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RESEARCH ARTICLE

A novel T cell-based vaccine capable of stimulating long-term functional CTL memory against B16 melanoma *via* CD40L signaling

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The ultimate goal of antitumor vaccines is to develop memory CD8$^+$ cytotoxic T lymphocytes (CTLs), which are critical mediators of antitumor immunity. We previously demonstrated that the ovalbumin (OVA)-specific CD4$^+$ T cell-based (OVA-T$_{EXO}$) vaccine generated using OVA-pulsed dendritic cell (DC$_{OVA}$)-released exosomes (EXO$_{OVA}$) stimulate CTL responses *via* IL-2 and costimulatory CD80 signaling. To assess the potential involvement of other costimulatory pathways and to define the key constituent of costimulation for memory CTL development, we first immunized wild-type (WT) C57BL/6 and gene-knockout mice with WT CD4$^+$ OVA-T$_{EXO}$ cells or OVA-T$_{EXO}$ cells with various molecular deficiencies. We then assessed OVA-specific primary and recall CTL responses using PE-H-2K^b/OVA$_{257-264}$ tetramer and FITC-anti-CD8 antibody staining by flow cytometry. We also examined antitumor immunity against the OVA-expressing B16 melanoma cell line BL6-10$_{OVA}$. We demonstrated that CD4$^+$ OVA-T$_{EXO}$ cells stimulated more efficient CTL responses compared to DC$_{OVA}$. By assessing primary and recall CTL responses in mice immunized with OVA-T$_{EXO}$ or with OVA-T$_{EXO}$ lacking the costimulatory molecules CD40L, 4-1BBL or OX40L, we demonstrated that these costimulatory signals are dispensable for CTL priming by OVA-T$_{EXO}$. Interestingly, CD40L, but not 4-1BBL or OX40L, plays a crucial role in the development of functional memory CTLs against BL6-10$_{OVA}$ tumors. Overall, this work suggests that a novel CD4$^+$ T cell-based vaccine that is capable of stimulating long-term functional CTL memory *via* CD40L signaling may represent a novel, efficient approach to antitumor vaccination.
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INTRODUCTION

Vaccines that are capable of stimulating CD8$^+$ cytotoxic T lymphocyte (CTL) responses and memory have been extensively studied in an effort to control pathogen-induced diseases and tumor development. Stimulation of T-cell responses by antigen-presenting cells (APCs) involves at least two signaling events: one elicited by T-cell receptors (TCRs) recognizing peptide-major histocompatibility complexes (pMHC) and another triggered by costimulatory molecules (e.g., CD40, CD80, 4-1BBL and OX40L)[1] associated with the immunological synapse between the APC and the CD8$^+$ T cell,[2] a multiprotein signaling complex responsible for controlling T-cell activation.[1,3] APCs also provide an additional polarization signal (signal 3), such as IL-12,[4,5] which selectively drives the development of type I or type II immunity, each characterized by a distinct microenvironment promoting a particular set of effector mechanisms.[4,6] Following the recognition of foreign antigen (Ag), CD8$^+$ T cells undergo three distinct developmental phases: (i) a proliferation (primary) phase, in which naive CD8$^+$ T cells undergo autonomous clonal expansion and develop into effector CTLs; (ii) a contraction phase, in which approximately 95% of the effector CTLs undergo activation-induced cell death through apoptosis, allowing the remaining approximately 5%–10% of the CTLs to develop into memory CTLs; and (iii) a maintenance phase, in which memory CTLs survive for a prolonged duration. Unlike their naive counterparts, memory CTLs display enhanced responses to subsequent Ag encounters, undergoing rapid proliferation and having heightened effector functions during recall responses.

Dendritic cells (DCs) represent the most effective type of APC. DCs process exogenous Ags in their endosomal compartments, such as multivesicular endosomes,[7] which can later fuse with the plasma membrane, releasing Ag-presenting vesicles called 'exosomes'.[8,9] Exosomes (EXOs) are 50–90 nm diameter vesicles containing Ag-presenting molecules, including MHC class I, MHC class II, CD1 and hsp70–90, tetraspan molecules (CD9, CD63 and CD81), adhesion molecules (CD11b and CD54) and costimulatory molecules (CD80 and CD86).[10,11] Together, these molecules comprise the molecular machinery required for generating potent immune responses. Recently, we have shown that Con A-stimulated CD4$^+$ T cells derived from ovalbumin (OVA)-specific TCR transgenic OTII mice can internalize OVA-pulsed, DC-released EXO (EXO$_{OVA}$) *via* TCR/MHC and LFA-1/CD54 interactions. EXO$_{OVA}$-loaded CD4$^+$ T cells

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can be used as OVA-T_{EXO}-cell vaccines capable of stimulating $CD4^+$ T cell-independent $CD8^+$ T-cell priming *via* pMHC-I-mediated targeting and *via* IL-2 and CD80 costimulatory signaling.[12] Additional costimulations *via* 4-1BBL/4-1BB, OX40L/OX40 and CD40/CD40L interactions between DCs and $CD8^+$ T cells have also been found to promote effector $CD8^+$ T-cell responses during the priming phase[13,14] and $CD8^+$ Tm-cell development in the contraction phase.[15–18] However, whether these costimulatory events are also involved in priming and memory development in OVA-T_{EXO}-stimulated $CD8^+$ T cells is currently not well understood.

In this study, we assessed the potential role of costimulatory molecules, including CD40L, 4-1BBL and OX40L, in OVA-T_{EXO} vaccine-induced $CD8^+$ CTL responses and memory development against OVA-expressing B16 melanoma in wild-type (WT) C57BL/6 mice.

MATERIALS AND METHODS

Reagents, cell lines and animals

The biotin-labeled or fluorescein isothiocyanate (FITC)-labeled antibodies (Abs) specific for CD11c (HL3), CD40L (MR1), CD80 (16-10A1), 4-1BBL (TKS-1), OX40L (RM134L) or H-$2K^b$/$OVA_{257–264}$ (OVAI) (pMHC-I) (25D.1) were obtained from BD Pharmingen Canada Inc. (Missisauga, Ont., Canada) and Biolegend (San Diego, CA, USA). The depleting anti-CD20 (AISB12) and anti-plasmacytoid DC (120G8) Abs were obtained from eBioscience (San Diego, CA, USA). PE-labeled H-$2K^b$/$OVA_{257–264}$[12] tetramer was obtained from Beckman Coulter (Mississauga, Ont., Canada). Female WT C57BL/6 (B6), transgenic OTII and various gene knockout (KO) mice (except for 4-$1BBL^{-/-}$ mice, obtained from Amgen, Seattle, WA, USA) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Homozygous OTII/H-$2K^{b-/-}$, OTII/$CD40L^{-/-}$, OTII/4-$1BBL^{-/-}$ and OTII/$OX40L^{-/-}$ mice were generated by backcrossing the designated gene KO mice onto the OTII background. All mice used in the experiments were 6–8 weeks old and were treated according to the animal care committee guidelines of the University of Saskatchewan.

DC and EXO preparation

The generation of bone marrow-derived mature OVA-pulsed DCs (DC_{OVA}) (from WT C57BL/6 mice) in the presence of GM-CSF/IL-4 (20 ng/ml) has been described previously.[11] DC_{OVA} derived from 4-$1BBL^{-/-}$ and $OX40L^{-/-}$ mice were termed (4-$1BBL^{-/-}$)DC_{OVA} and ($OX40L^{-/-}$)DC_{OVA}, respectively. EXOs derived from the culture supernatants of DC_{OVA}, (4-$1BBL^{-/-}$)DC_{OVA} and ($OX40L^{-/-}$)DC_{OVA} were termed EXO_{OVA}, (4-$1BBL^{-/-}$)EXO_{OVA} and ($OX40L^{-/-}$)EXO_{OVA}, respectively.

$CD4^+$ T_{EXO} preparation

To generate active $CD4^+$ T cells, spleen cells from OTII 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 cells (ConA-T) were then purified using MACS anti-CD4 microbeads, as previously described.[19] ConA-T cells derived from OTII/$CD40L^{-/-}$, OTII/4-$1BBL^{-/-}$ and OTII/$OX40L^{-/-}$ mice were termed ($CD40L^{-/-}$)T, (4-$1BBL^{-/-}$)T and ($OX40L^{-/-}$)T cells. ConA-T cells were incubated with EXO_{OVA} (10 μg/1×10^6 T cells) at 37 °C for 3 h and were termed OVA-specific T_{EXO} (OVA-T_{EXO}).[20] ($CD40L^{-/-}$)T cells that had internalized EXO_{OVA} were termed ($CD40L^{-/-}$)T_{EXO}, and (4-$1BBL^{-/-}$)T and ($OX40L^{-/-}$)T cells that had internalized (4-$1BBL^{-/-}$)EXO_{OVA} and ($OX40L^{-/-}$)EXO_{OVA} were termed (4-$1BBL^{-/-}$)T_{EXO} and ($OX40L^{-/-}$)T_{EXO}, respectively.

Assessment of CTL responses by PE-tetramer staining

C57BL/6 or various gene KO mice (6/group) were injected intravenously (*i.v.*) with DC_{OVA}, WT OVA-T_{EXO} or OVA-T_{EXO} with a specific molecular deficiency (3×10^6 cells/mouse). To assess recall responses, immunized mice were boosted *i.v.* with DC_{OVA} (0.5×10^6 cells/mouse) on day 30 after the primary immunization. Six or four days after the primary immunization or the boost, peripheral blood samples from the mice were incubated with PE-H-$2K^b$/$OVA_{257–264}$ and FITC-anti-CD8 Ab for 30 min at room temperature, and then analyzed by flow cytometry.

In vivo cytotoxicity assay

C57BL/6 mouse splenocytes were labeled with either high (3.0 μM, $CFSE^{high}$) or low (0.6 μM, $CFSE^{low}$) concentrations of CFSE. $CFSE^{high}$ and $CFSE^{low}$ cells were then pulsed with OVAI ($OVA_{257–264}$, SIINFEKL) and irrelevant Mut1 (FEQNTAQP) peptides, which served as internal OVA-specific target cells and internal control target cells, respectively. These peptide-pulsed target cells were co-injected *i.v.* at a 1 : 1 ratio into mice immunized with either OVA-T_{EXO} or OVA-T_{EXO} harboring a specific molecular deficiency (3×10^6 cells/mouse) 6 days after the immunization. Sixteen hours post-injection, the residual antigen-specific $CFSE^{high}$ and control $CFSE^{low}$ target cells remaining in the recipients' spleens were analyzed by flow cytometry.[19]

Animal studies

To examine antitumor protective immunity, WT C57BL/6 or $CD40^{-/-}$ mice (6/group) were injected *i.v.* with WT OVA-T_{EXO} or OVA-T_{EXO} with a specific molecular deficiency (3×10^6 cells/mouse). Thirty days after the immunization, mice were challenged *i.v.* with

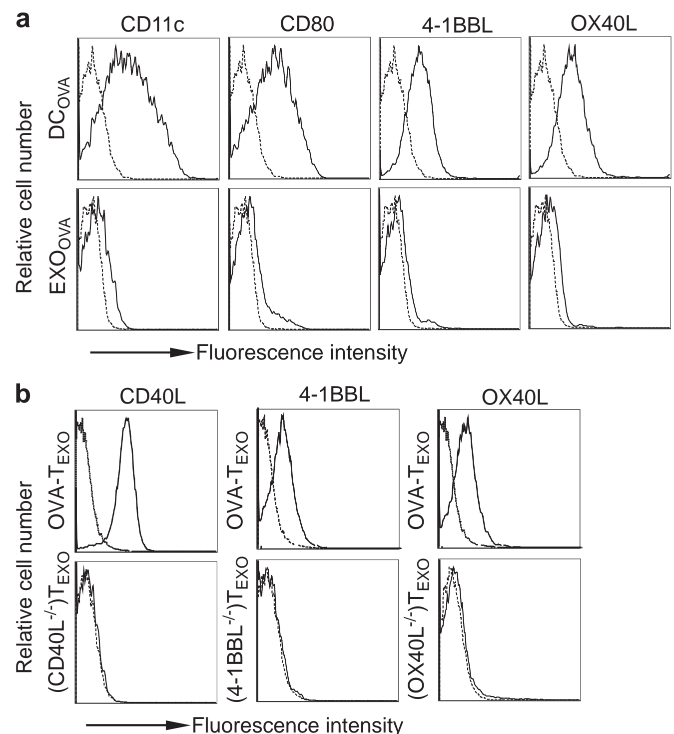

Figure 1 Flow cytometric analysis. (**a**) DC_{OVA} and EXO_{OVA} and (**b**) OVA-T_{EXO} or OVA-T_{EXO} with indicated molecular deficiencies were stained with a panel of specific Abs (solid lines) or isotype-matched irrelevant Abs (dotted lines) and analyzed by flow cytometry. One representative experiment of two is shown. Ab, antibody; DC, dendritic cell; EXO, exosome; OVA, ovalbumin.

0.5×10^{6} OVA-expressing B16 melanoma BL6-10$_{OVA}$ cells.[19] The mice were euthanized 3 weeks following tumor cell challenge, and the lung metastatic tumor colonies were counted in a blind fashion. Metastases on freshly isolated lungs appeared as discrete, black-pigmented foci in BL6-10$_{OVA}$ tumors. Metastatic foci that were too numerous to count were assigned an arbitrary value of >300.[19]

Statistical analysis

Statistical analyses were performed using the Student's *t*-test or the Mann–Whitney *U* test[20] for comparison of variables from different groups. A value of $P<0.05$ or <0.01 was considered to be statistically significant or very significant.

RESULTS AND DISCUSSION

CD4^{+} OVA-T$_{EXO}$ cells stimulate efficient CD8^{+} T-cell responses

OVA-pulsed DCs (DC$_{OVA}$ expressed CD11c (a marker of DCs), the costimulatory molecules CD80, 4-1BBL, and OX40L and pMHC-I complexes, whereas DC$_{OVA}$-released EXO (EXO$_{OVA}$) displayed these molecules to a much lesser extent (Figure 1a). OVA-T$_{EXO}$ cells derived from ConA-stimulated OTII CD4^{+} T lymphocytes (ConA-T) that had internalized EXO$_{OVA}$ expressed 4-1BBL, OX40L and CD40L (Figure 1b) and secreted IFN-γ (approximately 2.6 ng/ml/10^{6} cells/24 h) and IL-2 (approximately 2.1 ng/ml/10^{6} cells/24 h) but did not secrete IL-4 or IL-10. To examine the stimulatory effect of WT OVA-T$_{EXO}$, we immunized C57BL/6 mice with OVA-T$_{EXO}$ cells or DC$_{OVA}$ as a control. The efficiency of the CD8^{+} T-cell responses were then assessed using FITC-anti-CD8 Ab and PE-H-2K^{b}/OVA$_{257–264}$ tetramer staining, followed by flow cytometry on day 6 post-immunization. We found that OVA-T$_{EXO}$ immunization induced an OVA-specific CD8^{+} T-cell response (2.30% of the total CD8^{+} T-cell population) *in vivo* that was more efficient than the DC$_{OVA}$-stimulated CTL response (1.95% of the total CD8^{+} T-cell population) (Figure 2a).

CD40L, 4-1BBL and OX40L signaling are dispensable for priming effector CD8^{+} CTL responses

We previously demonstrated that CD80 costimulation plays an important role in OVA-T$_{EXO}$-initiated priming of CD8^{+} T-cell responses.[12] To assess the potential role of other costimulatory molecules, such as CD40L, 4-1BBL and OX40L, we immunized C57BL/6 mice with (CD40L$^{-/-}$)T$_{EXO}$, (4-1BBL$^{-/-}$)T$_{EXO}$ or (OX40L$^{-/-}$)T$_{EXO}$. We found that (CD40L$^{-/-}$)T$_{EXO}$-, (4-1BBL$^{-/-}$)T$_{EXO}$- and (OX40L$^{-/-}$)T$_{EXO}$-stimulated OVA-specific CD8^{+} T-cell responses (6 days post-immunization) were similar to responses observed in mice immunized with WT OVA-T$_{EXO}$ cells (2.30%) of total CD8^{+} T cells) ($P>0.05$) (Figure 2a). This finding indicates that CD40L, 4-1BBL and OX40L signaling are not involved in OVA-T$_{EXO}$-induced

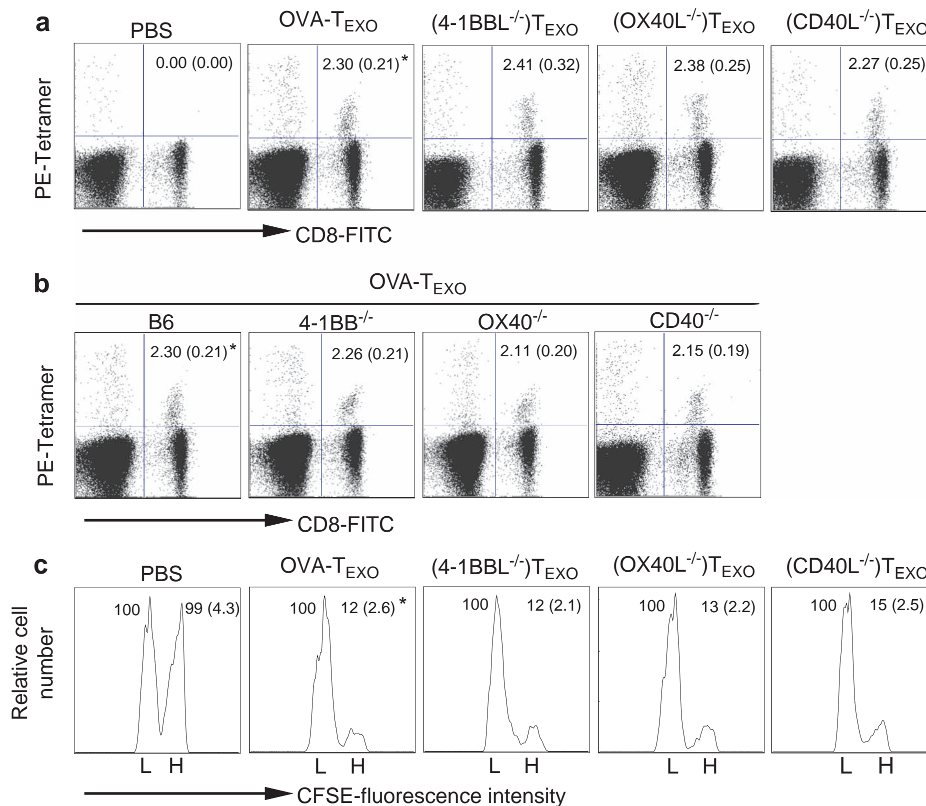

Figure 2 CD40L signaling by OVA-T$_{EXO}$ is not involved in effector CD8^{+} T-cell priming. (**a**) C57BL/6 or (**b**) gene KO mice were immunized with DC$_{OVA}$, WT OVA-T$_{EXO}$ or OVA-T$_{EXO}$ with indicated molecular deficiencies. Six days after the immunization, tail-blood samples were stained with PE-H-2K^{b}/OVAI peptide tetramer and FITC-anti-CD8 Ab and analyzed by flow cytometry. Values represent the mean percentage of OVA-specific CD8^{+} CTLs, with standard deviations in parentheses. *$P>0.05$ *vs.* cohorts of other groups (Student's *t*-test). (**c**) For *in vivo* cytotoxicity assays, OVA-specific CFSE^{high} and irrelevant-peptide CFSE^{low} splenocytes were injected *i.v.* into WT or gene KO OVA-T$_{EXO}$-immunized C57BL/6 mice 6 days after immunization. Sixteen hours later, the percentages of residual CFSE^{high} (H) and CFSE^{low} (L) target cells remaining in the recipients' spleens were analyzed by flow cytometry. Values represent the mean percentage of CFSE^{high} *vs.* CFSE^{low} target cells remaining in the spleen. One representative experiment of two is shown. Ab, antibody; CTL, cytotoxic T lymphocyte; EXO, exosome; *i.v.*, intravenously; KO, knockout; OVA, ovalbumin; WT, wild-type.

CD8$^+$ T-cell priming. This result was further confirmed by analyzing CTL priming in WT B6 and gene KO mice that had been immunized with WT CD4$^+$ OVA-T_{EXO} cells. Consistent with our earlier findings, there was also no significant difference between the WT B6 and gene KO groups ($P>0.05$) (Figure 2b). To assess the effector function of primed CD8$^+$ T cells, we next performed an *in vivo* cytotoxicity assay. In this assay, OVAI peptide-pulsed/CFSE^{high} and irrelevant Mut1 peptide-pulsed/CFSE^{low} C57BL/6 mouse splenocytes (which served as OVA-specific and control target cells, respectively) were co-injected *i.v.* at a 1 : 1 ratio into C57BL/6 mice that had been immunized with WT OVA-T_{EXO} or OVA-T_{EXO} carrying various molecular deficiencies six days after the immunization. Sixteen hours post-injection, the residual OVA-specific CFSE^{high} and control CFSE^{low} target cells remaining in the recipients' spleens were analyzed by flow cytometry. We found that 88% of the OVA-specific CFSE^{high} target cells, but none of the irrelevant control Mut1 peptide-pulsed (CFSE^{low}) target cells, were killed in the 16 h following the transfer of the target cells into WT OVA-T_{EXO}-immunized mice (Figure 2c). This result indicates that OVA-T_{EXO} can efficiently stimulate the differentiation of CD8$^+$ T-cells into CTL effectors. Similar to WT OVA-T_{EXO}, (CD40L$^{-/-}$)T_{EXO}, (4-1BBL$^{-/-}$)T_{EXO} and (OX40L$^{-/-}$)T_{EXO} cells also actively stimulated the differentiation of CD8$^+$ T cells into CTL effectors ($P>0.05$) (Figure 2c), suggesting that CD40L, 4-1BBL and OX40L signaling by CD4$^+$ OVA-T_{EXO} cells is dispensable for priming effector CD8$^+$ CTL responses.

CD40L-, but not 4-1BBL- or OX40L-induced signaling, is required for functional CTL memory development

To assess whether CD8$^+$ memory CTLs are functional, the mice were further boosted with DC_{OVA} 30 days after the immunization, at a timepoint when the OVA-T_{EXO}-stimulated effector CD8$^+$ CTLs would have developed into memory T cells. We observed a 20-fold increase in boosted CD8$^+$ T cells 4 days after the boost of DC_{OVA} in mice previously immunized with OVA-specific OVA-T_{EXO},

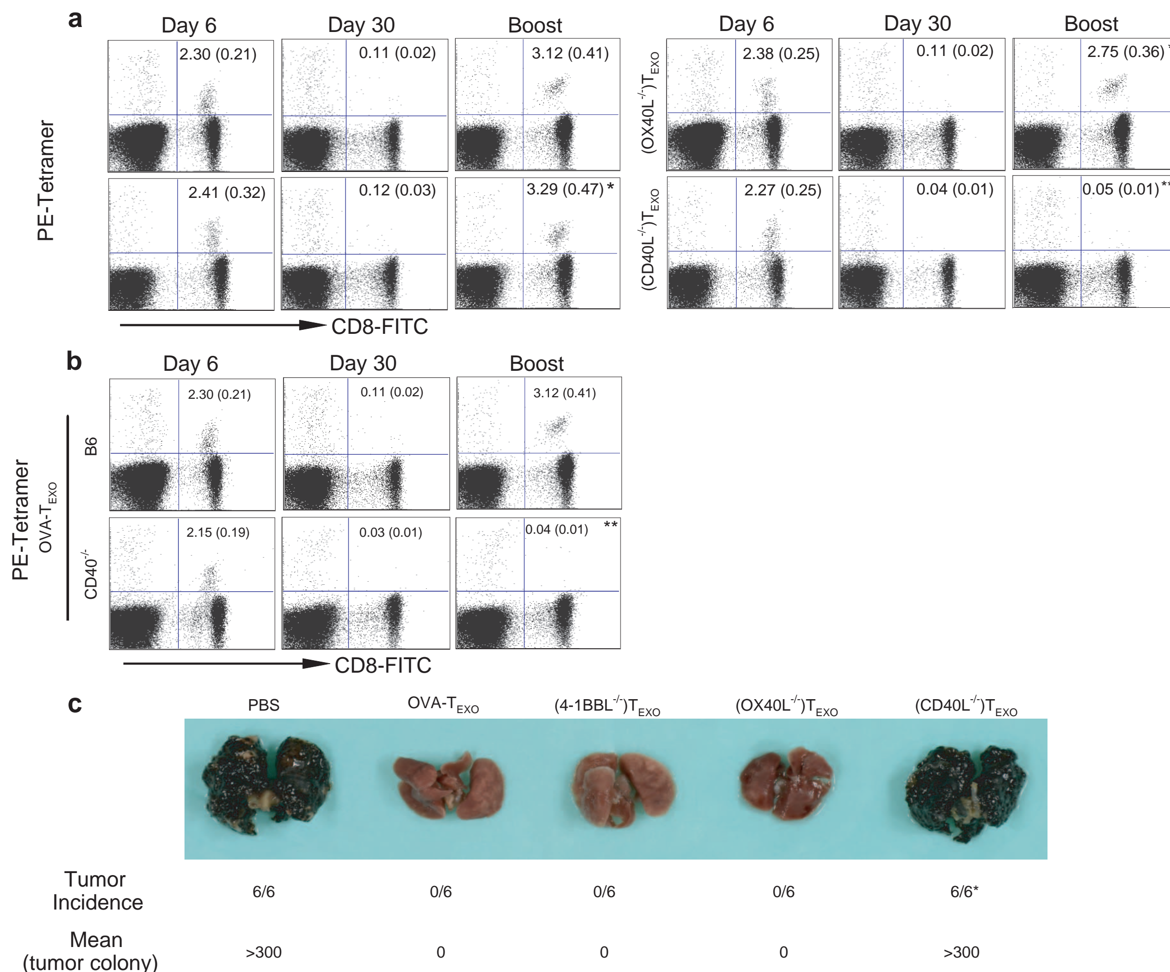

Figure 3 CD40L signaling by OVA-T_{EXO} is involved in CD8$^+$ T-cell memory development. (**a**) Thirty days after immunization with WT OVA-T_{EXO} or OVA-T_{EXO} harboring indicated molecular deficiencies, the tail-blood samples of immunized C57BL/6 mice were analyzed for OVA-specific memory CTL responses by flow cytometry. (**b**) Thirty days after the immunization of C57BL/6 or CD40$^{-/-}$ mice with WT OVA-T_{EXO}, the tail-blood samples of immunized mice were analyzed for OVA-specific memory CTL responses by flow cytometry. All of the above immunized mice were further boosted with DC_{OVA}. Four days after the boost, the tail-blood samples of immunized mice were analyzed for OVA-specific memory CTL recall responses by flow cytometry. Values represent the mean percentage of OVA-specific CD8$^+$ CTLs, with standard deviations in parentheses. **P*>0.05 and ***P*<0.05 *vs.* cohorts of the OVA-T_{EXO} group (**a**) or C57BL/6 mice (**b**) (Student's *t*-test). (**c**) The immunized mice were challenged with BL6-10_{OVA} tumor cells at day 30 following the primary immunization, and mice were euthanized 3 weeks subsequent to challenge. Lungs with black tumor nodules are shown. **P*<0.01 *vs.* cohorts of the OVA-T_{EXO} group (Mann–Whitney *U* test). One representative experiment of two is shown. CTL, cytotoxic T lymphocyte; DC, dendritic cell; EXO, exosome; OVA, ovalbumin; WT, wild-type.

$(OX40L^{-/-})T_{EXO}$ and $(4\text{-}1BBL^{-/-})T_{EXO}$ cells ($P>0.05$) (Figure 3a). Interestingly, there was no increase in boosted $CD8^+$ T cells in mice previously immunized with $(CD40L^{-/-})T_{EXO}$ ($P<0.05$) (Figure 3a), suggesting that CD40L-, but not 4-1BBL- or OX40L-signaling by $CD4^+$ T_{EXO} cells is required for functional $CD8^+$ T-cell memory development. This observation was further confirmed by our finding that $CD40^{-/-}$ CTLs also had defects in their recall responses in $CD40^{-/-}$ mice that had previously been primed by WT OVA-T_{EXO} (Figure 3b). To assess the antitumor immunity in these mice, we further challenged the immunized mice with the OVA-expressing B16 melanoma cell line BL6-10_{OVA} 30 days after the primary immunization. We found that all OVA-T_{EXO}-immunized mice were significantly more protected against BL6-10_{OVA} tumor challenge compared to $(CD40L^{-/-})T_{EXO}$-immunized mice ($P<0.01$) (Figure 3c), confirming the loss of functional CTL memory derived from $(CD40L^{-/-})T_{EXO}$-immunization.

CD40L signaling by $CD4^+$ T cells has been found to be important in licensing DCs to induce CTL responses *via* interactions between CD40 on DCs and CD40L on $CD4^+$ T cells.[21] In addition, Munroe *et al.*[22] demonstrated a functional costimulation of T cells through CD40 *via* the induction of kinases and transcription factors. Furthermore, Bourgeois *et al.*[23] reported that CD40 deficiency has a major impact on CTL memory responses, suggesting that help provided by $CD4^+$ T cells during $CD8^+$ T-cell responses may involve direct cell-cell interactions between $CD4^+$ and $CD8^+$ T cells in DC-$CD4^+$–$CD8^+$ T-cell clusters. However, these reports did not provide any direct *in vivo* evidence for the role of CD40L expressed on $CD4^+$ T cells in CTL memory development. In this study, we demonstrate that although CD40L signaling by $CD4^+$ OVA-T_{EXO} cells is dispensable for $CD8^+$ T-cell priming, this signaling event plays an important role in functional $CD8^+$ T-cell memory development. The loss of the functional recall responses and antitumor immunity in C57BL/6 mice 30 days after the primary immunization with $CD4^+$ $(CD40L^{-/-})T_{EXO}$ cells clearly indicates the critical role of CD40L signaling by $CD4^+$ OVA-T_{EXO} cells in long-term functional CTL memory development.

Dendritic cells (DCs are the most effective APCs and have been extensively used in tumor vaccines in clinical trials.[24] Unfortunately, these attempts have demonstrated only limited efficacy, mainly due to the silencing effect imposed on DCs by the tumor-tolerogenic microenvironment.[25] In addition, similar to many other types of vaccines, such as DNA, tumor lysates, tumor antigens or peptides, DC vaccines rely on $CD4^+$ T cell-dependent CTL responses and antitumor immunity. In clinical practice, a wealth of data indicate that antitumor immunity directed against a wide variety of malignancies is suppressed in cancer patients.[26] Suppressive $CD4^+$ T cells, including Tr1, Th2 and natural $CD4^+25^+$ Tr cells, play a critical role in the mediation of the suppression.[27,28] In cancer patients, enrichment of $CD4^+25^+$ Tr cell populations has been observed both in the circulation and in the tumor microenvironment.[29,30]

We previously showed that (i) the novel EXO-targeted OVA-T_{EXO} cells were able to directly stimulate CTL responses in the absence of host $CD4^+$ T-cell help and trigger more efficient CTL responses compared to DC_{OVA}; (ii) pMHC-I, CD80 signaling and IL-2 signaling play important roles in $CD4^+$ OVA-T_{EXO}-stimulated CTL responses; and (iii) OVA-T_{EXO} cells triggered OVA-specific CTL responses in C57BL/6 mice, even following the transfer of $CD4^+25^+$ Tr cells, by counteracting $CD4^+25^+$ Tr tolerance.[12] In this study, we further demonstrate that $CD4^+$ T cell-based OVA-T_{EXO} vaccination stimulates functional long-term CTL memory *via* CD40L signaling, indicating that (i) the three signals required for OVA-T_{EXO} to induce a T-cell response (pMHCI/TCR, costimulatory signal such as CD80/CD28 and CD40L/CD40, and IL-2) are distinct from the three signals required for DCs (pMHCI/TCR, CD80/CD28 and IL-12);[4,5] and (ii) CD40L signaling plays an important role in OVA-T_{EXO}-stimulated functional memory CTL development.

Overall, this work suggests that a novel $CD4^+$ T cell-based vaccine capable of stimulating long-term functional CTL memory *via* CD40L signaling may represent a novel and efficient approach to antitumor vaccination.

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