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Novel Exosome-Targeted CD4⁺ T Cell Vaccine Counteracting CD4⁺25⁺ Regulatory T Cell-Mediated Immune Suppression and Stimulating Efficient Central Memory CD8⁺ CTL Responses

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T cell-to-T cell Ag presentation is increasingly attracting attention. In this study, we demonstrated that active CD4⁺ T (aT) cells with uptake of OVA-pulsed dendritic cell-derived exosome (EXO OVA) express exosomal peptide/MHC class I and costimulatory molecules. These EXO OVA-uptaken (targeted) CD4⁺ aT cells can stimulate CD8⁺ T cell proliferation and differentiation into central memory CD8⁺ CTLs and induce more efficient in vivo antitumor immunity and long-term CD8⁺ T cell memory responses than OVA-pulsed dendritic cells. They can also counteract CD4⁺25⁺ regulatory T cell-mediated suppression of in vitro CD8⁺ T cell proliferation and in vivo CD8⁺ CTL responses and antitumor immunity. We further elucidate that the EXO OVA-uptaken (targeted) CD4⁺ aT cell’s stimulatory effect is mediated via its IL-2 secretion and acquired exosomal CD80 costimulation and is specifically delivered to CD8⁺ T cells in vivo via acquired exosomal peptide/MHC class I complexes. Therefore, EXO-targeted active CD4⁺ T cell vaccine may represent a novel and highly effective vaccine strategy for inducing immune responses against not only tumors, but also other infectious diseases. The Journal of Immunology, 2007, 179: 2731–2740.

D endritic cells (DC)³ process exogenous Ags in endosomal compartments such as multivesicular endosomes (1) which can fuse with plasma membrane, thereby releasing Ag-presenting vesicles called “exosomes” (EXO) (2, 3). EXO are 50- to 90-nm diameter vesicles containing Ag-presenting (MHC class I, class II, CD1, heat shock protein 70–90), tetraspan (CD9, CD63, CD81), adhesion (CD11b, CD54), and costimulatory (CD86) molecules (4, 5), i.e., the necessary machinery required for generating potent immune responses.

Zitvogel et al. (3) first demonstrated vaccination of DC-derived EXO in eradication of tumors in animal models. Subsequently, EXO-based vaccines have been confirmed to stimulate strong CTL responses and induce antitumor immunity in different animal models (6, 7). However, its therapeutic efficiency is still limited to only production of prophylactic immunity against tumors possibly due to lacking capacity of breaking immune tolerance (8). Exosomal peptide/MHC class I and II (pMHC I and II) complexes are functional, but require transfer to DC to promote T cell activation leading to tumor eradication (9–11). Therefore, the potential pathway of in vivo EXO-mediated antitumor immunity may be through uptake of EXO by host immature DC that, in turn, stimulate Ag-specific T lymphocytes via the pMHC complexes and costimulatory molecules on EXO-uptaken DC. In addition, Kennedy et al. (12) have also demonstrated that CD4⁺ T cells can acquire APC membrane molecules in vivo and induce memory CTL responses. We have recently demonstrated that mature DC with uptake of OVA-pulsed DC (DC OVA)-derived EXO (EXO OVA) express a higher level of pMHC I and costimulatory CD40, CD54, and CD80 molecules and can strongly stimulate OVA-specific CD8⁺ CTL responses and antitumor immunity (13). Recently, we have also demonstrated that CD4⁺ Th cells can acquire membrane molecules from DC via DC activation, and act as Th-APCs (14). These Th-APC with acquired pMHC I and costimulatory CD54 and CD80 molecules can stimulate tumorspecific CD8⁺ CTL responses and induce antitumor immunity. One of the potential mechanisms of CD4⁺ T cell acquisition of DC molecules is uptake of DC-released EXO by CD4⁺ T cells. Therefore, these results clearly indicate that DC-derived EXO can transfer the Ag-presenting activity of DCs to either DC or CD4⁺ T cells through EXO uptake by these cells. However, the EXO-targeted CD4⁺ T cell vaccine and its potential immune mechanism in induction of antitumor CTL responses have not been studied and elucidated.

In this study, we demonstrated that EXO OVA-uptaken (targeted) active CD4⁺ T (aT EXO) cells can 1) stimulate central memory CD8⁺ CTL responses, more efficient antitumor immunity, and T cell memory than DC OVA and 2) counteract CD4⁺25⁺ regulatory T (Tr) cell-mediated immune suppression. We also elucidated that
the aTEXO stimulatory effect is mediated via its IL-2 secretion and acquired exosomal CD80 costimulation and is specifically delivered to CD8^+ T cells in vivo via acquired exosomal pMHC I complexes.

Materials and Methods

Reagents, cell lines, and animals

OVA was obtained from Sigma-Aldrich. OVA I (SIINFEKL) and OVA II (ISQAVHAAHEINEAGR) which are OVA peptides specific for H-2K^b and I^a, respectively (15, 16). Mut I (FEQNTAQP) peptide is specific for H-2K^b of an irrelevant 3LL lung carcinoma (11). All peptides were synthesized by Multiple Peptide Systems. Biotin-labeled or FITC-labeled Abs specific for H-2K^b (AF6-88.5), I^a (AF6-120.1), CD4 (GK1.5), CD8 (53-6.7), CD11c (HL3), CD25 (7D4), CD40 (IC10), CD44 (IM7), CD54 (3E2), CD62L (MEL-14), CD69 (H1.2F3), CD80 (16-10A1), IL-7R (4G3), and Vε2Vβ^5^ TCR (MR9-4), as well as FITC-conjugated avidin, were all obtained from BD Pharmingen. Anti-LFA-1 Ab, CTLA-4/lig fusion protein, recombinant mouse IL-4, and GM-CSF were purchased from R&D Systems. The mouse thymoma cell line EL4 and OVA-transfected EL4 (EG7) cell line were obtained from American Type Culture Collection. The highly lung metastasis OVA-transfected B6-1OVA melanoma cell line was generated in our own laboratory (14). Female C57BL/6 (B6, CD45.2^+^), C57BL/6.1 (B6.1, CD45.1^+^), OVA-specific TCR-transgenic OT I and OT II mice, H-2K^b, I^a, IL-2, IFN-γ, TNF-α, CD40, CD54, and CD80 gene knockout (KO) mice were obtained from The Jackson Laboratory. Homozygous OT II/H-2K^b^ cells were obtained from The Jackson Laboratory. OVA-transfected B6-1OVA melanoma cell line was generated in our own laboratory (14). Female C57BL/6 (B6, CD45.2^+^), C57BL/6.1 (B6.1, CD45.1^+^), OVA-specific TCR-transgenic OT I and OT II mice, H-2K^b^ mice, I^a^, IL-2, IFN-γ, TNF-α, and OT II/TNF-α/γ mice were generated by backcrossing the designated gene KO mice onto the OT II background. All mice were treated according to animal care committee guidelines of the University of Saskatchewan.

DC generation

DCs were generated as described previously (17). Briefly, spleen cells were prepared in PBS with 5 mM EDTA, washed, and incubated in culture medium with 7% FCS at 37°C for 2 h. Nonadherent cells were removed by gentle pipetting with warm serum-free medium. Adherent cells were cultured overnight in medium with 1% normal mouse serum, GM-CSF (1 ng/ml), and OVA (0.3 mg/ml). These DCs were termed as DC_OVA. DC generated from H-2K^b^, CD54, CD40, and CD80 gene KO mice were referred to as (K^b^) DC_OVA, (CD40^−/−^)DC_OVA, (CD54^−/−^)DC_OVA, and (CD80^−/−^)DC_OVA, respectively.

EXO preparation

Preparation and purification of EXO derived from the culture supernatants of DC_OVA were previously described (13), an average of 5 μg of EXO was recovered from an overnight culture of 1 × 10^7 DC_OVA in 1 mL of AIM-V serum-free medium containing GM-CSF (10 ng/ml). EXO derived from DC_OVA were termed EXO_OVA. Similar to DC_OVA, EXO_OVA also expressed MHC class I (H-2K^b^) and class II (I^a^), CD11c, CD40, CD54, and CD80, and the pMHC I complex, but in a lesser content, compared with DC_OVA (13). EXO derived from (K^b^) DC_OVA, (CD40^−/−^)DC_OVA, (CD54^−/−^)DC_OVA, and (CD80^−/−^)DC_OVA were termed (K^b^)EXO_OVA, (CD40^−/−^)EXO_OVA, (CD54^−/−^)EXO_OVA, and (CD80^−/−^)EXO_OVA, respectively. To generate CSF-expressed EXO_OVA were stained with 0.5 μM CFSE at 37°C for 20 min (16) and washed three times with PBS, and then pulsed with OVA protein in AIM-V serum-free medium overnight. The CFSE-labeled EXO (EXO_CFSE) was harvested and purified from the culture supernatants as previously described above.

CD4^+ T cell preparation and characterization

Naive OVA-specific T (nT) cells were isolated from OVA-specific TCR-transgenic OT I and OT II mouse spleens, enriched by passage through nylon wool columns (C&A Scientific), and then purified by negative selection using anti-mouse CD8(Ly2) or CD4 (L3T4) paramagnetic beads (Dynal Biotech) to yield populations that were >98% CD4^+OVA^+ B or CD8^+OVA^+ B, respectively (14). To generate active OT II CD4^+ T cells, the spleen cells from OT II mice were cultured in RPMI 1640 medium containing IL-2 (20 U/ml) and Con A (1 μg/ml) for 3 days. The Con A-activated CD4^+ T (aT) cells were purified using nylon wool columns and then CD4 microbeads (Miltenyi Biotec). The CD4^+ aT cells derived from OT II/H-2K^b^, OT II/CD40^−/−^, OT II/CD54^−/−^, OT II/CD80^−/−^ were termed aT(H-2K^b^), aT(12E40^−/−^), aT(12CD54^−/−^), and aT(12CD80^−/−^), respectively. The above naive and Con A-activated CD4^+ T cells were analyzed using a panel of Abs by flow cytometry. To further determine the potential T cell populations in the T cell preparation, the OVA peptide-specific OT I and OT II T cells were stained with PE-anti-CD25 (clone PC61.5), FITC-CD4 (clone GK1.5), and energy-coupled dye (ECD)-Foxp3 (clone FJK-16S) using Regulatory T Cell Staining kit No. 3 (eBioscience), and then analyzed by flow cytometry.

Exosomal molecule uptake by CD4^+ T cells

First, the OT II CD4^+ nT and aT cells were incubated with EXO_CFSE (10 μg/1 × 10^6^ T cells in 200 μl of AIM-V serum-free medium containing IL-2 (20 U/ml) at 37°C for 5 h and then analyzed for CFSE staining by flow cytometry. In another set of experiments, the OT II CD4^+ aT cells were incubated with EXO_CFSE under the above same condition for 1, 3, and 5 h, and then assessed by confocal fluorescence microscopy. To further determine the transfer of exosomal molecules to T cells, the CD4^+ nT and aT cells from OT II mice or OT II mice with different gene KO were incubated with EXO_OVA and then analyzed for expression of H-2K^b^, CD40, CD54, CD80, and pMHC I by flow cytometry or analyzed for expression of CD40 and CD80 by confocal fluorescence microscopy. The OT II CD4^+ nT and aT cells cocultured with EXO_OVA were termed nT EXO and aT_EXO cells, respectively. The CD4^+ aT(H-2K^b^), aT(CD40^−/−^), aT(CD54^−/−^), aT(12CD80^−/−^) cells were cocultured with (K^b^)EXO_OVA, (CD40^−/−^)EXO_OVA, (CD54^−/−^)EXO_OVA, and (CD80^−/−^)EXO_OVA and termed CD4^+ aT_EXO (K^b^), aT_EXO(CD40^−/−^), aT_EXO(CD54^−/−^), and aT_EXO(CD80^−/−^) cells, respectively. The CD4^+ aT cells from OT II/H-2K^b^, OT II/IFN-γ, and OT II/TNF-α/γ mice were cocultured with EXO_OVA and termed CD4^+ aT_EXO(H-2K^b^), aT_EXO(IFN-γ), and aT_EXO(TNF-α/γ) cells, respectively. The cytokine profiles of aT_EXO(K^b^), aT_EXO(12CD40^−/−^), aT_EXO(12CD54^−/−^), and aT_EXO(12CD80^−/−^) cells are similar to that of aT_EXO cells, whereas the cytokine production of aT_EXO(IL-2^−/−^), aT_EXO(IFN-γ^−/−^), and aT_EXO(TNF-α/γ^−/−^) cells are also similar to that of aT_EXO cells except for the specific cytokine (IL-2 or IFN-γ or TNF-α) deficiency (data not shown). For blocking assays, OT II CD4^+ aT cells were incubated with anti-H-2K^b^, anti-I^a^, and anti-LFA-1 Abs (12 μg/ml) or CTTA-4-lg (12 μg/ml), respectively, on ice for 30 min, then were cocultured with EXO_CFSE for 5 h at 37°C. The cells were harvested and analyzed for CFSE expression by flow cytometry.
**FIGURE 2.** EXO uptake by CD4⁺ T cells. a, Both naive and active OT II and C57BL/6 CD4⁺ T cells (with thick solid lines) and without (thin dotted lines) uptake of EXOCFSE were analyzed for CFSE expression by flow cytometry. b, Kinetic study of EXO uptake by CD4⁺ T cells. Active OT II CD4⁺ T cells (2 × 10⁶ cells) were incubated with EXOCFSE (20 μg) in DMEM (0.2 ml) containing 10% FCS and IL-2 (10 U/ml) at 37°C for different times. CFSE-positive T cells were detected by fluorescence microscopy and the percentages of CFSE-positive T cells were calculated. In another set of experiments, active OT II CD4⁺ T cells with incubation of EXOCFSE for 5 h were cultured in DMEM containing 10% FCS and IL-2 (10 U/ml) at 37°C for different times. CFSE-positive T cells were detected by fluorescence microscopy and the percentages of CFSE-negative T cells were calculated. c and e, Both naive and active OT II CD4⁺ T cells (with thick solid lines) and without (thin dotted lines) uptake of EXOCFSE were analyzed for expression of a panel of surface molecules including H-2Kb, CD40, CD54, and CD80, and pMHC I by flow cytometry. Irrelevant isotype-matched Abs were used as controls (thin dotted lines). d and f, Both naive and active OT II CD4⁺ cells from H-2Kb, CD40, CD54, and CD80 gene KO mice were also cocultured with (thick solid lines) and without (thick dotted lines) EXOCFSE and then analyzed for expression of H-2Kb, CD40, CD54, and CD80 by flow cytometry, respectively. Irrelevant isotype-matched Abs were used as controls (thin dotted lines). g, In the blocking assay, OT II CD4⁺ T cells were treated with anti-H-2Kb, anti-I-Ab, and anti-LFA-1 Abs, and CTLA-4/Ig fusion protein, a mixture of these reagents or a mixture of matched isotype Abs (as control reagents) or PBS (as positive control) on ice for 30 min, respectively, and then incubated with EXOCFSE. In addition, OT II CD4⁺ T cells with CD54 deficiency were incubated with EXOCFSE derived from CFSE-labeled (CD54⁻/⁻) DCOVA. The fractions of CFSE-positive T cells were analyzed after coculture for 5 h at 37°C. * p < 0.05 vs cohorts of the positive control (aT/EXO CFSE) (Student’s t test). One representative experiment of three is displayed.

**T cell proliferation assays**

To assess the functional effect of CD4⁺ nTEXO and aTEXO cells, we performed an in vitro CD8⁺ T cell proliferation assay. CD4⁺ nTEXO and aTEXO (0.3 × 10⁶ cells/well) cells derived from OT II mice and their 2-fold dilutions were cultured with a constant number of naive OT I CD8⁺ T cells (1 × 10⁶ cells/well) in presence or absence of CD4⁺ CD25⁺ Tr cells (0.3 × 10⁶ cells/well) derived from C57BL/6 mice. To examine the molecular mechanism, a panel of reagents including anti-H-2Kb, I-Ab, and LFA-I Abs and CTLA-4/Ig fusion protein (each 10 μg/ml), a mixture of the above reagents (as mixed reagents), and a mixture of isotype-matched irrelevant Abs (as control reagents) were added to the cell cultures, respectively. After culturing for 3 days, thymidine incorporation was determined by liquid scintillation counting (19). In the in vivo OVA-specific CD8⁺ T cell proliferation assay, C57BL/6 mice (six mice per group) were i.v. immunized with irradiated (4000 rad) stimulators including DC₃₀ᵥ, CD4⁺ aT, and aTEXO cells (1 × 10⁶ cells/mouse) with different gene KO. In another set of experiments, C57BL/6 mice (six mice per group) were i.v. immunized with irradiated (4000 rad) DC₃₀ᵥ, CD4⁺ aT, and aTEXO cells (1 × 10⁶ cells/mouse) alone or together with CD4⁺ CD25⁺ Tr cells (3 × 10⁶ cells/mouse). For evaluation of in vivo OVA-specific CD8⁺ T cell proliferation, the tail blood samples derived from mice 6 days after immunization were incubated with PE-H-2Kb/OVA tetramer and FITC-anti-CD8 Ab (Beckman Coulter) and analyzed by flow cytometry (13).

**Cytotoxicity assay**

In vivo cytotoxicity assay was performed as previously described (14). Briefly, wild-type C57BL/6 or I-A⁻/⁻ mice (six mice per group) were i.v. immunized with the above stimulator cells (1 × 10⁶ cells/mouse), respectively. In another set of experiments, C57BL/6 mice (six mice per group)
were i.v. injected with irradiated (4000 rad) DC<sub>OVA</sub> and CD4<sup>+</sup> aT<sub>EXO</sub> cells (1 × 10<sup>6</sup> cells/mouse) alone or together with CD4<sup>+</sup> 25<sup>+</sup> Tr cells (3 × 10<sup>6</sup> cells/mouse). Splenocytes were harvested from naïve mouse spleens and incubated with either high (3.0 μM, CFSE<sup>high</sup>) or low (0.6 μM, CFSE<sup>low</sup>) concentrations of CFSE, to generate differentially labeled target cells. The CFSE<sup>high</sup> cells were pulsed with OVA I peptide, whereas the CFSE<sup>low</sup> cells were pulsed with Mut 1 peptide and served as internal controls. These peptide-pulsed target cells were washed extensively to remove free peptides, and then i.v. injected at a 1:1 ratio into the immunized mice 6 days after immunization. Sixteen hours after the target cell delivery, the spleens of immunized mice were removed and residual CFSE<sup>high</sup> and CFSE<sup>low</sup> target cells remaining in the recipients’ spleens were analyzed by flow cytometry.

**Animal studies**

To examine the antitumor protective immunity conferred by EXO-targeted CD4<sup>+</sup> T cells, wild-type C57BL/6 mice (n = 8) were injected i.v. with irradiated (4000 rad) DC<sub>OVA</sub>, at, and aT<sub>EXO</sub> cells (1 × 10<sup>6</sup> cells/mouse) with or without coinjection of CD4<sup>+</sup> 25<sup>+</sup> Tr cells (3 × 10<sup>6</sup> cells/mouse) aT<sub>EXO</sub> cells with various gene KO (1 × 10<sup>6</sup> cells/mouse), respectively. The mice were injected with PBS as a control. To assess the antitumor immunity, the immunized mice were challenged i.v. with 0.5 × 10<sup>6</sup> BL6-10OVA tumor cells 6 days subsequent to the immunization. To assess the long-term antitumor immunity, wild-type C57BL/6 mice were first immunized with irradiated (4000 rad) DC<sub>OVA</sub> and aT<sub>EXO</sub> cells (1 × 10<sup>6</sup> cells/mouse). The immunized mice were then challenged i.v. with 2 × 10<sup>6</sup> BL6-10OVA cells 3 mo subsequent to the immunization to assess development of tumor-specific CD8<sup>+</sup> memory T (Tm) cells. The mice were sacrificed 4 wk after tumor cell injection and two individuals counted the lung metastasis tumor colonies in a blind fashion. BL6-10OVA-derived metastases on freshly isolated lungs appeared as discrete black-pigmented foci that could be distinguishable from normal lung tissues and all were confirmed by histological examination. Metastasis foci too numerous to count were assigned an arbitrary value of >100 (14).

**Results**

**CD4<sup>+</sup> T cells uptake exosomal pMHC I and costimulatory molecules in both Ag-specific and nonspecific manners**

The nT and Con A-stimulated aT cells derived from transgenic OT II mice expressed both CD4 and TCR. The latter ones also expressed active T cell marker cells (CD25 and CD69) (Fig. 1) and secreted IL-2 (~2.4 ng/ml/10<sup>6</sup> cells/24 h), IFN-γ (~2.0 ng/ml/10<sup>6</sup> cells/24 h), and TNF-α (~1.7 ng/ml/10<sup>6</sup> cells/24 h), but no IL-4 and IL-10, indicating that they are active type Th1 cells. In addition, there was no CD11-positive splenic DC contamination in the purified CD4<sup>+</sup> nT population (Fig. 1). To assess EXO uptake, CD4<sup>+</sup> nT and aT cells derived from OT II and wild-type C57BL/6 (B6) mice were incubated with EXO<sub>CFSE</sub> expressing CFSE (Fig. 2a) for various times and then analyzed by flow cytometry and confocal fluorescence microscopy. As shown in Fig. 2a, the CFSE dye was minimally and apparently detectable on OT II CD4<sup>+</sup> nT and aT cells, respectively. The uptake of EXO<sub>CFSE</sub> by OT II CD4<sup>+</sup> aT cells increased with incubation time and reached a maximal level (80% CFSE-positive cells) after a 5-h incubation (Fig. 2b), which was also confirmed by confocal fluorescence microscopic analysis (Fig. 3a). The CFSE-positive cells declined with time when culturing them in medium, but were still detectable ≥3 days in culture, indicating that the uptaken exosomal molecules on CD4<sup>+</sup> T cells are quite stable, which is consistent with a previous report by Undale et al. (20). Similar to CFSE dye, other exosomal molecules such as H-2K<sup>+</sup>, CD40, CD54, and CD80 molecules were also minimally and apparently transferred from EXO<sub>OVA</sub> onto OT II CD4<sup>+</sup> nT and aT cells, respectively (Fig. 2, c and e). In addition, the pMHC I complexes were also transferred onto CD4<sup>+</sup> aT cells. To further confirm it, we incubated EXO<sub>OVA</sub> with OT II CD4<sup>+</sup> T cells with different gene KO and then analyzed by flow cytometry or confocal fluorescence microscopy. The original OT II CD4<sup>+</sup> nT and aT cells with different gene KO did not express endogenous H-2K<sup>+</sup>, CD40, CD54, and CD80, respectively. However, after incubation with EXO<sub>OVA</sub>, T cells (especially the CD4<sup>+</sup> aT cells) did display the above exosomal molecules (Fig. 2, d and f), indicating that increased expression of the above molecules on CD4<sup>+</sup> T cells is due to an uptake of EXO molecules rather than an endogenous up-regulation. This was also confirmed by confocal fluorescence microscopic analysis. As shown in Fig. 3b, CD4<sup>+</sup> aT cells with deficiency of endogenous CD40 and CD80 expression displayed CD40 and CD80 expression after uptake of EXO<sub>OVA</sub>. To elucidate the molecular mechanism involved in EXO uptake, we used a panel of reagents in a blocking assay. The anti-Ia<sup>+</sup> and LFA-1 Abs, but not the CTLA-4/Ig fusion protein and the anti-H-2K<sup>+</sup> Ab, were able to significantly block EXO uptake (p < 0.05) (Fig. 2g). This was further confirmed by a significant decrease in EXO uptake (p < 0.05) when incubation of aT(CD54<sup>−/−</sup>), but not aT(CD40<sup>−/−</sup>) and aT(CD80<sup>−/−</sup>) cells, with EXO<sub>CFSE</sub> derived from CFSE-labeled (CD54<sup>−/−</sup>)DC<sub>OVA</sub>, (CD40<sup>−/−</sup>)DC<sub>OVA</sub>, and (CD80<sup>−/−</sup>)DC<sub>OVA</sub>, respectively, indicating that the EXO<sub>OVA</sub> uptake by CD4<sup>+</sup> T cells is mediated by both OVA-specific Ia<sup>+</sup>/TCR and nonspecific CD54/LFA-1 interactions, but not by H-2K<sup>+</sup>/TCR and CD80/CD28 interactions, which is consistent with previous reports by Hwang et al. (21, 22).

**CD4<sup>+</sup> aT<sub>EXO</sub> cells counteract in vitro CD4<sup>+</sup> 25<sup>+</sup> Tr cell-mediated suppression of naive CD8<sup>+</sup> T cell proliferation**

We then examined the stimulatory effect of EXO<sub>OVA</sub>-targeted active CD4<sup>+</sup> T (aT<sub>EXO</sub>) cells. As shown in Fig. 4a, EXO<sub>OVA</sub> could prime CD8<sup>+</sup> T cell proliferation in vitro, which is consistent with previous reports by Hwang et al. (21, 22), but in a lesser extent compared with DC<sub>OVA</sub>. Of interest, CD4<sup>+</sup>
aTEXO is a stronger stimulator in CD8+ T cell proliferation than DCOVA, whereas nTEXO is a relatively weak stimulator. CD4+25+ Tr cells expressing CD4, CD25, and Foxp3 (Fig. 4b) inhibited DCOVA-stimulated CD8+ T cell proliferation (Fig. 4a). However, aTEXO cells maintained their stimulatory effect in presence of CD4+25+ Tr cells, indicating that aTEXO cells can counteract in vitro CD4+25+ Tr cell-mediated suppression of naive CD8+ T cell proliferation.
CD4+ aTEXO cells stimulate in vitro CD8+ T cell proliferation and differentiation into central memory T cells

We next conducted phenotypic characterization of the above in vitro aTEXO-primed CD8+ T cells. Our data showed that DC_OVA or aTEXO priming resulted in several cycles of CD8+ CFSE-T cell division, whereas nontreated CD8+ CFSE-T cells were not divided (Fig. 4c). The primed T cells displayed expression of CD25, CD44 (Tm marker) (23) and CD62L (Fig. 4c). However, aTEXO-primed CD8+ T cells expressed IL-7R and higher CD62L than DC_OVA-primed ones with no IL-7R expression, indicating that they may be prone to becoming long-lived Tm cells. We then examined whether aTEXO-primed CTL exhibited any other functional traits prone to becoming long-lived Tm cells. We then examined whether aTEXO-primed CD44+CD62LlowIL-7R- and aTEXO-primed CD44+CD62LhighIL-7R+ CTLs, which have high and low cytotoxicity to tumor cells, are consistent with typical effector and central memory CTL (emCTL and cmCTL), respectively (12, 25).

CD4+ aTEXO cells stimulate in vivo CD4+ T cell-independent CD8+ T cell proliferation

We then performed a tetramer staining assay to detect OVA-specific CD8+ T cells in wild-type C57BL/6 (B6) or MHC class II (IaAg) gene KO mice. As shown in Fig. 5a, DC_OVA and aTEXO cells stimulated proliferation of H-2KbOVA57–264 tetramer-positive CD8+ T cells accounting for 1.03 and 2.24% of the total spleen CD8+ T cell population in B6 mice, respectively, indicating that aTEXO is a significantly stronger stimulator than DC_OVA (p < 0.05). In IaAg gene KO mice lacking CD4+ T cells, however, only aTEXO cells (2.01%), but not DC_OVA, could stimulate OVA-specific CD8+ T cell responses, indicating that the aTEXO-induced CD8+ T cell response is CD4+ T cell independent, whereas those of DC_OVA are CD4+ T cell dependent.

CD4+ aTEXO cell-induced CTL responses and antitumor immunity are mediated by IL-2 and acquired exosomal CD80 specifically delivered via acquired exosomal pMHC I

To elucidate the immune mechanism involved in aTEXO-induced CTL responses, we performed animal studies by using aTEXO with

![Diagram](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/article-pdf/2017/7/2736/2736_2756/2736_2756.pdf)
different gene KO. The stimulation of OVA-specific CD8\(^+\) T cell responses by aTEXO(CD54\(^{-/}\)) was reduced to more than half (1.08%) of the original one by aTEXO (2.24%) (Fig. 5c), again confirming that the acquired pMHC I complexes play a critical role in targeting the CD4\(^+\) aTEXO stimulatory effect is mediated by its IL-2 secretion and acquired CD80 costimulation. In addition, the CD4\(^+\) aTEXO(K\(^b/-\))-vaccinated mice did not display any killing activity (3\%), again confirming that the acquired pMHC I complexes play a critical role in targeting the CD4\(^+\) aTEXO stimulatory effect to OVA-specific CD8\(^+\) T cells in vivo. Furthermore, the results derived from our in vivo antitumor immunity studies are also consistent with the above aTEXO-induced in vivo CD8\(^+\) CTL responses (Fig. 5a). Our data showed that 1) CD4\(^+\) aTEXO cells induced stronger antitumor immunity than DC\(_{OVA}\) (Expt. I of Table I), and 2) IL-2 and acquired exosomal CD80 of CD4\(^+\) aTEXO are specifically delivered to CD8\(^+\) T cells in vivo via acquired exosomal pMHC I (Expt. II of Table I).

CD4\(^+\) aTEXO cells induce efficient long-term OVA-specific CD8\(^+\) T cell memory

Active CD8\(^+\) T cells can become long-lived Tm cells after adoptive transfer in vivo (26). We then assessed whether these CD4\(^+\) aTEXO-primed CD8\(^+\) T cells can also become long-lived Tm cells. As shown in Fig. 6a, we still detected 0.46% OVA-specific CD8\(^+\) T cells in peripheral blood of mice immunized with aTEXO, which substantially lost in CD4\(^+\) aTEXO(II-2\(^{-/}\))-(2\%) and aTEXO(CD80\(^{-/}\))-immunized (15\%) mice and significantly less than the CD4\(^+\) aTEXO(II-2\(^{-/}\))-(89\%), aTEXO(TNF-\(\alpha/-\))-(90\%), and aTEXO(CD40\(^{-/}\))-immunized (88\%) mice, thus further confirming that CD4\(^+\) the aTEXO stimulatory effect is mediated by its IL-2 secretion and acquired CD80 costimulation. In addition, the CD4\(^+\) aTEXO(K\(^b/-\))-vaccinated mice did not display any killing activity (3\%), again confirming that the acquired pMHC I complexes play a critical role in targeting the CD4\(^+\) aTEXO stimulatory effect to OVA-specific CD8\(^+\) T cells in vivo. Furthermore, the results derived from our in vivo antitumor immunity studies are also consistent with the above aTEXO-induced in vivo CD8\(^+\) CTL responses (Fig. 5a). Our data showed that 1) CD4\(^+\) aTEXO cells induced stronger antitumor immunity than DC\(_{OVA}\) (Expt. I of Table I), and 2) IL-2 and acquired exosomal CD80 of CD4\(^+\) aTEXO are specifically delivered to CD8\(^+\) T cells in vivo via acquired exosomal pMHC I (Expt. II of Table I).

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**Table I.** EXO-targeted CD4\(^+\) T cell vaccine protects against lung tumor metastases

<table>
<thead>
<tr>
<th>Vaccines</th>
<th>Tumor Growth Incidence (%)</th>
<th>Median Number of Lung Tumor Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC(_{OVA})</td>
<td>2/8 (25)</td>
<td>17 ± 6</td>
</tr>
<tr>
<td>aTEXO</td>
<td>0/8 (0)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>PBS</td>
<td>8/8 (100)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aTEXO(II-2(^{-/}))</td>
<td>7/8 (88)</td>
<td>78 ± 20</td>
</tr>
<tr>
<td>aTEXO(IFN-(\gamma/-))</td>
<td>0/8 (0)</td>
<td>0</td>
</tr>
<tr>
<td>aTEXO(TNF-(\alpha/-))</td>
<td>0/8 (0)</td>
<td>0</td>
</tr>
<tr>
<td>aTEXO(CD80(^{-/}))</td>
<td>5/5 (63)</td>
<td>47 ± 23</td>
</tr>
<tr>
<td>aTEXO(K(^b/-))</td>
<td>7/8 (88)</td>
<td>84 ± 17</td>
</tr>
<tr>
<td>PBS</td>
<td>8/8 (100)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Expt. 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC(_{OVA})</td>
<td>4/8 (50)</td>
<td>14 ± 9</td>
</tr>
<tr>
<td>aTEXO</td>
<td>0/8 (0)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>PBS</td>
<td>8/8 (100)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Expt. 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC(_{OVA})</td>
<td>2/8 (25)</td>
<td>17 ± 6</td>
</tr>
<tr>
<td>DC(_{OVA}) plus Tr cells</td>
<td>8/8 (100)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>aTEXO</td>
<td>0/8 (0)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>aTEXO plus Tr cells</td>
<td>0/8 (0)</td>
<td>0</td>
</tr>
<tr>
<td>PBS</td>
<td>8/8 (100)</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

*In experiment 1, the wild-type C57BL/6 (B6) mice (n = 8) were i.v. immunized with DC\(_{OVA}\), aTEXO, and PBS. In experiment 2, the wild-type C57BL/6 mice (n = 8) were i.v. immunized with aTEXO or PBS. Three months after the immunization, each mouse was challenged s.c. with 1 x 10\(^6\) C57BL/6 tumor cells and challenged p.o. with OVA-expressing (BL6-10 OVA) tumor cells (0.5 x 10\(^6\) cells/mouse). Six days after the immunization, each mouse was challenged i.v. with OVA-expressing (BL6-10 OVA) tumor cells (0.5 x 10\(^6\) cells/mouse). Six days after the immunization, each mouse was challenged i.v. with OVA-expressing (BL6-10 OVA) tumor cells (0.5 x 10\(^6\) cells/mouse). All the mice were sacrificed 4 wk after tumor cell challenge and the numbers of lung metastatic tumor colonies were counted. One representative experiment of three is shown.*
Because we demonstrated above the counteraction of in vitro CD4$^+$ T cell proliferation and differentiation in vivo. Wild-type C57BL/6 mice were i.v. immunized with irradiated (a) DC$_{OVA}$, aT, and aTE$_{EXO}$, alone or with CD4$^{+}$ 25$^+$ Tr cells, respectively. Six days after immunization, the tail blood samples of immunized mice were incubated with PE-H-2K$^b$/OVA tetramer and FITC-anti-CD8 Ab according to the company’s protocol, then analyzed by flow cytometry. The value in each panel represents the percentage of tetramer-positive CD8$^+$ T cells vs the total CD8$^+$ T cell population. The value in parentheses represents the SD. *p < 0.05 vs cohorts of the positive control (aTE$_{EXO}$ in absence of Tr cells) (Student’s t test). b. In an in vivo cytotoxicity assay, the above immunized mice were i.v. coinfected at a 1:1 ratio of splenocytes labeled with high (0.0.05), indicating that CD4$^+$ suppress the antitumor immunity.

Discussion

According to the progressive linear differentiation hypothesis (27), T cell differentiation involves a phase of proliferation preceding the acquisition of fitness and effector function. Primed CD8$^+$ T cells reach a variety of differentiation stages that contain effector cells as well as cells that have been arrested at intermediate levels of differentiation. Thus, they retain a flexible gene imprinting. T cells that may survive after the retraction phase of an immune response can be resolved into distinct subsets of either cmCTLs representing cells at intermediate levels of differentiation or fully differentiated emCTLs with effector capacity (28, 29). It has been shown that a strong Ag presentation stimulates development of effector CTL, whereas a less efficient Ag presentation can lead to generation of central memory CTL (30). In this study, we demonstrated that CD4$^+$ aTE$_{EXO}$ cells were able to stimulate naive CD8$^+$ T cell differentiation into CD8$^{+}$44$^{+}$62$^{low}$IL-7R$^-$ cmCTLs with less cytotoxicity and longer survival capacity leading to strong memory T cell responses, compared with DC$_{OVA}$-primed CD8$^{+}$44$^{+}$62$^{low}$IL-7R$^-$ emCTLs with high cytotoxicity and shorter survival capacity in vivo. Therefore, an EXO-targeted
CD4+ T cell vaccine using peripheral blood CD4+ T cells of a cancer patient incubated with tumor cell-derived EXO from ascites of a cancer patient may be a useful strategy for EXO-based treatment of cancer.

Administration of attenuated T lymphocytes to animals has been shown to stimulate immune suppression and to prevent the development of experimental autoimmune diseases (31, 32). Vaccination using myelin basic protein autoreactive T cells has also been applied to clinical trial in multiple sclerosis (33). In this study, we clearly showed that CD4+ aTEXO cells can more strongly stimulate OVA-specific immunogenic CD8+ CTL responses, antitumor immunity, and CD8+ T cell memory in wild-type mice than EXO and DC OVA. Interestingly, the CD8+ CTL responses stimulated by aTEXO are found to be CD4+ T cell independent. Thus, EXO-targeted T cell vaccines may be very useful in induction of anti-HIV immunity in HIV patients with CD4+ T cell deficiency because HIV-1 infection is characterized by a gradual loss of CD4+ T cells and progressive immune deficiency (34). Furthermore, we also elucidated the molecular mechanism involved in aTEXO vaccine, which includes 1) the IL-2 secretion and the acquired exosomal CD80 costimulation that mediate the aTEXO stimulatory effect and 2) the acquired exosomal pMHC I complexes that play a critical role in targeting the aTEXO stimulatory effect to CD8+ T cells in vivo.

CD4+ 25+ Tr cells develop in the thymus and then enter peripheral tissues where they suppress activation of other self-reactive T cells (35, 36). It has been reported that an elevated number of Tr cells was detected in tumors (37, 38) which suppressed the antitumor immune responses by inhibition of CD4+ T cell proliferation and a helper effect (39–41) as well as DC maturation (42). Therefore, how to combat immune tolerance becomes a critical challenge in cancer immunotherapy (43). A variety of stimuli that increase the potency of T cell stimulation have been shown to abrogate CD4+ Tr cell function including high Ag dose, TLR signals, CD28 engagement, and provision of IL-2 (44–48). In this study, we demonstrated that CD4+ aTEXO cells, but not DC OVA, can stimulate CD8+ T cell proliferation in the presence of CD4+ 25+ Tr cells in vitro and in vivo. These CD4+ aTEXO cells expressing exosomal pMHC I and CD80 and secreting IL-2 can break Tr cell-mediated immune tolerance, possibly due to its capacity to 1) directly stimulate CD8+ T cell responses in absence of CD4+ Th cells and DC, thus bypassing the above Tr cell-mediated suppressive pathways or 2) directly counteract CD4+ Tr suppression via CD28 engagement and IL-2 stimulation (44–48).

Taken together, our data show that DC OVA-derived EXO OVA can be uptaken by CD4+ T cells. EXO OVA-targeted CD4+ aTEXO cells expressing acquired exosomal pMHC I and CD80 can stimulate CD8+ cmCTL responses, more efficient in vivo antitumor immunity and long-term CD8+ T cell memory than DC OVA, and counteract CD4+ 25+ Tr cell-mediated suppression of OVA-specific CTL responses and antitumor immunity. The aTEXO stimulatory effect is mediated via its IL-2 secretion and acquired exosomal CD80 costimulation and is specifically targeted to CD8+ T cells in vivo via acquired exosomal pMHC I complexes. Therefore, an EXO-targeted active CD4+ T cell vaccine may represent a novel and highly effective vaccine strategy for inducing immune responses not only against tumors, but also other infectious diseases.

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Disclosures
The authors have no financial conflict of interest.


