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

Siguon Hao,* Yongqing Liu,* Jinying Yuan,* Xueshu Zhang,* Tianpe He,* Xiaochu Wu,† Yangdou Wei,† Deming Sun,‡ and Jim Xiang2*

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.

Dendritic 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 antigen-susceptible 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 DC activation, and act as Th-APCs. 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 (SINFEKL) and OVA II (ISQAVHAAHAEINEAGR) which are OVA peptides specific for H-2Kb and Iaα, respectively, were used as antigens to prime CD4+ T cells. The highly specific for H-2Kb of an irrelevant 3LL lung carcinoma (11). All peptides were synthesized by Multiple Peptide Systems. Biotin-labeled or FITC-labeled Abs specific for H-2Kb (AF6-88.5), Iaα (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 V beta2 Vβ5+ TCR (MR9-4), as well as FITC-conjugated avidin, were all obtained from BD Pharmingen. Anti-IL-1α Ab, CTLA-4/1g 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-2Kb, Iaα, IL-2, IFN-γ, TNF-α, CD40, CD54, and CD80 gene knockout (KO) mice were obtained from The Jackson Laboratory. Homozygous OT II/H-2Kb+, OT II/CD40−, OT II/CD54−, OT II/CD80−, OT II/IFN-γ−, and OT II/TNF-α− mice were generated by backcrossing the designated gene KO 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-2Kb, CD40, and CD80 gene KO mice were referred to as (Kb−)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 μl of EXO was recovered from an overnight culture of 1 × 108 DC OVA in 1.0 ml of AIM-V serum-free medium containing 10% mouse serum (10 μg/ml). EXO derived from DC OVA were termed EXO OVA. Similar to DC OVA, EXO OVA also expressed MHC class I (H-2Kb) and class II (Iaα), CD11c, CD40, CD54, and CD80, and the pMHC I complex, but in a lesser content, compared with DC OVA (13). EXO derived from (Kb−)DC OVA, (CD40−/−)DC OVA, (CD54−/−)DC OVA, and (CD80−/−)DC OVA were termed (Kb−)EXO OVA, (CD40−/−)EXO OVA, (CD54−/−)EXO OVA, and (CD80−/−)EXO OVA, respectively. To generate CSFE-labeled EXO, DC OVA were stained with 0.5 μM CSFE 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 CSFELabeled EXO (EXO CSFE) was harvested and purified from the culture supernatants as previously described above.

CD4+ T cell preparation and characterization

Naive OVA-specific T (OT) 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+ or CD8+/OVA+ (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-2Kb+, OT II/CD40−, OT II/CD54−, OT II/CD80− mice were termed aT(H-2Kb+), aT(CD40−), aT(CD54−), and aT(CD80−), respectively. The above naive and Con A-activated CD4+ T cells were analyzed using a panel of Abs by flow cytometry. To assess the potential counteraction of CD4+ T cells on DCs, naive CD4+ T cells were cocultured with DC OVA 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 spleen cells from OT II mice were cultured in RPMI 1640 medium containing 1% normal mouse serum, GM-CSF, and OVA (0.3 mg/ml). These DCs were termed aTEXO. aTEXO(IL-2+), aTEXO(IFN-γ+), and aTEXO(TNF-α+) cells were cocultured with (Kb−)EXO OVA, (CD40−/−)EXO OVA, (CD54−/−)EXO OVA, and (CD80−/−)EXO OVA and termed aTEXO/(Kb−), aTEXO/(CD40−/−), aTEXO/(CD54−/−), and aTEXO/(CD80−/−) cells, respectively. The above naive and Con A-activated CD4+ 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+ T cells were incubated with EXO CSFE (10 μg/ml) 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 CSFE staining by flow cytometry. In another set of experiments, the OT II CD4+ T cells were incubated with EXO CSFE 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+ T cells were cocultured with EXO OVA and analyzed for expression of H-2Kb, CD40, CD54, and CD80, and the pMHC I by flow cytometry. To analyze the expression of CD40 and CD80 by confocal fluorescence microscopy. The OT II CD4+ 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.
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 x 10^6 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 x 10^6 cells/well) in the presence or absence of CD4+ CD25+ Tr cells (0.3 x 10^6 cells/well) derived from C57BL/6 mice. To examine the immune mechanism, a panel of reagents including anti-H-2Kb, I-Ab, and LFA-1 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 DCOVA, CD4+ CD25+ Tr cells (3 x 10^6 cells/mouse), or CD4+ aTExo cells (1 x 10^6 cells/mouse), or CD4+ aTExo cells (1 x 10^6 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) DCOVA, CD4+ aT, and aTExo cells (1 x 10^6 cells/mouse) alone or together with CD4+ CD25+ Tr cells (3 x 10^6 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 Ia+/− mice (six mice per group) were i.v. immunized with the above stimulator cells (1 x 10^6 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>OV</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 naive 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 OVA1 peptide, whereas the CFSE<sup>low</sup> cells were pulsed with Mut1 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>OV</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>OV</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. 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> aT 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 endogenous H-2K<sup>b</sup>, CD40, CD54, and CD80, respectively. However, after incubation with EXO<sub>OV</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>OV</sub>. To elucidate the molecular mechanism involved in EXO uptake, we used a panel of reagents in a blocking assay. The anti-Ia<sup>b</sup> and LFA-1 Abs, but not the CTLA-4/Ig fusion protein and the anti-H-2K<sup>b</sup> Ab, were able to significantly block EXO uptake (p < 0.05) (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>OV</sub>. To elucidate the molecular mechanism involved in EXO uptake, we used a panel of reagents in a blocking assay. The anti-Ia<sup>b</sup> and LFA-1 Abs, but not the CTLA-4/Ig fusion protein and the anti-H-2K<sup>b</sup> Ab, were able to significantly block EXO uptake (p < 0.05) when incubation of aT(CD4<sup>+</sup>CD54<sup>−</sup>) and aT(CD80<sup>−</sup>), but not aT(CD4<sup>−</sup>CD54<sup>−</sup>) and aT(CD80<sup>−</sup>), cells with EXO<sub>CFSE</sub> derived from CFSE-labeled (CD54<sup>−</sup>)DC<sub>OV</sub>, (CD40<sup>−</sup>)DC<sub>OV</sub>, and (CD80<sup>−</sup>)DC<sub>OV</sub>, respectively, indicated that the EXO<sub>OV</sub> uptake by CD4<sup>+</sup> T cells is mediated by both OVA-specific Ia<sup>b</sup>/TCR and nonspecific CD54/LFA-1 interactions, but not by H-2K<sup>b</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>OV</sub>-targeted active CD4<sup>+</sup> T (aT<sub>EXO</sub>) cells. As shown in Fig. 4a, EXO<sub>OV</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 to a lesser extent compared with DC<sub>OV</sub>. Of interest, CD4<sup>+</sup>
aTEXO is a stronger stimulator in CD8+T cell proliferation than DC_OVA, whereas nTEXO is a relatively weak stimulator. CD4+25+ Tr cells expressing CD4, CD25, and Foxp3 (Fig. 4b) inhibited DC_OVA-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.

FIGURE 4. Stimulation of CD8+ memory T cell responses in vitro. a. In vitro CD8+ cell proliferation assay. EXO_OVA (10 μg/ml), DC_OVA, nTEXO, aTEXO, and Con A-activated OTI T (aT) cells and their 2-fold dilutions were cocultured with a constant number of OT I CD8+ T cells in presence or absence of CD4+25+ Tr cells. After 3 days, the proliferation response of CD8+ T cells was determined by [3H]thymidine uptake assay. b. Phenotypic analysis of CD4+25+ Tr cells. The CD4+25+ Tr cells were purified from C57BL/6 mouse splenocytes using CD25 microbeads, stained with PE-CD25, FITC-CD4, and ECD-Foxp3 Abs, and then analyzed by flow cytometry. The FITC-CD4+ and PE-CD25-positive T cells were grouped for analysis of ECD-Foxp3 expression (solid line). Irrelevant isotype-matched Ab was used as a control (dotted line). c. Phenotypic analysis of in vitro aTEXO-primed CD8+ T cells. CFSE-labeled naive T cells derived from OT I mice were primed with irradiated DC_OVA and aTEXO for 2 days in vitro and stained for CD8, CD44, CD62L, and IL-7R, respectively. Dot plots of CFSE-positive CD8+ T cells stained with PE-anti-CD8 Ab are shown, indicating that the CFSE-labeled CD8+ T cells underwent some cycles of cell division, and were sorted by flow cytometry. The in vitro DCOVA- and aTEXO-activated OT I CD8+ CD45.1+ T cells were purified using biotin anti-CD45.1 Ab and anti-biotin microbeads (Miltenyi Biotec) and are referred to as DCOVA/OT I6.1 and aTEXO/OT I6.1, respectively. They were then incubated with irradiated (4,000 rad) EG7 and EL4 for 24 h. The supernatants in wells containing DC_OVA/OT I6.1 and aTEXO/OT I6.1 were examined for IFN-γ expression by ELISA. e. T cell proliferation assay. In vitro DCOVA- and aTEXO-activated CD8+ CD45.1+ T cells (0.4 × 10^5 cells/well) derived from OT I/B6.1 mouse OTI CD8+ T cells, primed on day 0 with irradiated (f) or aTEXO (Œ), were maintained in cultures for 1 wk with the indicated cytokines (IL-2 (50 U/ml) and IL-7 (10 ng/ml)) added on days 3 and 5. Live CD8+ T cells with trypan blue exclusion for each culture done in triplicate were counted at the indicated time points. f. In vitro cytotoxicity assay. The above DCOVA/OT I6.1 (f) and aTEXO/OT I6.1 (Œ) cells were used as effector cells, whereas 51Cr-labeled EG7 or EL4 cells were used as target cells in a chromium release assay. One representative experiment of three is displayed.
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 CTL exhibited any other functional traits prone to becoming long-lived Tm cells.

In an in vivo cytotoxicity assay, the above immunized mice were i.v. coinfected at a 1:1 ratio of splenocytes labeled with high (3.0 μM, CFSE⁻) and low (0.6 μM, CFSE⁺) concentrations of CFSE and pulsed with OVA and Mut 1 peptide, respectively, 6 days after immunization with aTEXO, and with various gene KO, respectively. Sixteen hours after target cell delivery, the residual CFSE⁻ and CFSE⁺ target cells remaining in the recipients’ spleens were sorted and analyzed by flow cytometry. The value in each panel represents the percentage of CFSE⁻ and CFSE⁺ cells remaining in the spleens. * p < 0.05 vs cohorts of the positive control (aTEXO) (Student’s t test).

FIGURE 5. Stimulation of CD8⁺ T cell proliferation and differentiation into CTL in vivo. Wild-type C57BL/6 or Ia⁻/⁻ gene KO mice were i.v. immunized with irradiated (a) DC_OVA, aT and aTEXO, and (b) aTEXO with various gene KO, respectively. Six days after immunization, the tail blood samples of immunized mice were incubated with PE-H-2Kb/OVA257–264 tetramer and FITC-anti-CD8 Ab (Beckman Coulter) 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 represent the SD. *, p < 0.05 vs cohorts of the positive control (aTEXO) (Student’s t test). c, In an in vivo cytotoxicity assay, the above immunized mice were i.v. coinfected at a 1:1 ratio of splenocytes labeled with high (3.0 μM, CFSE⁻) and low (0.6 μM, CFSE⁺) concentrations of CFSE and pulsed with OVA and Mut 1 peptide, respectively, 6 days after immunization with aTEXO, and with various gene KO, respectively. Sixteen hours after target cell delivery, the residual CFSE⁺ and CFSE⁻ target cells remaining in the recipients’ spleens were sorted and analyzed by flow cytometry. The value in each panel represents the percentage of CFSE⁺ and CFSE⁻ cells remaining in the spleens. * p < 0.05 vs cohorts of the positive control (aTEXO) (Student’s t test).

For aTEXO cell-induced CTL responses and antitumor immunity, IL-2 and acquired exosomal CD80 specifically delivered via acquired exosomal pMHC I.
different gene KO. The stimulation of OVA-specific CD8+ T cell responses by aTEXO(CD54−/−) was reduced to more than half (10.8%) of the original one by aTEXO (2.24%) (Fig. 5b), possibly due to lacking CD54/LFA-1 interaction leading to decreased EXO uptake by aTEXO(CD54−/−) cells. Interestingly, the stimulation of OVA-specific CD8+ T cell responses by aTEXO(IL-2−/−) (0.24%) and aTEXO(IFN-γ−/−) (0.42%) cells, but not by aTEXO(IFN-γ−/−) (2.15%), aTEXO(TNF-α−/−) (2.13%) and aTEXO(CD40−/−) (2.21%), was greatly lost and significantly less than that by aTEXO (p < 0.05), indicating that the aTEXO stimulatory effect is mediated by its IL-2 secretion and acquired CD80 costimulation. In addition, the CD4+ aTEXO(Kb−/−)-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 (Exp. 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 (Exp. 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

### Table I. EXO-targeted CD4+ T cell vaccine protects against lung tumor metastases

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<th>Vaccines</th>
<th>Tumor Growth Incidence (%)</th>
<th>Median Number of Lung Tumor Colonies</th>
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<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>0</td>
</tr>
<tr>
<td>aT</td>
<td>8/8 (100)</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>
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<tr>
<td>aTEXO(IL-2−/−)</td>
<td>7/8 (88)</td>
<td>78 ± 20</td>
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<td>aTEXO(IFN-γ−/−)</td>
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<td>0</td>
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<tr>
<td>aTEXO(TNF-α−/−)</td>
<td>0/8 (0)</td>
<td>0</td>
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<tr>
<td>aTEXO(CD54−/−)</td>
<td>3/8 (38)</td>
<td>26 ± 8</td>
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<tr>
<td>aTEXO(CD80−/−)</td>
<td>5/8 (63)</td>
<td>47 ± 23</td>
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<tr>
<td>aTEXO(Kb−/−)</td>
<td>7/8 (88)</td>
<td>84 ± 17</td>
</tr>
<tr>
<td>PBS</td>
<td>8/8 (100)</td>
<td>&gt;100</td>
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<td>0</td>
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<tr>
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<td>&gt;100</td>
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<tr>
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<tr>
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<td>aTEXO</td>
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<td>0</td>
</tr>
<tr>
<td>PBS</td>
<td>8/8 (100)</td>
<td>&gt;100</td>
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*In experiment 1, the wild-type C57BL/6 (B6) mice (n = 8) were i.v. immunized with DC OVA, aT and aTEXO cells, or PBS. In experiment 2, the wild-type C57BL/6 mice (n = 8) were i.v. immunized with either aTEXO cells or aTEXO cells with various gene KO. Six days after the immunization, each mouse was challenged i.v. with OVA-expressing (BL6–10OVA) tumor cells (0.5 × 106 cells/mouse). In experiment 3, the wild-type C57BL/6 mice (n = 8) were i.v. immunized with DC OVA, aTEXO cells alone or together with CD4+ 25T Tr cells. Six days after the immunization, each mouse was challenged i.v. with OVA-expressing (BL6–10OVA) tumor cells (0.5 × 106 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.

![Figure 6. Development of Ag-specific CD8+ memory T cells.](http://www.jimmunol.org/)

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*Note: The document is a continuation of the discussion on the immune system, specifically focusing on the role of exosomes in adaptive immunity. The text provided is a summary of the content, with the full document available for further reading.*
is significantly stronger than that (0.12%) in mice immunized with DCOVA 3 mo after the immunization (*p < 0.05). These OVA-specific CD8$^+$ T cells were also CD44$^+$, indicating that they are OVA-specific CD8$^+$ Tm cells. In addition, the survived CD4$^+$ aTEXO-primed CD8$^+$ Tm cells are nearly 4-fold compared with the survived DCOVA-stimulated ones, indicating that CD4$^+$ aTEXO-primed CD44$^+$CD62L$^{high}$IL-7R$^+$ CTL with low cytotoxicity to tumor cells are long survival cmCTL. The recall responses were then assessed on day 4 after the boost of immunized mice with DCOVA. As expected, CD8$^+$ Tm cells were expanded by 10-fold in these immunized mice after the boost (Fig. 6b), indicating that these CD8$^+$ Tm cells are functional. In another set of experiments, the above immunized mice were challenged with a high dose (2 × 10$^6$ cells/mouse) of BL6–10OVA tumor cells. Only four of eight (50%) mice immunized with DCOVA were tumor free, whereas all eight of eight (100%) mice immunized with CD4$^+$ aTEXO cells did not have any lung metastasis (Expt. III of Table I), indicating that CD4$^+$ aTEXO cells can induce more efficient long-term CD8$^+$ T cell memory than DCOVA.

CD4$^+$ aTEXO cells counteract in vivo CD4$^+$25$^+$ Tr cell-mediated suppression of OVA-specific CD8$^+$ T cell proliferation and antitumor immunity

Because we demonstrated above the counteraction of in vitro CD4$^+$25$^+$ Tr cell-mediated suppression, we wanted to further assess the potential effect of CD4$^+$ aTEXO cells in counteraction of in vivo CD4$^+$25$^+$ Tr cell-mediated suppression. We performed in vivo proliferation and cytotoxicity assays as described above. As shown in Fig. 7a, the stimulation of OVA-specific CD8$^+$ T cell responses in mice by DCOVA (1.03%) was almost completely lost (0.10%) when mice were coinjected with CD4$^+$25$^+$ Tr cells (p < 0.05), indicating that CD4$^+$25$^+$ Tr cells can significantly inhibit DCOVA-stimulated CD8$^+$ T cell responses. However, the OVA-specific CD8$^+$ T cell responses in mice with CD4$^+$ aTEXO stimulation in presence or absence of CD4$^+$25$^+$ Tr cells maintained at a similar level (2.24%). In the in vivo cytotoxicity assay, the effector CTL responses derived from DCOVA, but not CD4$^+$ aTEXO cells, in presence of CD4$^+$25$^+$ Tr cells were lost (Fig. 7b). Furthermore, the results derived from our in vivo antitumor immunity studies (Expt. IV of Table I) are also consistent with the above aTEXO-induced in vivo CD8$^+$ CTL responses (Fig. 5). Our data demonstrated that CD4$^+$ aTEXO cells, but not DCOVA$^+$ induced efficient antitumor immunity in mice even with coinjection of CD4$^+$25$^+$ Tr cells, which was against the challenge of BL6–10OVA tumor cells from developing lung tumor metastasis (Expt. IV of Table I), indicating that CD4$^+$ aTEXO cells can counteract in vivo CD4$^+$25$^+$ Tr cell-mediated immune suppression, possibly through bypassing the CD4$^+$25$^+$ Tr cell-mediated suppressive pathway.

**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$^+$ aTEXO cells were able to stimulate naive CD8$^+$ T cell differentiation into CD8$^+$44$^{62high}$IL-7R$^+$ cmCTLs with low cytotoxicity and longer survival capacity leading to strong memory T cell responses, compared with DCOVA-primed CD8$^+$44$^{62low}$IL-7R$^-$ 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.

References


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