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RESEARCH ARTICLE

$CD4^+$ T cell-released exosomes inhibit $CD8^+$ cytotoxic T-lymphocyte responses and antitumor immunity

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T cells secrete bioactive exosomes (EXO), but the potential immunoregulatory effect of T-cell EXO is largely unknown. In this study, we generated activated ovalbumin (OVA)-specific $CD4^+$ T cells *in vitro via* coculture of OVA-pulsed dendritic cells (DC_{OVA}) with naive $CD4^+$ T cells derived from OVA-specific T-cell receptor (TCR) transgenic OTII mice. $CD4^+$ T-cell EXO were then purified from the $CD4^+$ T-cell culture supernatants by differential ultracentrifugation. $CD4^+$ T-cell EXO exhibited the 'saucer' shape that is characteristic of EXO with a diameter between 50 and 100 nm, as assessed by electron microscopy, and contained the EXO-associated proteins LAMP-1, TCR and lymphocyte function associated antigen-1 (LFA-1), as determined by western blot. Flow cytometric analysis showed that $CD4^+$ T-cell EXO expressed $CD4^+$ T-cell markers (CD4, TCR, LFA-1, CD25 and Fas ligand), but to a lesser extent than $CD4^+$ T cells. We demonstrated that DC_{OVA} took up $CD4^+$ T-cell EXO *via* peptide/major histocompatibility complex (pMHC) II/TCR and CD54/LFA-1 interactions. OVA-specific $CD4^+$ T-cell EXO from OTII mice, but not ConA-stimulated polyclonal $CD4^+$ T-cell EXO from wild-type C57BL/6 mice inhibited DC_{OVA}-stimulated *in vitro* $CD4^+$ T-cell proliferation and *in vivo* $CD8^+$ cytotoxic T lymphocyte (CTL) responses and antitumor immunity against OVA-expressing B16 melanoma BL6-10_{OVA} cells. In addition, EXO derived from a T-cell hybridoma cell line, MF72.2D9, expressing an OVA-specific $CD4^+$ TCR, had a similar inhibitory effect as OTII $CD4^+$ T-cell EXO on CTL-mediated antitumor immunity. Taken together, our data indicate that antigen-specific T-cell EXO may serve as a new type of immunosuppressive reagent for use in transplant rejection and treatment of autoimmune diseases.
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INTRODUCTION

Exosomes (EXO) are small membrane vesicles (50–100 nm in diameter) of endocytic origin that are formed by the fusion of multivesicular endosomes (MVE) with the plasma membrane, followed by exocytosis.[1] EXO can be constitutively or inductively secreted by a variety of cell types, such as dendritic cells (DCs), macrophages, T cells, B cells, reticulocytes, mastocytes and tumor cells.[2] Extensive studies have shown that DC- and tumor cell-released EXO can trigger strong tumor-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses and antitumor immunity.[3–7] Thus, DC- and tumor cell-derived EXO are attractive molecules for immunotherapy and have been used for antitumor vaccines.

Activated T cells can release bioactive EXO-bearing TCR/CD3 complexes,[8] Fas ligand (FasL) and APO2 ligand,[9] which are implicated in immune regulation *via* the induction of activation-induced cell death of T cells.[10] In addition, T-cell EXO can be taken up by DCs or B cells by intercellular molecule transfer from T cells to antigen-presenting cells (APCs), leading to modulation of the APC and regulation of T-cell responses.[11] However, the potential effect of activated T-cell EXO on modulation of DC-induced immune responses is still largely unclear.

In this study, we activated $CD4^+$ T cells derived from OVA-specific T-cell receptor (TCR) transgenic OTII mice[12] *in vitro* with ovalbumin (OVA)-pulsed dendritic cells (DC_{OVA}) and purified EXO from culture supernatants of the $CD4^+$ T cells by differential ultracentrifugation. We assessed the effect of EXO on DC_{OVA}-mediated $CD8^+$ CTL responses and antitumor immunity. We found that DC_{OVA} took up $CD4^+$ T-cell EXO bearing the OVA-specific TCR and lymphocyte function associated antigen-1 (LFA-1) *via* peptide/major histocompatibility complex (pMHC) II/TCR and CD54/LFA-1 interactions. We demonstrated that OVA-specific $CD4^+$ T-cell EXO, but not ConA-stimulated polyclonal $CD4^+$ T-cell EXO from C57BL/6 mice, inhibited *in vitro* DC_{OVA}-stimulated $CD4^+$ T-cell proliferation and *in vivo* $CD8^+$ CTL responses and antitumor immunity against OVA-expressing B16 melanoma BL6-10_{OVA} cells. In addition, EXO derived from the T-cell hybridoma cell line MF72.2D9 expressing an OVA-specific $CD4^+$ TCR had an inhibitory effect similar to that of OTII $CD4^+$ T-cell EXO on $CD8^+$ CTL-mediated antitumor immunity.

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MATERIALS AND METHODS

Reagents, cell lines and animals

Fluorescein isothiocyanate (FITC)- or biotin-labeled antibodies (Abs) specific for CD4, Vβ5.1, 5.2 TCR, LFA-1, CD25, H-2K^b, Ia^b, CD11c, CD40, CD54, CD80, Fas, FasL, LAMP-1 and Calnexin, and an FITC-conjugated avidin Ab were all obtained from BD Biosciences (Mississauga, Ont., Canada). A biotin-labeled anti-H-2K^b/OVA$_{257–264}$ (OVAI) (SIINFEKL) (pMHC I) Ab was obtained from eBioscience (San Diego, CA, USA), and a phycoerythrin (PE)-labeled H-2K^b/OVAI tetramer and FITC-labeled anti-mouse CD8 Ab were obtained from Beckman Coulter (San Diego, CA, USA). The MHC class I (H-2K^b)-restricted OVAI peptide and irrelevant Mut1 peptide (FEQNTAQP) were synthesized by Multiple Peptide Systems (San Diego, CA, USA). Recombinant mouse interleukin (IL)-2, IL-4 and granulocyte macrophage-colony-stimulating factor (GM-CSF) were obtained from Endogen (Woburn, MA, USA). The mouse T-cell hybridoma cell line MF72.2D9 expressing a TCR specific for Ia^b/OVA$_{323-339}$ (ISQAVHAAHAEINEAGR) was obtained from Dr K. Rock (University of Massachusetts Medical Center, Worcester, MA, USA). The highly lung metastatic B16 mouse melanoma (BL6-10) and OVA-transfected BL6-10 (BL6-10$_{OVA}$) cell lines were generated in our laboratory.[12] Female C57BL/6 (B6, H-2K^b) mice were obtained from Charles River Laboratories (St Laurent, Que., Canada). The OVA$_{323–339}$-specific TCR-transgenic (OTII) mice on a C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, MA, USA). All mice were housed in the animal facility at the Saskatoon Cancer Center. All animal experiments were performed in accordance with the Canadian Council for Animal Care guidelines.

Preparation of EXO

OVA-pulsed bone marrow-derived dendritic cells (DC$_{OVA}$) from C57BL/6 mice were generated in the presence of GM-CSF/IL-4 (20 ng/ml) and pulsed with OVA protein (0.1 mg/ml) overnight, as previously described.[12] To generate activated OVA-specific CD4$^+$ T cells, naive CD4$^+$ T cells (4×10^5 cells/ml) from OTII mice were stimulated for 72 h with irradiated (4000 rad) DC$_{OVA}$ (1×10^5 cells/ml) in the presence of IL-2 (20 U/ml).[12] The OVA-specific CD4$^+$ T cells were incubated with or without 0.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) and then cultured in serum-free AIM-V medium (Invitrogen Corp., Carlsbad, CA, USA) with IL-2 (20 U/ml) for 24 h. EXO were purified from the T-cell culture supernatants by differential ultracentrifugation, and protein content was quantified by Bradford assay.[13] DC$_{OVA}$-activated OTII CD4$^+$ T cell-released EXO and CFSE-labeled OTII CD4$^+$ T cell-released EXO were termed EXO and EXO$_{CFSE}$, respectively. EXO derived from the T-cell hybridoma cell line MF72.2D9 expressing an OVA-specific CD4$^+$ TCR were termed EXO$_{MF}$, and EXO generated from ConA-stimulated C57BL/6 CD4$^+$ T cells were used as control EXO (EXOcont).

Electron microscopic analysis

EXO samples were fixed in 4% paraformaldehyde and then loaded onto carbon-coated formvar grids. After incubation in a moist atmosphere for 20 min, the samples were washed twice in phosphate-buffered saline (PBS) and then fixed for 5 min in 1% glutaraldehyde. After three washes, the EXO sample-loaded grids were stained for 10 min with saturated aqueous uranyl. EXO samples were then examined with a Zeiss EM10C electron microscope at 60 kV.

Western blot analysis

EXO samples (10 μg/lane) were loaded onto 12% acrylamide gels, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and subsequently transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). Membranes were blocked by incubation for 2 h at room temperature with Odyssey blocking buffer (LI-COR Bioscience, Lincoln, NE, USA) and immunoblotted with a panel of Abs specific for LAMP-1, Vβ5.1, 5.2 TCR, LFA-1 and Calnexin at 4 °C overnight. After three washes with PBS containing 0.05% (v/v) Tween 20, membranes were further incubated with goat anti-rat/-mouse IRDyeR800CW and scanned using an Odyssey instrument according to the manufacturer's instructions (LI-COR Bioscience).

Phenotypic analysis by flow cytometry

For phenotypic analysis, DC$_{OVA}$-activated CD4$^+$ T and MF72.2D9 cells were stained with a panel of Abs specific for CD4, Vβ5.1, 5.2 TCR, LFA-1, CD25, H-2K^b, Ia^b, CD11c, CD40, CD54, CD80, pMHC I, Fas and FasL and analyzed by flow cytometry as described previously.[13] EXO samples (10 g/100 μl PBS) were incubated with FITC-Abs (2 μl, 1 mg/ml) specific for CD4, Vβ5.1, 5.2 TCR, LFA-1, CD25 and FasL on ice for 30 min and then analyzed by flow cytometry.[13] EXO were first gated using calibrated polystyrene latex microbeads (3.8 μm) bound to fluorescent dyes (Sigma, St Louis, MO, USA) and analyzed for expression of the above molecules by flow cytometry.

Uptake of EXO by DCs

For assessment of exosomal uptake, DC$_{OVA}$ were incubated with CFSE-EXO (10 μg/1×10^6 DC$_{OVA}$ cells) in serum-free AIM-V medium at 37 °C for 4 h, washed with PBS and then analyzed for CFSE expression by flow cytometry and confocal fluorescence microscopy. To examine the molecular mechanism involved in EXO uptake, DC$_{OVA}$ were incubated with a panel of Abs specific for H-2K^b, Ia^b and LFA-1 on ice before and during coculture with CFSE-EXO in AIM-V medium at 37 °C for 4 h. After being washed with PBS, the CFSE-positive cells were detected and counted by confocal fluorescence microscopy.

T-cell proliferation assay

To assess *in vitro* CD4$^+$ T-cell proliferation, purified naive CD4$^+$ T cells (0.4×10^5 cells/well) from OTII mice were incubated with serial dilutions of irradiated (4000 rad) DC$_{OVA}$ (0.1×10^5 cells/well) in the absence or presence of different concentrations (1, 5 and 20 μg/ml) of EXO or EXOcont. After incubation for 48 h, CD4$^+$ T-cell proliferation was measured by adding 1 μCi ^{3}H-thymidine (1 mCi/ml; GE Healthcare, Waukesha, WI, USA) to each well. After overnight incubation, ^{3}H-thymidine incorporation was determined by liquid scintillation counting. For the *in vivo* CD8$^+$ T-cell proliferation assay, C57BL/6 mice (eight mice per group) were immunized intravenously (i.v.) with DC$_{OVA}$ (1×10^6 cells/mouse) alone or together with OTII CD4$^+$ T-cell EXO, EXOcont or EXO$_{MF}$ (30 μg/mouse). Six days later, cells from tail blood samples of immunized mice were stained with a PE-H-2K^b/OVAI tetramer and FITC-anti-CD8 Ab according to the manufacturer's protocol and analyzed by flow cytometry.[12]

Cytotoxicity assay

To assess killing activity of DC$_{OVA}$-induced CD8$^+$ CTL, C57BL/6 mice were immunized i.v. with DC$_{OVA}$ (1×10^6 cells/mouse), alone or together with OTII CD4$^+$ T-cell EXO, EXOcont or EXO$_{MF}$ (30 μg/mouse). Spleen cells from C57BL/6 mice pulsed with OVAI peptide were labeled with a high concentration of CFSE (CFSE^{high}, 3.0 μM) and served as OVA-specific target cells, whereas spleen cells pulsed with the irrelevant Mut1 peptide were labeled with a low concentration of CFSE (CFSE^{low}, 0.6 μM) and served as nonspecific target cells. Six days later, the immunized mice were then injected i.v. with a 1 : 1

(CFSE^{high}/CFSE^{low}) mixture of splenocyte targets. Sixteen hours after target cell delivery, spleens of the recipient mice were removed, and the relative proportions of CFSE^{high} and CFSE^{low} target cells in the spleens were analyzed by flow cytometry.[12]

Animal studies

For evaluation of antitumor immunity, C57BL/6 mice (eight mice per group) were vaccinated i.v. with DC_{OVA} (1×10^6 cells/mouse) alone or DC_{OVA} (1×10^6 cells/mouse) together with OTII CD4$^+$ T-cell EXO, EXOcont or EXO_{MF} (30 μg/mouse). Eight days after immunization, mice were challenged with a subcutaneous injection of 0.3×10^6 $BL6\text{-}10_{OVA}$ tumor cells. Tumor growth was monitored daily for up to 60 days; for ethical reasons, all mice with tumors that reached a size of 1.5 cm in diameter were killed.

Statistical analysis

Student's *t* test was used for a comparison of variables from different groups in both *in vitro* and *in vivo* experiments, and a log rank test was used for comparison of animal survival in different groups. Statistical analyses were performed using Prism software (GraphPad Software, Inc., San Diego, CA, USA).[14] *P* values of <0.05 were considered significant.

RESULTS

T-cell EXO express CD4$^+$ T-cell molecules, such as CD4, TCR, LFA-1, CD25 and FasL

In vitro DC_{OVA}-stimulated CD4$^+$ T cells derived from OVA-specific TC-transgenic OTII mice expressed CD4, Vβ5.1, 5.2 TCR and CD25 (Figure 1a), indicating that they were OVA-specific CD4$^+$ T cells. They also expressed the T-cell adhesion molecule, LFA-1 and FasL. EXO released from *in vitro* DC_{OVA}-stimulated OTII CD4$^+$ T cells and ConA-stimulated polyclonal CD4$^+$ T cells derived from wild-type C57BL/6 mice were purified by differential ultracentrifugation and termed EXO and EXOcont, respectively. These EXO were then subjected to analysis by electron microscopy, flow cytometry and western blot. As shown in Figure 1b, EXO had a typical exosomal 'saucer' or round shape with a diameter between 50 and 100 nm. Flow cytometric analysis showed that OTII CD4$^+$ T-cell EXO expressed T-cell markers (CD4, Vβ5.1, 5.2 TCR, LFA-1, CD25 and FasL), but to a much lesser extent than OTII CD4$^+$ T cells (Figure 1a). Except for Vβ5.1, 5.2 TCR expression, EXOcont had a similar phenotype as OTII CD4$^+$ T cell-released EXO (data not shown). We also demonstrated that EXO-associated proteins, including LAMP-1, TCR and LFA-1, were abundant, but no apoptotic marker, including Calnexin,[15] was found in OTII CD4$^+$ T-cell EXO by western blot analysis (Figure 1c), indicating that apoptotic vesicles were absent in the EXO samples. In addition, ConA-stimulated polyclonal C57BL/6 CD4$^+$ T cell-released EXOcont had a similar morphological and biochemical profile as OTII CD4$^+$ T-cell EXO, and EXOcont also contained EXO-associated proteins, such as LAMP-1 and LFA-1, but not Vβ5.1, 5.2 TCR or Calnexin (data not shown).

DCs take up T-cell EXO *via* pMHC II/TCR and CD54/LFA-1 interactions

DCs can take up T-cell EXO. To assess whether T-cell EXO could be taken up by DCs, DC_{OVA} were incubated with CFSE-labeled OTII CD4$^+$ T cell-released EXO (CFSE-EXO or EXO_{CFSE}) for 4 h and then analyzed by flow cytometry and confocal fluorescence microscopy. As

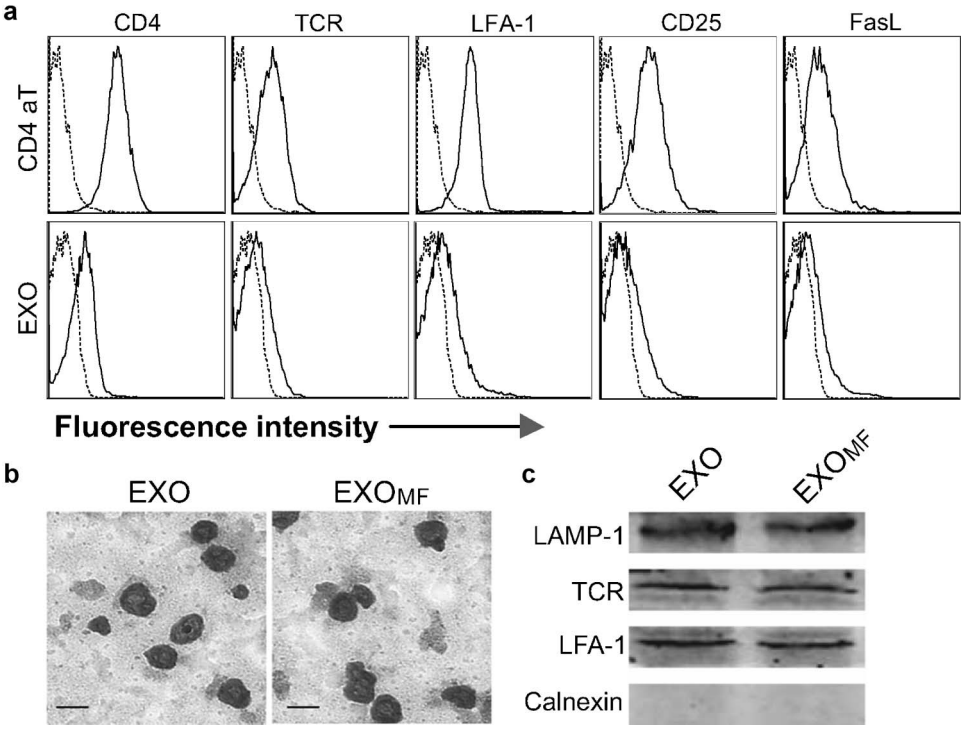

Figure 1 Phenotypic analysis of DC_{OVA}-activated CD4$^+$ T cells and CD4$^+$ T cell- or MF72.2D9 T cell hybridoma-released EXO. (**a**) Flow cytometric analysis. DC_{OVA}-stimulated OTII CD4$^+$ T cells and activated OTII CD4$^+$ T cell-released EXO were stained with a panel of Abs (solid lines) or isotype-matched irrelevant antibodies (dotted lines) and then analyzed by flow cytometry. (**b**) Analysis by electron microscopy of OTII CD4$^+$ T-cell EXO and EXO_{MF}. Representative electron micrographs of EXO are shown. Bar, 100 nm. (**c**) Western blot analysis of OTII CD4$^+$ T-cell EXO and EXO_{MF} using a panel of Abs. One representative experiment of two is shown. Ab, antibody; DC_{OVA}, OVA-pulsed dendritic cell; EXO, exosomes; EXO_{MF}, EXO derived from the T-cell hybridoma cell line, MF72.2D9, expressing an OVA-specific CD4$^+$ TCR.

shown in Figure 2a and b, DC_{OVA} expressed CFSE after incubation with CFSE-EXO, but not with CFSE-EXOcont, indicating that DC_{OVA} took up OTII $CD4^+$ T-cell EXO. To assess the molecular mechanism of uptake of $CD4^+$ T-cell EXO by DC_{OVA}, we added blocking reagents to the above mixture of DC_{OVA} and EXO_{CFSE}, and CFSE-positive DC_{OVA} cells with EXO_{CFSE} uptake were further assessed by confocal fluorescence microscopy. We found that both anti-Ia^b and anti-LFA-1 Abs, but not the control anti-H-$2K^b$ Ab, significantly inhibited the uptake of EXO_{CFSE} by DC_{OVA} ($P<0.05$) (Figure 2c), indicating that both pMHC II/TCR and CD54/LFA-1 interactions mediated DC_{OVA} uptake of $CD4^+$ T-cell EXO.

T-cell EXO inhibit *in vitro* $CD4^+$ T-cell proliferation

Bone marrow-derived DC_{OVA} from C57BL/6 mice expressed CD11c, CD40, CD80 and Ia^b (Figure 3a), indicating that they were mature DCs. In addition, DC_{OVA} expressed CD54, the pMHC I and Fas. To assess the potential effect of T-cell EXO, we performed an *in vitro* DC_{OVA}-stimulated $CD4^+$ T-cell proliferation assay. We found that DC_{OVA} efficiently stimulated OTII $CD4^+$ T-cell proliferation (Figure 3b), whereas OTII $CD4^+$ T-cell EXO, but not polyclonal $CD4^+$ T-cell EXOcont, significantly inhibited DC_{OVA}-mediated $CD4^+$ T-cell proliferation ($P<0.05$) in a dose-dependent manner (Figure 3c). However, OTII $CD4^+$ T-cell EXO did not inhibit *in vitro* CD3/28-mediated T-cell proliferation (data not shown). OTII $CD4^+$ T-cell EXO did not induce Annexin V (an apoptosis marker) expression in naive T cells, whereas irradiation did (Figure 3d), indicating that EXO had no direct ability to kill naive $CD4^+$ T cells, and the reduced T-cell proliferation seen in Figure 3c was therefore not due to the killing activity of EXO.

T-cell EXO suppress *in vivo* OVA-specific $CD8^+$ CTL responses and antitumor immunity

DC_{OVA} also stimulated *in vivo* OVA-specific $CD8^+$ T-cell responses, as detected by FITC-CD8 and PE-tetramer staining in wild-type C57BL/6 mice (Figure 4). However, they failed to stimulate $CD8^+$ T-cell responses in $Ia^{b-/-}$ mice lacking $CD4^+$ T cells, indicating that the $CD8^+$ CTL responses were $CD4^+$ T cell dependent. To assess whether T-cell EXO could suppress *in vivo* DC_{OVA}-activated $CD4^+$ T cell-dependent $CD8^+$ CTL responses, we immunized C57BL/6 mice i.v. with DC_{OVA} alone or DC_{OVA} together with T-cell EXO. As shown in Figure 5a, DC_{OVA} stimulated 1.58% of tetramer-positive $CD8^+$ T cells from the total $CD8^+$ T-cell population in the blood at day 6 after immunization. However, co-immunization of DC_{OVA} with OTII $CD4^+$ T-cell EXO, but not with polyclonal $CD4^+$ T-cell EXOcont, stimulated 0.62% of tetramer-positive $CD8^+$ T cells from the total $CD8^+$ T-cell population ($P<0.05$), indicating that OTII $CD4^+$ T-cell EXO expressing an OVA-specific TCR significantly inhibited DC_{OVA}-induced OVA-specific $CD8^+$ T-cell responses. To assess the effector function of OVA-specific $CD8^+$ T cells, we adoptively transferred OVAI peptide-pulsed splenocytes that had been labeled with $CFSE^{high}$ as well as Mut1 control peptide-pulsed splenocytes that had been labeled with $CFSE^{low}$ (1 : 1) into recipient mice that had been vaccinated with DC_{OVA} or DC_{OVA} plus EXO. We then analyzed loss of OVAI-specific $CFSE^{high}$ target cells in the recipient mice, which represented the killing activity of OVA-specific effector $CD8^+$ CTLs in the recipient mice. As shown in Figure 5b, no $CFSE^{high}$ target cell loss was observed in mice immunized with PBS. As expected, there was a substantial loss (81.3%) of OVA-specific target ($CFSE^{high}$) cells in mice immunized with DC_{OVA}, indicating that DC_{OVA} induced $CD8^+$ T-cell differentiation into OVA-specific CTL effectors *in vivo*. Similar to the

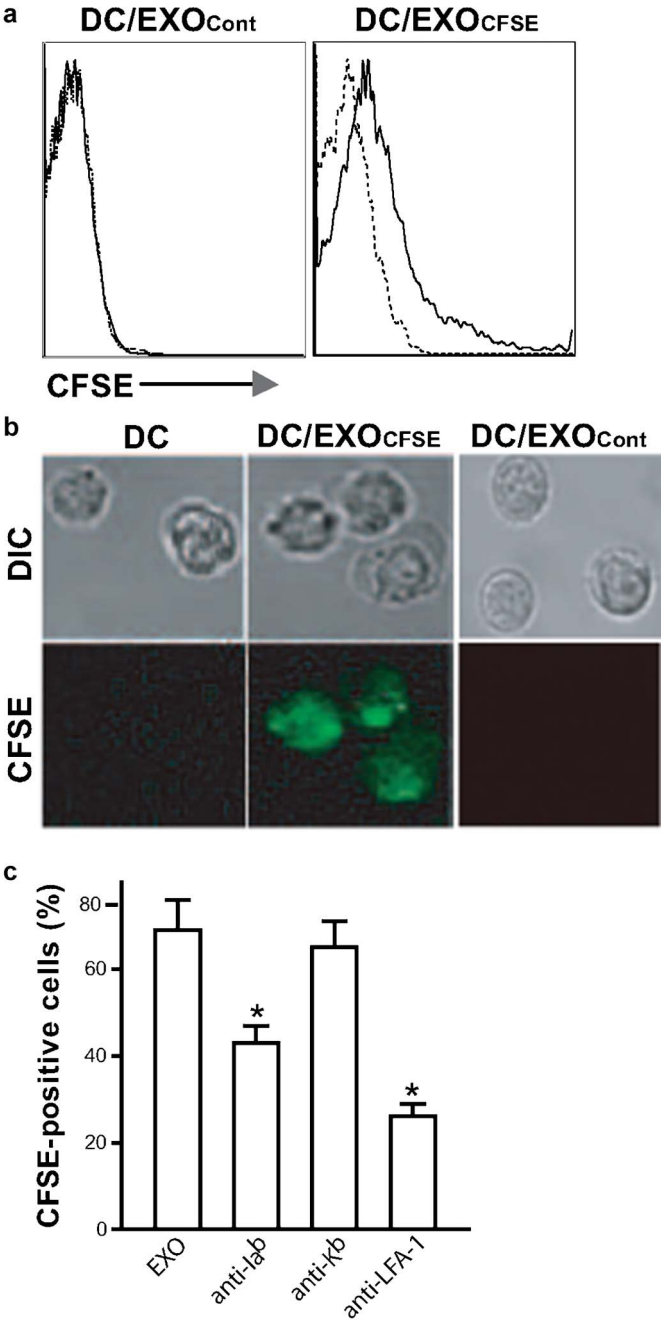

Figure 2 DC_{OVA} take up T-cell EXO *via* pMHC II/TCR and CD54/LFA-1 interactions. (**a**) Analysis of CFSE expression by flow cytometry. DC_{OVA} were cocultured with (solid line) or without (dotted line) CFSE-EXO or CFSE-EXOcont and then analyzed by flow cytometry. (**b**) Analysis of CFSE expression by confocal fluorescence microscopy. DC_{OVA} were cocultured with or without CFSE-EXO or CFSE-EXOcont and then analyzed by confocal fluorescence microscopy. Representative pictures are shown. (**c**) CFSE-positive cells counted by confocal fluorescence microscopy. DC_{OVA} were incubated with CFSE-EXO in the absence or presence of a panel of Abs, including anti-K^b, -Ia^b and -LFA-1. CFSE-positive cells were then detected and counted by confocal fluorescence microscopy. *$P<0.05$ versus cohorts of EXO or EXO plus the anti-H-$2K^b$ Ab (Student's *t* test). One representative experiment of three is shown. Ab, antibody; CFSE, carboxyfluorescein diacetate succinimidyl ester; DC_{OVA}, OVA-pulsed dendritic cell; EXO, exosomes; EXOcont, control EXO; pMHC, peptide/major histocompatibility complex; TCR, T-cell receptor.

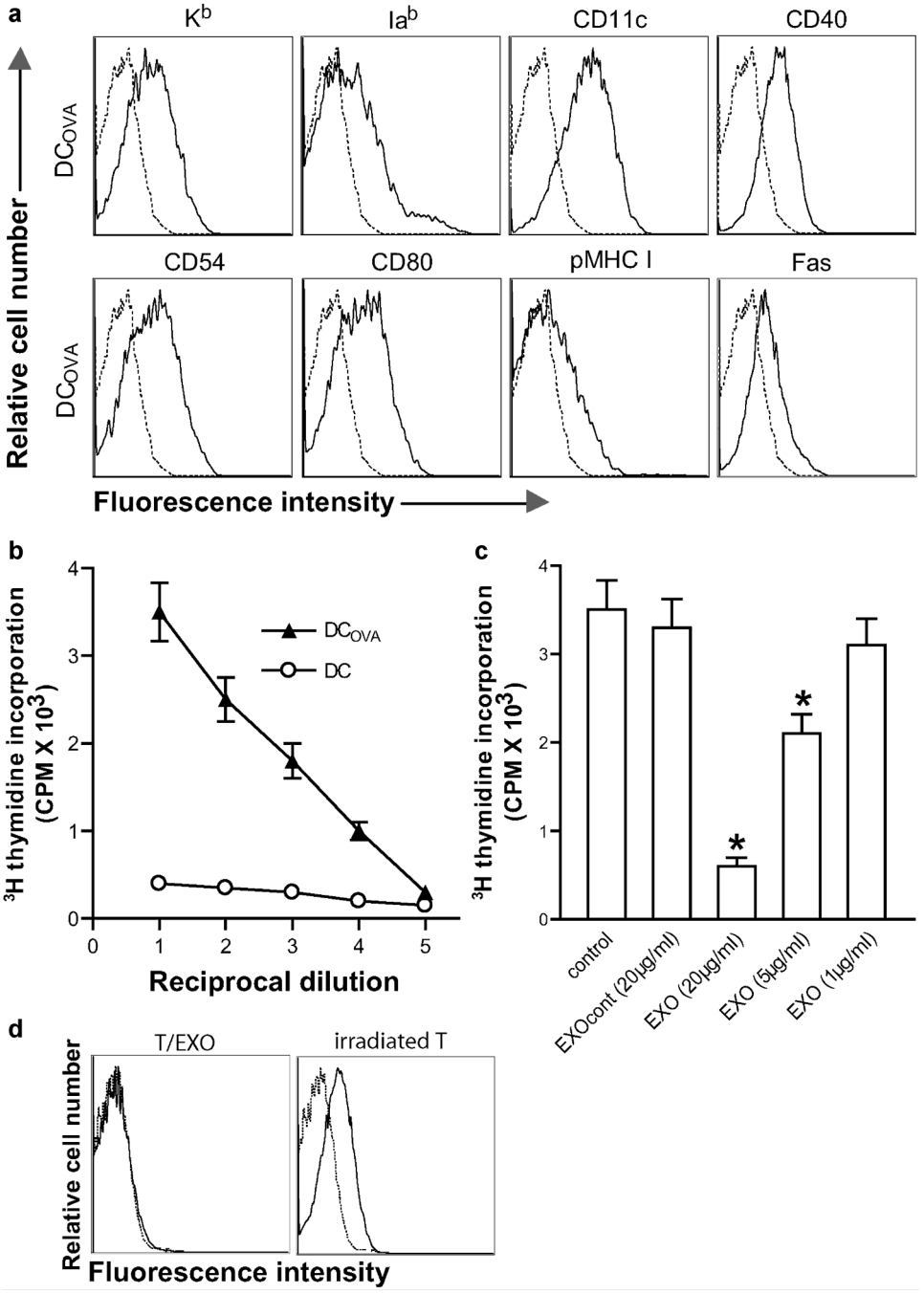

Figure 3 T-cell EXO inhibit *in vitro* DC_{OVA}-stimulated $CD4^+$ T-cell proliferation. (**a**) Phenotypic analysis of DC_{OVA} by flow cytometry. DC_{OVA} cells were stained with a panel of Abs (solid lines) or isotype-matched irrelevant Abs (dotted lines) and then analyzed by flow cytometry. (**b**, **c**) *In vitro* $CD4^+$ T-cell proliferation assay. Naive OTII $CD4^+$ T cells were incubated with irradiated DC_{OVA} in the absence or presence of different concentrations of OTII $CD4^+$ T-cell EXO or EXOcont in a ^{3}H-thymidine uptake assay. *$P<0.05$ versus cohorts of DC_{OVA} or DC_{OVA} plus EXOcont (Student's *t* test). (**d**) Naive OTII $CD4^+$ T cells (2×10^6 cells) were incubated with (solid line) or without (dotted lines) OTII $CD4^+$ T-cell EXO (30 μg/ml) in DMEM plus 10% FCS and IL-2 (20 U/ml), or irradiated (2000 rad) naive OTII $CD4^+$ T cells were cultured in DMEM plus 10% FCS and IL-2 (20 U/ml) for 8 h (solid line). T cells were then harvested and stained with FITC-Annexin V for flow cytometric analysis. One representative experiment of three is shown. Ab, antibody; DC_{OVA}, OVA-pulsed dendritic cell; DMEM, Dulbecco's modified Eagle's medium; EXO, exosomes; EXOcont, control EXO; FCS, fetal calf serum; FITC, fluorescein isothiocyanate.

DC_{OVA}-immunized group, DC_{OVA} plus EXOcont immunization resulted in a substantial loss (78.8%) of OVA-specific $CFSE^{high}$ cells. However, DC_{OVA} plus OTII $CD4^+$ T-cell EXO immunization killed only 52.5% of OVA-specific $CFSE^{high}$ cells ($P<0.05$), indicating that OTII $CD4^+$ T-cell EXO expressing an OVA-specific TCR inhibited DC_{OVA}-stimulated effector $CD8^+$ CTL responses. Generation of effective $CD8^+$ CTL responses requires $CD4^+$ T help,[16,17] suggesting that OTII $CD4^+$ T-cell EXO might also specifically suppress *in vivo* DC_{OVA}-induced $CD4^+$ T-cell activation, leading to inhibition of OVA-specific $CD8^+$ CTL responses. To assess whether OTII $CD4^+$

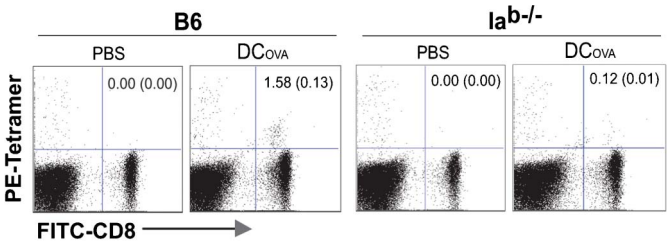

Figure 4 DC_{OVA} stimulate $CD8^+$ CTL responses in a $CD4^+$ T cell-dependent manner. Six days after immunization of C57BL/6 and $Ia^{b-/-}$ mice with DC_{OVA}, cells from tail blood samples of immunized mice were stained with a PE-H-$2K^b$/OVAI tetramer and FITC-anti-CD8 Ab, and DC_{OVA}-induced *in vivo* $CD8^+$ T-cell proliferation was then analyzed by flow cytometry. The value in each panel represents the percentage of tetramer-positive $CD8^+$ T cells versus the total $CD8^+$ T-cell population. The value in parentheses represents the SD. Naive $CD4^+$ T cells or irradiated (4000 rad) naive $CD4^+$ T cells were incubated with or without EXO in the presence of IL-2 (20 U/ml) for 16 h. T cells were then stained with PE-Annexin V (solid lines) or an irrelevant PE-conjugated Ab (dotted lines) and analyzed by flow cytometry. One representative experiment of two is shown. Ab, antibody; CTL, cytotoxic T lymphocyte; DC_{OVA}, OVA-pulsed dendritic cell; FITC, fluorescein isothiocyanate; OVAI, $OVA_{257–264}$; PE, phycoerythrin; SD, standard deviation.

T-cell EXO inhibited antitumor immunity, C57BL/6 mice were immunized i.v. with DC_{OVA} or DC_{OVA} plus EXO. Eight days later, the immunized mice were challenged subcutaneously with BL6-10_{OVA} tumor cells. As shown in Figure 5c, all the mice injected with PBS died from the malignancy within 21 days after tumor cell challenge. The DC_{OVA} vaccine protected 8/8 (100%) mice from tumor growth. However, DC_{OVA} plus OTII $CD4^+$ T-cell EXO, but not DC_{OVA} plus EXOcont, significantly inhibited DC_{OVA}-mediated immune protection against OVA-expressing BL6-10_{OVA} tumors, with 5/8 surviving mice ($P<0.05$), indicating that OTII $CD4^+$ T-cell EXO also inhibited OVA-specific antitumor immunity.

T-cell hybridoma-released EXO similarly inhibit DC-stimulated $CD8^+$ CTL responses and antitumor immunity

EXO derived from the T-cell hybridoma cell line, MF72.2D9, expressing an OVA-specific $CD4^+$ TCR (EXO_{MF}), were purified from culture supernatants by differential ultracentrifugation. The EXO_{MF} were then phenotypically characterized by electron microscopy, western blot and flow cytometry. As shown in Figure 1b, EXO_{MF} had a typical exosomal 'saucer' or round shape with a diameter between 50 and 100 nm. We also confirmed that LAMP-1, Vβ5.1, 5.2 TCR and LFA-1 were expressed in EXO_{MF} samples (Figure 1c). In addition, EXO_{MF} expressed T-cell markers (Vβ5.1, 5.2 TCR, LFA-1 and FasL), but to a much lesser extent than MF72.2D9 hybridoma cells (data not shown). To assess its inhibitory effect, EXO_{MF} released from MF72.2D9 hybridoma cells were further used in *in vivo* experiments as described above. We found that MF72.2D9 T-cell hybridoma-derived EXO_{MF} expressing an OVA-specific $CD4^+$ TCR also inhibited DC_{OVA}-stimulated OVA-specific $CD8^+$ CTL responses (Figure 5a and b) and antitumor immunity against BL6-10_{OVA} tumor cells (Figure 5c). This result indicated that EXO_{MF} had a similar inhibitory effect as OTII $CD4^+$ T-cell EXO on *in vivo* DC_{OVA}-induced OVA-specific $CD8^+$ CTL responses and antitumor immunity.

DISCUSSION

The exchange of plasma membrane components between immune cells is common during cellular encounters, and this activity could play an important role in physiologic, pathologic and therapeutic settings.[18,19] The detailed mechanisms of trogocytosis have yet to be elucidated, but likely require contact between donor and acceptor cells and formation of an immunologic synapse.[20,21] Surface molecule transfer has been predominantly reported for the transfer of DC surface molecules to T cells in a unidirectional manner.[22,23] As a result, T cells with acquired DC molecules have been shown to be either immunogenic[12,24] or tolerogenic[25,26] in their effect on immune responses. In addition, T cells with uptake of DC-released EXO have been used as T-cell vaccines for stimulation of antitumor immunity.[22,27] Recently, we have shown that molecular transfer between DCs and T cells is bidirectional, and DCs can also acquire T-cell molecules *via* a dissociation-associated pathway.[28]

It has recently been reported that transfer of T-cell molecules to DCs occurs when DCs activate $CD4^+$ T cells *via* two distinct mechanisms, including an early antigen-independent mechanism of T-cell activation and a late antigen-specific mechanism of T-cell activation.[29] The direction of plasma membrane exchange between lymphocytes and accessory cells by trogocytosis is influenced by the nature of accessory cells.[30] Another potential mechanism for trogocytosis is *via* exosome fusion to acceptor cells or internalization and recycling of exosomal molecules on the acceptor cells.[31] The exosomal molecules on acceptor cells are quite stable and can last as long as 12 days in culture.[32] Activated T cells can also secrete bioactive EXO,[8–10] which can be taken up by DCs or B cells *via* direct intercellular molecule transfer from T cells to APCs.[11] However, the potential effect of T-cell EXO on the regulation of immune responses is not clear.

In this study, we demonstrated that activated $CD4^+$ T cells secreted EXO-expressing T-cell markers and that DCs took up $CD4^+$ T-cell EXO *via* pMHC II/TCR and CD54/LFA-1 interactions. OVA-specific $CD4^+$ T-cell EXO from OTII transgenic mice, but not ConA-stimulated polyclonal $CD4^+$ T-cell EXO from wild-type C57BL/6 mice, inhibited OVA-specific DC-stimulated *in vitro* $CD4^+$ T-cell proliferation and *in vivo* $CD8^+$ CTL responses and antitumor immunity against OVA-expressing B16 melanoma BL6-10_{OVA} cells. DCs receiving TCR molecules are less efficient at priming T-cell responses, possibly because the transferred TCR molecule mask antigen-bearing MHCs and consequently reduce their accessibility to antigen-specific T cells.[29] It has also been demonstrated that pMHC I expression on DCs can be downregulated after interaction with antigen-specific $CD8^+$ T cells *via* the TCR-mediated internalization pathway.[20,33] In addition, FasL-expressing tumor cell-released EXO can induce T-cell apoptosis *via* the Fas/FasL pathway.[34–36] In our study, OTII $CD4^+$ T-cell EXO bearing an OVA-specific TCR and expressing FasL specifically inhibited DC_{OVA}-mediated $CD4^+$ T-cell stimulation, and this may be associated with the downregulation or masking of the pMHC II on DC_{OVA} by $CD4^+$ T-cell EXO and induction of apoptosis in Fas-expressing DC_{OVA} cells *via* the Fas/FasL pathway. Importantly, we also demonstrated that EXO derived from the T-cell hybridoma cell line, MF72.2D9, expressing an OVA-specific $CD4^+$ TCR had a similar inhibitory effect as OTII $CD4^+$ T-cell EXO on $CD8^+$ CTL-mediated antitumor immunity. Autoimmune diseases and rejection of transplanted organs are mainly mediated by DC-induced cellular CTL responses.[37,38] Antigen-specific T-cell EXO derived from immortal tumor cell lines, engineered to express both antigen-specific TCRs and FasL, could thus be used as a new type of immunosuppressive reagent to inhibit autoimmune diseases and prolong organ transplantation by masking or killing the antigen-bearing DC.

Taken together, our data demonstrate that antigen-specific T cell-secreted EXO inhibit antigen-specific $CD8^+$ CTL responses and thus indicate that antigen-specific T-cell EXO might be used as a new type

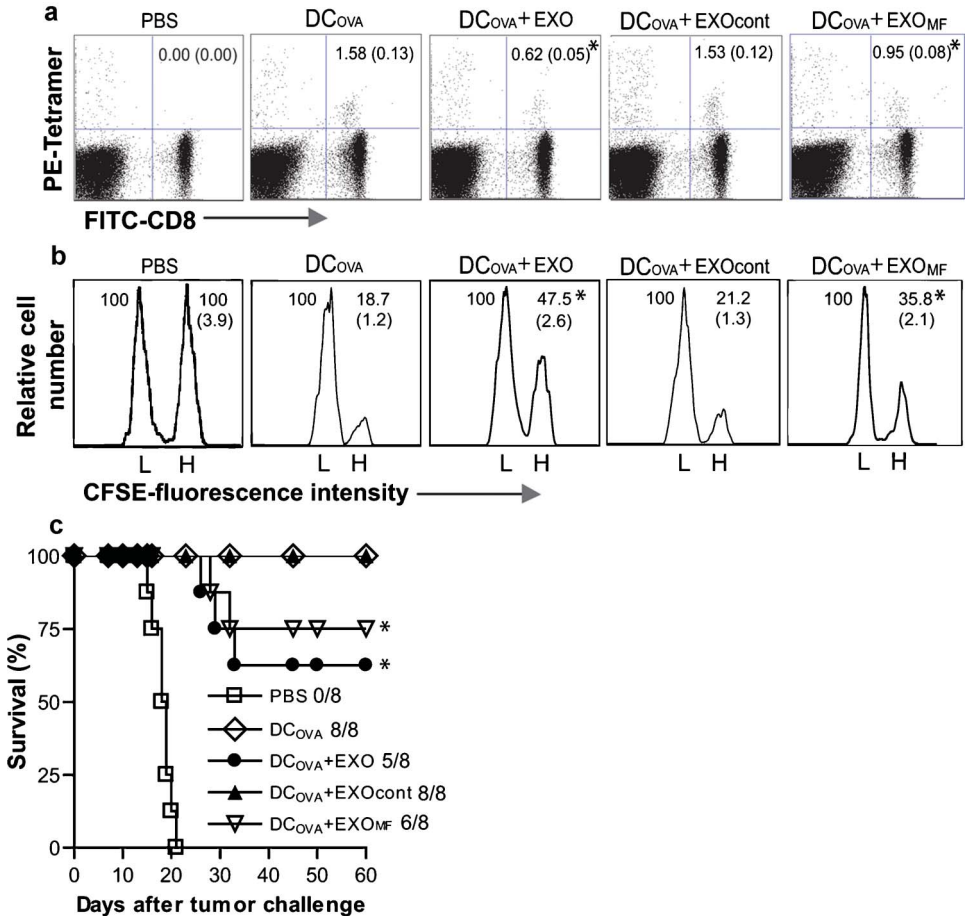

Figure 5 T-cell EXO and EXO_{MF} suppress $CD8^+$ CTL responses and antitumor immunity. (**a**) *In vivo* $CD8^+$ T-cell proliferation assay. Six days after immunization of C57BL/6 mice with DC_{OVA} alone, DC_{OVA} plus OTII $CD4^+$ T-cell EXO, EXOcont or EXO_{MF}, respectively, cells from tail blood samples of immunized mice were stained with a PE-H-$2K^b$/OVAI tetramer and FITC-anti-CD8 Ab and then analyzed by flow cytometry. The value in each panel represents the percentage of tetramer-positive $CD8^+$ T cells versus the total $CD8^+$ T-cell population. The value in parentheses represents the SD. (**b**) *In vivo* cytotoxicity assay. The spleens of immunized mice after injection of a 1 : 1 mixture of $CFSE^{high}$- and $CFSE^{low}$-labeled splenocytes that had been pulsed with OVAI or Mut1 peptides, respectively, were removed and the percentages of the residual $CFSE^{high}$ (H) and $CFSE^{low}$ (L) target cells were analyzed by flow cytometry. The value in each panel represents the percentage of $CFSE^{high}$ versus $CFSE^{low}$ target cells in the spleen. The value in parentheses represents the SD. *$P<0.05$ versus cohorts of DC_{OVA} or DC_{OVA} plus EXOcont (Student's *t* test). (**c**) Animal studies. C57BL/6 mice were vaccinated i.v. with DC_{OVA} alone, DC_{OVA} plus OTII $CD4^+$ T-cell EXO, EXOcont or EXO_{MF}, respectively. Eight days later, the immunized mice were inoculated s.c. with BL6-10_{OVA} tumor cells. Animal mortality and tumor growth were monitored daily for up to 60 days. *$P<0.05$ versus cohorts of DC_{OVA} or DC_{OVA} plus EXOcont (log rank test). One representative experiment of two is shown. Ab, antibody; $CFSE^{high}$, high concentration of CFSE; $CFSE^{low}$, low concentration of CFSE; CTL, cytotoxic T lymphocyte; DC_{OVA}, OVA-pulsed dendritic cell; EXO, exosomes; EXOcont, control EXO; EXO_{MF}, EXO derived from the T-cell hybridoma cell line, MF72.2D9, expressing an OVA-specific $CD4^+$ TCR; FITC, fluorescein isothiocyanate; OVAI, $OVA_{257–264}$; PE, phycoerythrin; s.c., subcutaneously; SD, standard deviation.

of immunosuppressive reagent for treatment of autoimmune diseases and use in transplant rejection.

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