Article

# Dendritic Cell-Derived Exosomes Stimulate Stronger $CD8^+$ CTL Responses and Antitumor Immunity than Tumor Cell-Derived Exosomes

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**Exosomes (EXO) derived from dendritic cells (DC) and tumor cells have been used to stimulate antitumor immune responses in animal models and in clinical trials. However, there has been no side-by-side comparison of the stimulatory efficiency of the antitumor immune responses induced by these two commonly used EXO vaccines. In this study, we selected to study the phenotype characteristics of EXO derived from a transfected EG7 tumor cells expressing ovalbumin (OVA) and OVA-pulsed DC by flow cytometry. We compared the stimulatory effect in induction of OVA-specific immune responses between these two types of EXO. We found that OVA protein-pulsed $DC_{OVA}$-derived EXO ($EXO_{DC}$) can more efficiently stimulate naïve OVA-specific $CD8^+$ T cell proliferation and differentiation into cytotoxic T lymphocytes *in vivo*, and induce more efficient antitumor immunity than EG7 tumor cell-derived EXO ($EXO_{EG7}$). In addition, we elucidated the important role of the host DC in EXO vaccines that the stimulatory effect of EXO is delivered to T cell responses by the host DC. Therefore, DC-derived EXO may represent a more effective EXO-based vaccine in induction of antitumor immunity. *Cellular & Molecular Immunology*. 2006;3(3):205-211.**

**Key Words:** dendritic cell, exosome, tumor cell, cytotoxic T lymphocyte, antitumor immunity

## Introduction

Tumor cells express an array of antigens recognized by cytotoxic T lymphocytes (CTL) (1, 2). However, the nature of the tumor antigens that actually mediate efficient immune responses leading to tumor rejection remains unclear (3). Tumor transplantation studies in mice using carcinogen-induced neoplasia demonstrate that antigens resulting from mutations incidental to the oncogenic process are responsible for protective immunity in tumor bearing animals (4, 5). Shared tumor antigens can serve as tumor-rejection antigens (6).

One general characteristic of tumour cells is releasing or sheding membrane vesicular, nowadays, called exosomes (EXO), which was initially described by Taylor et al. 25 years ago (7). Recently, tumour derived EXO have attracted much attention as a source of tumour antigens for vaccines (8-10). EXO are small (~100 nm in diameter), membrane-bound vesicles of the endocytic pathway that are externalized by a variety of cell types. They are formed by the fusion of multivesicular bodies with the plasma membrane, followed by exocytosis (11, 12). Such EXO display a discrete set of proteins involved in antigen presentation, that is, major histocompatibility complex class I and II (MHC-I and MHC-II), costimulatory (CD80, CD86) and tetraspan molecules (CD63, CD82) and are selectively enriched in molecules potentially involved in effector cell targeting, that is, CD11b, lactadherin and CD9 molecules (13, 14). These tumor cell-derived EXO and tumor-derived EXO isolated from malignant effusions can transfer tumor antigens to DC and induce specific CTL responses and antitumor immunity (15-17).

Peptide-pulsed dendritic cell (DC)-derived EXO elicited potent antitumor immune responses in tumor-bearing mice (18). It has been shown that DC-derived EXO contain tumor antigens and have antigen-presentation capability of making them become a potentially attractive vehicle for immuno-therapy (19-21). It has been demonstrated that DC-derived EXO can trigger potent $CD8^+$ T-cell dependent antitumor responses (16, 20, 21) and induce antitumor immunity (15, 22-25) *via* transfer of exosomal molecules to DCs. In recent

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clinical trials, tumor specific immune responses were observed by using these DC-derived EXO (26, 27). However, there has been no side-by-side comparison of the stimulatory efficiency of the antitumor immune responses induced by these two commonly used EXO.

In this study, we investigated the phenotype characteristics of EXO derived from a transfected EG7 tumor cells expressing ovalbumin (OVA) and OVA-pulsed DC ($DC_{OVA}$) by flow cytometry. We also compared the efficiency of EG7 cells-derived EXO ($EXO_{EG7}$) and DC-derived EXO ($EXO_{DC}$) in stimulation of OVA-specific CTL responses and antitumor immunity. In addition, we elucidated the important role of the host DC in EXO vaccines.

## Materials and Methods

*Reagents, cell lines and animals*

Ovalbumin (OVA) was obtained from Sigma (St. Louis, MO). OVA I (SIINFEKL) and OVA II (ISQAVHAAHAEINEAGR), are OVA peptides specific for H-$2K^b$ and $Ia^b$, respectively (28, 29). Mut I (FEQNTAQP) peptide is specific for H-$2K^b$ of an irrelevant 3LL lung carcinoma (30). All peptides were synthesized by Multiple Peptide Systems (San Diego, CA). Biotin-labeled or fluorenscein isothiocyanate (FITC)-labeled antibodies (Abs) specific for H-$2K^b$ (AF6-88.5), $Ia^b$ (AF6-120.1), CD40 (IC10), CD54 (3E2) and CD80 (16-10A1) as well as FITC-conjugated avidin were all obtained from Pharmingen Inc. (Mississauga, Ontario, Canada). The anti-H-$2K^b$/OVA I complex (pMHC I) Ab was obtained from Dr. Germain (National Institute of Health, Bethesda, MD) (31). The recombinant mouse IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from R&D Systems Inc. (Minneapolis, MN). The highly lung metastatic B16 melanoma cell line BL/6-10 and the OVA-transfected BL6-10 ($BL6\text{-}10_{OVA}$) cell line expressing OVA as a surrogate tumor antigen were generated in our own laboratory (32). The mouse thymoma cell line EL4 and OVA-transfected EL4 (EG7) cell line were obtained from American Type Culture Collection (ATCC). Female C57BL/6 and the diphtheria toxin receptor (DTR) transgenic mice were obtained from the Jackson Laboratory (Bar Harbor, MA). Mice were treated according to animal care committee guidelines of the University of Saskatchewan.

*Generation of bone marrow-derived DC*

The generation of bone marrow (BM)-derived DC has been described previously (32). DCs at day 6 in culture were further pulsed with OVA protein (0.3 mg/ml) in fetal calf serum (FCS)-free AIM-V medium (GIBCO) for overnight culture and termed $DC_{OVA}$.

*Generation and purification of exosomes*

EXO were isolated as described previously (25, 33). Briefly, culture supernatants of $DC_{OVA}$ cultured overnight in FCS-free AIM-V medium containing OVA (0.3 mg/ml) were subjected to four successive centrifugations at 300× g for 5 min to remove cells, 1,200× g for 20 min and 10,000× g for 30 min to remove cellular debris and 100,000× g for 1 h to pellet EXO. The EXO pellets were washed twice in a large volume of PBS and recovered by centrifugation at 100,000× g for 1 h. The amount of exosomal proteins recovered was measured using Bradford assay (Bio-Rad, Richmond, CA). EG7 cells were cultured in FCS-free AIM-V medium, and the supernatants were harvested and underwent above procedures for isolation of EXO. EXO derived from $DC_{OVA}$ of wild-type C57BL/6 and EG7 tumor cells were termed as $EXO_{DC}$ and $EXO_{EG7}$.

*Phenotypic characterization of DC, EG7 cells, $EXO_{DC}$ and $EXO_{EG7}$*

For phenotypic analysis of DC and EG7 cells, both DC and EG7 cells were stained with a panel of biotin-labeled and FITC-labeled Abs and analyzed by flow cytometry. For phenotypic analysis of EXO, both $EXO_{DC}$ and $EXO_{EG7}$ (25-40 μg) were incubated with a panel of FITC-conjugated Abs on ice for 30 min, and then analyzed by flow cytometry as previously described (34). To determine the optimal voltage suitable for EXO analysis, Dynal M450 beads with a size of 4.5 μm in diameter (DYNAL Inc, Lake Success, NY) were used as a size control by flow cytometric analysis (34) using FACScan (Coulter EPICS XL, Beckman Coulter, San Diego, CA). Isotype-matched biotin-labeled or FITC-conjugated Abs were used as control.

*Tetramer staining*

C57BL/6 mice were *i.v.* immunized with $EXO_{DC}$ and $EXO_{EG7}$ (10 μg/mouse), respectively. The mice injected with PBS were considered as control. After 6 days of immunization, the tail blood samples were harvested and were incubated with ten microliters of PE-conjugated H-$2K^b$/$OVA_{257\text{-}264}$ tetramer (Beckman Coulter, Mississauga, Ontario, Canada) and FITC-conjugated anti-CD8 (PK135) for 30 min at room temperature. The erythrocytes were then lysed using lysis/fixed buffer (Beckman Coulter). The cells were analyzed by flow cytometry.

*Cytotoxicity assays*

In *in vitro* cytotoxicity assays, red blood cell-depleted splenic lymphocytes from the mice immunized with $EXO_{DC}$ and $EXO_{EG7}$ were harvested after 7 days of immunization. Splenic lymphocytes ($5 \times 10^6$) were co-cultured with γ-irradiated (6,000 rad) EG7 cells ($1 \times 10^5$) in 2 ml of DMEM plus 10% FCS in each well of a 24-well plate (Costar, Cambridge, MA) respectively. Three days subsequently, T cells were harvested. These T cells were used as effector cells in the chromium release assay. While $^{51}$Cr-labeled EG7 or control EL-4 tumor cells were used as target (T) cells. Ten thousands of labeled target cells per well were mixed with effector cells at various effector/target cell ratios in triplicate and were incubated for 6 h. Percentage of specific lysis was calculated as: 100 × [(experimental CPM - spontaneous CPM) / (maximal CPM - spontaneous CPM)]. The maximal count per minute (CPM) was released by adding 1% Triton X-100 to wells in experiments (35). *In vivo* cytotoxicity assays were performed as previously described (32). Briefly, C57BL/6 mice were *i.v.*

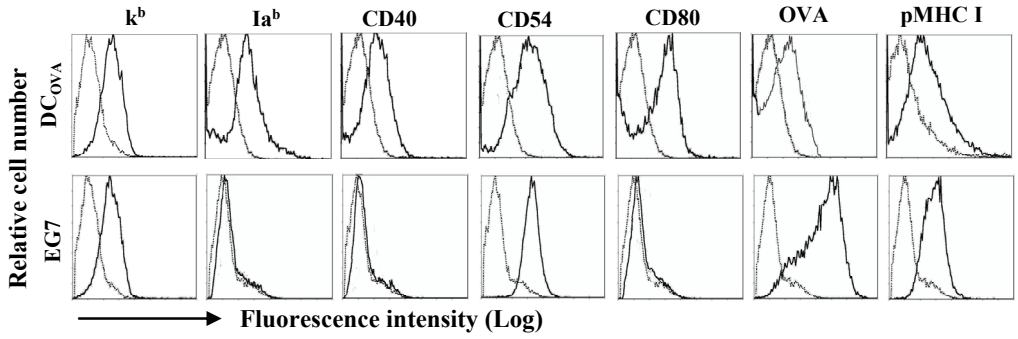


**Figure 1. Phenotypic analysis of DC and EG7 cells.** BM-derived DC pulsed with OVA ($DC_{OVA}$) and EG7 tumor cells (solid lines) were stained with a panel of Abs, and then analyzed by flow cytometry. These cells (thin dotted lines) were also stained with isotype-matched irrelevant Abs, and employed as control populations. One representative experiment of two is displayed.

immunized with $EXO_{DC}$ and $EXO_{EG7}$, respectively. C57BL/6 splenocytes were harvested from naïve mouse spleens and incubated with either high (3.0 μM, $CFSE^{high}$) or low (0.6 μM, $CFSE^{low}$) concentrations of CFSE, to generate differentially labeled target cells. The $CFSE^{high}$ cells were pulsed with OVA I peptide, whereas the $CFSE^{low}$ cells were pulsed with Mut 1 peptide and served as internal controls. These peptide-pulsed target cells were *i.v.* injected at 1:1 ratio into the above immunized mice 6 days after immunization. Sixteen hours later, the spleens of immunized mice were removed and residual $CFSE^{high}$ and $CFSE^{low}$ target cells remaining in the recipients' spleens were analyzed by flow cytometry.

*Animal studies*

To examine whether $EXO_{DC}$ and $EXO_{EG7}$ can induce protective antitumor immunity, wild-type C57BL/6 and DC knockout (KO) mice (n = 8) were injected *i.v.* with $EXO_{DC}$ and $EXO_{EG7}$ (10 μg/mouse), respectively. The immunized mice were *i.v.* challenged with $0.5 \times 10^6$ BL6-$10_{OVA}$ 6 days later. To investigate the involvement of the host DC in EXO vaccines, we also used diphtheria toxin receptor (DTR) transgenic mice, in which, $CD11c^+$ DC sensitive to diphtheria toxin (DT) (36). The DTR transgenic and wild-type C57BL/6 mice were injected single dose of DT (1.5 ng/g weight), after 2 days, almost no $CD11c^+$ DC were detectable in spleen (36). The treated mice (n = 8) were then injected *i.v.* with $EXO_{DC}$ and $EXO_{EG7}$ (10 μg/mouse), respectively, and then *i.v.* challenged with $0.5 \times 10^6$ BL6-$10_{OVA}$ 6 days later. The mice were sacrificed 4 weeks after tumor cell injection and the lung metastatic tumor colonies were counted in a blind fashion. Metastases on freshly isolated lungs appeared as discrete black pigmented foci that were easily distinguishable from normal lung tissues and confirmed by histological examination. Metastatic foci too numerous to count were assigned an arbitrary value of >100 (32).

## Results

*Phenotypical characterization of DC and EG7 tumor cells*

Bone marrow-derived $DC_{OVA}$ pulsed with OVA expressed MHC class I and II, CD40, CD54, CD80 and OVA molecules. In addition, $DC_{OVA}$ also expressed pMHC I (Figure 1). Similar to DC, EG7 cell line also expressed OVA, CD54 and pMHC I molecules, but not MHC class II, CD40 and CD80 molecules.

*Phenotypical characterization of $EXO_{DC}$ and $EXO_{EG7}$*

To Phenotypically characterize EXO, EXO derived from $DC_{OVA}$ and EG7 tumor cells were stained with a panel of Abs and analyzed by flow cytometry. As shown in Figure 2, $DC_{OVA}$-derived $EXO_{DC}$ displayed expression of molecules (MHC I and II, CD40, CD54, CD80, OVA and pMHC I) similar to, but in less extent to $DC_{OVA}$. EG7-derived $EXO_{EG7}$ did not show any MHC II, CD40 and CD80 molecules, indicating that $EXO_{DC}$ may be more immunogenic than $EXO_{EG7}$.

*$EXO_{DC}$ stimulate stronger $CD8^+$ T cell proliferation via the host DC than $EXO_{EG7}$*

Since EXO harbor immune molecules, they have potent effect on stimulation of $CD8^+$ T cells (20). To assess whether EXO can stimulate $CD8^+$ T cell proliferation *in vivo*, we performed tetramer staining assays (20). As shown in Figure 3, $EXO_{EG7}$ immunization was able to induce 0.85% tetramer-positive $CD8^+$ T cells of the total $CD8^+$ T cell population at day 6 after immunization, indicating that $EXO_{EG7}$ can activate naïve OVA-specific $CD8^+$ T cell responses *in vivo*. However, $EXO_{DC}$ stimulated 1.09% tetramer-positive $CD8^+$ T cell responses, which is stronger than $EXO_{EG7}$. The diphtheria toxin (DT) treatment itself did not affect the tetramer-positive $CD8^+$ T cell responses since a similar amount of the tetramer-positive $CD8^+$ T cell responses (data not shown) was detected in DT-treated C57BL/6 mice as seen in untreated C57BL/6 mice. Interestingly, no tetramer-positive $CD8^+$ T cells were detected in DT-treated DTR transgenic mice immunized with these two types of EXO, indicating that the induction of $CD8^+$ T cell activation by EXO is dependent upon the host DC to deliver EXO's stimulatory effect to the host T cells.

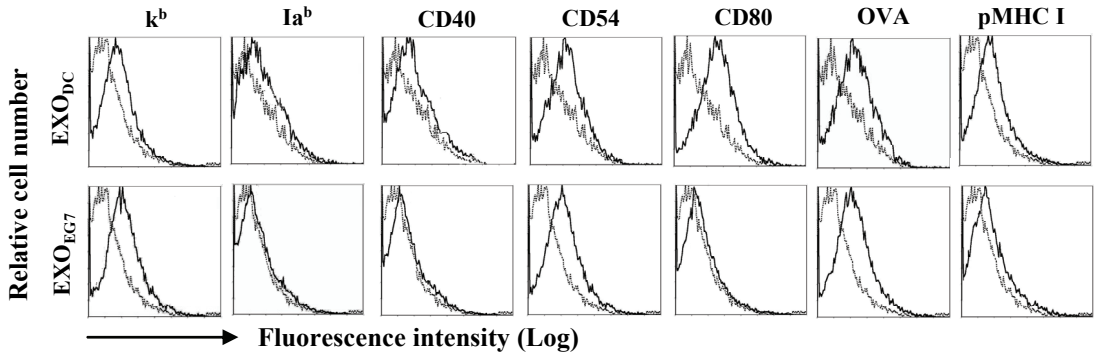


**Figure 2. Phenotypic analysis of exosomes.** OVA-pulsed DC-derived and EG7 tumor cell-derived EXO ($EXO_{DC}$ and $EXO_{EG7}$) (solid lines) were stained with a panel of FITC-Abs, and then analyzed by flow cytometry. These EXO (thin dotted lines) were also stained with isotype-matched irrelevant FITC-Abs, respectively, and employed as control populations. One representative experiment of two is displayed.

*$EXO_{DC}$ stimulate stronger $CD8^+$ T cell differentiation into CTL effectors than $EXO_{EG7}$*

To examine the functionality of these EXO, we tested their ability to induce $CD8^+$ T cell differentiation into CTL effector, as determined using *in vitro* $^{51}Cr$ release assays. CTLs from the mice immunized with $EXO_{DC}$ showed stronger killing activities against OVA-expressing EG7 tumor cells (30% killing; E:T ratio, 25:1) than those from the mice immunized with $EXO_{EG7}$ (21%; E: T ratio, 12:1) (Figure 4A). No killing activity against its parental EL4 tumor cells was detectable, indicating that the killing activity of these CTLs is OVA specific. To assess whether EXO can also stimulate $CD8^+$ T cell differentiation into CTL effectors *in vivo*, we adoptively transferred OVA I peptide-pulsed splenocytes that had been strongly labeled with CFSE ($CFSE^{high}$) as well as the control Mut I peptide-pulsed splenocytes that had been weakly labeled with CFSE ($CFSE^{low}$) into the recipient mice that had been vaccinated with $EXO_{DC}$ and $EXO_{EG7}$, respectively. As shown in Figure 4B, no $CFSE^{high}$ target cells loss (> 2%) was observed in mice immunized with PBS. As expected, there was a substantial loss of the $CFSE^{high}$ cells (54% losses of the $CFSE^{high}$ target cells) in mice immunized with $EXO_{EG7}$, indicating that $EXO_{EG7}$ can also stimulate $CD8^+$ T cell differentiation into CTL effectors *in vivo*. However, there was a more substantial loss of the $CFSE^{high}$ cells (66% losses of the $CFSE^{high}$ target cells) in mice immunized with $EXO_{DC}$, indicating that $EXO_{DC}$ stimulate stronger $CD8^+$ T cell differentiation into CTL effectors than $EXO_{EG7}$. Interestingly, our data also showed that both $EXO_{DC}$ and $EXO_{EG7}$ did not induce any losses of $CFSE^{high}$ target cells in DT-treated DTR transgenic mice with DC deficiency, indicating that the stimulatory effect of $EXO_{DC}$ and $EXO_{EG7}$ to $CD8^+$ T cell differentiation is mediated by the host DC.

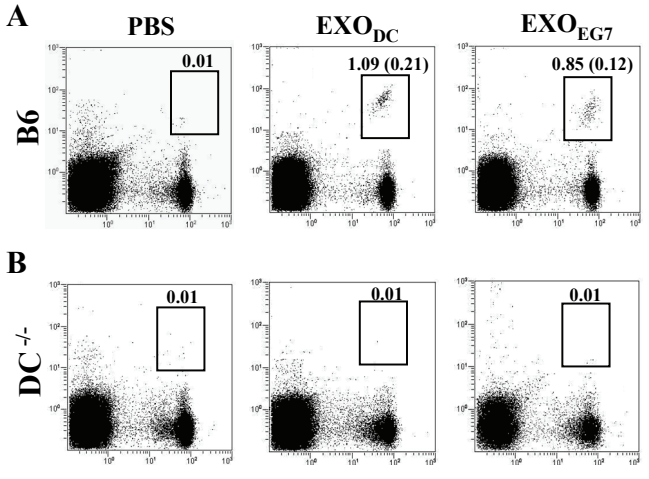


**Figure 3. Stimulation of T cell proliferation *in vivo*.** C57BL/6 and DTR transgenic mice were immunized *i.v.* with $EXO_{DC}$ and $EXO_{EG7}$, respectively. Six days after immunization, the tail blood samples were taken from (A) the immunized C57BL/6 mice or (B) the immunized DTR transgenic mice with DT-treatment, and stained with PE-H-$2K^b$/OVA tetramer and FITC-anti-CD8 Ab. The expression of PE-H-$2K^b$/OVA tetramer-specific TCR and CD8 molecules was examined by flow cytomery. The results presented are representative of 4 separate mice per group. The value in parenthesis represents the standard deviation. One representative experiment of three is shown.

*$EXO_{DC}$ induce stronger immunity against lung tumor metastases than $EXO_{EG7}$*

To investigate the induction of antitumor immunity, mice were *i.v.* immunized with $EXO_{DC}$ and $EXO_{EG7}$, respectively. Six days after the immunization, the immunized mice were *i.v.* challenged with $BL6\text{-}10_{OVA}$ tumor cells. As shown in Exp I of Table 1, all the mice injected with PBS had large numbers (>100) of lung metastatic tumor colonies. $EXO_{DC}$ vaccine protected 5/8 (63%) mice. Similar to $EXO_{DC}$, $EXO_{EG7}$ also protected 2/8 (25%) mice, indicating that $EXO_{EG7}$ can also induce antitumor protection against tumor challenge, but a weaker protection than $EXO_{DC}$. The specificity of the protection was confirmed with the observation that both $EXO_{DC}$ and $EXO_{EG7}$ did not protect against BL6-10 tumors that did not express OVA, with all mice having large numbers (>100) of lung metastatic tumor colonies after tumor cell challenge. The diphtheria toxin (DT)

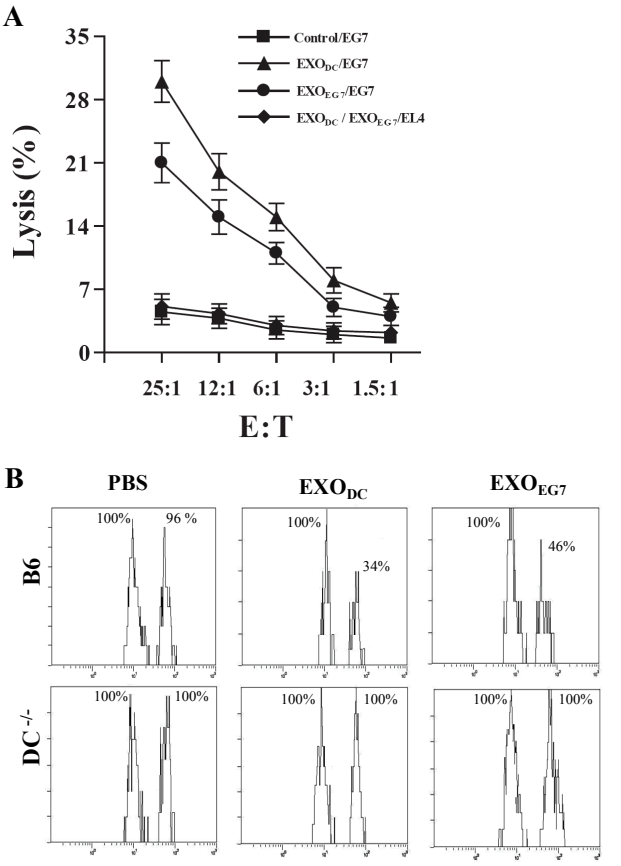


**Figure 4. Development of antigen-specific CTL activities *in vivo*.** (A) In *in vitro* cytotoxicity assays, splenic lymphocytes from the mice immunized with $EXO_{DC}$ and $EXO_{EG7}$ were harvested after 7 days of immunization and co-cultured with γ-irradiated EG7 cells. Three days subsequently, T cells derived from mice vaccinated with $EXO_{DC}$ (▲) and $EXO_{EG7}$ (●) were harvested and used as effector (E) cells in the chromium release assay, while $^{51}$Cr-labeled EG7 tumor cells were used as target (T) cells. T cells derived from naïve mice were used as control effector cells (♦). To confirm that T-cell cytotoxicity is OVA specific, we also included EL4 tumor cells as a target control (■). Each point represents the mean of triplicates. (B) In *in vivo* cytotoxicity assays, C57BL/6 splenocytes were harvested from naïve mouse spleens and incubated with either high (3.0 μM, $CFSE^{high}$) or low (0.6 μM, $CFSE^{low}$) concentrations of CFSE, to generate differentially labeled target cells. The $CFSE^{high}$ cells were pulsed with OVA I peptide, whereas the $CFSE^{low}$ cells were pulsed with Mut I peptide and served as internal controls. These peptide-pulsed target cells were *i.v.* injected at 1:1 ratio into the above immunized C57BL/6 mice or DTR ($DC^{-/-}$) transgenic mice with DT treatment 6 days after immunization of $DC_{EXO}$ and $EG7_{EXO}$, respectively. Sixteen hrs later, the spleens of immunized mice were removed and the percentages of the residual $CFSE^{high}$ (H) and $CFSE^{low}$ (L) target cells remaining in the recipients' spleens were analyzed by flow cytometry. The value in parenthesis represents the standard deviation. One representative experiment of three is shown.

treatment did not affect the OVA-specific antitumor immunity derived from EXO vaccination since a similar extent of the antitumor immunity (data not shown) was found

**Table 1.** Exosome vaccine protects against lung tumor metastases

| Vaccines | Tumor cell challenge | Tumor growth incidence (%) | Median number of lung tumor colonies |
|---|---|---|---|
| Exp. I. | | | |
| $EXO_{DC}$ | BL6-$10_{OVA}$ | 2/8 (25) | 21 ± 5 |
| $EXO_{EG7}$ | BL6-$10_{OVA}$ | 5/8 (63)* | 29 ± 8 |
| PBS | BL6-$10_{OVA}$ | 8/8 (100) | >100 |
| $EXO_{DC}$ | BL6-10 | 8/8 (100) | >100 |
| $EXO_{EG7}$ | BL6-10 | 8/8 (100) | >100 |
| Exp. II. | | | |
| $EXO_{DC}$ (B6) | BL6-$10_{OVA}$ | 2/8 (25) | 21 ± 5 |
| $EXO_{EG7}$ (B6) | BL6-$10_{OVA}$ | 5/8 (63) | 29 ± 8 |
| $EXO_{DC}$ (DC KO) | BL6-$10_{OVA}$ | 8/8 (100) | >100 |
| $EXO_{EG7}$ (DC KO) | BL6-$10_{OVA}$ | 8/8 (100) | >100 |

In experiment I, wild-type C57BL/6 (n = 8) were *i.v.* immunized with $DC_{OVA}$-derived $EXO_{DC}$ and EG7 tumor cell-derived $EXO_{EG7}$ (10 μg/mouse) or PBS. Six days after immunization, each mouse was challenged *i.v.* with OVA transgene-expressing BL6-$10_{OVA}$ or wild-type BL6-10 tumor cells. In experiment II, the diphtheria toxin receptor (DTR) transgenic mice (n = 8) were *i.v.* treated with DT (1.5 ng/g weight). Two days later, these treated mice (termed DC knockout mice) were immunized with $EXO_{DC}$ and $EXO_{EG7}$. Six days after immunization, each mouse was then *i.v.* challenged with BL6-$10_{OVA}$ tumor cells. The mice were sacrificed 4 weeks after tumor cell challenge and the numbers of lung metastatic tumor colonies were counted. *$p < 0.05$ (nonparametric Mann-Whitney U test) (41) *vs* cohorts of mice immunized with the same number of $EXO_{DC}$. One representative experiment of three is shown.

in DT-treated C57BL/6 mice as seen in untreated C57BL/6 mice. Interestingly, our data also showed that all EXO-immunized DTR transgenic mice with depletion of $CD11c^+$ DC by DT treatment having large numbers (>100) of lung metastatic tumor colonies after tumor cell challenge (Exp II of Table 1), indicating that the antitumor immunity induced by these two types of EXO is mediated by the host DC.

## Discussion

In recent years, exosomes (EXO) research has attracted more attention by the finding that APC such as B lymphocytes and DC secrete EXO during exocytic fusion of multivesicular MHC class II compartments with the cell surface (18, 19). It has been demonstrated that DC-derived EXO harboring many important immunological molecules, such as MHC class I and class II, costimulatory molecules can elicit antitumor immunity (15, 22-25). Human and mouse tumor cells constitutively release membrane vesicles, similar to DC-derived EXO in their morphology, density and expression of certain membrane markers (16). However, there is no study on side-by-side comparison of stimulatory efficiency in induction of antitumor immune responses by EXO derived from DC and tumor cells. In this study, we selected to study $EXO_{DC}$ derived from bone marrow-derived

DC pulsed with OVA and $EXO_{EG7}$ derived from EG7 tumor cells expressing transgene OVA. We demonstrated that both $EXO_{DC}$ and $EXO_{EG7}$ displayed expression of OVA, MHC class I, CD54 and pMHC I complexes. In addition, $EXO_{DC}$, but not $EXO_{EG7}$ also expressed the costimulatory molecules such as CD40 and CD80, indicating that $EXO_{DC}$ may be more immunogenic than $EXO_{EG7}$.

As EXO derived from tumor cells and tumor peptide-pulsed DC were found to be immunogenic (16-18), we further compared the immunogenicity of $EXO_{DC}$ and $EXO_{EG7}$ in eliciting OVA-specific $CD8^+$ CTL immune responses. In this study, we demonstrated that $EXO_{DC}$ can more strongly stimulate $CD8^+$ T cell proliferation and differentiation into CTL effectors *in vivo*, and induce stronger antitumor immunity against tumor cell challenge both *in vitro* and *in vivo* than $EG7_{EXO}$. The less immunogenicity of $EXO_{EG7}$ may be possibly due to lacking expression of the costimulatory molecules CD40 and CD80 on $EXO_{EG7}$, since both molecules have been repeatedly shown to be key elements in the initiation of primary immune responses (37). For example, the interaction of CD40 on DC with CD40 ligand on naïve T cells is critical for generation of antigen-specific T cell responses (37-39). It is also recognized that stimulation of T cells by DC involves at least two signaling events: one elicited by T cell receptor recognition of pMHC complexes and the other by costimulatory molecule signaling (e.g., T cell CD28/DC CD80) (40).

It has been previously reported that EXO may need the host DC as an adjuvant for induction of immune responses based only upon *in vitro* experiments (23, 30). However, there is no direct evidence on the role of the host DC in EXO-based vaccines. Jung et al. have demonstrated the importance of the host DC in priming of $CD8^+$ T cell responses *in vivo* by cross-presentation of exogenous cell-associated antigens using a novel diphtheria toxin-based system that allows the inducible, short-term ablation of DC *in vivo* (36). In this study, we showed that both $EXO_{DC}$ and $EXO_{EG7}$ completely lost their stimulatory effects on induction of $CD8^+$ T cell proliferation and differentiation into CTL effectors and on induction of antitumor immunity. Therefore, for the first time, we provided clear evidence that EXO needs the host DC for delivery of their stimulatory effect to $CD8^+$ CTL responses *in vivo*.

Taken together, our data showed that OVA protein-pulsed $DC_{OVA}$-derived $EXO_{DC}$ can more efficiently stimulate naïve OVA-specific $CD8^+$ T cell proliferation and differentiation into CTL effectors *in vivo*, and induce more efficient antitumor immunity than EG7 tumor cell-derived $EXO_{EG7}$. The stimulatory effect of EXO is delivered to T cell responses by the host DC. Therefore, DC-derived EXO may represent a more effective EXO-based vaccine on induction of antitumor immunity.

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