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RESEARCH ARTICLE

Novel exosome-targeted T-cell-based vaccine counteracts T-cell anergy and converts CTL exhaustion in chronic infection via CD40L signaling through the mTORC1 pathway

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CD8$^+$ cytotoxic T lymphocyte (CTL) exhaustion is a chief issue for ineffective virus elimination in chronic infectious diseases. We generated novel ovalbumin (OVA)-specific OVA-Texo and HIV-specific Gag-Texo vaccines inducing therapeutic immunity. To assess their therapeutic effect in chronic infection, we developed a new chronic infection model by i.v. infecting C57BL/6 mice with the OVA-expressing adenovirus AdVova. During chronic AdVova infection, mouse CTLs were found to express the inhibitory molecules programmed cell-death protein-1 (PD-1) and lymphocyte-activation gene-3 (LAG-3) and to be functionally exhausted, showing a significant deficiency in T-cell proliferation, IFN-γ production and cytolytic effects. Naive CD8$^+$ T cells upregulated inhibitory PD-ligand 1 (PD-L1), B- and T-lymphocyte attenuator and T-cell anergy-associated molecules (Grail and Itch) while down-regulating the proliferative response upon stimulation in mice with chronic infection. Remarkably, the OVA-Texo vaccine counteracted T-cell anergy and converted CTL exhaustion. The latter was associated with (i) the upregulation of a marker for CTL functionality, diacetylated histone-H3 (diAcH3), (ii) a fourfold increase in CTLs, occurring independent of host DCs or CD4$^+$ T cells, and (iii) the restoration of CTL IFN-γ production and cytotoxicity. *In vivo* OVA-Texo-stimulated CTLs upregulated the activities of the mTORC1 pathway-related molecules Akt, S6, eIF4E and T-bet, and treatment of the CTLs with an mTORC1 inhibitor, rapamycin, significantly reduced the OVA-Texo-induced increase in CTLs. Interestingly, OVA-Texo-mediated CD40L signaling played a critical role in the observed immunological effects. Importantly, the Gag-Texo vaccine induced Gag-specific therapeutic immunity in chronic infection. Therefore, this study should have a serious impact on the development of new therapeutic vaccines for human immunodeficiency virus (HIV-1) infection.
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INTRODUCTION

CD8$^+$ cytotoxic T lymphocytes (CTLs) are important effector T cells that are actively involved in the immune response against various viral infections. Following an acute viral infection, CTL immunity develops in three phases: expansion, contraction and memory.[1] In brief, the initial encounter with viral antigens (Ags) derived from an infectious agent triggers the activation and proliferation of Ag-specific CTLs.[2,3] CTL responses often peak 7 days after an infection, followed by the contraction phase, when the majority (>90%) of effector CTLs (eCTLs) dies of activation-induced cell death.[4,5] The remaining 5–10% of the CTLs go on to seed the memory pool and become long-term memory CTLs (mCTLs).[6] These mCTLs are capable of responding robustly upon re-encounter with the

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same viral Ags. In patients with chronic human immunodeficiency virus (HIV) infection, the immune system is often functionally deficient. For example, patients with progressive HIV infection often have $CD4^+$ T-cell deficiency and dysfunctional dendritic cells (DCs) due to the binding of the HIV Gp120 protein to CD4 on $CD4^+CCR5^+$ T cells and the C-type lectin DC-SIGN on DCs, leading to $CD4^+$ T-cell death and the impairment of DCs by the viral cytopathic effect.[7–10] In addition, Ag-specific $CD8^+$ T cells that initially acquired effector function also gradually lose their functionality as chronic HIV infection progresses. This loss of function, known as 'exhaustion,' is hierarchical, with properties such as proliferative potential and IL-2 production affected early and IFN-γ production reduced later in the course of infection.[11,12] T-cell exhaustion is probably one of the chief issues leading to ineffective virus control in HIV patients.

Previous therapeutic vaccines (using peptides, DNA or AdV vectors) requiring the engagement of the host DCs and $CD4^+$ T cells often failed to stimulate therapeutic immunity.[13] Distinct from these small biologic molecules, immune cell-based therapeutics have become a new third pillar of therapeutics. DCs are the most potent antigen-presenting cells (APCs). They have been used as vaccines to stimulate HIV-specific CTL responses in animal models[14–16] as well as in clinical trials on HIV patients.[17–21] In these trials, the safety profile has been excellent, with minor local side-effects observed. However, the immunotherapeutic efficacy of DC vaccines was found to be relatively poor in HIV patients with a deficiency in $CD4^{\pm}$ T-cell help. Nevertheless, some inhibitory effects on viral titers and disease progression have been found,[19–21] warranting a search for more potent cell-based HIV-specific therapeutic vaccines.

We previously developed a new 'T-APC' concept by demonstrating that $CD4^+$ helper T cells became 'T-APCs' capable of directly stimulating $CD8^+$ CTL responses after acquiring Ag-specific DC membrane molecules.[22] On the basis of this concept, we developed a novel ovalbumin (OVA)-specific, DC (DC_{OVA})-released, exosome (EXO)-targeted, T-cell-based (OVA-Texo) vaccine expressing various cytokines such as IL-2, TNF-α and IFN-γ[23,24] and demonstrated that non-specific T cells with uptake of Ag-specific, DC-released EXO via a CD54/LFA-1 interaction were able to directly stimulate Ag-specific CTL responses in the absence of $CD4^+$ T-cell help.[23,24] The OVA-Texo vaccine acts more potently than the DC_{OVA} approach by counteracting $CD4^+25^+FoxP3^+$ regulatory T (Treg) cell suppression.[23,24] To develop an HIV-specific T-cell-based vaccine, we constructed a recombinant HIV Gp120-expressing adenoviral vector (AdV_{Gp120}) and generated a Gp120-specific Gp120-Texo vaccine using AdV_{Gp120}.[25] We found that the Gp120-Texo vaccine stimulated Gp120-specific CTL responses, leading to therapeutic immunity.[25,26] Because HIV Gag is more suitable than Gp120 for vaccine-induced CTL immunity against HIV,[27] we developed an HIV Gag-specific, EXO-targeted, T-cell-based (Gag-Texo) vaccine using AdV_{Gag} and showed that the Gag-Texo vaccine was capable of activating Gag-specific CTL responses and immunity in transgenic HLA-A2 mice.[28] To enhance its immunogenicity, we generated a 4-1BBL-expressing Gag-Texo vaccine and demonstrated that it elicited more efficient CTL responses and therapeutic immunity against Gag-expressing tumor challenges than our original Gag-Texo vaccine.[29]

In this study, we initially infected C57BL/6 mice with the OVA-expressing adenovirus AdVova to establish a mouse AdV-induced chronic infection model. Two months after AdV infection, we assessed OVA-specific CTL responses by flow cytometry. We found that OVA-specific memory CTLs expressing CD40 along with the inhibitory programmed cell-death protein-1 (PD-1) and inhibitory lymphocyte-activation gene 3 (LAG-3) proteins and were functionally exhausted, which was indicative of an OVA-specific chronic infection in AdVova-infected mice. In addition, naive $CD8^+$ T cells expressed CD40, inhibitory programmed death-ligand 1 (PD-L1) and B- and T-lymphocyte attenuator (BTLA) molecules[30] and became anergic in mice with AdVova-induced chronic infection. To assess the immunogenicity of a 4-1BBL-expressing OVA-Texo (termed OVA-Texo) vaccine, C57BL/6 mice were infected with the β-galactosidase (Gal)-expressing adenovirus AdV_{Gal}, which also creates a chronic infection model, showing rapid functional exhaustion and CTL deletion.[31] Some of these mice were immunized with the OVA-Texo vaccine. Interestingly, our work revealed that OVA-Texo vaccine stimulated potent $CD8^+$ T-cell responses in an AdV_{Gal}-induced chronic infection model by counteracting naive $CD8^+$ T-cell anergy. To assess the potential conversion of CTL exhaustion by OVA-Texo, AdVova-infected C57BL/6 mice were immunized with the OVA-Texo vaccine. Our experiments showed that OVA-Texo directly converted CTL exhaustion independent of the host $CD4^+$ T cells and DCs via the activation of the mTORC1 pathway. Consistent with this, treatment of CTLs with an mTORC1 inhibitor, rapamycin, significantly reduced the proliferation of functionally exhausted CTLs upon OVA-Texo stimulation. Furthermore, our observations revealed that OVA-Texo signaling through CD40L played a critical role in these immunological effects. To examine whether the Gag-Texo vaccine is capable of effectively stimulating Gag-specific immune responses in chronic infection, we immunized AdVova-infected C57BL/6 mice with the HIV Gag-specific Gag-Texo vaccine and determined that this vaccination induced therapeutic immunity against Gag-expressing tumors in chronic infection.

MATERIALS AND METHODS

Reagents, cell lines and animals

OVA was obtained from Sigma (St Louis, MO, USA). The OVAI ($OVA_{257-264}$, SIINFEKL) peptide (H-$2K^b$-restricted OVA antigen epitope) and Mut1 (FEQNTAQP) peptide (H-$2K^b$-restricted tumor antigen epitope of an irrelevant 3LL lung carcinoma) were synthesized by Multiple Peptide Systems (San Diego, CA, USA). Biotin-labeled antibodies (Abs) specific for CD44 (IM7) and CD40 (3/23) and phycoerythrin (PE)-CD45RA (14.8) and PE/Cy5-anti-IFN-γ (XMG1.2) Abs

were obtained from BD Biosciences (Mississauga, ON, Canada). Biotin-labeled Abs against CD62L (MEL-14), IL-7R (SB/199), lymphocyte-activation gene 3 (LAG-3) (C9B7W), diacetylated histone 3 (diAcH3) (Poly6019), and T-bet (Poly6235) and PE/Cy5-conjugated streptavidin were obtained from Biolegend (San Diego, CA, USA). FITC- or PE/Cy5-CD8 (53-6.7) and biotin-labeled programmed cell-death protein 1 (PD-1) (J43), programmed death-ligand 1 (PD-L1) (1-111A), BTLA (8F4) and Ki67 (SolA15) Abs were obtained from eBioscience (San Diego, CA). Anti-phospho-Akt (pAkt) 1/2/3 (Ser 473), GRAIL (H-91) and ITCH (H-110) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-phospho-eukaryotic translation initiation factor 4E (peIF4E) (Ser209), anti-phospho-S6 (pS6) ribosomal protein (Ser235/236) (D57.2.2E) and anti-β-Actin (D6A8) polyclonal Abs were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). PE-labeled H-2K^b/OVA$_{257\text{-}264}$ tetramer (YTS 169.4) was obtained from Beckman Coulter (Miami, FL, USA). Fluorescein isothiocyanate (FITC)-labeled CD8 (53-6.7) was obtained from Caltag, Burlingame, CA, USA. FITC-conjugated AffiniPure goat anti-rabbit IgG (H+L) was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Carboxyfluorescein succinimidyl ester (CFSE) was obtained from Molecular Probes (Eugene, OR, USA). The depleting anti-CD4 antibody was purified from the ascites of the hybridoma cell line GK1.5. Rat anti-mouse PD-L1 (10F.9G2) was obtained from BioXCell, Inc. (West Lebanon, NH, USA).[32] The recombinant adenoviral vectors AdVova, AdV$_{Gal}$, AdV$_{Gag}$ and AdV$_{41BBL}$, expressing OVA, β-galactosidase (Gal), HIV-1 Gag and 41BBL, respectively, were previously constructed in our laboratory.[29,33] Recombinant *Listeria monocytogenes* rLmOVA[34] expressing OVA was obtained from DMX Inc. (West Chester, PA, USA). The highly lung metastatic OVA-expressing BL6-10$_{OVA}$ and Gag-expressing BL6-10$_{Gag}$ tumor cell lines were generated in our lab.[22,33] Female wild-type (WT) C57BL/6 (B6), OVA-specific CD8$^+$ and CD4$^+$ T-cell receptor (TCR)-transgenic (Tg) OTI and OTII, transgenic diphtheria toxin receptor (DTR)-CD11c and various gene knockout (KO) mice were obtained from Jackson Laboratory (Bar Harbor, MA, USA). The animals were housed in the University of Saskatchewan Animal Facility (accreditation SCA-001). All experiments were performed according to protocols and guidelines approved by the Animal Research Ethics Board, University of Saskatchewan (Protocol# 20130020).

Dendritic cell and exosome preparations

Bone marrow-derived DCs were obtained by culturing bone marrow cells from WT B6 mice in culture medium containing granulocyte monocyte colony-stimulating factor (GM-CSF) (20 ng/ml) and IL-4 (20 ng/ml) for 6 days as previously described.[24] The DCs were pulsed with OVA (0.5 mg/ml) overnight or infected with AdV$_{Gag}$ and termed DC$_{OVA}$ or DC$_{Gag}$ cells. DC$_{OVA}$ or DC$_{Gag}$-released exosomes (EXO$_{OVA}$ or EXO$_{Gag}$) were then purified from DC$_{OVA}$ or DC$_{Gag}$ culture supernatants by differential ultracentrifugation.[24]

T-cell preparation

Naive or memory CD8$^+$ T cells were isolated from naive or AdV$_{Gag}$- or AdVova-infected WT B6 and OVA-specific TCR transgenic OTI mouse spleens, enriched by passage through nylon wool columns (C&A Scientific, Manassas, VA, USA), and purified by negative selection using anti-mouse CD4 paramagnetic beads (DYNAL, Lake Success, NY, USA). To generate active CD8$^+$ T cells, the spleen cells from naive C57BL/6 mice were cultured in RPMI 1640 medium containing IL-2 (20 U/ml) and ConA (1 μg/ml) for 3 days. CD8$^+$ T cells were then purified from ConA-activated T (ConA-T) cells using MACS anti-CD8 microbeads (Miltenyi Biotech, Auburn, CA, USA) to yield T-cell populations with $>98\%$ purity.[22] ConA-T cells derived from IL-2, TNF-α and CD40L KO mice were termed (IL-2$^{-/-}$), (TNF-α$^{-/-}$) and (CD40L$^{-/-}$) ConA-T cells, respectively.

Preparation of OVA-Texo and Gag-Texo vaccines

OVA- and Gag-specific T-cell-based vaccines were generated by the incubation of CD8$^+$ ConA-T cells with EXO$_{OVA}$ and EXO$_{Gag}$ and subsequent transfection with AdV$_{4\text{-}1BBL}$, as previously described.[29] The 41BBL-expressing, Gag-specific, T-cell-based vaccine is termed Gag-Texo, whereas the 41BBL-expressing, OVA-specific, T-cell-based vaccine is termed OVA-Texo. The 4-1BBL-expressing OVA-Texo cells derived from WT B6, (IL-2$^{-/-}$)-, (TNF-α$^{-/-}$)- and (CD40L$^{-/-}$) -ConA-T cells were termed OVA-Texo, and OVA-Texo (IL-2$^{-/-}$), OVA-Texo(TNF-α $^{-/-}$) and OVA-Texo(CD40L $^{-/-}$) vaccines lacking IL-2, TNF-α and CD40L, respectively.

Establishment of a chronic infection animal model

To establish an acute infection animal model, we infected B6 mice i.v. with rLmOVA (2000 c.f.u./mouse).[34] To establish a chronic infection animal model, we infected these B6 mice with AdVova (2×10^6 p.f.u./mouse) or AdV$_{Gal}$ (1×10^8 p.f.u./mouse), followed by a kinetic study of OVA-specific CD8$^+$ T-cell responses by flow cytometry. Mouse blood samples were collected at different time points post immunization and either double stained with FITC-anti-CD8 antibody (FITC-CD8) and PE-H-2K^b/OVA$_{257\text{-}264}$ tetramer (PE-tetramer) or triple stained with FITC-CD8, PE-tetramer and PE-Cy5-antibodies for various immune molecules, and then the cells were analyzed by flow cytometry. For intracellular staining, the splenocyte samples were re-stimulated with 2 μM OVAI peptide and subjected to intracellular staining (BD Biosciences) for IFN-γ, as described previously.[35] To assess CTL recall responses, mouse splenic T-cell populations (containing mCTLs) from the chronically and acutely infected mice were adoptively transferred into WT B6 mice, and then the mice with similar amounts of adoptive mCTLs were i.v. boosted with DCova (1×10^6 cells/mouse). Four days after the boost, OVA-specific CTL responses were analyzed by flow cytometry. In addition, we also established a more stringent model of chronic infection by i.v. injecting B6 mice with anti-CD4 antibody (300 μg/mouse) to deplete CD4$^+$ helper T (Th) cells, followed by i.v. infection with AdVova 1 day after antibody treatment for the

assessment of the conversion effect of OVA-Texo vaccination on CTL exhaustion, in which 'helpless' $CD8^+$ T cells demonstrated stronger functional defects.[36]

Cytotoxicity assay

The *in vivo* cytotoxicity assay was performed in immunized mice with transfer of both OVAI-pulsed CFSE (3.0 μM $CFSE^{high}$)-labeled (H) and control irrelevant Mut1-pulsed CFSE (0.6 μM $CFSE^{low}$)-labeled (L) target splenocytes at a ratio of 1:1 (each 4×10^6 cells), as previously described.[22] Sixteen hours after the cell transfer, the residual $CFSE^{high}$ (H) and $CFSE^{low}$ (L) target cells remaining in the recipients' spleens were analyzed by flow cytometry.[23]

RNA array analysis

Naïve $CD8^+$ T cells from naive or chronically infected WT B6 mice spleens were purified using an EasySep Mouse $CD8^+$ T-Cell Isolation Kit (StemCell Technologies, Vancouver, BC, Canada), followed by Biotin-CD45RA (BD Biosciences) and MACS anti-biotin microbeads (Miltenyi Biotech). RNA isolation was performed using an RNeasy Plus Mini Kit (Qiagen Inc., Toronto, ON, Canada), cDNA was synthesized immediately using an RT^2 First Strand Kit (Qiagen Inc.). A panel of primers specific for T-cell anergy-associated genes (Ikaros, Grail, Casp3, EGR2, Grg4 and Itch) was synthesized.[37] Real-time PCR was carried out with RT^2 SYBR Green qPCR Mastermix (Qiagen) using a StepOnePlus Real-time PCR system (Applied Biosystems, Burlington, ON, Canada).[38] The expression of each gene was normalized to β-actin. The fold induction represents the ratio of mRNA expression in $CD8^+$ T cells from chronically AdV-infected mouse spleens to that in naive mice spleens.

Western blot analysis

Cell lysates (10 μg/lane) were loaded onto 12% acrylamide gels, subjected to sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred onto a nitrocellulose membrane (Millipore, Bedford, MA, USA). The membrane was blocked by incubation for 2 h at room temperature with ODYSSEY blocking buffer (LI-COR Bioscience, Lincoln, NE, USA) and immunoblotted with Abs specific for GRAIL and ITCH at 4 °C overnight. After three washes with PBS containing 0.05% (V/V) Tween 20, the membrane was further incubated with goat anti-rabbit IRDye800CW and scanned using an ODYSSEY instrument according to the manufacturer's instructions (LI-COR Bioscience).[38]

Flow cytometric analysis

To assess the counteraction of naive $CD8^+$ T-cell anergy by OVA-Texo, B6 mice (4–6 per group) were i.v. infected with AdV_{Gal} (1×10^8 p.f.u./mouse) and 2 months later were further i.v. immunized with DCova and OVA-Texo cells (1×10^6 cells/mouse). Six days after the immunization, mouse blood samples were stained with FITC-CD8 Ab and PE-tetramer, and OVA-specific CTL responses were analyzed by flow cytometry.[39] To assess CTL recall responses, the vaccinated mice were then i.v. boosted with rLmOVA (1000 c.f.u.) 30 days after immunization and measured for OVA-specific CTL responses 4 days following the boost by flow cytometry.[39] To assess the molecular mechanism for OVA-Texo-mediated counteraction of anergy, B6 mice (4–6 per group) with AdV_{Gal}-induced chronic infection were i.v. immunized with OVA-Texo cells; OVA-Texo cells lacking IL-2, TNF-α and CD40L (2×10^6 cells/mouse); or OVA-Texo plus anti-CD40L antibody (200 μg/mouse). The OVA-specific CTL responses in the immunized mice were assessed 6 days after immunization by flow cytometry.

T-cell proliferation assays

In the *in vitro* T-cell proliferation assay, naive $CD8^+$ T cells purified from the splenocytes of AdV_{Gal}- and AdVova-infected B6 mice using biotin-anti-CD45RA antibody and MACS anti-biotin microbeads (Miltenyi Biotech) followed by an EasySep Mouse $CD8^+$ T-Cell Isolation Kit (StemCell Technologies) were labeled with CFSE (3 mM). CFSE-labeled T cells were then incubated with CD3/CD28 microbeads in the presence of IL-2 (40 U/ml) and beta-mercaptoethanol (2-ME; 50 μM). Three days after incubation, the active $CD8^+$ T cells were harvested and analyzed for the determination of the number of CFSE-labeled T-cell divisions by flow cytometry. In one *in vivo* T-cell proliferation assay, naive $CD8^+$ T cells were purified from naive OTI mice or OTI mice chronically infected with AdV_{Gal}, labeled with CFSE (4 μM), and adoptively transferred (1×10^6/mouse) into naive B6 recipients. The DCova or OVA-Texo vaccine was injected on the following day to activate the CFSE-labeled OTI-$CD8^+$ T cells *in vivo*. Seventy-two hours later, the splenocytes were collected and analyzed by flow cytometry. In another *in vivo* T-cell proliferation assay, exhausted mCTLs purified from chronically AdVova-infected mice using PE-tetramer and MACS anti-PE-microbeads (Miltenyi Biotech) were cultured in culture medium with or without rapamycin (100 nM) for 6 h[40] and transferred into mice with AdV_{Gal}-induced chronic infection, followed by OVA-Texo (1×10^6) vaccination. Four days after vaccination, the OVA-specific CTL responses were assessed by flow cytometry.

Conversion of CTL exhaustion by the OVA-Texo vaccine

To assess the conversion of mCTL exhaustion, chronically AdVova-infected B6 mice (8 per group) with CTL exhaustion were given i.v. OVA-Texo cells (1×10^6 cells/mouse) or OVA-Texo ($CD40L^{-/-}$) cells, with defective CD40L (1×10^6 cells/mouse), 45–50 days after infection. To determine whether the OVA-Texo-induced conversion of CTL exhaustion in chronic infection was dependent on DCs and $CD4^+$ T-cell help, we repeated the above experiments using OVA-Texo vaccination of AdVova-infected DTR-CD11c mice, who received i.p. diphtheria toxin (DT) (300 ng/mouse) treatment once every other day starting 3 days before vaccination to deplete $CD11c^+$ DCs for a total of three times, or AdVova-infected WT B6 mice who received a single i.p. treatment with

anti-CD4 antibody (500 μg/mouse) 1 day before vaccination to deplete $CD4^+$ T cells.[41] Four days after vaccination, the mouse blood samples were collected, stained with FITC-CD8 Ab and PE-tetramer, and analyzed by flow cytometry. To assess OVA-specific CTLs in the spleens and lungs, mouse splenocytes and lung cell suspensions prepared by mincing lung tissues into small fragments and digesting them with collagenase D (1 mg/ml, Worthington Biochemical, Freehold, NJ, USA) at 37 °C for 30 min[42] were stained with FITC-CD8 Ab and PE-tetramer and analyzed by flow cytometry. For intracellular staining, mouse splenocytes were first incubated with FcR-blocking anti-16/32 Ab (eBioscience) for 30 min on ice to eliminate any nonspecific staining. The cells were then stained with FITC-CD8 Ab and PE-tetramer, followed by fixation and permeabilization with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. The cells were further stained with PE/Cy5-antibodies specific for various molecules, and the intracellular expression of these molecules was assessed by flow cytometry. For the intracellular staining of IFN-γ, the mouse splenocytes were first incubated in culture medium containing OVAI peptide (2 μg/ml) and Golgistop (0.7 μg/ml) (BD Biosciences) at 37 °C for 5 h, followed by incubation with an FcR-blocking anti-16/32 Ab for 30 min on ice. The cells were then stained with FITC-CD8 Ab and PE-tetramer, followed by fixation and permeabilization with Cytofix/Cytoperm (BD Biosciences). Intracellular staining for IFN-γ was conducted using PE/Cy5-anti-IFN-γ Ab, and the intracellular expression of IFN-γ was assessed by flow cytometry. Sample data were acquired on a FACSCalibur (BD Bioscience) and analyzed with FlowJo software (TreeStar, San Diego, CA, USA).

Animal studies

To assess the functional effect of mCTLs in chronic infection, splenocytes from chronically AdVova- or rLmOVA-infected B6 mice 45–50 days after infection were transferred into naive B6 mice, and the recipient mice (8 per group) were injected with 0.5×10^6 BL6-10_{OVA} cells on the following day. The mice were killed 3 weeks after tumor cell challenge, and the lung metastatic tumor colonies were counted in a blind fashion. To examine the protective antitumor immunity conferred by the Gag-Texo vaccine, B6 mice (8/group) with AdVova-induced chronic infection were i.v. injected with Gag-Texo or DC_{Gag} cells (2×10^6 cells/mouse). The immunized mice were i.v. challenged with 0.5×10^6 BL6-10_{Gag} cells 6 days subsequent to the immunization. To examine the therapeutic antitumor immunity conferred by the Gag-Texo vaccine, chronically infected B6 mice (8 per group) were first i.v. injected with 0.5×10^6 BL6-10_{Gag} cells. Three days after the tumor cell inoculation, the B6 mice were then i.v. injected with Gag-Texo or DC_{Gag} cells (1×10^6 cells/mouse). The mice were killed 3 weeks after tumor cell challenge, and the lung metastatic tumor colonies were counted by an individual blinded to the study conditions. Metastases on freshly isolated lungs appeared as discrete black-pigmented foci that were easily distinguishable from normal lung tissues and confirmed by histological examination. Metastatic foci too numerous to count were assigned an arbitrary value of > 300.[24]

Statistical analyses

Unless stated otherwise, the data are expressed as the mean (with s.d.). Statistical analyses were performed using the Mann–Whitney *U*-test for the comparison of variables from different groups in animal studies or Student's *t*-test for the comparison of variables from different groups in all other studies. Probability values of $P > 0.05$, $P < 0.05$ and $P < 0.01$ are considered statistically not significant, significant and very significant, respectively.[39]

RESULTS

OVA-expressing adenoviral vector induces chronic infection in mice

Distinctive $CD8^+$ T-cell responses ranging from immunogenic CTL responses to CTL exhaustion or depletion have been reported to be elicited post vaccination with adenoviral vectors (AdVs) expressing different antigens, at various doses, or through different routes.[31,43–48] For example, the persistence of adenoviral transgene expression in AdV-infected mice led to CTL inflation,[46] and mice i.v. infected with β-galactosidase (Gal)-expressing AdV_{Gal} developed a chronic infection with CTL exhaustion and deletion.[31] Unfortunately, the Gal-specific PE tetramer, which is useful for detecting Gal-specific CTLs, is not commercially available, which makes it difficult to study chronic infection in this model. To assess whether a replication-deficient transgene, OVA-expressing AdVova,[33] induces chronic infection in mice, C57BL/6 (B6) mice were i.v. infected with AdVova. Our analysis demonstrated that 60 days later, AdVova infection resulted in a dose-dependent (Supplementary Figure 1) OVA-specific memory $CD8^+$ T-cell expansion (Figure 1a). The expanded mCTLs had decreased cell surface T-cell memory markers, CD62L and IL-7R, when compared with mCTLs developed in rLmOVA-immunized mice (Figure 1b). Interestingly, we found that these mCTLs expressed cell surface CD40 (Figure 1b). Importantly, we also found that these mCTLs upregulated inhibitory molecules, such as PD-1, PD-L1 and LAG-3 (Figure 1b), indicating that these mCTLs may be exhausted. To assess the functional traits of these potentially exhausted CTLs, including their capacity for Ag-specific cell killing and CTL recall responses, we performed flow cytometric analyses to measure cellular IFN-γ expression, cytolytic effectiveness and CTL recall responses following antigen boost and also conducted animal studies to analyze protective immunity against OVA-expressing tumor (BL6-10_{OVA}) challenge. We determined that only 17% (1.37% vs 8.11%) of these CTLs possessed intracellular IFN-γ in comparison to nearly 75% (5.16/7.06) of IFN-γ-positive CTLs observed in rLmOVA-immunized controls (Figure 1c). This observation clearly indicates that CTLs display a significant reduction in the expression of an effector cytokine, IFN-γ, during chronic infection. In addition, these CTLs also showed defects in the cytolytic response against OVA-specific, highly

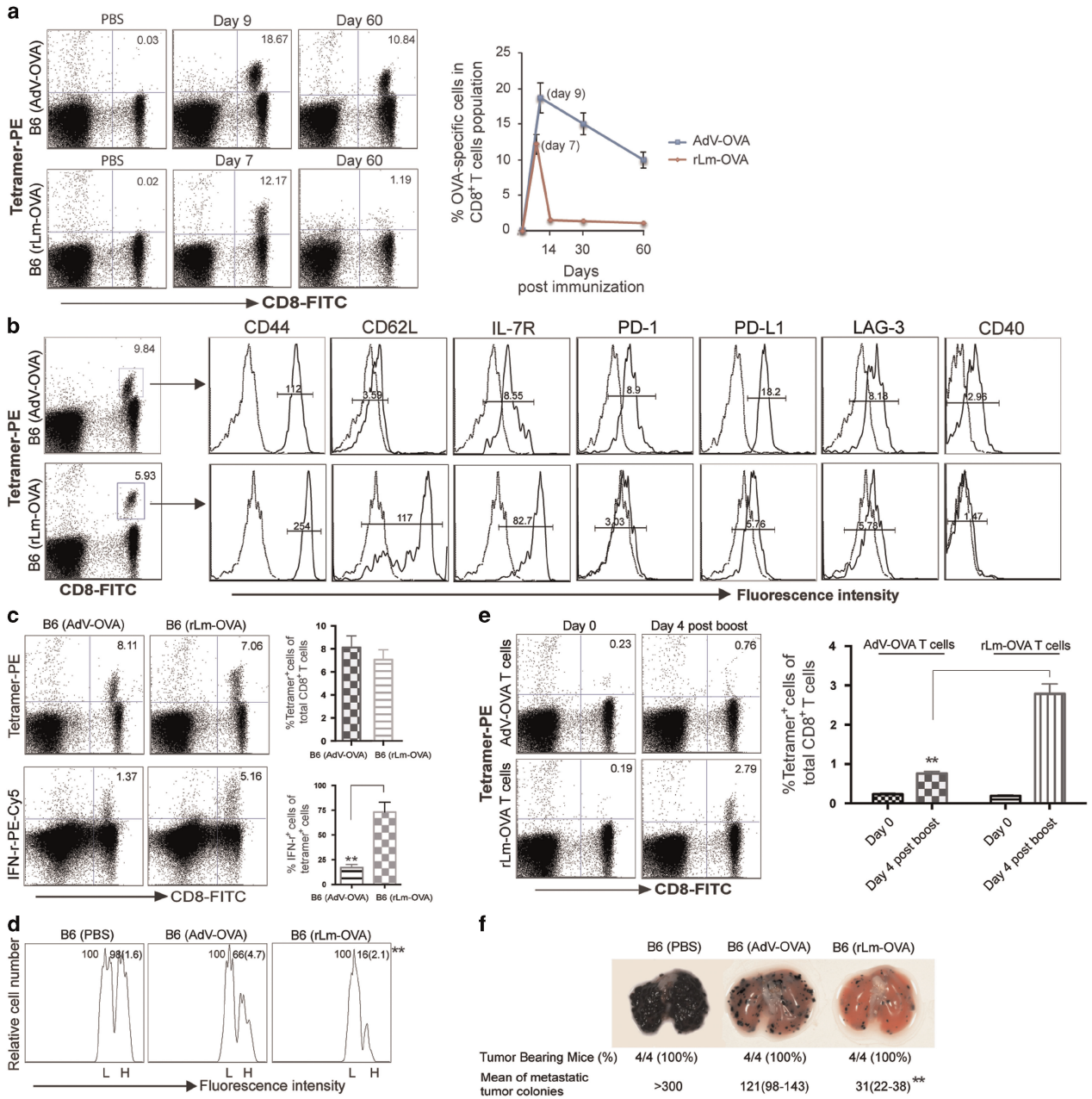

Figure 1 AdVova induces chronic infection in mice. (**a**) OVA-specific CTL responses were analyzed at the indicated days post-AdVova or -rLmOVA infection by flow cytometry. The value in each panel represents the percentage of OVA-specific (tetramer-positive) $CD8^+$ T cells in the total $CD8^+$ T-cell population. (**b**, **c**) To enhance mCTLs in rLmOVA-infected mice, C57BL/6 mice were first transferred with 1×10^4 naive $CD4^+$ OTII and 1×10^4 naïve OTI $CD8^+$ T cells to increase the numbers of precursor T cells before rLmOVA infection. Sixty days after the infection, we performed flow cytometric analyses. (**b**) $CD8^+$ T cells with positive PE-tetramer and FITC-CD8 staining were gated (rectangle) for further assessment of the expression of the indicated molecules (solid lines on the right). Mean fluorescence intensity (MFI) numbers are indicated in each panel. Dotted lines (on the left) represent isotype-matched controls. The control MFI numbers for the upper and lower panels were 2.16 and 3.28, respectively. (**c**) $CD8^+$ T cells were permeabilized for the assessment of intracellular IFN-γ by flow cytometry. The value in each panel represents the percentage of $CD8^+$ T cells producing IFN-γ in the total $CD8^+$ T-cell population. (**d**) *In vivo* cytotoxicity assay. Sixty days after the infection, we performed an *in vivo* cytotoxicity assay as described in Materials and methods. The value in each panel represents the percentage of $CFSE^{high}$ vs $CFSE^{low}$ target cells remaining in the spleen. (**e**, **f**) Sixty days after the infection, splenocytes from AdVova-infected mice were adoptively transferred into C57BL/6 mice, and 1 day after transfer, the mice had 0.23% OVA-specific mCTLs, similar to the 0.19% OVA-specific mCTLs in rLmOVA-infected mice. (**e**) These mice were boosted with DCova. Four days after the boost, their peripheral blood samples were analyzed for OVA-specific memory CTL recall responses using flow cytometry. The value in each panel represents the percentage of PE-tetramer-positive $CD8^+$ T cells in the total $CD8^+$ T-cell population. (**f**) The mice were i.v. injected with $BL6\text{-}10_{OVA}$ tumor cells and were killed 3 weeks later. The numbers of lung metastatic tumor colonies were counted. $^*P<0.05$, $^{**}P<0.01$. Error bars represent s.d. One representative experiment of two to three is shown.

CFSE-labeled (H) target cells (Figure 1d) in mCTL recall responses upon DC_{OVA} boost (Figure 1e) and in their immune response to OVA-expressing BL6-10_{OVA} tumors (Figure 1f), indicating that these OVA-specific mCTLs are indeed functionally exhausted in an AdVova-induced chronic infection model. Taken together, our observations suggest that AdVova-infected mice represent a new chronic infection mouse model with mCTL inflation and exhaustion. In addition, we also assessed mCTLs in AdV_{Gal}-infected mice using mouse peripheral blood samples stained with antibodies for CD8 and CD44 (memory T-cell marker). We demonstrated that these $CD8^+CD44^+$ mCTLs also expressed inhibitory PD-1 and PD-L1 (Supplementary Figure 2), confirming that AdV_{Gal}-infected mice also represent a chronic infection animal model useful for assessing the immunogenicity of our OVA-Texo vaccine.

$CD8^+$ T-cell anergy in chronic infection

A bystander chronic infection associated with some level of immune tolerance often enhances the susceptibility of chronically infected hosts to a variety of co-infections.[49] However, the underlying mechanism is not clear. To assess potential immune tolerance in our chronic infection model, we examined OVA-specific CTL responses derived following vaccination with DCova in wild-type (WT) B6 and chronically AdV_{Gal}-infected B6 mice. This analysis revealed that DCova induced ~3-fold (0.62% vs 2.24%) less OVA-specific CTL priming at day 6 post immunization and ~9-fold (2.24% vs 21.79%) less mCTL recall responses measured at day 4 after the boost in mice with chronic infection (Figure 2Ac and d), compared with the responses observed in WT B6 mice (Figure 2Aa and b). These data indicate that some immune tolerance occurs in the presence of the AdV_{Gal}-induced chronic infection. To assess whether the immune tolerance involves $CD8^+$ T cells, we performed an *in vitro* T-cell proliferation assay. In the assay, naive $CD8^+$ T cells purified from chronically infected B6 mice or WT B6 mice were labeled with CFSE and activated with anti-CD3/CD28 beads and IL-2 *in vitro*. Three days later, these T cells were analyzed by flow cytometry. Naive $CD8^+$ T cells derived from chronically AdVova-infected B6 mice underwent less cell division (78.3%) (Figure 2Bc) than those (95.2%) from WT B6 mice (Figure 2Bb), indicating that naive $CD8^+$ T cells are anergic during chronic infection. Similar data were also observed in naive $CD8^+$ T cells labeled with CFSE and stimulated with CD3/28 beads and IL-2 in chronically AdV_{Gal}-infected B6 mice (Supplementary Figure 3a). In addition, we also performed an *in vivo* T-cell proliferation assay. In the assay, naive $CD8^+$ T cells purified from OTI mice or AdV_{Gal}-infected OTI mice were labeled with CFSE and transferred into WT B6 mice. One day after the transfer, the mice were immunized with DCova, and the mouse splenocytes were analyzed 3 days later by flow cytometry. This experiment demonstrated that CFSE-labeled OTI $CD8^+$ T cells derived from AdV_{Gal}-infected OTI mice underwent fewer cell divisions (72.7%) (Figure 2Ce) than those (95.2%) derived from uninfected OTI mice (Figure 2Cb), thus confirming that naive $CD8^+$ T cells with a chronic infection background are indeed anergic. It has been shown that naive splenocytes containing B and T cells and DCs upregulated inhibitory PD-L1 in the course of chronic infection, but the subset of immune cells expressing PD-L1 was not defined.[36] To assess the phenotype of anergic naive $CD8^{\pm}$ T cells in our AdVova-induced chronic infection animal model, we performed flow cytometric analysis. Interestingly, we found that naive $CD45RA^+CD8^+$ T cells[50] in B6 mice with AdVova-induced chronic infection had increased inhibitory PD-L1 and BTLA molecules compared with cells derived from uninfected naive B6 mice (Figure 2D). In support of this, we found that naive $CD8^+$ T cells in AdV_{Gal}-induced chronic infection also upregulated inhibitory PD-L1, BTLA, B7H3 and LAG3 (Supplementary Figure 3b). Interestingly, we demonstrated that ~44% of the naive $CD8^{\pm}$ T cells in chronic infection, but not in healthy naive B6 mice, expressed cell surface CD40 (Figure 2D). In addition, we also performed RT-PCR analysis to assess the expression of T-cell anergy-associated genes and showed that the naive $CD8^+$ T cells in an AdVova-induced chronic infection model upregulated the T-cell anergy-associated genes Grail and Itch (Figure 2E), which implies that $CD8^+$ T-cell tolerance in chronic infection is associated with naive $CD8^+$ T-cell anergy. This was further confirmed by western blotting analysis, demonstrating the upregulation of the Grail and Itch molecules at the protein level in naive $CD8^+$ T cells derived from mice with AdVova- and AdV_{Gal}-induced chronic infection (Figure 2F, Supplementary Figure 3c).

OVA-Texo counteracts naive $CD8^+$ T-cell anergy in chronic infection

To evaluate whether the OVA-Texo vaccine counteracts the immune tolerance in our chronic infection model, we assessed OVA-specific CTL responses derived from OVA-Texo vaccination of WT B6 and chronically AdV_{Gal}-infected B6 mice. In contrast to significantly decreased OVA-specific CTL responses in DCova-vaccinated mice with chronic infection, relatively smaller, but still comparable, CTL priming (1.67%) and mCTL recall (11.91%) responses were found in OVA-Texo-immunized mice with chronic infection (Figure 2Ag and h) when compared with those in WT B6 mice (2.78 and 18.70%) (Figure 2Ae and f). In contrast to the significant reduction of DCova-induced CTL priming and recall responses in mice with chronic infection, OVA-Texo stimulated comparable CTL priming and recall responses in mice with chronic infection compared with WT mice, indicating that the OVA-Texo vaccine can counteract T-cell tolerance caused by chronic infection. Interestingly, the OVA-Texo vaccine was also found to induce comparable proliferation (94.4%) of CFSE-labeled $CD8^+$ T cells transferred from AdV_{Gal}-infected OTI mice (Figure 2Cf) compared with that (92.3%) of CFSE-labeled $CD8^+$ T cells transferred from WT OTI mice (Figure 2Cc). Our data thus indicate that the OVA-Texo vaccine is capable of counteracting naïve $CD8^+$ T-cell anergy in chronic infection.

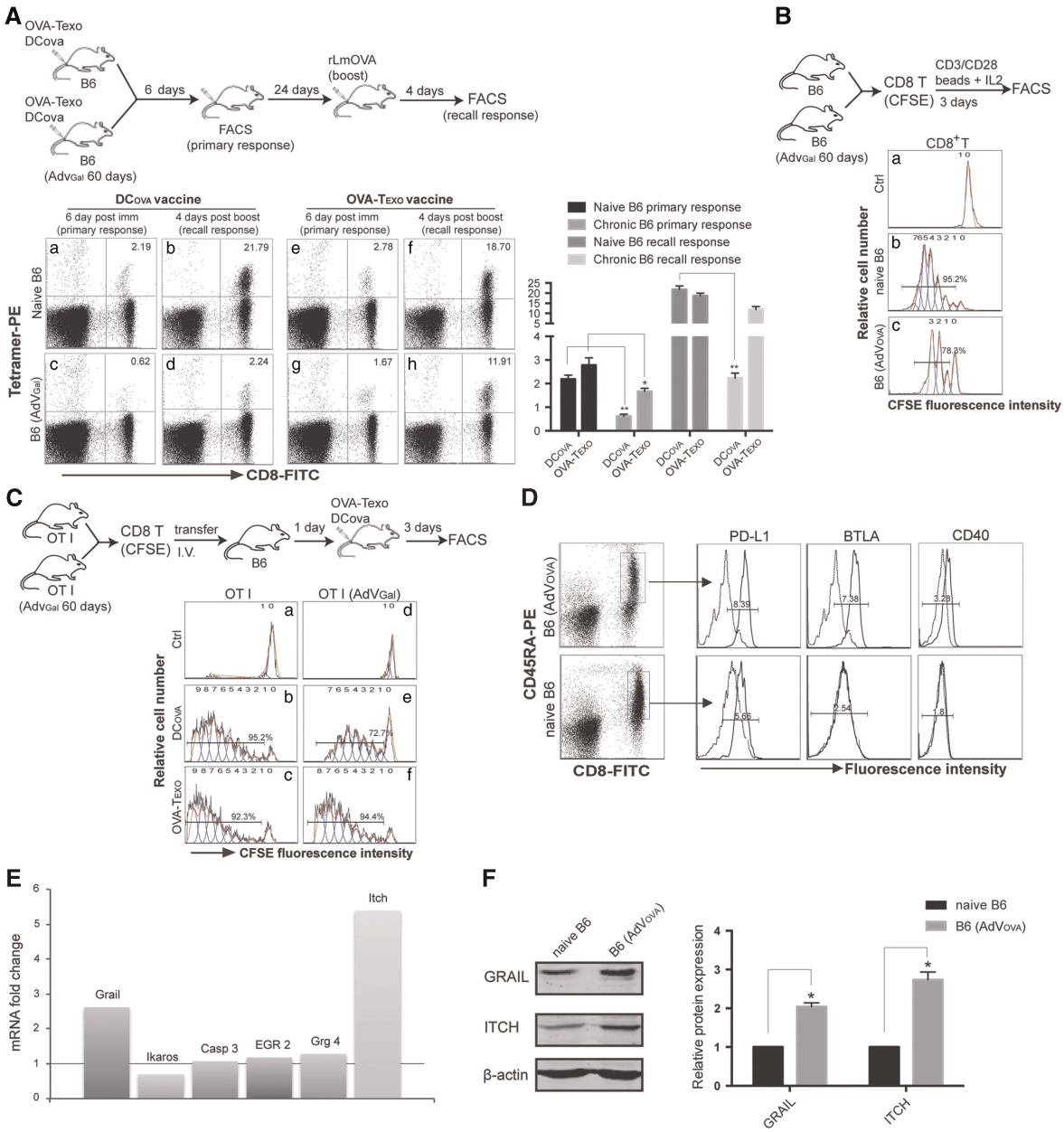

Figure 2 T-cell anergy in chronic infection. (**A**) We assessed CTL priming and recall responses as described in the diagram. The value in each panel represents the percentage of OVA-specific (tetramer-positive) $CD8^+$ T cells in the total $CD8^+$ T-cell population. (**B**, **C**) We assessed cell proliferation as described in the diagrams. Cell divisions were monitored by analyzing CFSE dilution. Histograms show the FACS profiles of (**B**) $CD8^+$/$CFSE^+$ T cells from the *in vitro* proliferation assay or (**C**) PE-$tetramer^+$/$CFSE^+$ T cells from the *in vivo* proliferation assay. The numbers of cell divisions were indicated on top of each panel, and the percentages of dividing cells were indicated on top of the number of divisions. (**D**) Peripheral blood samples from naive mice or chronically AdVova-infected mice were stained with PE-CD45RA, FITC-CD8 and PE-Cy5-labeled Abs and then analyzed by flow cytometry. Naive $CD8^+$ T cells with positive PE-CD45RA and FITC-CD8 staining were gated (rectangle) for further measurement of the expression of co-stimulatory or inhibitory molecules (solid lines on the right). Mean fluorescence intensity (MFI) numbers are indicated in each panel. Dotted lines (on the left) represent isotype-matched controls. The control MFI numbers for the upper panel's PD-L1/BTLA and CD40 were 1.28 and 1.80, respectively, while the control MFI numbers for the lower panel's PD-L1/BTLA and CD40 were 2.51 and 1.80, respectively. (**E**) The expression of anergy-associated genes was assessed by RT-PCR using mRNA purified from naive $CD8^+$ T cells derived from naive or chronically infected mouse splenocytes. The numbers of mRNA fold changes (the ratio of mRNA expression of each gene in naive $CD8^+$ T cells derived from AdVova-infected mice vs WT B6 mice) are indicated. (**F**) Western blotting analysis. The protein extracts were analyzed by western blotting. Each protein band intensity was quantitated using a computational densitometer in the ODYSSEY software. The expression ratio (ER) represents the protein expression of each gene normalized to the matching β-actin control. The relative protein expression (RPE) represents the ratio of the ER of each gene in AdVova-infected B6 mice vs that in naive B6 mice. $*P<0.05$, $**P<0.01$. Error bars represent the s.d. One representative experiment of two to three is shown.

OVA-Texo-induced counteraction of CD8$^+$ T-cell anergy depends upon CD40L signaling

It has been demonstrated that the cytokines IL-2 and TNF-α along with CD40L co-stimulation counteract T-cell anergy or tolerance.[51–53] To assess a potential molecular mechanism for the OVA-Texo-mediated counteraction of CD8$^+$ T-cell anergy, we generated a panel of OVA-Texo vaccines with various molecular defects, such as OVA-Texo(IL-2$^{-/-}$), OVA-Texo (TNF-α$^{-/-}$) and OVA-Texo(CD40L$^{-/-}$), lacking the expression of IL-2, TNF-α and CD40L, respectively. Mice with AdV$_{Gal}$-induced chronic infection were individually immunized with each of these vaccines, and their OVA-specific CTL responses were assessed by flow cytometry on day 6 after immunization. This approach demonstrated that CD40L deficiency alone reduced OVA-Texo-stimulated OVA-specific CTL responses during chronic infection by 85%, though the CTL responses were also significantly downregulated when using the OVA-Texo(TNF-α$^{-/-}$) and OVA-Texo(IL-2$^{-/-}$) vaccines (Figure 3a). Our data indicate that the OVA-Texo-induced counteraction of CD8$^+$ T-cell anergy in chronic infection is mainly mediated by CD40L signaling. In addition, a blockade of CD40L using an antagonistic anti-CD40L antibody also significantly inhibited OVA-Texo-stimulated CTL responses in mice with chronic infection (Figure 3b), thus confirming the above observation. Taken together, our data determine that the OVA-Texo vaccine is capable of counteracting naïve CD40-expressing CD8$^+$ T-cell anergy/tolerance in chronic infection via signaling through CD40L.

The OVA-Texo vaccine converts CTL exhaustion in chronic infection

In our further experiments, we examined whether the OVA-Texo vaccine would enhance the responses of functionally exhausted CD8$^+$ T cells in mice with AdVova-induced chronic infection. This is a more stringent model of chronic infection because the primary infection with AdVova is performed in the absence of CD4$^{\pm}$ T cells, which are initially eliminated using an anti-CD4 Ab. The exhausted CTLs derived from 'helpless' CD8$^{\pm}$ T cells in the T-cell priming phase of the infection have stronger functional defects in IFN-γ production and cytotoxicity.[54] Mice with AdVova-induced chronic infection were boosted with the OVA-Texo vaccine. Four days after the boost and the cell number increased, IFN-γ expression and cytolytic activity of OVA-specific CTLs were analyzed by flow cytometry. These experiments demonstrated that there were ~4-fold (10.85% vs 2.70%) more of OVA-specific CTLs in OVA-Texo-boosted mouse peripheral blood at day 4 after the boost (Figure 4a), indicating that the OVA-Texo vaccine can directly convert CTL exhaustion. The 4-fold increase in OVA-specific CTLs was likely generated from the previously exhausted mCTLs because the primary OVA-specific CTLs derived from the OVA-Texo boost did not occur in the peripheral blood at day 4, but peaked at day 6 post stimulation (Supplementary Figure 4). To further confirm this, we transferred exhausted mCTLs derived from chronically AdVova-infected B6 (CD45.2$^+$) mice into chronically AdV$_{Gal}$-

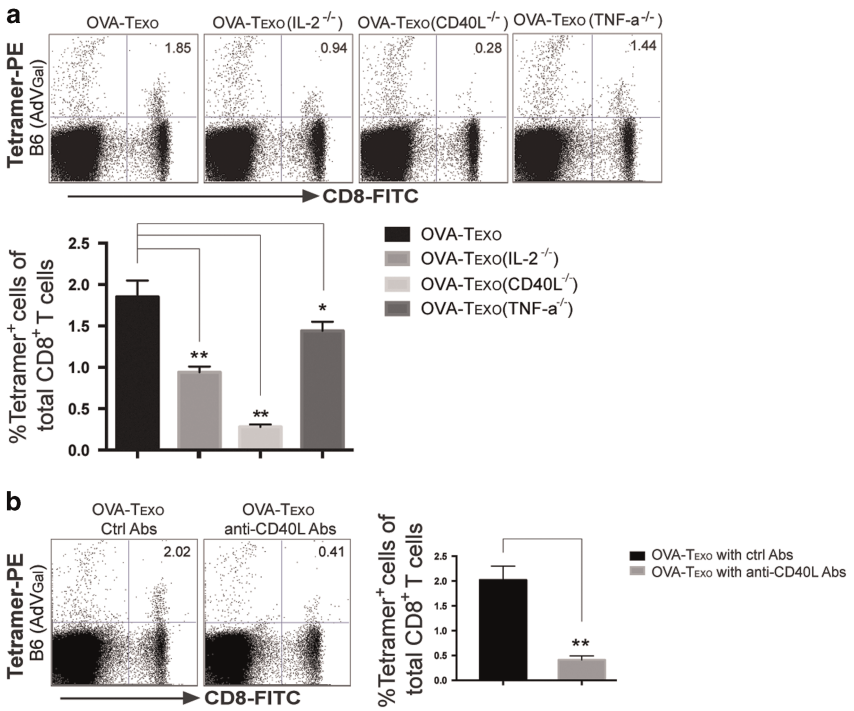

Figure 3 OVA-Texo counteracts CD8$^+$ T-cell anergy via CD40L signaling. AdV$_{Gal}$-infected C57BL/6 mice were immunized with (**a**) the OVA-Texo vaccine or the OVA-Texo vaccine deficient for one of several molecules or (**b**) the OVA-Texo vaccine plus anti-CD40L antibody treatment. Six days after the immunization, mouse peripheral blood samples were stained with PE-tetramer and FITC-CD8, and analyzed for the assessment of OVA-specific CTLs by flow cytometry. The value in each panel represents the percentage of PE-tetramer-positive CD8$^+$ T cells in the total peripheral CD8$^+$ T-cell population. $*P<0.05$, $**P<0.01$. Error bars represent the s.d. One representative experiment of two is shown.

infected B6.1 (CD45.1$^+$) mice and performed a OVA-Texo boost. As expected, all OVA-specific CTLs with an ~4-fold increase at day 4 post boost exclusively expressed CD45.2, but not CD45.1 (Figure 4b), thus confirming that this increased cell population represents the OVA-Texo-induced expansion of exhausted CD45.2$^+$ mCTLs. Our data thus suggest that the OVA-Texo vaccine is capable of efficiently converting CTL exhaustion in chronic infection by significantly increasing the number of previously exhausted CTLs. In addition to the CTL increase in the peripheral blood, there were also ~4-fold more OVA-specific CTLs in the spleens and lungs following the boost (Figure 4c). Consistent with a previous report,[36] no significant change in the expression of inhibitory molecules (PD-1, PD-L1 and LAG3) could be observed on CTLs after the OVA-Texo boost (Figure 4a). We next measured the OVA-Texo-induced expression of an effector cytokine, IFN-γ, in exhausted CTLs on a 'per-cell' basis by intracellular staining of IFN-γ. We observed that 88% of the OVA-specific CTLs were IFN-γ$^+$ in OVA-Texo-treated mice, compared with only ~20% of IFN-γ-producing OVA-specific CTLs in PBS control mice (Figure 4d). In addition, we also used flow cytometry to measure the intracellular level of a useful marker for memory T-cell activation and functionality, diacetylated histone-H3 (diAcH3).[55] Our experiments revealed that OVA-Texo-stimulated, OVA-specific CTLs had increased diAcH3 levels (Figure 4a). Because in chronic infection, exhausted CTLs have a functional defect in their ability to lyse target cells (Figure 1d),[11] we attempted to determine whether OVA-Texo-converted CTLs had restored cytolytic effectiveness. To achieve this, we performed an *in vivo* cytotoxicity assay by transferring highly and lowly CFSE-labeled OVA-specific (H) and non-specific (L) target cells in a 1:1 ratio into OVA-Texo-immunized mice and monitoring the lysis of OVA-specific (H) target cells by flow cytometry. The results showed a significant increase in OVA-specific cytolytic responses (93.5% lysis) in OVA-Texo-treated mice compared with weak target cell lysis (13.5%) in untreated mice (Figure 4e). Taken together, our data indicate that the OVA-Texo vaccine efficiently converts CTL exhaustion by not only increasing the number of CTLs but also restoring their function.

OVA-Texo-induced conversion of CTL exhaustion is relatively independent of host CD4$^+$ T cells and completely independent of host DCs

We showed previously that our OVA-Texo vaccine stimulated OVA-specific CTL responses relatively independently of host CD4$^+$ T cells, but completely independent of host DCs in WT B6 mice.[23] To assess whether the OVA-Texo vaccine also converts CD4$^+$ T/DC-independent CTL exhaustion in chronic infection, we repeated our experiments in AdVova-infected B6 mice in the presence of anti-CD4 antibody treatment to deplete CD4$^+$ T cells or in AdVova-infected transgenic DTR-CD11c mice treated with diphtheria toxin (DT) to deplete CD11c$^+$ DCs, as we previously described.[41] We determined that the OVA-Texo vaccine stimulated slightly less CTL responses (6.0%) in anti-CD4 antibody-treated C57BL/6, but similar CTL responses (8.3%) in DT-treated DTR-CD11c mice with chronic infection 4 days post vaccination, compared with CTL responses in matching control mice (7.8% and 8.2%, respectively; Figure 4f). These data indicate that our OVA-Texo vaccine is capable of directly converting CTL exhaustion in the absence of CD4$^+$ T cells and DCs in chronic infection.

OVA-Texo-induced conversion of CTL exhaustion depends upon CD40L signaling

Because CD40L signaling by the OVA-Texo vaccine was found to be important for the counteraction of CD8$^+$ T-cell tolerance, we wanted to examine whether CD40L has a role in the conversion of CTL exhaustion by the OVA-Texo vaccine. Chronically AdVova-infected B6 mice with CTL exhaustion were immunized with the OVA-Texo(CD40L$^{-/-}$) vaccine, which lacks CD40L signaling. Interestingly, CD40L deficiency alone caused a significant decrease in the ability of the vaccine to induce T-cell proliferation; the induced proliferation went from a ~4-fold increase with OVA-Texo (2.70% vs 10.85%) down to no change with OVA-Texo(CD40L$^{-/-}$) (2.87% vs 3.25%) (Figure 5a), indicating that the OVA-Texo-induced conversion of CTL exhaustion occurs mainly via CD40L signaling. We also measured the OVA-Texo stimulation-induced expression of an effector cytokine, IFN-γ, in exhausted CTLs on a 'per-cell' basis. This revealed that 86% of the total OVA-specific CTL population produced IFN-γ in the OVA-Texo-treated mice, compared with only 26% of the OVA-specific CD8$^+$ T cells producing IFN-γ in the OVA-Texo(CD40L$^{-/-}$)-stimulated mice (Figure 5b), which was similar to the 22% of OVA-specific CD8$^+$ T cells producing IFN-γ in chronically infected control PBS-treated mice (Figure 4d). Our data thus indicate that CD40L signaling has a critical role in the conversion of CTL exhaustion by the OVA-Texo vaccine.

OVA-Texo converts CTL exhaustion through activation of the mTORC1 pathway

It has been shown that CD40L signaling assures T-cell activation via the mTORC1 pathway.[56] To assess whether the OVA-Texo vaccine activates the mTORC1 pathway, we analyzed CTLs by flow cytometry for the phosphorylation status and intracellular expression of a panel of molecules associated with the mTORC1 pathway, including Akt, mTORC1-regulated S6, eIF4E and T-bet. We determined that OVA-specific CTLs upregulated their levels of pAkt, pS6, peIF4E and T-bet as well as Ki67 (a protein associated with cell cycle progression) in chronically AdVova-infected B6 mice following OVA-Texo vaccination (Figure 5c). This indicates that the OVA-Texo vaccine stimulates the proliferation of exhausted CTLs at least in part by activating the PI3K/Akt/mTORC1 pathway. In addition, OVA-specific CTLs did not show any upregulation of mTORC1-related molecules in OVA-Texo (CD40L$^{-/-}$)-stimulated mice (data not shown), suggesting that OVA-Texo converts CTL exhaustion via the CD40L-induced activation of the mTORC1 pathway. Rapamycin is a well-described inhibitor for the mTORC1 pathway.[36] To assess

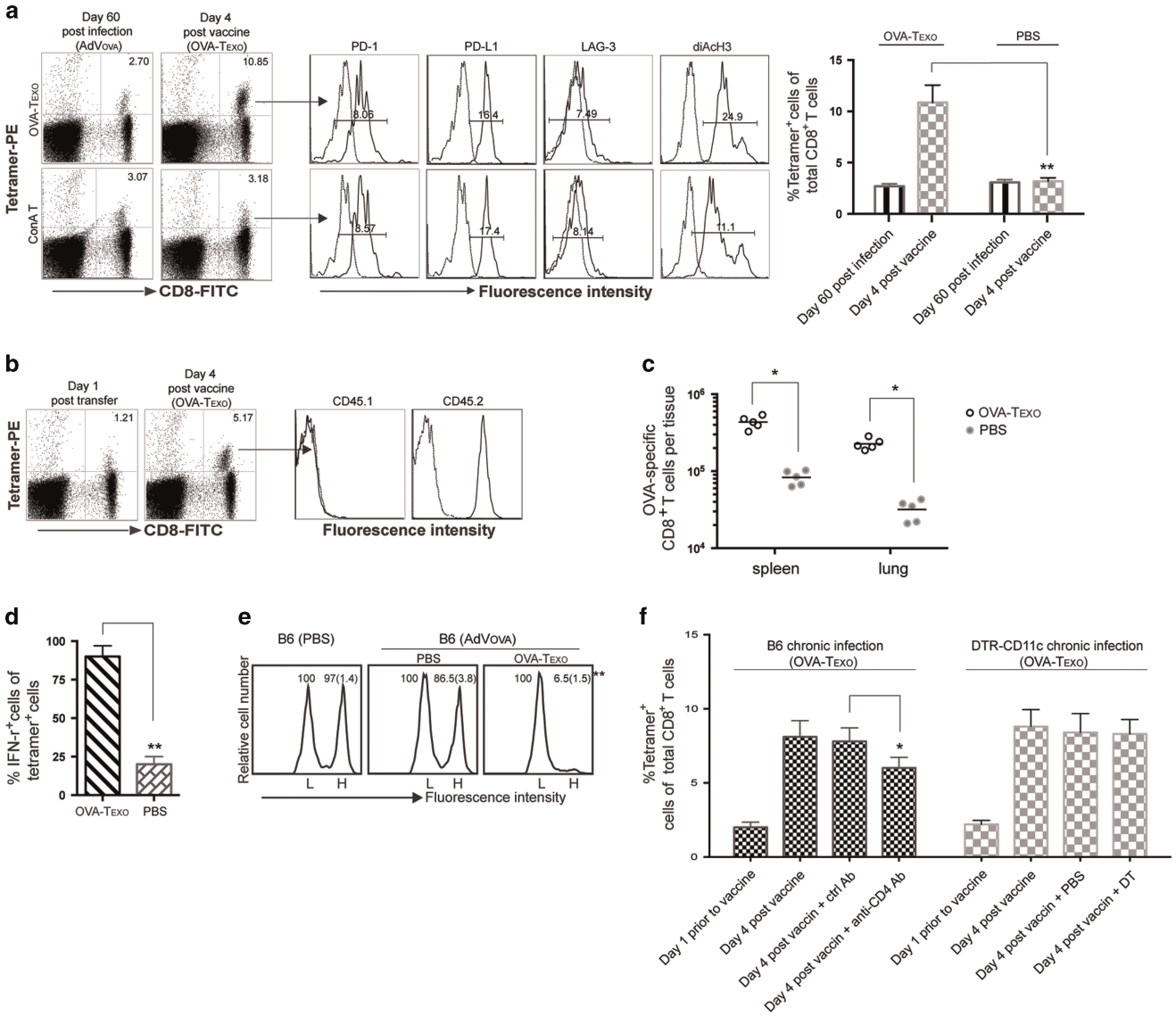

Figure 4 The OVA-Texo vaccine converts T-cell exhaustion. (**a**) OVA-specific CTL responses were analyzed on the indicated days by flow cytometry. OVA-specific $CD8^+$ T cells with positive PE-tetramer and FITC-CD8 staining were gated (arrow) for the further assessment of PD-1, PD-L1 and diAcH3 expression (solid lines on the right). The mean fluorescence intensities (MFI) are indicated. Dotted lines (on the left) represent isotype-matched controls. The value in each panel represents the percentage of PE-tetramer-positive $CD8^+$ T cells in the total $CD8^+$ T-cell population. (**b**) OVA-specific CTLs were purified from the splenocytes of chronically AdVova-infected B6 mice ($CD45.2^+$) using PE-tetramer staining followed by anti-PE microbeads and then transferred into B6.1 ($CD45.1^+$) mice with AdV_{Gal}-induced chronic infection. The transferred OVA-specific CTLs in the mice were assessed by flow cytometry 1 day post transfer. The mice were also boosted with the OVA-Texo vaccine and assessed for CTL responses 4 days post boost using triple staining for PE-tetramer, FITC-CD8 and PE-Cy5-CD45.1 or CD45.2 by flow cytometry. The OVA-specific $CD8^+$ T cells with positive PE-tetramer and FITC-CD8 staining were gated (arrow) for further measurement of the expression of CD45.1 and CD45.2 (solid lines on the right). Dotted lines (on the left) represent isotype-matched antibody controls. (**c**) The total number of PE-$tetramer^+$/FITC-$CD8^+$ T cells in the spleens and lungs following OVA-Texo immunization were analyzed by flow cytometry using splenocytes and lung tissue cell suspensions. (**d**) The percentage of IFN-γ producing cells in the PE-$tetramer^+$/FITC-$CD8^+$ T-cell population was also analyzed in the indicated conditions, as described in Figure 1c. (**e**) An *in vivo* cytotoxicity assay was performed in chronically AdVova-infected OVA-Texo-treated mice, as described in Figure 1d. The value in each panel represents the percentage of $CFSE^{high}$ vs $CFSE^{low}$ target cells remaining in the spleen. (**f**) AdVova-infected B6 mice with anti-CD4 antibody treatment or AdVova-infected DTR-CD11c mice with diphtheria toxin (DT) treatment were vaccinated with OVA-Texo 1 day after treatment. At day 4 following the vaccination, blood samples were collected, double stained with FITC-CD8 and PE-tetramer, and analyzed by flow cytometry. The percentages of PE-tetramer-positive $CD8^+$ T cells in the total $CD8^+$ T-cell population in anti-CD4 antibody- and DT-treated mice vs those in matching untreated control mice are indicated. $^*P<0.05$, $^{**}P<0.01$. Error bars represent the s.d. One representative experiment of two to three is shown.

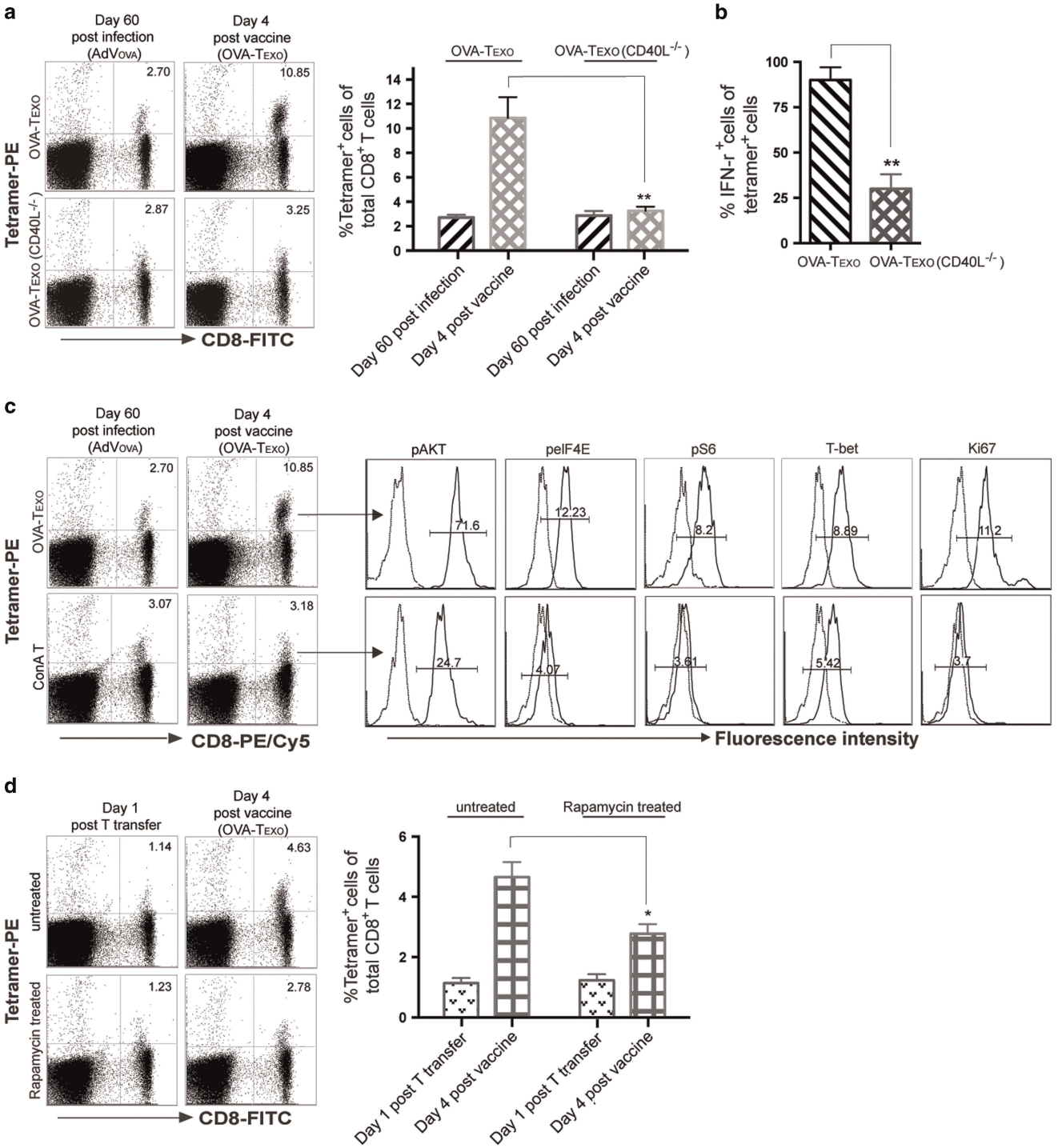

Figure 5 The OVA-Texo vaccine converts CTL exhaustion through CD40L signaling via the mTORC1 pathway. (**a**) AdVova-infected C57BL/6 mice were immunized with OVA-Texo 60 days after primary infection. Prior to and 4 days post immunization, mouse peripheral blood was analyzed for OVA-specific CTLs by flow cytometry. The value in each panel represents the percentage of PE-tetramer-positive $CD8^+$ T cells in the total $CD8^+$ T-cell population. (**b**) The percentage of IFN-γ producing cells in the PE-$tetramer^+$ and FITC-$CD8^+$ T-cell population was analyzed in each treatment group. (**c**) The mouse splenocytes from (**a**) were triple-stained with PE-Tetramer, FITC-CD8 and PE/Cy5-labeled Abs. The OVA-specific $CD8^+$ T cells with positive PE-tetramer and FITC-CD8 staining were gated (arrow) and assessed for the expression of pAKT, peIF4E, pS6, T-bet and Ki67 (solid lines on the right). The mean fluorescence intensity (MFI) numbers of solid lines are indicated. Dotted lines (on the left) represent isotype-matched controls. The MFI numbers of the dotted lines in the upper panels were similar to those in the lower panels. (**d**) Rapamycin-treated or untreated CTLs purified from chronically AdVova-infected B6 mice were transferred to B6 mice with AdV_{Gal}-induced chronic infection, followed by OVA-Texo vaccination 1 day post transfer. The OVA-specific CTLs were detected by flow cytometry. The value in each panel represents the percentage of PE-tetramer-positive $CD8^+$ T cells in the total peripheral $CD8^+$ T-cell population. $^*P<0.05$, $^{**}P<0.01$. Error bars represent the s.d. One representative experiment of two is shown.

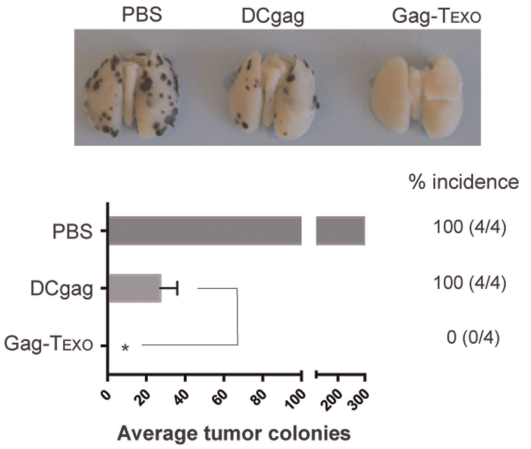

Figure 6 Gag-Texo vaccine induces therapeutic antitumor immunity in mice with chronic infection. AdVova-infected mice were i.v. injected with BL6-10_{Gag} cells. Three days later, the mice were vaccinated with Gag-Texo and DC_{Gag}. The mice were killed 3 weeks after tumor cell challenge, and lung-metastatic tumor colonies with black color were counted in the lungs. $^*P<0.05$. Error bars represent the s.d. One representative experiment of two is shown.

whether activation of the mTORC1 pathway is responsible for OVA-Texo-induced conversion of CTL exhaustion, we treated exhausted CTLs purified from chronically infected mice with rapamycin and transferred them into chronically AdV_{Gal}-infected B6 mice, followed by OVA-Texo vaccination. Interestingly, rapamycin treatment significantly reduced the efficiency of the OVA-Texo boost from the original ~4-fold (4.63% vs 1.14%) down to only ~2-fold (2.78% vs 1.23%) (Figure 5d). Our data thus confirm that OVA-Texo directly converts CTL exhaustion either mostly or at least partly through the activation of the mTORC1 pathway.

Gag-Texo vaccine induces Gag-specific therapeutic immunity in a chronic infection model

To assess potential therapeutic immunity in this model, chronically AdVova-infected B6 mice were injected with Gag-expressing BL6-10_{Gag} melanoma cells. Three days after tumor cell challenge, the chronically infected mice were immunized with the Gag-Texo or DC_{Gag} vaccine. Notably, we determined that Gag-Texo, but not the DC_{Gag} vaccine, completely eradicated all established BL6-10_{Gag} lung metastases, even in the presence of chronic infection (Figure 6), indicating that our Gag-Texo vaccine is capable of inducing therapeutic immunity against established Gag-expressing tumors in AdVova-induced chronic infection.

DISCUSSION

Vaccines based on the recombinant human E1/E3-depleted adenovirus-5 (AdV-5) vector are well characterized and have been shown to be effective at inducing $CD8^+$ CTL responses due to their strong immunogenicity.[57] Adenoviral vectors are easy to manipulate to infect different types of cells, and their safety is well defined because the vector genome does not integrate into the cellular DNA.[58] However, adenoviral transgene expression could still persist in a host at low levels for a relatively long time[43,44,46] and even for one year post-AdV infection.[43,44] Exhausted CTLs, resulting from excessive or persistent Ag stimulation, often present a significant barrier to immune responses and managing chronic infection.[59] For example, in the exhausted state of LCMV-infected mice, LCMV-specific CTLs become subject to multiple inhibitory signals, such as PD-1, LAG-3, CD160 and 2B4, and lose their functional effectiveness in a stepwise fashion.[12,60] In this study, we i.v. infected C57BL/6 mice with our OVA-expressing AdVova and phenotypically and functionally characterized OVA-specific CTLs. We observed mCTL expansion and mCTLs with defects in proliferation, IFN-γ expression and cytotoxicity in the presence of AdVova-induced chronic infection, indicating that these OVA-specific mCTLs were functionally exhausted. These exhausted mCTLs upregulated their expression of inhibitory PD-1, PD-L1 and LAG-3, which is consistent with the CTL inflation and exhaustion observed in an LCMV clone 13-induced chronic infection model.[12,60] Therefore, our AdVova-infected mice may be utilized as a new chronic infection model to experimentally evaluate immunotherapeutics against chronic infection because of the commercial availability of the PE-H-$2K^b/OVA_{257-264}$ tetramer, an immune reagent, allowing the detection of OVA-specific CTLs by flow cytometry.

A chronic infection with persisting pathogens can impair immune responses to unrelated infections and vaccines, and a dysfunctional immunity (a type of well-known bystander immunity in chronic infection) might be responsible for the increased susceptibility to various co-infections.[49] However, our current understanding of the bystander chronic infection is mainly based upon epidemiological evidence, with limited insight into the molecular mechanisms. DC defects in maturation, cytokine production and antigen presentation[61–63] and T-cell defects in proliferation[64–66] have been reported in animal models and humans with chronic infections. However, it is not clear whether the T-cell proliferation defect is intrinsic to T cells themselves or is derived from the functional deficiency of DCs. The upregulation of the inhibitory molecule PD-L1 was originally observed on splenocytes from chronically LCMV-infected mice, but the subset of immune cells expressing PD-L1 in splenocytes was not defined.[36] Later, reports confirmed that PD-L1 expression was high in the spleen tissues, especially on spleen $CD11b^+F4/80^+$ macrophages and $B220^+CD19^+$ B cells, but low in the bone marrow.[67] In our study, we demonstrate that naive $CD8^+$ T lymphocytes upregulate their expression of the inhibitory molecule PD-L1 and the immunoglobulin-like co-inhibitory receptor BTLA[68] and overexpress T-cell anergy-associated Grail and Itch molecules,[69] suggesting that these cells may become anergic in the presence of chronic infection. In addition, we show that during chronic infection, naive $CD8^+$ T cells also express cell surface CD40, which is absent on WT naive $CD8^+$ T cells. Naive $CD8^+$ T-cell anergy in our chronic infection model is further confirmed by showing that these naive $CD8^+$ T cells

have some degree of proliferation defect both in *in vitro* and *in vivo*. Therefore, our study is the first report demonstrating that naive $CD8^+$ T-cell anergy is responsible for a naive T-cell proliferation defect during chronic infection, thus revealing a new mechanism of $CD8^+$ T-cell anergy during the well-known bystander immunity in chronic infection. Nevertheless, the molecular mechanism for naive T-cell anergy in chronic infection needs to be further elucidated. Interestingly, our OVA-Texo vaccine, but not the DCova vaccine, is capable of counteracting naive T-cell anergy associated with chronic infection.

Co-stimulation with CD40L and inflammatory cytokines such as IL-2 and TNF-α has been reported to counteract T-cell anergy and tolerance.[53] To assess a potential molecular mechanism for the counteraction of T-cell anergy by OVA-Texo, we prepared a panel of OVA-Texo vaccines, OVA-Texo(IL-$2^{-/-}$), OVA-Texo(TNF-$\alpha^{-/-}$) and OVA-Texo ($CD40L^{-/-}$), which are deficient in IL-2, TNF-α or CD40L, respectively, and compared the OVA-specific CTL responses in AdV-infected B6 mice vaccinated with each of these preparations. We demonstrate here that the deficiency in CD40L alone, but not in other signaling components of the OVA-Texo vaccine, nearly completely abolishes its stimulatory effect on T-cell proliferation in our model of chronic infection, indicating that CD40L signaling has a central role in counteracting naive $CD8^+$ T-cell anergy. This was also supported by our experiments, showing that treatment with an antagonistic anti-CD40L antibody significantly reduced the stimulatory effect of OVA-Texo on T-cell proliferation. When considered together with our new finding that during chronic infection, naive $CD8^\pm$ T cells express cell surface CD40, these data allow us to speculate that CD40L has a central role in counteracting the anergy of naive CD40-expressing $CD8^\pm$ T cells via CD40/CD40L-initiated signaling.

CD40 signaling was originally found to activate and license DCs that then provide second signals to naïve $CD8^+$ T cells upon antigen presentation.[70–72] We have previously demonstrated that CD40L signaling by our OVA-Texo vaccine did not enhance CTL priming, but promoted memory CTL development in WT B6 mice.[39] To assess a potential role of co-stimulatory CD40L-initiated signaling in the OVA-Texo-induced conversion of CTL exhaustion, we immunized AdVova-infected mice with OVA-Texo($CD40L^{-/-}$) cells lacking CD40L. We demonstrate that the absence of CD40L in the OVA-Texo vaccine strongly decreases its stimulating effect on CTL proliferation and its converting effect on CTL exhaustion in chronic infection, indicating that CD40L signaling has a crucial role in converting CD40-expressing CTL exhaustion in chronic infection. Therefore, we speculate that the OVA-Texo-induced conversion of CTL exhaustion occurs through breaking exhaustion via CD40L signaling, leading to an increased number of CTLs. Combined stimulation with exosomal OVA-specific peptide/MHC-I (pMHC-1), CD80 molecules, and cellular cytokines derived from OVA-Texo cells may contribute to the stimulation of CTLs independent of host DCs and $CD4^\pm$ T-cell help. Importantly, our data suggest that our Gag-specific T-cell-based vaccine may also convert CTL exhaustion in HIV-1 patients with dysfunctional DCs and $CD4^\pm$ T-cell deficiency.[7–10]

The inhibitory PD-1 molecule is expressed on active T cells following TCR engagement and then declines. During chronic infection, PD-1 expression is sustained on CTLs due to persistent antigen stimulation, leading to CTL exhaustion. It has been demonstrated that PD-1 suppresses T-cell functions by antagonizing TCR signaling by recruiting phosphatases[73,74] and modulating the Ras and the PI3K/Akt/mTORC1 pathways regulating T-cell cycle, metabolism, proliferation and effector function,[75,76] leading to CTL exhaustion. It has also been shown that CD40L stimulates T-cell activation by inducing the recruitment of adaptor proteins, including the TNF receptor-associated factor, leading to the activation of the PI3K/Akt/mTORC1 pathway.[56] To assess whether the OVA-Texo vaccine activates the PI3K/Akt/mTORC1 pathway, we analyzed CTLs for the intracellular expression of molecules associated with this signaling cascade. Our experiments demonstrate that after immunization of chronically AdVova-infected mice with the OVA-Texo vaccine, the OVA-specific CTLs upregulate the expression or phosphorylation of pI3K, mTORC1-regulated pS6, peIF4E and T-bet, which control T-cell activation, and increase the expression of a protein associated with cell cycle progression, Ki67. This strongly suggests that the conversion of CTL exhaustion by the OVA-Texo vaccine may occur via the activation of the PI3K/Akt/mTORC1 pathway. This is further supported by our data showing that the treatment of CTLs purified from chronically infected mice with an inhibitor of the mTORC1 pathway, rapamycin, significantly reduces their proliferation upon the OVA-Texo boost. Our data thus provide the first evidence that our novel T-cell-based vaccine is capable of converting CTL exhaustion in chronic infection, either mostly or at least in part via its CD40L-induced signaling through the mTORC1 pathway.

$CD8^+$ CTLs are important effector T cells capable of directly destroying HIV-1-infected cells, and they have a critical role in controlling HIV-1 proliferation and disease progression even in the absence of neutralizing antibodies.[77,78] Recently, efforts to purge latent reservoirs have focused on reactivating latent proviruses without inducing global T-cell activation.[79] Stimulation of HIV-1-specific CTLs has been reported to facilitate the elimination of latent viral reservoirs.[80,81] In this study, we demonstrated that HIV-1 Gag-specific, exosome-targeted, T-cell-based vaccine Gag-Texo is capable of inducing therapeutic immunity against Gag-expressing tumors during chronic infection, suggesting that our Gag-Texo vaccine may also induce the responses of CTLs that destroy HIV-1-infected cells. Humanized mouse models with HIV-1 infection, which mimic the immunopathology in HIV-1-infected human patients, have become useful for studying the mechanisms of HIV-1 immunopathogenesis and for developing novel immune-based therapies.[82,83] We are also planning to develop an HIV-1-infected humanized mouse model to further assess the potential therapeutic efficacy of our Gag-Texo vaccine.

In summary, our data demonstrate that our novel antigen-specific, exosome-targeted, T-cell-based vaccine is capable of counteracting the anergy of naive CD40-expressing $CD8^+$ T cells and converts the exhaustion of CD40-expressing CTLs independent of DCs and $CD4^+$ T cells. It achieves these effects by acting through CD40L-triggered signaling, inducing the activation of the mTORC1 pathway and thus efficiently stimulating Gag-specific therapeutic immunity in the presence of chronic infection. Therefore, this study is likely to produce a strong impact on the development of new therapeutic vaccines against HIV-1 and other chronic infectious diseases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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