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RESEARCH PAPER

Co-transfection Gene Delivery of Dendritic Cells Induced Effective Lymph Node Targeting and Anti-tumor Vaccination

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ABSTRACT

Purpose Successful genetically engineered Dendritic Cell (DC) can enhance DC's antigen presentation and lymph node migration. The present study aims to genetically engineer a DC using an efficient non-viral gene delivery vector to induce a highly efficient antigen presentation and lymph node targeting *in vivo*.

Methods Spermine-dextran (SD), a cationic polysaccharide vector, was used to prepare a gene delivery system for DC engineering. Transfection efficiency, nuclear trafficking, and safety of the SD/DNA complex were evaluated. A vaccine prepared by engineering DC with SD/gp100, a plasmid encoding melanoma-associated antigen, was injected subcutaneously into mice to evaluate the tumor suppression. The migration of the engineered DCs was also evaluated *in vitro* and *in vivo*.

Results SD/DNA complex has a better transfection behavior *in vitro* than commercially purchased reagents. The DC vaccine co-transfected with plasmid coding CCR7, a chemokine receptor essential for DC migration, and plasmid coding gp100 displayed superior tumor suppression than that with plasmid coding gp100 alone. Migration assay demonstrated that DC transfected with SD/CCR7 can promote DC migration capacity.

Conclusions The study is the first to report the application of nonviral vector SD to co-transfect DC with gp100 and CCR7-coding plasmid to induce both the capacity of antigen presentation and lymph node targeting.

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KEY WORDS CCR7 · dendritic cell · gene delivery · immunotherapy · spermine-dextran

ABBREVIATIONS

APC	antigen presenting cell
CCL21	C-C Chemokine ligand 21
CCR7	C-C chemokine receptor type7
CLSM	confocal laser scanning microscopy
DC	dendritic cell
EGFP	enhanced green fluorescent protein
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony stimulating factor
MHC	major histocompatibility complex
MβCD	methyl-β-cyclodextrin
PI	propidium iodide
SD	spermine-dextran
TAA	tumor associated antigen

INTRODUCTION

Cancer is one of the leading causes of death worldwide (1), and immunotherapy is expected to be a promising approach to conquer this disease. Recent findings suggest that anti-tumor vaccines based on molecular identities of tumor-associated antigens (TAAs) are feasible and promising (2,3).

The dendritic cell (DC) is a professional antigen-presenting cell essential in initiating and regulating adaptive immune responses (4). The major function of DCs is to capture foreign- and self-antigen and present these antigens to T cells when infection or cancer occurs (5). In 1990, it was first reported that injection of DC with protein antigens *in vivo* can promote antigen-specific response in animal models (6). The DC networking system has emerged as an attractive approach in cancer therapy (7,8).

Several methods are used to load DC with TAAs including tumor-associated peptides impulse, whole tumor lysate impulse, TAA RNA transfection, and TAA DNA transfection

(9). Referring to the methods of using tumor co-culture, tumor-lysate and tumor peptides as TAA, the disadvantage that presence of irrelevant antigens could cause autoimmune responses should be concerned. After transfecting DC with DNA TAA, it expresses specific protein which is well known in both structure and function, and avoids autoimmune responses in immunotherapy. Several reports have quoted this design and method to improve DC capacity or prepare DC vaccine (10–12). Plasmids encoding, cytokines, and chemotactic cytokines (chemokines) are also widely used in DC engineering to enhance the immunological function of DCs (13).

A successful gene delivery depends on the safety and efficiency of the transfection vector, such that the improvement of DC gene transfection efficiency *in vitro* has been a research hotspot. Gene delivery vectors are commonly divided into viral and nonviral vectors. The former are efficient but pose several safety risks (14). By contrast, nonviral vectors, such as liposomes and polymers, have been promoted recently because of their low immune response, ease of synthesis under controllable conditions, and accessibility of modification according to special needs (7). Previous studies that focused on nonviral gene delivery vectors applied in DC engineering produced several achievements (15,16). However, the main disadvantage of nonviral vectors is the relatively low efficiency in transfection, resulting in a moderately successful achievement of DC transfection with nonviral vector/DNA delivery system (17).

DC migration to secondary lymphoid tissues is essential in antigen presentation. Chemokines are small molecule cytokines that enable cell migration. A chemokine receptor can bind specifically to chemokine and mediate migration. CCR7 belongs to CC chemokine receptor and its ligand secondary lymphoid is CCL21/19, highly expressed in the endothelial lymphatic duct of the lymph nodes (18). In engineering a potential DC vaccine, a chemokine receptor coding plasmid is loaded to induce a lymphoid tissue targeting *in vivo*. In the previous study, CCR7 gene has been utilized to genetically promote DC capacity by viral vector to induce more intensive T cell specific response based on the function of chemokine in the up-regulation of DC maturation and migration capability (19). In the present study, plasmid encoding CCR7 was used to co-transfect DC by non-viral gene delivery system.

Here, spermine-dextran (SD), a cationic polysaccharide vector, was used as the gene vector for DC transfection. Our previous study on tumor cells showed that SD displayed an efficient capacity for gene delivery and was biocompatible (20). DC engineered by SD/DNA complexes and intratumorally injected in mice showed significant anti-tumor activity (16). However, the SD/DNA complex intracellular and intranuclear mechanisms remain unknown. Furthermore, whether the enhancement of DC migration capacity would induce enhanced tumor suppression efficiency still needs further study. In the current study, SD was synthesized and SD/DNA complexes were prepared by direct incubation. Physicochemical properties, transfection efficiency, cytotoxicity, nuclear trafficking, and endocytosis pathway of SD/DNA complexes were investigated. A DC vaccine was engineered by co-transfection with SD/gp100, and CCR7 was subcutaneously injected to protect against melanoma solid tumor (Fig. 1). This study is the first report to engineer a DC by gp100 and CCR7 coding plasmid as a lymphoid tissue targeting vaccine using a SD/DNA delivery system. Though the transfection efficiency is not as high as adenoviral system reported previously (19,21), our study offers an alternatively promising approach for genetically enhancing the immunological functions of DCs via nonviral gene delivery vector.

MATERIALS AND METHODS

Animals and Materials

C57BL/6 mice (6- to 8-week-old, male) were purchased from the Animal Experiment Center of Zhejiang University, China. All animals were maintained under constant temperature 25°C ± 1°C on a 12 h light/dark cycle, with free access to food and water. All experimental procedures were in accordance with guidelines of the Zhejiang University for the welfare of experimental animals.

Plasmid DNA (pGL3 and pEGFP-N1) was kindly provided by the Institute of Infectious Diseases, Zhejiang University, China. Plasmid DNA (pHM5/gp100) and DNA (pCMV-CCR7) were kindly provided by Professor Shinsaku Nakagawa (Graduate School of Pharmaceutical Sciences, Osaka University, Japan). Dextran, with a weight-average molecular weight of 74 000, and spermine were purchased from Sigma (St. Louis, MO, USA). FITC-DNA was purchased from Sangon Biotechnology Inc. (China). Lipofectamine2000 (Lipo) was purchased from Invitrogen (USA). All other chemicals were of analytical grade. B16BL6 melanoma cell line derived from C57BL/6 mice was kindly provided by the Institute of Chemical Biology and Pharmaceutical Chemistry, Zhejiang University, China, and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Synthesis of Spermine-Dextran

Synthesis of spermine-dextran (SD) was performed according to the procedure described in the previous report (16). Dextran (50 mg) was dissolved in 5 mL DMSO and activated by 225 mg CDI for 5 min. The activated dextran was then gradually added to 45 mL DMSO containing 1873.5 mg spermine and reacted for 18 h at room

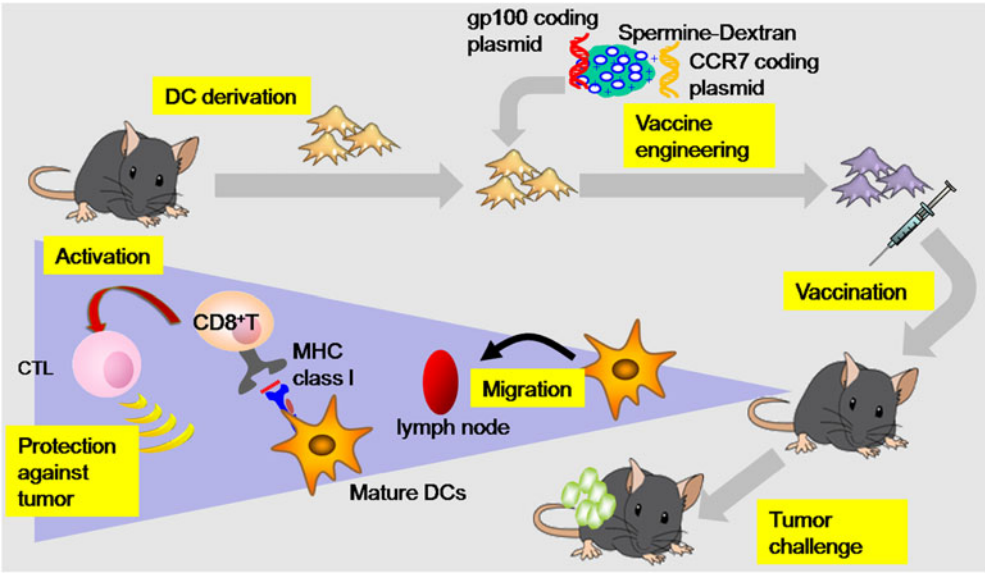

Fig. 1 Representative graph for the research strategy.

temperature. The product was dialyzed in ultrapure water for 48 h (MWCO 12,000 to 14,000) and lyophilized. The grafted rate of SD was then detected by elemental analysis.

Preparation of SD/DNA Complexes

SD/DNA complexes were temporally prepared for each experiment. The mass ratio of the SD/DNA complexes was 3:1, determined according to previous experimental data (16). Complexes were prepared by merging polymer with equal volumes of pGL3-DNA (for size and zeta potential measurements), pEGFP-N2 DNA (for *in vitro* GFP transfection), or FITC-DNA in PBS solution (for CLSM observation). The mixture was then gently vortexed and incubated at room temperature for 15 min.

Preparation and Culture of DCs

Mouse immature DC were obtained according to conventional procedure, as previously reported (22), with several modifications. Briefly, bone marrow cells (BMC) were harvested from the femur and tibia of the male C57BL/6 mice. Erythrocytes among BMC were lysed with Tris-NH_4Cl (pH7.4) solution. The cells were finally resuspended in RPMI-1640 (Gibco, USA) containing penicillin (100 U/ml), streptomycin (100 μg/ml), 10% FBS (HyClone, USA), and 20 ng/ml recombinant mouse-derived granulocyte-macrophage colony-stimulating factor (rmGM-CSF) (R&D Systems, Inc., USA) on day 0. On day 2, fresh RPMI1640 containing 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10 ng/ml rmGM-CSF was added after complete removal of the medium. The dishes were replaced with half volume fresh media every 2 day. Cells cultured for 9 day to 14 day were collected as mouse immature DC in all experiments. Cell phenotype of DCs was assessed by detecting the expression of DC surface markers, which proved the presence of DCs.

Transfection Efficiency Evaluation

DCs were seeded into a 24-well plate at a density of 1×10^5 cells/well. SD/DNA complexes (mass ratio = 3:1) and Lipo/DNA complexes (mass ratio = 3:1) were added with 500 μl fresh serum-free medium after 24 h to replace the previous culture medium. The serum-free medium was replaced with a medium containing 10% FBS after 6 h of incubation. Fluorescence signal was observed using a fluorescence-inverted microscope after an additional 18 h of incubation. The ratio of Lipo/DNA complexes were according to the instructions of the manufacturer. Each well contained 1 μg of DNA.

Nuclear Trafficking of SD/DNA Nanocomplexes on the DCs

DCs were plated on glass slips in 12-well plates at 1×10^5 cells/well and cultured for 24 h. The medium was then replaced with serum-free medium containing SD/DNA-FITC complexes (mass ratio = 3:1) and Lipo/DNA-FITC (mass ratio = 3:1). After 4 h of incubation, cells were washed by PBS and fixed with 4% paraformaldehyde. The nucleus was stained by PI for 20 min at room temperature, and then washed three times with PBS. Afterward, the slips were mounted reversely on slides and sealed with resinene for observation under CLSM (MRC-1024, Bio-Rad, UK).

Endocytosis Pathway Investigation

DCs were stained with CM-Dil before being seeded in 12-well plates at 1×10^6/well in serum-free medium, which were either

untreated or pretreated with chlorpromazine (10 μg/ml), methyl-β-cyclodextrin (13.3 mg/ml), amiloride hydrochloride (15.1 μg/ml), and NaN_3 to inhibit the clathrin-mediated pathway, caveolae-mediated pathway, macropinocytosis, and respiratory energy chain for 30 min. SD/FITC-DNA complexes were added and incubated with DC for 1 h. After two washings with PBS, the DCs were fixed by 4% paraformaldehyde for 20 min and observed under CLSM.

Cell Viability Assays

For the cell viability assay, DCs were seeded in 96-well plates at a density of 1×10^5 cells/well. The culture medium was replaced with fresh serum-free medium after 24 h. SD/DNA (mass ratio :1) and Lipo/DNA (mass ratio = 3:1) complexes were then added for a 6-h incubation. The serum-free medium was replaced with a culture medium with 10% FBS. After 18 h of incubation, 20 μl of MTT solution (5 mg/ml) was added, and the OD value at 570 nm was measured according to the instructions of the manufacturer. The control group was treated with naked plasmid DNA. All experiments were repeated to ascertain reproducibility. Data was expressed as mean± standard deviation (n=6). Statistical analysis was performed using the Student's t-test. Differences were considered statistically significant at $P<0.05$.

Vaccine Efficacy of SD-gp100/DCs and SD-CCR7-gp100/DCs Against B16BL6 Tumor Challenge

SD/gp100 and SD/CCR7 were prepared with corresponding vectors at a mass ratio = 3:1, and were added to DCs for an incubation of 6 h in tube. The serum-free medium was then replaced by a culture medium containing 10% FBS for additional incubation for 18 h for transfection. C57BL/6 mice were immunized three times by intradermal injection of engineered DCs at 1×10^6 cells/100 μl, with one week interval. One week after the last vaccination, 3×10^5 B16BL6 melanoma cells were inoculated into the mice. The size of the tumors was assessed using microcalipers and expressed as tumor volume, as calculated by the following formula:

$$\text{Tumor volume}\left(\text{mm}^3\right) = \text{Major axis(mm)} \times \text{Minor axis(mm)}^2 \times 0.5.$$

On day 21 post-inoculation, tumor-bearing mice were euthanized, and the tumors were weighed.

In Vitro Migration Assay

Chemotaxis of DCs was examined in 24-well chemotaxis chambers (HTS FluoroBlok™ Multiwell insert systems; BD Biosciences, Franklin Lakes, NJ) with an 8 μm pore size membrane separating the upper from the lower compartment. DCs were seeded into 24-well plates at 1×10^5 cells/well. After 24 h, SD/CCR7 complexes were prepared, as previously described. SD/CCR7 (mass ratio = 3:1) and Lipo/DNA (mass ratio = 3:1) complexes were added with 500 μl fresh serum-free medium to replace the previous culture medium. After 6 h of incubation, the serum-free medium was replaced with a culture medium with 10% FBS. After 18 h of incubation, 1×10^6 DCs were washed by PBS and seeded in the upper wells of the chamber. Lower compartments were filled with RPMI-1640 supplemented with 1% FBS CCL21 chemokines, at a final concentration of 500 ng/ml (23). After 4 h of incubation at 37°C, DC migration to the lower chambers was determined by a hemocytometer (Hausser Scientific). The chemotactic index (CI), a measure of the specificity of migration, was calculated as follows: (number of DCs migrating to chemokines)/(number of cells that migrated to medium alone).

In Vivo Migration Assay

DCs were seeded into a 12-well plate at 1×10^6 cells/well. After 24 h of culture, SD/CCR7 complexes were prepared as previously described. SD/CCR7 complexes (mass ratio = 3:1) and Lipo/DNA complexes (mass ratio = 3:1) were added with 500 μl fresh serum-free medium to replace the previous culture medium. After 6 h of incubation, the serum-free medium was replaced with a culture medium with 10% FBS. After 18 h of incubation, DCs were washed by PBS and stained with CM-Dil. The mice were subcutaneously injected with 1×10^6 DCs. The mouse inguinal lymph nodes were then isolated after 24 h and observed by intravital fluorescence microscopy (Maestro™ *In-vivo* Imaging System, CRi, USA).

RESULTS

Synthesis and Characterization of SD and SD/DNA

The reaction involved in the synthesis of SD is illustrated in Fig. 2a. The process is characterized by elemental analysis shown in the table. According to the elemental analysis result, theoretically, each monosaccharide of dextran has three hydroxyl groups that can be grafted by the amino group of spermine. Using elemental analysis, 12.3% hydroxyl group of dextran was calculated to be conjugated with spermine when nitrogen% was 7.84% according to a previous report (20). Previous experiments showed that the best transfection efficiency would be obtained at an N/P ratio of 3 for SD and DNA (16) or a mass ratio close to 3. Fig. 2b shows the average particle size of the SD/DNA complexes determined by Zetasizer. The particle sizes of the complexes decreased with the increase in SD/DNA mass ratio. The average particle size was 1500 nm when SD/DNA (w/w) ratio was 1. However, the sizes of the complexes decreased

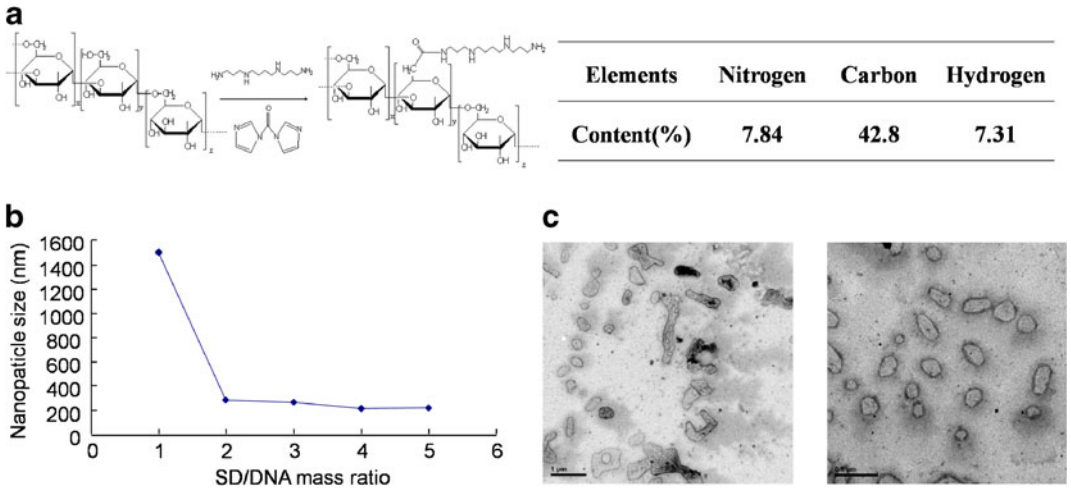

Fig. 2 (**a**) Reaction pathway for the synthesis of SD. CDI was used as a linking agent. Polymerization was performed at room temperature for 18 h. Product was purified by dialysis and stored in 4°C after lyophilization. (**b**) Mean particle size of SD/DNA complexes determined by Zetasizer. The mass ratio of SD/DNA was from 1:1 to 5:1; (**c**) SD/DNA complexes observed by TEM. Left panel The mass ratio of SD/DNA was 1:1 The Right panel SD/DNA was 3:1.

to less than 300 nm and eventually maintained at approximately 200 nm when the ratio was increased, as reconfirmed by TEM images shown in Fig. 2c. At a SD/DNA mass ratio = 1, the SD and DNA cannot perform homogeneously with a particle-like structure in Fig. 2c, left panel. Fig. 2c right panel shows that the SD/DNA complexes maintained a round shape with similar ellipsoid and sphere homogeneous structures when the SD/DNA mass ratio was 3:1. These results indicated that SD and DNA can successfully form a complex when the SD/DNA mass ratio exceeds 2.

Transfection Efficiency and Cytotoxicity of SD/DNA Complexes

In evaluating the transfection efficiency of SD/DNA complexes, plasmid pEGFP-N1-encoding green fluorescent protein (GFP) was applied to evaluate the GFP expression in DCs. The DCs were transfected with SD delivering pEGFP-N1 using Lipo/DNA as positive control. Fig. 3a and b depict the transfection efficiency of the complexes on the DCs. Fluorescence signal was observed in DCs transfected by Lipo/DNA for 24 h (Fig. 3b). The percentage of GFP positive SD/DNA engineered DC in five fields of vision was 16±4%, while Lipo/DNA-DC was 6±3%. By contrast, higher positive rate and stronger fluorescent intensity were observed in those transfected by SD/DNA (Fig. 3a). This result indicated that, the plasmid in the SD/DNA delivery system can be smoothly expressed within the DCs. Hence, SD exhibited a relatively higher delivering efficiency in engineered DCs than that of commercially purchased reagent.

Cytotoxicity is one of main concerns for polymers. The results of the *in vitro* cytotoxicity evaluation of SD were detected by MTT assay. Fig. 3c demonstrates the viability of DC incubated with Lipo/DNA and SD/DNA complexes for 24 h. The cell viability of DC incubated with Lipo/DNA complexes was 75%, significantly less than that of the naked DNA control and SD/DNA complex groups. By contrast, the viability of DCs after incubation with SD/DNA complexes suffered negligible cytotoxicity, with cell survival rate of 93%, similar to that of the naked DNA group.

Endocytosis Pathway and Nuclear Trafficking Observation of SD/DNA Complexes

DCs (stained by CM-Dil) were pretreated with inhibitors of clathrin- and caveolae-mediated pathway (chlorpromazine and MβCD, respectively) as well as with amiloride hydrochloride, an inhibitor of macropinocytosis, for 1 h to delineate the endocytosis mechanism of SD/DNA complexes applied in this study (24,25). DCs were then allowed to internalize the SD/FITC-DNA complexes for 1 h, in the presence of inhibitors, prior to fixation. Fig. 4a and b demonstrate that the pretreatment with amiloride hydrochloride and chlorpromazine did not block the endocytosis pathway of the complexes; a bunch of green fluorescence of FITC-DNA can be observed intracellularly in these inhibitor-treated groups. Interestingly, the group pretreated with cells containing MβCD demonstrated a remarkable inhibition of FITC-DNA entrance (Fig. 4c). Moreover, pretreatment with NaN_3, a cytochrome oxidase inhibitor by binding irreversibly to the heme cofactor in a process similar to the action of carbon monoxide, showed definite inhibition (Fig. 4d). These results suggest a significant SD/DNA complex entrance by caveolae-mediated pathway, which is respiratory chain energy-dependent.

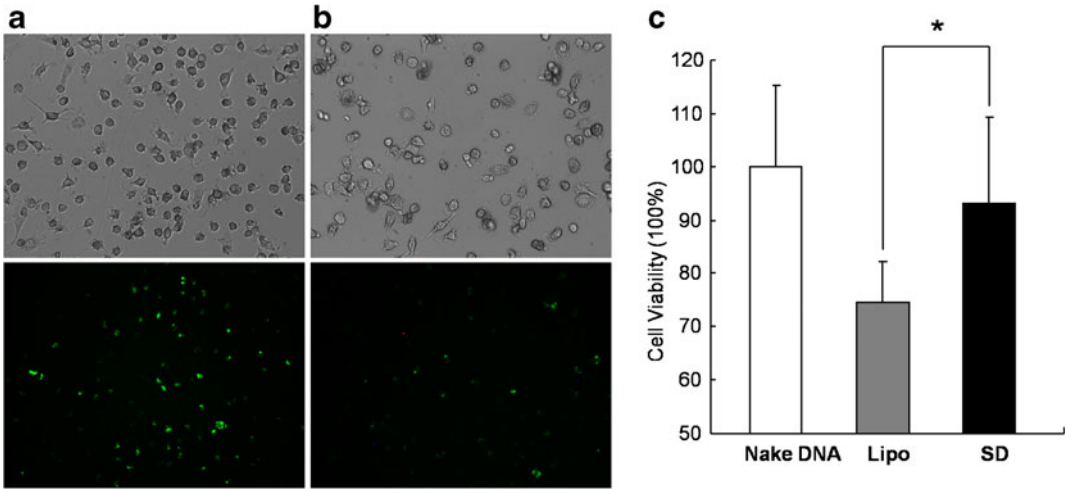

Fig. 3 Transfection efficiency and viability of SD/DNA (eGFP-control) complex DCs after 24 h of transfection with Lipo/DNA and SD/DNA complexes. Transfection with (**a**) SD/DNA and (**b**) with Lipo/DNA complexes. The top panel shows images from an ordinary light microscope, and the panel below shows those from the fluorescence microscope. The green plots show the GFP signal; (**c**) Viability of DC transfection ($n = 6$) as mean ± standard deviation ($n = 6$). Statistical analysis was performed using the Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

Nuclear trafficking of SD/DNA complexes in DCs was observed under CLSM. Fig. 4e-f illustrates that SD can help DNA to enter into the nucleus successfully within 4 h (Fig. 4e). By contrast, DNA carried by commercially purchased reagents Lipofectamine 2000 failed to enter into the nucleus (Fig. 4f). The trafficking of the DNA complexes from the ectoplasm to nucleus was inferred to be important to transfection efficiency. SD/DNA complexes can enter into the DC cytoplasm, escape from endosomes, and traffic into the nucleus, supporting the high transfection efficacy as previously shown.

Vaccine Efficacy of SD-gp100/DCs and SD-CCR7-gp100/DCs Against B16BL6 Tumor Challenge

DCs transfected with SD/gp100 or co-transfected with SD/gp100 and SD/CCR7 were utilized to vaccinate mice against B16BL6 solid tumor to evaluate the immune effect of the DC vaccine *in vivo*. The results are shown in Fig. 5. The tumor inhibition curve (Fig. 5a) shows that each DC-vaccinated group exhibited relative tumor suppression. Compared with the control group, the DC vaccine engineered through SD/gp100

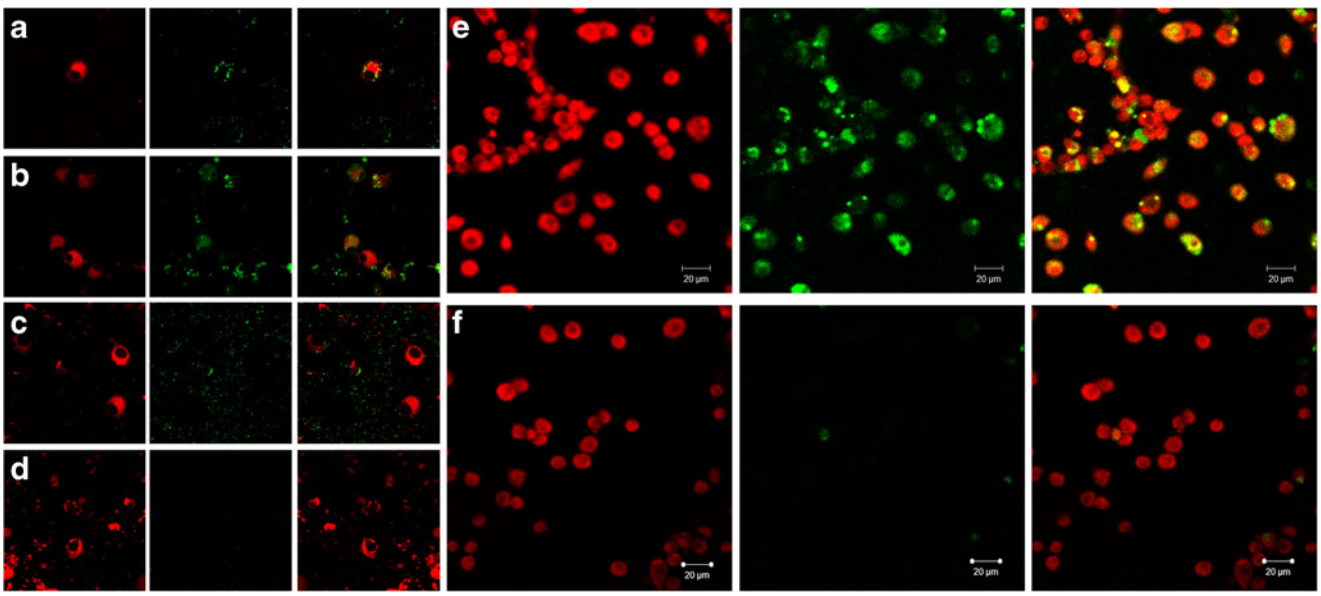

Fig. 4 Intracellular distribution and nuclear trafficking observation of SD/DNA complexes into DCs was observed under confocal laser scanning microscopy. (**a-d**) Observation of FITC-DNA carried by SD/DNA complexes in DCs by CLSM after 1 h transfection. DCs pre-treated by (**a**) amiloride hydrochloride, (**b**) chlorpromazine, (**c**) MβCD, and (**d**) NaN_3. The left panel shows the membrane (*red*) and the middle panel shows the FITC-plasmid (*green*). The right panel shows the over-lay of the left and the middle. (**e-f**) Nuclear trafficking observation of SD/DNA complexes into DCs by CLSM after 4 h of transfection. (**e**) SD/DNA complex; (**f**) Lipo/DNA complexes as control. The left panel shows the nucleus (*red*), and the middle panel shows the FITC-plasmid (*green*). The right panel shows the over-lay of the left and the middle.

transfection or SD/CCR7/gp100 co-transfection exhibited significant tumor suppressive activity. Fig. 5b illustrates the tumor weight calculation when the tumor-bearing mice were killed on 21 day. The statistical data imply that the average tumor weight in each DC-vaccine group was less than that of the PBS control group. The average tumor weight in SD/CCR7/gp100 co-transfection was significantly less than that of the control group. This result can be confirmed from the tumor suppression curve. Fig. 5c shows the statistical data of the survival rate in each group, and the tumor volume greater than 2000 mm^3 was judged to lead to death. At 15 day after inoculation of tumor cells, death occurred in the PBS control and intact DC vaccine groups. No death occurred within 17 day in the SD/gp100 transfection or SD/CCR7/gp100 co-transfection groups, whereas the survival rate in the PBS and intact DC vaccine groups decreased by 37.5% and 62.5%, respectively. Subsequently, death increased in each group over time. On day 21, when all mice were sacrificed, the survival rates of the PBS, intact DC, SD/gp100 transfection, and SD/CCR7/gp100 co-transfection groups were 25%, 33.3%, 66.7%, and 55.6%, respectively. These results indicated that the DC vaccine prepared via SD/gp100 transfection or SD/CCR7/gp100 co-transfection can significantly prolong the life span of mice and suppress tumor growth. Fig. 5d illustrates the body weight detection in each group after DC vaccination. The body weight in each group did not decrease significantly. The body mass index did not indicate any toxicity of the DC vaccine within the mice. The *in vivo* vaccination data demonstrate that the DC vaccine engineered by SD/DNA delivery system possesses a promising efficacy in tumor immunotherapy.

In Vitro and *In Vivo* Migration Assay

An *in vitro* chemotaxis was performed to determine whether the increased CCR7-expression by SD-mediated transfection

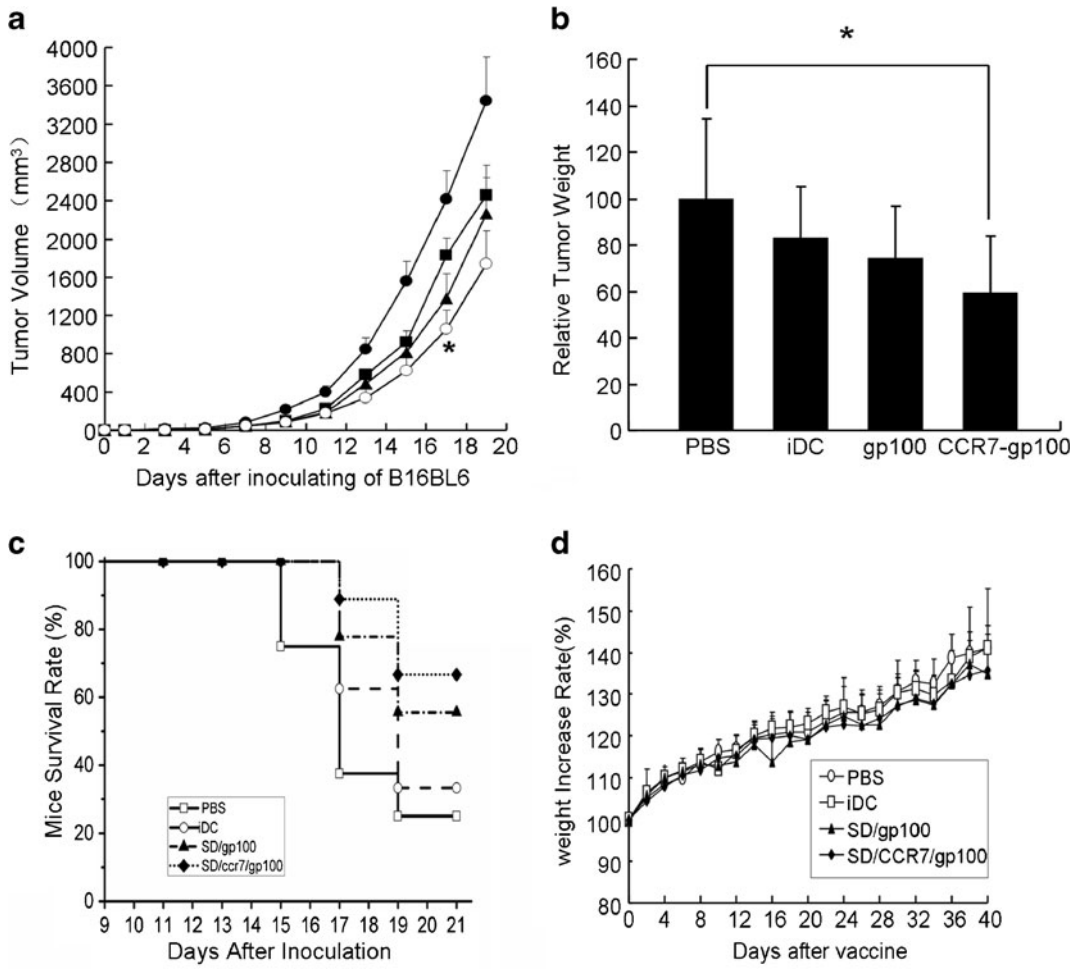

Fig. 5 (**a**) Time-course of tumor volume change after intradermal injection of PBS (●), unmodified DC (■), DC transfected with SD and plasmid gp100 (▲) complexes, or plasmid gp100 and CCR7 (○) by i.d. ($n = 8$) *$P < 0.05$ *versus* the tumor volume of intact DC group; (**b**) Average tumor weight in each group on 21 day post-inoculation of B16BL6 ($n = 8$). *$P < 0.05$ *versus* the tumor weight in the PBS group; (**c**) Survival rate of immune subsets responsible for the protective immunity induced by vaccination with DCs engineered by SD/gp100 and SD/gp100/CCR7; (**d**) Mouse average body weight increase curve after immunization: PBS (○), unmodified DC (□), DC transfected with SD and plasmid gp100 (▲) complexes, or plasmid gp100 and CCR7 (●).

can enhance the migration ability of DCs (Fig. 6a). The migration results showed that, DCs transfected with SD/CCR7 under CCL21 stimulation performed a better migration capacity than intact DCs and Lipo/CCR7-transfected DCs. The chemotactic index (CI) of the SD/CCR7 group to intact DC group was approximately 1.7, representing a relatively high migration ratio (26).

Inguinal LN cells were isolated for the observation of CM-Dil-stained DC migration to test whether the migration capacity to secondary lympoid tissues was enhanced by DCs transfected with SD/CCR7. Lymph nodes were harvested at 24 h post-DC inoculation and detected by an *in vivo* bioluminescence system. Fig. 6b shows that CM-Dil-stained DCs are detectably present in the regional lymph nodes after transfection with Lipo/CCR7 (Fig. 6b, row 3) or SD/CCR7 (Fig. 6b, row 4). In particular, high-density fluorescence was observed in the regional lymph nodes of the group transfected with SD/CCR7. However, low-density fluorescence was observed in the lymph nodes of the untreated group (Fig. 6b, row 2). Though there is no quantitive data but microscopy imagines of Fig. 6b, it is obvious that migration of SD/CCR7-DC group is more efficient than that induced by PBS or unmodified DC group. This result is consistent with the *in vitro* migration result. The *in vivo* migration results further illustrated that the plasmid CCR7 delivered by SD can promote the DC migration capacity *in vivo* and provide a support for the effect of *in vivo* immunity induced by co-transfected DCs.

DISCUSSION

A vector that can effectively deliver a foreign gene to DCs is essential for the preparation of a genetically recombined DC vaccine. Safety, transfection efficiency, as well as the cytoplasmic and nuclear distribution of the nonviral vector should be considered in the design of an ideal vector for DC gene delivery. Studies reported that the nonviral vector SD can enhance the gene expression of stem cells (27,28) and DCs (16) by enhanced endocytosis. However, whether a transfected DC vaccine can function efficiently remains unexplored. Therefore, in the present study, we attempted to transfect DCs with SD/DNA complexes to prepare a DC gene vaccine (16). The MTT result showed that the SD/DNA complexes are safe for gene delivery compared with the commercially available transfection reagent (Lipofectamine 2000) used to transfect DCs (29,30), which exhibited cytotoxicity toward DCs to a certain extent. Gene delivery based on synthesized nonviral vectors or commercially available transfection reagents is inefficient in DC engineering because of low transfection efficiency. However, fluorescent images using SD/EGFP to characterize qualitatively the transfection result contrasted the two groups. The percentage of GFP-positive SD/DNA-engineered DC (16%±4%) was higher than that of lipo/DNA-DC (6%±3%), indicating that Lipofecatmine exhibits a relatively high toxicity and a low transfection efficiency in nondividing and primary cells.

Endocytosis is an essential cellular process for the internalization of macromolecules via phagocytosis or pinocytosis. Pinocytosis can be categorized into three pathways, namely, macropinocytosis, caveolae-mediated endocytosis, and clathrin-mediated endocytosis (31). The inhibition assays described in this study suggest that SD/DNA complexes undergo cellular entrance via caveolae-mediated pathway endocytosis depending on the energy. Several large molecules (~200 nm) are directly transported by caveolae (rather than clathrin-coated vesicles) into the cytosol (32,33).

The ability of a vector to escape the endosome and enter the nucleus crucially determines transfection efficiency. Thus, the nuclear trafficking observation of SD/DNA complexes by CLSM was performed to confirm the aforementioned

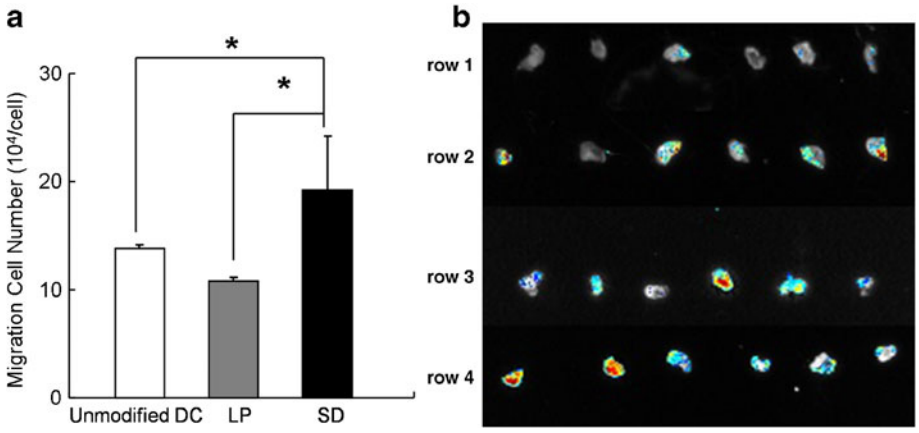

Fig. 6 *In vitro* and *in vivo* migration assay of DCs transfected with plasmid-encoding CCR7. (**a**) *In vitro* migration assay of DCs. The migratory capacity of DCs in response to CCL21 was determined *in vitro* using Transwell ($n=6$). Statistical analysis was performed using the Student's *t*-test. Differences were considered statistically significant at $p<0.05$. (**b**) *In vivo* migration assay of DCs. *In vivo* fluorescent observation of inguinal lymphnodes after 24 hDC vaccination: (**row 1**) PBS; (**row 2**) unmodified DC; (**row 3**) Lipo/CCR7-DC; and (**row 4**) SD/CCR7-DC vaccinations.

condition. The result showed that FITC-DNA can be detected after 4 h of transfection. Although endosome inhibition assay was not performed, nuclear trafficking suggests that SD/DNA complexes can overcome these intracellular barriers and enter the nucleus, which ensures DNA transcription in the nucleus.

Melanogenesis pathways have recently been used utilized to activate immunity for inducing the suppression of malignant melanoma (34,35). DC vaccination loaded with gp100-derived peptides has been used in clinical trials (36,37). Gp100 is a non-mutated self-differentiation antigen associated with melanin synthesis in normal melanocytes and a promising target antigen for specific immunotherapy (38,39). Mice vaccinated with murine gp100 may be tolerant (40). By contrast, immunization with human gp100 can overcome self-tolerance (41). The immunization of mice with gene-encoding human gp100 using an adenovirus can elicit a specific T cell response against B16BL6 cells *in vitro* and a protective tumor immunity in C57BL/6mice against B16BL6 challenge (40,41). A nonviral gene delivery system was developed in the present study to achieve transfection efficiency in DCs similar to that of the adenovirus (21). An SD-based gene delivery system can safely and efficiently deliver genetically modified DCs. Therefore, SD was used to transfer plasmid encoding gp100 to DCs to express specific TAA, present antigen to T cells, and induce adaptive anti-tumor immunity. This system eventually improves the protective therapeutic efficacy of DC-based vaccine in the mice B16BL6 melanoma model (Fig. 5).

The efficiency of DC vaccines depends on its ability to present antigen and migrate, and the chemokine family is essential in DC migration. Chemokine is a family of small cytokines or proteins secreted by cells. Chemokine/chemokine receptors mainly act as chemoattractants to guide the migration of cells. CCL21 is produced and secreted constitutively in lymphoid tissues and lymphatic vessels, whereas CCR7 is a seven-transmembrane domain G-protein-coupled receptor with its expression enhanced on the surface of maturing DCs. This chemokine–chemokine receptor coupling in DCs is necessary in The control of DC migration from the peripheral to the lymphoid tissues (42,43). Therefore, DCs genetically engineered by antigen genes can positively target lymphoid tissues and efficiently induce adaptive immunity by enhancing CCR7 expression. Efficient gene transfection to DCs of CCR7 plasmid can be a preparatory approach for this purpose. Thus, a TAA gp100 gene and a CCR7 gene co-transfected DC tumor vaccine by SD gene delivery vector was constructed, and the functions in tumor suppression (Fig. 5) and lymph node targeting *in vivo* (Fig. 6) were verified.

The therapeutic effect was not very strong and the therapeutic group exhibited no markedly significant difference from the control group. Despite these findings, the SD/DNA-transfected DC vaccine, especially the co-transfection group, showed a preferable trend in the treatment. In terms of tumor volume data (Fig. 5a), although the iDC group and SD/gp100-DC indicated no significant difference, the co-transfection DC and the iDC group exhibited a significant difference on day 17. This result indicates that co-transfection of CCR7-encoding plasmid improves immunotherapy. In terms of tumor weight data (Fig. 5b), no significant difference was observed between SDgp100-DC and the untreated group. However, a significant difference was found between co-transfection DCs and the untreated group, which indicates that co-transfection of the CCR7-encoding plasmid improves immunotherapy and suppresses tumor growth. Therefore, the co-transfection of CCR7 performs more efficiently compared with the SD/gp100-DC or the control group. Survival rates are very important for clinical prognosis and evaluation of drug effects. The SD/CCR7/gp100 group achieved a higher survival rate than did the other three groups (Fig. 5c), suggesting that co-transfected DC vaccine is preferable for promoting mice survival. The co-transfection of CCR7-coding plasmid may promote the capacity of DC vaccine, given these three aspects of tumor treatment, as well as the results related to the ascending migration capacity *in vitro* and *in vivo*.

Engineering DCs to enhance the migration capacity of a DC can target the second lymphoid, thus increasing tumor suppression. For the expression of CCR7 on DCs, the migration assays *in vitro* and vivo verify the CCR7 expression. The expression of gp100 can be partially ensured by the vaccine protection and the result of ELISA (data not shown). In this experiment, untreated DCs and the lipofectamine-transfected DC groups were designated as general controls to show that SD/CCR7 improves normal DC migration and is either equally or more efficient than commercially available reagents (Lipo and SD have the same effect, but the latter provides greater safety). For the Lipofectamine-transfected group, we used Lipofectamine2000 for all *in vitro* transfection experiments; thus, we used it as the control group in the *in vivo* experiment for consistency. A previous study showed that DCs cannot be activated by SD or naked plasmid (e.g., GFP) uptake (16). Another research (19) demonstrated that DC can express and upregulate CCR7 after transfection with DNA-encoding CCR7 and that DC migration is promoted by the expression of exogenous CCR7 rather than the upregulation of endogenous gene encoding CCR7. Therefore, we did not set an SD or SD/naked DNA control group. SD/gp100 and CCR7 constitute a promising delivery system for DC vaccine engineering. Low efficiency can induce a relatively stronger vaccine effect on SD/gp100 or SD/gp100/CCR7 groups probably because the cell-based immune response is a cascade amplification reaction and MHC loads expressing TAA are not dose-dependent. More work should be done to confirm this speculation. The results provided by the images sufficiently demonstrated the internalization and

nuclear trafficking experiments. DC-based immunotherapy is applied as a secondary therapy in accordance to clinical treatment schedule; DC-based immunotherapy via intratumoral injection (44,45) or engineered cytokines such as IL-12 (46,47) and IL-2 (48) is usually not sufficiently effective. Studies on the nonviral gene delivery system for DC engineering are rarely reported, and DC engineering by highly efficient viral vectors can exert a stronger therapeutic effect (49,50). In the present study, we utilized a nonviral vector that is biologically safe compared with other viral vectors for DC engineering. This kind of delivery system does not exhibit a markedly satisfactory effect, and a slightly significant difference is observed between the therapeutic group and the control group. The microenvironment and the growth mechanism of tumor are very complicated, and no precise and specific single indicator exists for the therapeutic effects of tumors. However, we consider the SD/DNA-engineered DC vaccine more effective, especially the co-transfection group. This conclusion was derived from four aspects: tumor volume suppression, tumor weight reduction, life prolongation, and improvement of DC migration.

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