immunoglobulin and mucin domain-3-containing molecule 3 (TIM3), and lymphocyte-activation gene 3 (LAG3) [26,28]. However, it is important to note that CD8+ T-cell

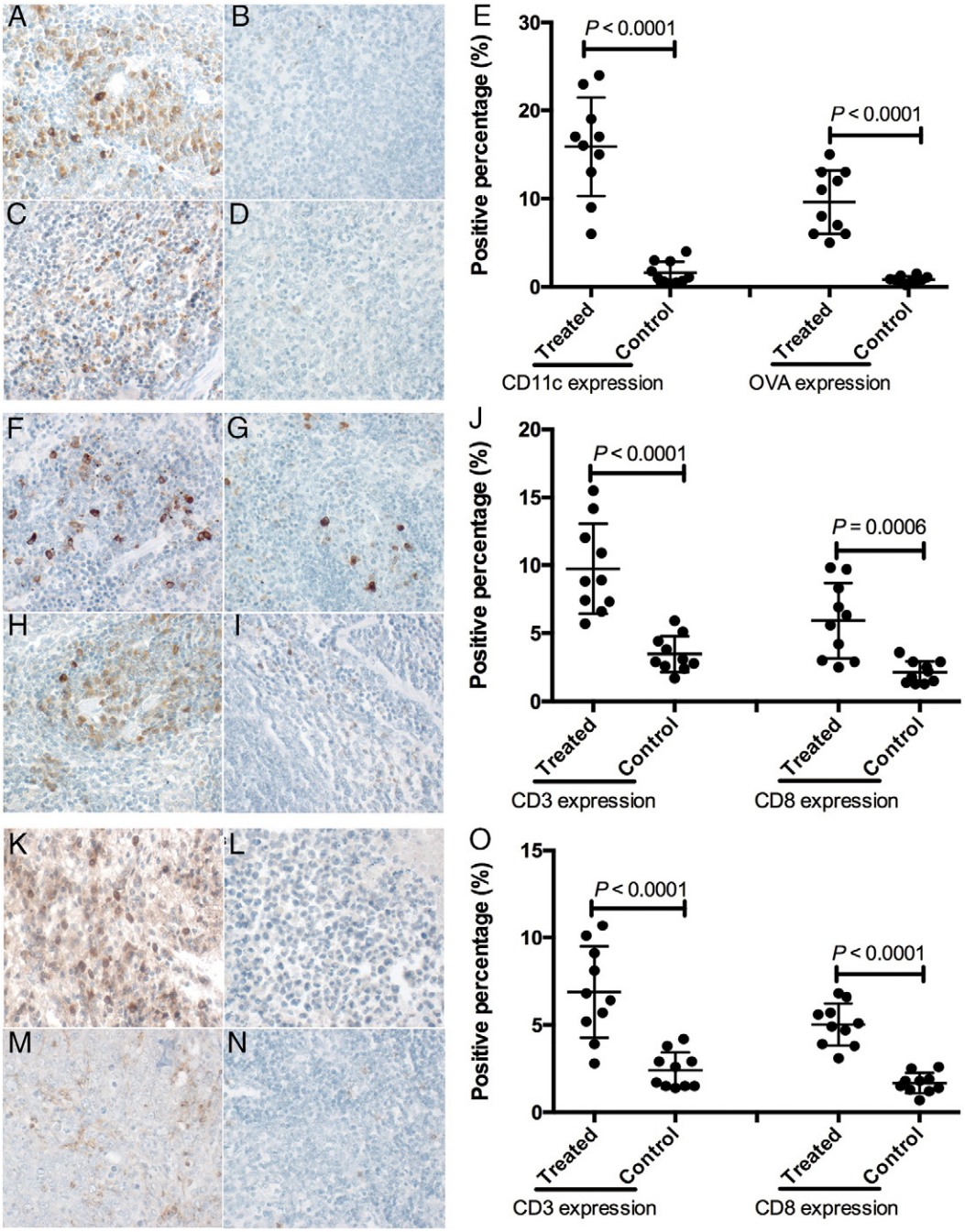

Figure 5. Modified DCs demonstrated enhanced homing to lymph nodes and resulted in significantly more cytotoxic T cells in both the lymph nodes and tumors. (A and B) CD11c staining for lymph nodes of DC-EGFP-OVA and DC-EGFP group, respectively. (C and D) For OVA staining. (E) Statistical analysis. Compared with the control, modified DC demonstrated enhanced homing to the lymph nodes (CD11c expression: 15.9% vs 1.6%, $P < .0001$; OVA expression: 9.6% vs 0.8%, $P < .0001$) (×400). (F–I) Representative staining of CD3 and CD8. (J) There were significantly more CD3-positive as well as CD8-positive T cells in the lymph nodes from mice immunized with DC-EGFP-OVA cells (CD3 positive rate: 9.7% vs 3.5%, $P < .0001$; CD8 positive rate: 5.9% vs 2.1%, $P = .0006$) (×400). (K–N) Staining of CD3- and CD8-positive T cells in the tumors. (O) Significantly more infiltration of both CD3- and CD8-positive T cells in the tumors of mice treated with DC-EGFP-OVA + T cells (CD3 positive rate: 6.9% vs 2.4%, $P < .0001$; CD8 positive rate: 5.0% vs 1.7%, $P < .0001$) (×400).

dysfunction in the tumor microenvironment is believed to be reversible, at least to some extent [26,28]. In preclinical cancer models, blockade of signaling through PD-1, TIM3, and LAG3 has been shown to improve CD8+ T-cell responses. It is therefore reasonable to speculate that combined DCs-based vaccine and antibodies of co-inhibitory receptors such as PD-1, TIM3, and LAG3 might generate even more robust antitumor effect.

IL-12 is a heterodimeric cytokine and exerts potent immune stimulatory effects on certain helper T cells as well as on CTL and natural killer cells. IL-12 promotes the proliferation and differentiation of activated CD8+ T cells into CTL effectors and stimulates the cytolytic activity of fully differentiated CTL [29,30]. In our present study, the serum concentration of IL-12 in mice treated with modified DC and T cells remained persistently at higher level, suggesting that

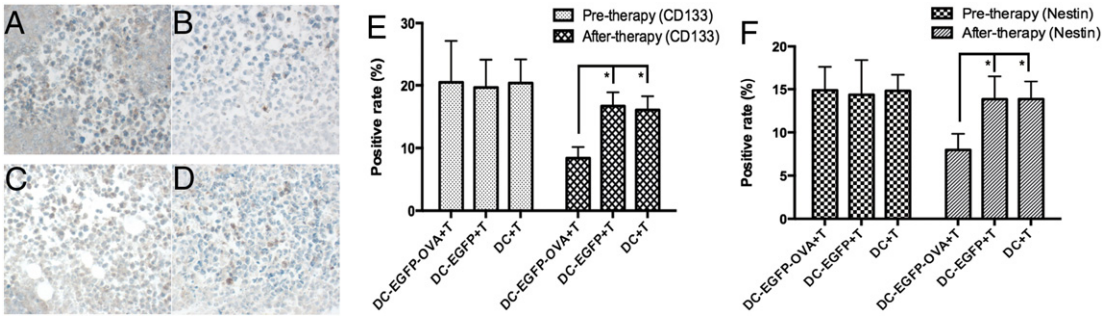

Figure 6. Modified DCs downregulated CSC markers in tumors. (A and B) Representative staining of CD133 before and after treatment in DC-EGFP-OVA + T group (×400). (C and D) Representative staining of nestin before and after treatment in DC-EGFP-OVA + T group (×400). (E) A comparison of CD133 positive rate pre- and posttherapy among different treatment groups. CD133 expression was reduced significantly only in the DC-EGFP-OVA + T group (20.7% vs 9.5%, $P < .05$). (F) A comparison of nestin positive rate pre- and posttherapy among different treatment groups. Nestin expression was reduced significantly only in the DC-EGFP-OVA + T group (14.8% vs 7.6%; $P < .05$).

OVA gene–modified DCs could also stimulate the production of key cytokines to enhance antitumor response. It will be interesting to test whether stronger antitumor effect can be achieved if DCs are modified to express both TAA and antitumor cytokine.

CSCs are critical for tumorigenesis and metastases, and are thought to be responsible for treatment resistance and disease recurrence [31]. Targeting CSC is thus imperative to achieve survival benefit and even curative intent that otherwise could be challenging through conventional therapies [32]. In the present study, using mice vaccinated with modified DCs, we demonstrated that DCs modified with *OVA* gene reduced significantly the population of cancer cells harboring stem cell markers, for example, CD133 and nestin, suggesting possible anti-CSC effect *in vivo* that is worth further exploration. This is consistent with the previous studies showing that bone marrow–derived DCs primed with breast CSC–derived antigen could significantly inhibit breast CSC proliferation both *in vitro* and *in vivo* [33]. Another study has also demonstrated that DCs loaded with antigens derived from glioma stem cells can effectively stimulate *naïve* T cells to form specific cytotoxic T cells to kill glioma cells [34]. These results indicated that DCs pulsed with CSC antigens might have unique therapeutic benefit.

In conclusion, our study demonstrated that *OVA* gene–modified DCs could stimulate robust T cell–mediated immunity. Modified DCs had enhanced homing to T cell–rich compartments and were capable of activating enhanced number of CTLs to kill the tumor cells. This vaccination approach has demonstrated its role in inhibiting tumor growth and prolonging the survival of tumor-bearing animals, as well as a potential adverse effect on CSCs that is worth further exploration.

Disclosure

The authors have declared no conflicts of interest.

Acknowledgments

Peilin Zhao designed the research project; Xiao Chen, Wei Zhou, and Tao Jiang supervised all experiments; Tao Jiang drafted the manuscript; Peilin Zhao, Caicun Zhou, Shengxiang Ren, and Tao Jiang discussed results, planned the study, and edited the manuscript. Guoxin Fan carried out statistical analysis; Caicun Zhou, Shengxiang Ren, and Jun. Zhang commented and revised the paper; all authors approved the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tranon.2016.12.002.

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