# Transgene IL-21-Engineered T Cell-Based Vaccine Potently Converts CTL Exhaustion via the Activation of the mTORC1 Pathway in Chronic Infection

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**How to cite this paper:** Xu, A.Z., Zhang, X.Y., Chibbar, R., Freywald, A., Tikoo, S., Zheng, C.Y. and Xiang, J. (2019) Transgene IL-21-Engineered T Cell-Based Vaccine Potently Converts CTL Exhaustion via the Activation of the mTORC1 Pathway in Chronic Infection. *World Journal of Vaccines*, **9**, 1-21.
https://doi.org/10.4236/wjv.2019.91001

**Received:** November 6, 2018
**Accepted:** December 22, 2018
**Published:** December 25, 2018



## Abstract

$CD8^+$ cytotoxic T lymphocyte (CTL) exhaustion is one of the major obstacles for the effectiveness of virus control in chronic infectious diseases. We previously generated novel ovalbumin (OVA)-specific 41BBL-expressing OVA-$T_{EXO}$ and human immunodeficiency virus (HIV-1) Gag-specific Gag-$T_{EXO}$ vaccines, inducing therapeutic immunity in wild-type C57BL/6 (B6) mice, and converting CTL exhaustion in recombinant OVA-specific adenovirus $AdV_{OVA}$-infected B6 ($AdV_{OVA}$-B6) mice with chronic infection. IL-21 cytokine plays an important role in controlling chronic infections. Therefore, in this study, we constructed recombinant transgene IL-21-expressing $AdV_{IL\text{-}21}$, and generated IL-21-expressing OVA-$T_{EXO/IL\text{-}21}$ and Gag-$T_{EXO/IL21}$ vaccines, or control vaccines (OVA-$T_{EXO/Null}$ and Gag-$T_{EXO/Null}$) by infecting OVA-$T_{EXO}$ and Gag-$T_{EXO}$ cells with $AdV_{IL\text{-}21}$ or the control $AdV_{Null}$, lacking transgene, and assessed their effects in B6 or $AdV_{OVA}$-B6 mice. We demonstrate that both OVA-$T_{EXO/IL\text{-}21}$ and control OVA-$T_{EXO/Null}$ vaccines are capable of converting CTL exhaustion in chronic infection. However, the OVA-$T_{EXO/IL\text{-}21}$ vaccine more efficiently rescues exhausted CTLs by increasing stronger CTL proliferation and effector cytokine IFN-$\gamma$ expression than the control OVA-$T_{EXO/Null}$ vaccine in $AdV_{OVA}$-B6 mice with chronic infection, though both vaccines stimulated comparable OVA-specific CTL responses and protective immunity

*Xu and Zhang made the same contribution to the work.

against OVA-expressing BL6-$10_{OVA}$ melanoma lung metastasis in wild-type B6 mice. *In vivo,* the OVA-$T_{EXO/IL-21}$-stimulated CTLs more efficiently up-regulate phosphorylation of mTORC1-controlled EIF4E and expression of mTORC1-regulated T-bet molecule than the control OVA-$T_{EXO/Null}$-stimulated ones. Importantly, the Gag-$T_{EXO/IL21}$ vaccine induces stronger Gag-specific therapeutic immunity against established Gag-expressing BL6-$10_{Gag}$ melanoma lung metastases than the control Gag-$T_{EXO/Null}$ vaccine in chronic infection. Therefore, this study should have a strong impact on developing new therapeutic vaccines for patients with chronic infections.

## Keywords

IL-21, Chronic Infection, CTL Exhaustion, Exosome, T Cell Vaccine

## 1. Introduction

During acute viral infections, both innate and adaptive immunity work together contribute to the clearance of the pathogens [1]. Many acute infections stimulate massive $CD8^+$ cytotoxic T lymphocyte (CTL) responses that play an important role in controlling invading viruses and these responses are divided into three phases: expansion, contraction and memory [1]. In the expansion phase, the infectious pathogen triggers proliferation of effector CTLs cytolytic to virus-infected cells. This is followed by the contraction phase, where 90% - 95% of effector CTLs die of apoptosis induced by cytolytic granzyme-B (GB)-mediated lethal hit, which is produced by regulatory T (Treg) cells [2]. Typically, only 5% - 10% of the expanded ensemble of CTLs survive and proceed in the final stage to constitute the long-term memory CTLs capable of turning on rapid responses, when re-encounting the same pathogen [1].

Although CTLs are effective in controlling acute viral infections, they often become functionally deficient or exhausted in chronic infections due to persistent pathogen presence [3]. The common characteristic of chronic infections, such as human immunodeficiency virus [4], hepatitis C virus (HCV) or hepatitis B virus (HBV) is that anti-virus CTLs are initially stimulated, but later become quantitatively and qualitatively defective leading to a stepwise progression of functional exhaustion and incapability of clearing pathogens [3]. Phenotypically, these exhausted CTLs express some immune inhibitory molecules, such as programmed death-1 (PD-1), T-cell Ig and mucin protein-3 (TIM-3), and lymphocyte-activation gene 3 (LAG-3) [3] [5] [6]. This makes CTLs ineffective in the stimulation-induced i) production of cytokines, including IL-2 and IFN-$\gamma$ [7], ii) cell proliferation, and iii) cytolytic effect on virus-infected cells [8] [9] [10] [11]. Severe CTL exhaustion often strongly correlates with high viral loads [12]. It has been found that longer duration of the chronic infection or severe loss of $CD4^+$ T cell help often leads to more serious CTL exhaustion [1] [13], and the final stage of CTL exhaustion often results in depletion of virus-specific CTLs [8] [10].

Consistent with this, the CTL exhaustion is one of the major reasons for ineffective HIV control in infected patients.

Mice with lymphocytic choriomeningitis virus (LCMV) infection are frequently used for investigating LCMV-specific CTL responses, since the nature of the virus makes it an excellent mouse infection model. For example, different LCMV strains can cause distinct viral infections both acute and chronic. The Armstrong strain induces an acute viral infection, whereas LCMV clone 13 infection results in viremia that can last up to 3 months with virus persisting in the brain and kidneys [10], leading to a chronic viral infection [14]. Interestingly, these two strains only differ from each other in 2 amino acids, but preserve all epitopes for T-cell receptors, thus allowing us to easily compare CTL responses between dominant and subdominant LCMV viral epitopes [15] [16]. In addition, the latter strain has been extensively applied to study the dynamics of CTL responses and CTL exhaustion, as well as to assess immune therapeutics for the conversion of CTL exhaustion in chronic infections [14] [17]. We have recently established an adenovirus-induced chronic infection model by i.v. infection of C57BL/6 mice with a recombinant adenovirus ($AdV_{OVA}$) expressing ovalbumin (OVA). Similar to the situation in the LCMV clone 13-induced chronic infection, our mice with the $AdV_{OVA}$-induced chronic infection demonstrated OVA-specific $CD44^+PD\text{-}1^+LAG\text{-}3^+$ memory CTL (mCTL) inflation. These mCTLs were also functionally defective and exhausted [18]. We also found that the PD-1 blockade efficiently converts CTL exhaustion in the OVA-specific chronic infection model [19].

IL-21 cytokine was originally found to be produced by $CD4^+$ T cells [20] and to serve as a "third" signal, functioning in concert with T cell receptor (TCR) activation and T cell co-stimulation to trigger $CD8^+$ T cell responses [21]. Recently, it has been shown that IL-21 plays a significant role in controlling chronic LCMV infection [22] [23] [24]. IL-21 enhances cytolytic and virus control abilities of HIV-specific CTLs in vitro [25] [26], and enhances the viral control in immunodeficiency virus [4]-infected rhesus macaques and HCV- and HIV-1-infected patients [7] [27] [28].

We previously developed a novel ovalbumin (OVA)-specific exosome (EXO)-targeted T cell-based ($OVA\text{-}T_{EXO}$) vaccine by using non-specific polyclonal T cells with the uptake of OVA-specific dendritic cell (DC)-released EXO *via* the CD54/LFA-1 interaction [29]. We demonstrated that the OVA-Texo vaccine was able to directly stimulate potent OVA-specific CTL responses in the absence of $CD4^+$ T cell help by counteracting $CD4^+25^+FoxP3^+$ regulatory T (Treg) cell suppression [29] [30]. We also developed an HIV-1 Gag-specific T cell-based vaccine, $Gag\text{-}T_{EXO}$, by using non-specific polyclonal T cells with the uptake of Gag-specific DC-released EXO and demonstrated that the $Gag\text{-}T_{EXO}$ vaccine triggered potent Gag-specific immunity against Gag-expressing tumors in transgenic HLA-A2 mice [31]. To enhance its immunogenicity, we generated 4-1BBL-expressing $OVA\text{-}T_{EXO}$ and $Gag\text{-}T_{EXO}$ vaccines, and demonstrated that

the former one triggered potent therapeutic immunity [32]. It also induced an efficient conversion of CTL exhaustion via its CD40L-dependent signaling activation of the mTORC1 pathway in chronic infection models [18].

In this study, we constructed a recombinant adenovirus ($AdV_{IL-21}$) expressing mouse IL-21 and generated new OVA-$T_{EXO/IL-21}$ and Gag-$T_{EXO/IL-21}$ vaccines engineered to express IL-21 by infection of the above OVA-$T_{EXO}$ and Gag-$T_{EXO}$ cells with $AdV_{IL-21}$ as previously described [32]. We assessed the effectiveness of the OVA-$T_{EXO/IL-21}$ vaccine in the conversion of CTL exhaustion and examined the effectiveness of the Gag-$T_{EXO/IL-21}$ vaccine in therapeutic immunity against Gag-expressing tumors in chronic infection model. We found that the OVA-$T_{EXO/IL-21}$ vaccination stronger rescued CTL exhaustion than the OVA-$T_{EXO}$ vaccine in chronic infection, while Gag-$T_{EXO/IL-21}$ vaccination triggered more potent therapeutic immunity against established Gag-expressing BL6-$10_{Gag}$ tumor lung metastases, when compared to the Gag-$T_{EXO}$ vaccine in chronic infection.

## 2. Materials and Methods

### 2.1. Reagents, Cell Lines and Animals

Chicken ovalbumin (OVA) was obtained from SIGMA-Aldrich Canada Ltd, Oakville, Ontario, Canada. Phycoerythrin (PE)-labeled anti-IFN-$\gamma$ antibody (Ab) was obtained from BD Biosciences (Mississauga, ON, Canada). Biotin-labeled anti-T-bet, anti-phosphor-EIF4E (S209), anti-CD44, anti-PD-1, anti-PD-L1 and anti-LAG3 Abs were obtained from eBioscience (San Diego, CA). PE-labeled H-$2K^b$/$OVA_{257-264}$ tetramer (PE-tetramer) and Fluorescein isothiocyanate (FITC)-labeled anti-CD8 Ab were obtained from Beckman Coulter (Miami, FL). Rabbit anti-CD9 and anti-LAMP-1 Abs were obtained from BD Bioscience. The H-$2K^b$-restricted OVAI ($OVA_{257-264}$, SIINFEKL) peptide and the control Lewis lung carcinoma H-$2K^b$-restricted MutI (FEQNTAQP) peptide were obtained from Multiple Peptide Systems (San Diego, CA). All cytokines were obtained from Peprotech (Peprotech, Rocky Hill, NJ). Recombinant adenoviral vectors, including transgene OVA-expressing $AdV_{OVA}$, transgene Gag-expressing $AdV_{Gag}$ and the no transgene-expressing $AdV_{Null}$ were available in our laboratory [18] [32]. Adenoviral vector $AdV_{IL-21}$ expressing mouse IL-21 was constructed by the insertion of IL-21 open reading frame of pUNO1-mIL-21 vector (InVivoGen, San Diego, CA) into the pShuttle-CMV vector (Stratagene, La Jolla, CA) by recombinant technology as we previously described [18] [32]. The Pmel-digested pShuttle-CMV-mIL-21 was transformed into BJ5183 *Escherichia coli* cells containing pAdEasy-1 backbone vector for the homologous recombination [18] [32]. The recombinant AdV vector was linealized by PacI digestion and transfected into HEK-293 cells using Lipofectamine 2000 (Invitrogene, Carlsbad, CA) to generate AdVIL-21 expressing transgene IL-21 [18] [32]. $AdV_{IL-21}$ was then amplified in HEK-293 cells and purified by a series of cesium chloride ultracentrifugation [18] [32]. The highly lung metastatic, OVA- and Gag-expressing BL6-$10_{OVA}$ and BL6-10Gag mouse B16 melanoma cell lines were

available in our laboratory [31] [32] [33]. IL-21 enzyme-linked immunosorbent assay (ELISA) kit was obtained from R & D Systems Inc, Minneapolis, MN. Female 6- to 8-week-old C57BL/6 (B6) (18 - 20 g) and OVA-specific T cell receptor (TCR) transgenic OTI mice were obtained from the Jackson Laboratory (Bar Harbor, MA). All experiments were performed according to protocols and guidelines approved by the Animal Research Ethics Board, University of Saskatchewan (Protocol No. 20130020).

### 2.2. Preparation of Dendritic Cells and Dendritic Cell-Released Exosomes

Bone marrow-derived dendritic cells (DCs) were obtained by culturing bone marrow cells of wild-type (WT) B6 mice in culture medium containing granulocyte monocyte colony-stimulating factor (GM-CSF) (20 ng/ml) and IL-4 (20 ng/ml) for six days, as previously described [29]. DCs were pulsed with OVA (0.5 mg/ml) for overnight and termed $DC_{OVA}$. $DC_{OVA}$ stained with various Abs were analysed by flow cytometry. $DC_{OVA}$-released exosomes ($EXO_{OVA}$) were then purified from $DC_{OVA}$ culture supernatants by differential ultracentrifugation [29]. In addition, DCs were also infected with $AdV_{Gag}$ (100 pfu/cell) for overnight in a humidified 37˚C, 5% $CO_2$ incubator, and termed $DC_{Gag}$. $DC_{Gag}$-released EXOs were purified from $DC_{Gag}$ culture supernatants by ultracentrifugation and termed $EXO_{Gag}$.

### 2.3. Preparation of ConA-Stimulated Polyclonal T Cells

Polyclonal naïve $CD8^+$ T cells were isolated from WT B6 mouse spleens, enriched by passage through nylon wool columns, and then purified by negative selection using anti-mouse CD4 paramagnetic beads (Life Technologies, Waltham, MA), as previously described [34] [35]. Purified polyclonal $CD8^+$ T cells were cultured for 3 days in RPMI 1640 medium, containing IL-2 (20 U/ml) and ConA (1 μg/ml). $CD8^+$ T cells were then purified from ConA-activated T cells using MACS anti-CD8 microbeads (Miltenyi Biotech, Auburn, CA) to yield $CD8^+$ T cell populations with >95% purity [29], and termed ConA-T cells.

### 2.4. Preparation of the OVA-$T_{EXO/IL\text{-}21}$ and Gag-$T_{EXO/IL\text{-}21}$ Vaccines and the Control OVA-$T_{EXO/Null}$ and Gag-$T_{EXO/Null}$ Vaccines

The OVA-$T_{EXO}$ and Gag-$T_{EXO}$ vaccines were generated by the incubation of $CD8^+$ ConA-T cells with $EXO_{OVA}$ or $EXO_{Gag}$ at $3 \times 10^6$ cells/10 μg for 1 hour at 37˚C, followed by the transfection of T cells with the uptake of $EXO_{OVA}$ or $EXO_{Gag}$ with $AdV_{41BBL}$ at 100 pfu/cell for another 2 hours to form the vaccines, as previously described [32]. To prepare the OVA-$T_{EXO/IL\text{-}21}$ or Gag-$T_{EXO/IL\text{-}21}$ and the control OVA-$T_{EXO/Null}$ or Gag-$T_{EXO/Null}$ vaccines, the above OVA-$T_{EXO}$ or Gag-$T_{EXO}$ cells were further transfected with $AdV_{IL\text{-}21}$ or $AdV_{Null}$ [100 pfu/cell] for 2 hours to form OVA-$T_{EXO/IL\text{-}21}$ or Gag-$T_{EXO/IL\text{-}21}$ and the control OVA-$T_{EXO/Null}$ or Gag-$T_{EXO/Null}$ vaccine respectively, as we previously described [32].

### 2.5. Establishment of $AdV_{OVA}$-Induced Chronic Infection Animal Model

To develop a chronic infection model, B6 mice (4/group) were intravenously (i.v.) injected with anti-CD4 Ab (300 μg/mouse) to deplete $CD4^+$ T cells, followed by i.v. infection of mice with $AdV_{OVA}$ ($1 \times 10^6$ pfu/mouse) one day after the anti-CD4 Ab treatment performed for $CD4^+$ T cell depletion. This experimental procedure was aimed to develop a more stringent mouse model of chronic infection, since $AdV_{OVA}$ infection of mice was performed under the condition of $CD4^+$ T cell depletion such that the 'helpless' $CD8^+$ T cells activated by $AdV_{OVA}$ infection became functionally exhausted with severe defects in IFN-$\gamma$ production and cytotoxicity [36]. These mice were chronically $AdV_{OVA}$-infected B6 ($AdV_{OVA}$-B6) mice and termed $AdV_{OVA}$-B6 mice with chronic infection. To develop an acute infection model, B6 mice (4/group) were i.v. infected with recombinant OVA-expressing rLmOVA bacteria [2000 colony forming unit (cfu)/mouse]. These mice were termed rLmOVA-B6 mice. After infections, OVA-specific $CD8^+$ CTL responses in $AdV_{OVA}$-B6 and rLmOVA-B6 mice were kinetically analyzed by flow cytometry.

### 2.6. Flow Cytometric Analysis

Peripheral blood samples derived from $AdV_{OVA}$-B6 and rLmOVA-B6 mice were double stained with FITC-CD8 Ab and PE-tetramer and analyzed by flow cytometry to assess $CD8^+$ T cell responses at different days after infection. To increase the amount of OVA-specific $CD8^+$ memory T (Tm) cells formed 30 days or over 30 days post infection, such that phenotypes of OVA-specific $CD8^+$ Tm cells can be more accurately analyzed, B6 mice were infected with an increased amount of $AdV_{OVA}$ ($1.2 - 1.4 \times 10^6$ pfu/mouse) for the development of chronic infection $AdV_{OVA}$-B6 mice, while B6 mice with prior i.v. injection of naïve $CD8^+$ T cells ($1 \times 10^3$/mouse) purified from OTI mouse spelnocytes were infected with rLmOVA bacteria for the development of acute infection rLmOVA-B6 mice [18]. To assess the phenotype of OVA-specific $CD8^+$ Tm cells, peripheral blood samples derived from $AdV_{OVA}$-B6 and rLmOVA-B6 mice 60 days post the primary infection were triply stained with FITC-CD8 Ab, PE-tetramer and PE-Cy5-Abs for various immune molecules, and then analyzed by flow cytometry. To assess the conversion of CTL exhaustion, $AdV_{OVA}$-B6 mice with chronic infection were i.v. immunized with OVA-$T_{EXO/IL\text{-}21}$ or the control OVA-$T_{EXO/Null}$ vaccine ($1 \times 10^6$ cells/mouse), followed by the analysis of OVA-specific $CD8^+$ T cell proliferation and phenotypes 4 days post immunization. To stain the intracellular molecules, mouse splenocytes derived from $AdV_{OVA}$-B6 and rLmOVA-B6 mice were first incubated with FcR blocking anti-16/32 Ab (eBioscience) for 30 min on ice for eliminating any nonspecific staining. The cells were then stained with FITC-CD8 Ab and PE-tetramer, followed by the fixation and permeabilization with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instruction. The cells were further stained with PE-Cy5-antibodies

specific for various molecules such as EIF4E and T-bet, and the expression of these molecules was assessed by flow cytometry. For intracellular staining of IFN-$\gamma$, mouse splenocytes were first incubated in culture medium containing OVAI peptide (2 μg/ml) and Golgi-stop (0.7 μg/ml) (BD Biosciences) at 37˚C for 5 hrs, followed by the incubation with 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 of IFN-$\gamma$ was conducted using PE-Cy5-anti-IFN-$\gamma$ Ab, and the expression of IFN-$\gamma$ was assessed by flow cytometry. Data were acquired by CytoFlex (Beckman Coulter) and analyzed with FlowJo software (TreeStar, San Diego, CA).

### 2.7. Western Blot Analysis

EXO (10 μg/well) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred onto the nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked with 5% bovine serum albumin (BSA) in PBS, immunoblotted with rabbit anti-CD9 or anti-LAMP-1 Ab, followed by the incubation with anti-rabbit IRDyeR800CW Ab, and then, scanning with the ODYSSEY imager according to manufacturer's instruction (Li-COR Bioscience, Lincoln, NE).

### 2.8. Electron Microscopy

EXO were fixed in 4% paraformaldehyde. The pellets were loaded onto carbon-coated formvar grids. The exosome sample-loaded grids were stained with saturated aqueous uranyl and then, examined with a JEOL 1200EX electron microscope at 60 kV.

### 2.9. Animal Studies

To examine the antitumor immunity conferred by the OVA-$T_{EXO/IL\text{-}21}$ vaccine, WT B6 mice (6/group) were i.v. immunized with OVA-$T_{EXO/IL\text{-}21}$ cells or the control OVA-$T_{EXO/Null}$ ($1 \times 10^6$ cells/mouse). Mice were i.v. injected with $0.5 \times 10^6$ BL6-$10_{OVA}$ cells six days post immunization. To assess therapeutic immunity of the Gag-$T_{EXO/IL\text{-}21}$ vaccine, chronic infection $AdV_{OVA}$-B6 mice (n = 6) with CTL inflation and functional exhaustion were first injected i.v. with $0.5 \times 10^6$ BL6-$10_{Gag}$ cells. Three days after tumor cell inoculation, AdVova-B6 mice were i.v. immunized with Gag-$T_{EXO/IL\text{-}21}$ or the control Gag-$T_{EXO/Null}$ cells ($1 \times 10^6$ cells/mouse). The mice were sacrificed 3 weeks after tumor cell injection, and the lung metastatic B16 melanoma colonies were counted in a blind fashion. Metastatic B16 melanoma colonies on freshly isolated lungs appeared as black color foci that were easily distinguishable from normal lung tissues. Metastasis was also confirmed by histological examination.

### 2.10. Statistical Analyses

Statistical analyses were performed with the Mann-Whitney *U* test using Prism

software (GraphPad Software, San Diago, CA) to compare variables of different groups in animal studies or with the Student *t* test to compare variables of different groups in flow cytometric analysis [32]. Unless stated otherwise, data are expressed as mean (with SD). A value of $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Preparation of the Transgene IL-21-Engineered T Cell-Based OVA-$T_{EXO/IL-21}$ Vaccine

We constructed a replication-deficient transgene IL-21-expressing recombinant adenovirus $AdV_{IL-21}$ by recombinant DNA technology as we described in MATERIALS & METHODS (**Figure 1(a)**) [18] [32]. B6 mouse dendritic cells were generated by culturing B6 mouse bone marrow cells in the culture medium containing GM-CSF and IL-4 for six days, pulsed with OVA for overnight and termed $DC_{OVA}$ [18] [32]. OVA-specific exosomes ($EXO_{OVA}$) were purified from $DC_{OVA}$ culture supernatants by ultracentrifugation, and analyzed by electron microscopy and Western blotting analyses, respectively. We demonstrated that $EXO_{OVA}$ had exosomal "saucer" or round shape with 50 - 90 nm in diameter (**Figure 1(b)**) and contained EXO-associated proteins such as CD9 and LAMP-1 (**Figure 1(c)**) [37]. We infected the OVA-$T_{EXO}$ cells [32] with $AdV_{IL-21}$ to generate a transgene IL-21-engineered T cell-based vaccine, OVA-$T_{EXO/IL-21}$. IL-21 secretion in OVA-$T_{EXO/IL-21}$ culture supernatants was estimated to be ~200 pg/ml using IL-21 ELISA kit. In contrast, there was no IL-21 detected in OVA-$T_{EXO}$ culture supernatants.

### 3.2. OVA-$T_{EXO/IL-21}$ Vaccine Stimulates OVA-Specific CTL Responses in Wild-Type C57BL/6 Mice

To examine the immunogenicity, we i.v. immunized B6 mice with OVA-$T_{EXO/IL-21}$ or the control OVA-$T_{EXO/Null}$ vaccine. We demonstrated that both OVA-$T_{EXO/IL-21}$

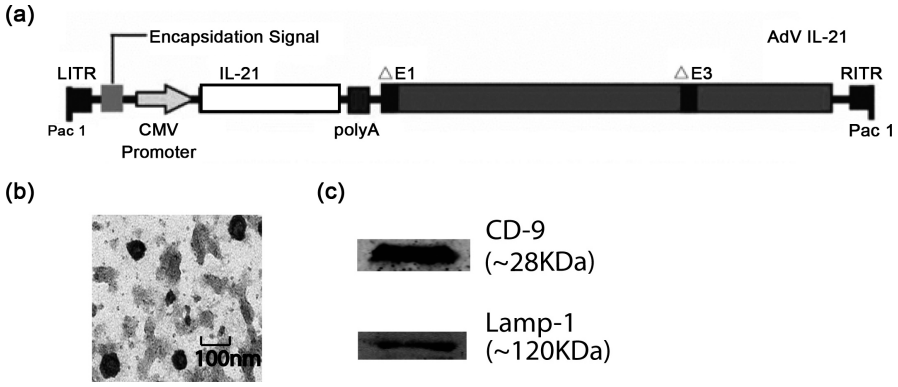


**Figure 1.** (a) Schematic representation of adenoviral (AdV) vector $AdV_{IL-21}$. The E1/E3 depleted replication-deficient AdV is under the regulation of the cytomegalovirus (CMV) early promoter. ITR, inverted terminal repeat. (b) Electron micrograph of $EXO_{OVA}$. Bar, 100 nm. (c) Western blot analysis of Western blot analysis of $EXO_{OVA}$ for expression of EXO markers CD9 and LAMP-1.

and control OVA-$T_{EXO/Null}$ vaccines stimulated comparable OVA-specific CTL responses (**Figure 2(a)**). To assess, whether these CTLs are of functional effect, we challenged immunized mice with OVA-expressing BL6-$10_{OVA}$ tumor cells and examined its protective immunity against lung BL6-$10_{OVA}$ tumor metastases. We showed that OVA-$T_{EXO/IL-21}$ or the control OVA-$T_{EXO/Null}$ vaccine induced complete protection from lung tumor metastases in all 6/6 mice (**Figure 2(b)** & **Figure 2(c)**), indicating that OVA-$T_{EXO/IL-21}$ vaccine stimulates comparable CTL responses compared to the control OVA-$T_{EXO}$ vaccine in wild-type B6 mice.

### 3.3. OVA-Expressing Adenovirus Induces an OVA-Specific Chronic Infection in Mice with CTL Exhaustion

We previously found that infection of B6 mice with $AdV_{OVA,}$ led to establishment of an OVA-specific chronic infection mouse model with OVA-specific CTL inflation and exhaustion [18]. To develop acute and chronic infection models, B6 mice were i.v. infected with OVA-expressing recombinant rLmOVA bacteria and $AdV_{OVA}$, respectively, followed by the kinetic analysis of OVA-specific CTL responses [18]. We demonstrated that $AdV_{OVA}$ infection resulted in OVA-specific memory $CD8^+$ T cell inflation, when compared to CTLs developed in rLmOVA-immunized mice (**Figure 3(a)**), suggesting that rLmOVA induces an acute infection, while $AdV_{OVA}$ induces a chronic infection with $CD8^+$ CTL inflation [18] [19]. To confirm it, the phenotypes of OVA-specific memory CTLs were analyzed by flow cytometry. We demonstrated that memory CTLs expressed cell

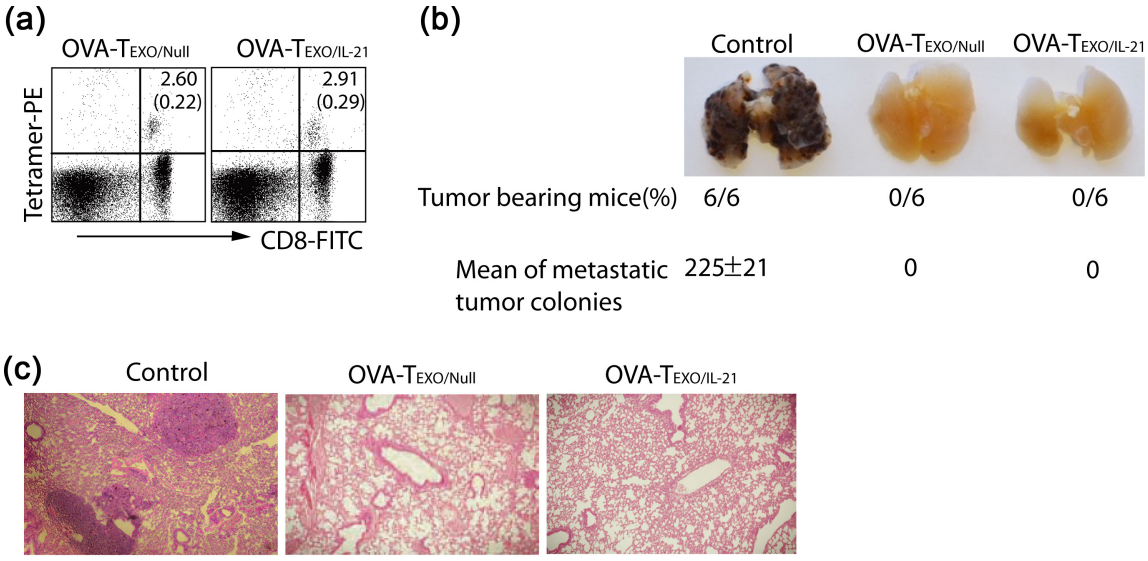


**Figure 2.** $AdV_{IL-21}$ does not enhance OVA-$T_{EXO}$ vaccine immunity in wild-type (WT) B6 mice. (a) WT B6 mice (n = 6) were injected with OVA-$T_{EXO/Null}$ or OVA-$T_{EXO/IL-21.}$ Six days after the injection, blood samples were collected, stained with FITC-CD8 Ab and PE-tetramer, and then analyzed by flow cytometry. The percentages of tetramer$^+$CD8$^+$ T cells in the total $CD8^+$ T cell population are indicated. (b) WT B6 mice were immunized with OVA-$T_{EXO/Null}$ or OVA-$T_{EXO/IL-21}$, followed by injection with $BL_{6\text{-}10/OVA}$ tumor cells 6 day later. Mice lungs were collected at 21 days after the tumor cell injection. Metastatic tumor colonies in lung tissues were counted. (c) The lung tissues of immunized mice were fixed in 10% neutral-buffered formalin and then embedded in paraffin. Tissue sections were stained with H&E and examined by microscopy. Magnification, ×100. One representative experiment of two is shown.

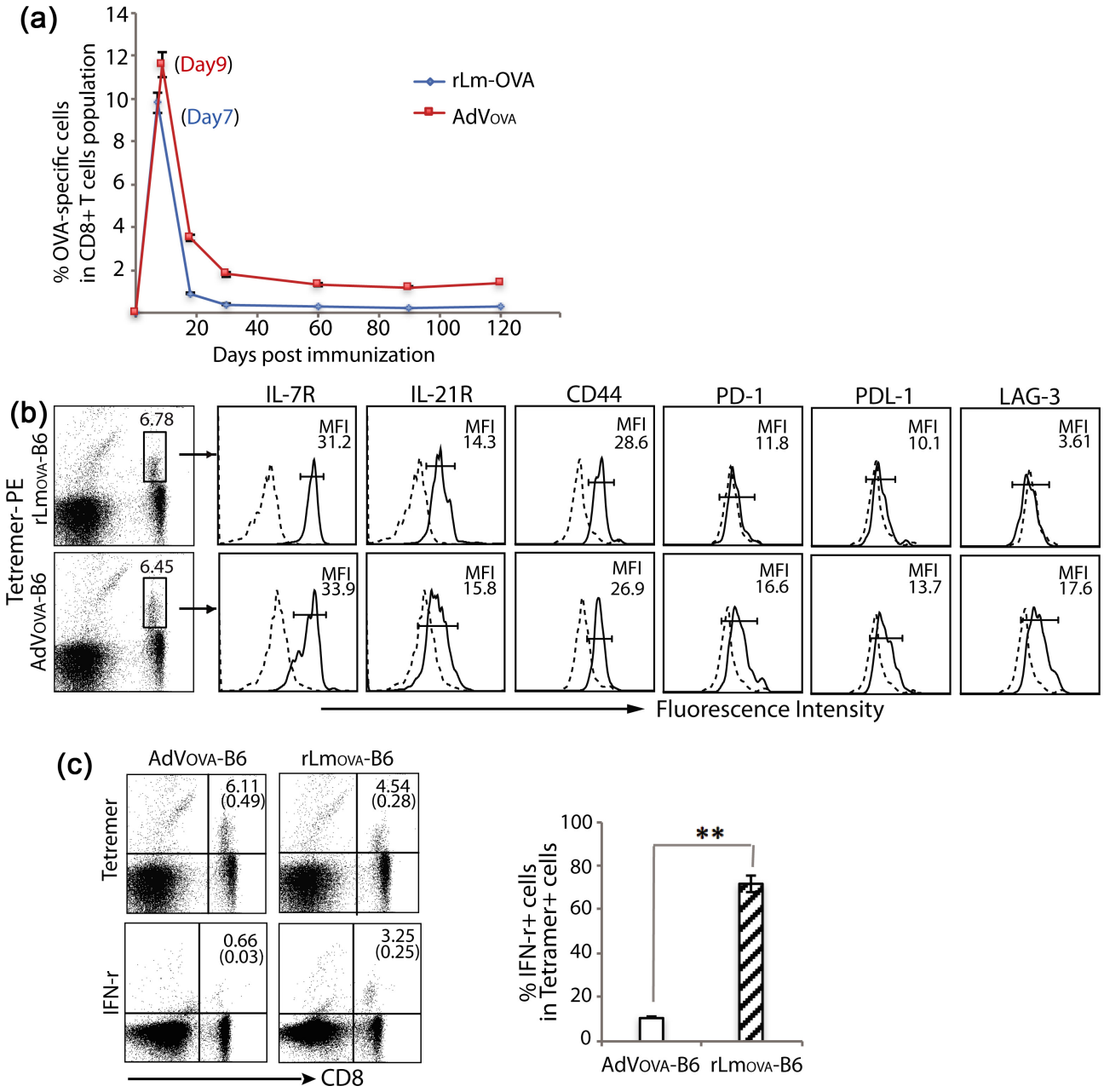


**Figure 3.** AdVova induces chronic infection in B6 mice. (a) OVA-specific CTL responses were analyzed at the indicated days post-AdVova or -rLmOVA infection by flow cytometry (n = 4). (b) Sixty days after the infection, flow cytometric analyses were performed. PE-tetramer and FITC-CD8 double positive cells were gated (rectangle) for further assessment of the expression of the indicated molecules (solid lines on the right). Mean fluorescence intensity (MFI) values are indicated in each panel. Dotted lines represent isotype controls. (c) Splenocytes were stained with PE-tetramer and FITC-CD8 or permeabilized for the assessment of intracellular IFN-$\gamma$ by flow cytometry. The value in each panel represents the percentage of $CD8^+$ T cells producing IFN-$\gamma$ in the total $CD8^+$ T-cell population. $^{**}p < 0.01$. One representative experiment of two is shown.

surface T-cell memory markers IL-7R and CD44 as well as IL-21R sixty days after infection in both $AdV_{OVA}$-B6 and rLmOVA-B6 mice (**Figure 3(b)**). Importantly, we also found that these CTLs developed in $AdV_{OVA}$-immunized mice up-regulated inhibitory molecules, such as PD-1, PD-L1 and LAG-3, when compared to CTLs developed in rLmOVA-immunized mice (**Figure 3(b)**),

indicating that these CTLs may be exhausted. To further confirm it, we assessed the functional effect (expression of the effector cytokine IFN-$\gamma$) of these CTLs by flow cytometry. We showed that ~72% OVA-specific CTLs were IFN-$\gamma$ positive in rLmOVA-B6 mice, compared to only ~10% OVA-specific CTLs were IFN-$\gamma$ positive in rLmOVA-B6 mice (**Figure 3(c)**), thus confirming that those OVA-specific CTLs in $AdV_{OVA}$-B6 mice are exhausted CTLs [18] [19].

### 3.4. The OVA-$T_{EXO/IL\text{-}21}$ Vaccine Converts CTL Exhaustion in Chronic Infection

We examined in our next set of experiments, whether the OVA-$T_{EXO/IL\text{-}21}$ vaccine converts CTL exhaustion in chronic infection. The $AdV_{OVA}$-B6 mice were boosted with OVA-$T_{EXO/IL\text{-}21}$ and the control OVA-$T_{EXO/Null}$ vaccine. Four days after the boost, cell proliferation and intracellular IFN-$\gamma$ expression of OVA-specific CTLs were assessed by flow cytometry. We demonstrated that there were ~6-fold (5.20% vs 0.81%) of CTL increase in OVA-$T_{EXO/IL\text{-}21}$-boosted mouse peripheral blood, which is more than ~3-fold of CTL increase in the control OVA-$T_{EXO/Null}$-boosted mouse peripheral blood at day 4 after the boost (**Figure 4(a)**), indicating that OVA-$T_{EXO/IL\text{-}21}$ vaccine can more potently convert CTL exhaustion by significantly stimulating the proliferation of previously exhausted CTLs. We next assessed expression of an effector T cell cytokine, IFN-$\gamma$, in exhausted CTLs on a "per-cell" basis by intracellular staining of T cell IFN-$\gamma$. We demonstrated that only ~20% of OVA-specific CTLs were IFN-$\gamma$ positive in $AdV_{OVA}$-B6 mice (**Figure 4(b)**). In OVA-$T_{EXO/Null}$- and OVA-$T_{EXO/IL\text{-}21}$-boosted $AdV_{OVA}$-B6 mice, however, we found ~60% and ~75% of IFN-$\gamma$-producing CTLs in the total OVA-specific CTLs, respectively (**Figure 4(b)**), confirming that OVA-$T_{EXO/IL\text{-}21}$ vaccine more potently converts CTL exhaustion in chronic infection by not only increasing the number of CTLs, but also restoring CTL functional effect.

### 3.5. OVA-Texo Converts CTL Exhaustion through the Activation of the mTORC1 Pathway

We previously showed that the OVA-$T_{EXO}$ vaccine rescued exhausted CTLs in chronic infection *via* its CD40L signaling, inducing the activation of the mTORC1 pathway [18]. Four days after the boost, cell phenotypes of the OVA-specific CTLs were assessed by flow cytometry to examine, whether the OVA-$T_{EXO/IL\text{-}21}$ vaccine activates the mTORC1 pathway. We analyzed CTLs for the phosphorylation status of mTORC1-regulated EIF4E (pEIF4E) and for the intracellular expression of the transcription factor T-bet by flow cytometry. We determined that OVA-specific CTLs up-regulated levels of phospho-EIF4E and T-bet in OVA-$T_{EXO/Null}$-boosted $AdV_{OVA}$-B6 mice (**Figure 4(a)**), which was consistent with our previous report [18]. Interestingly, the intracellular expression of pEIF4E and T-bet in OVA-specific CTLs was significantly higher in the OVA-$T_{EXO/IL\text{-}21}$-boosted $AdV_{OVA}$-B6 mice than that in the OVA-$T_{EXO/Null}$-boosted $AdV_{OVA}$-B6 mice (pEIF4E, $p = 0.02$; T-bet, $p = 0.001$) (**Figure 4(a)**), confirming

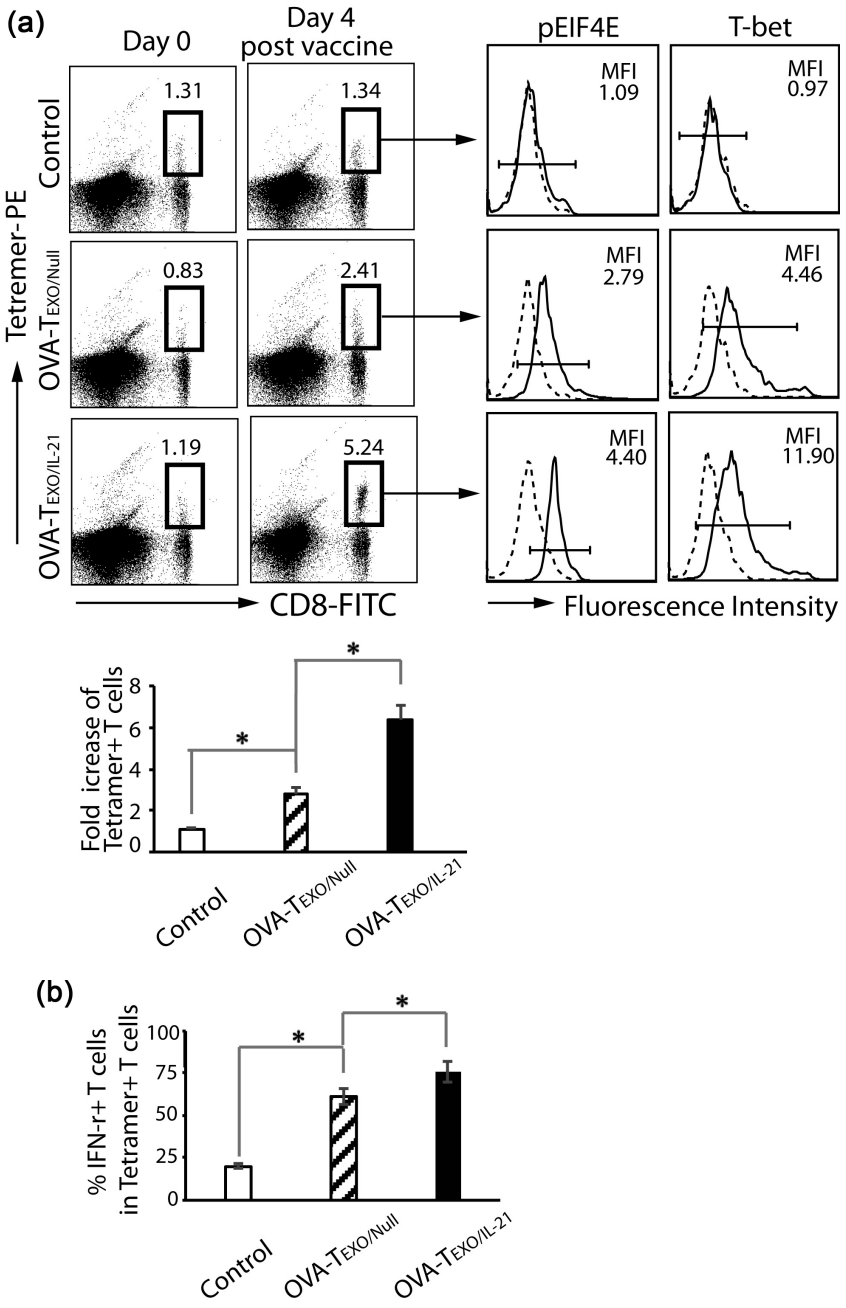


**Figure 4.** $AdV_{IL-21}$ enhance the capability of OVA-$T_{EXO}$ vaccine in converting CTL exhaustion. AdVova-infected B6 mice (n = 5) were immunized with OVA-$T_{EXO/Null}$ or OVA-$T_{EXO/IL-21}$ at day 60 after the infection. (a) One day prior to (day 0) and 4 days post the immunization, periphery blood samples were analyzed for OVA-specific CTL responses by flow cytometry. The value in each panel represents a percentage of PE-tetramer-positive $CD8^+$ T cells in the total peripheral $CD8^+$ T cell population. Four days after immunization, mouse splenocytes were stained with PE-Tetramer, FITC-CD8, and PE-Cy5-labeled Abs. The $tetramer^+CD8^+$ T cells were gated and assessed for expression of pelF4E and T-bet (solid lines). MFI values are indicated in each panel. The fold increases (%$Tetramer^+$ T cells on day 4 post vaccine/%$Tetramer^+$ T cells on day 0) of $tetramer^+CD8^+$ T cells are indicated in the graph. (b) Splenocytes were permeabilized for the assessment of intracellular IFN-$\gamma$ by flow cytometry at day 4 after immunization. *$p < 0.05$. One representative experiment of two is shown.

that OVA-$T_{EXO/IL-21}$ vaccine converts CTL exhaustion mostly through the activation of the mTORC1 pathway by both CD40L and IL-21 signaling.

### 3.6. The Gag-$T_{EXO/IL-21}$ Vaccine Induces Gag-Specific Therapeutic Immunity in Chronic Infection Model

To assess a potential therapeutic immunity of the Gag-$T_{EXO/IL-21}$ vaccine in chronic infection, $AdV_{OVA}$-B6 mice with chronic infection of $AdV_{OVA}$ were first i.v. injected with Gag-expressing BL6-$10_{Gag}$ melanoma cells, and four days post B16 melanoma cell challenge, mice were i.v. immunized with the Gag-$T_{EXO/IL-21}$ or the control Gag-$T_{EXO/Null}$ vaccine (**Figure 5(a)**). Seventeen days later, mouse lung tissues were collected for histopathological examination (**Figure 5(a)**). Importantly, the Gag-$T_{EXO/IL-21}$ vaccine demonstrated a complete eradication of established BL6-$10_{Gag}$ lung metastases in 5/6 $AdV_{OVA}$-B6 mice, thus stimulating more efficient therapeutic immunity against Gag-expressing BL6-$10_{Gag}$ melanoma than the control Gag-$T_{EXO/Null}$ vaccination (**Figure 5(b)**). Our data indicate that Gag-$T_{EXO/IL-21}$ vaccine is capable of inducing potent therapeutic immunity against established Gag-expressing tumors in the presence of chronic infection.

## 4. Discussion

The inhibitory PD-1 molecule was originally found on active T cells following TCR engagement and became a T cell-intrinsic mechanism for negatively regulating CTL responses [38] [39]. In chronic infection, PD-1 expression is sustained on CTLs due to persistent antigen stimulation. Expression of PD-1 suppresses T

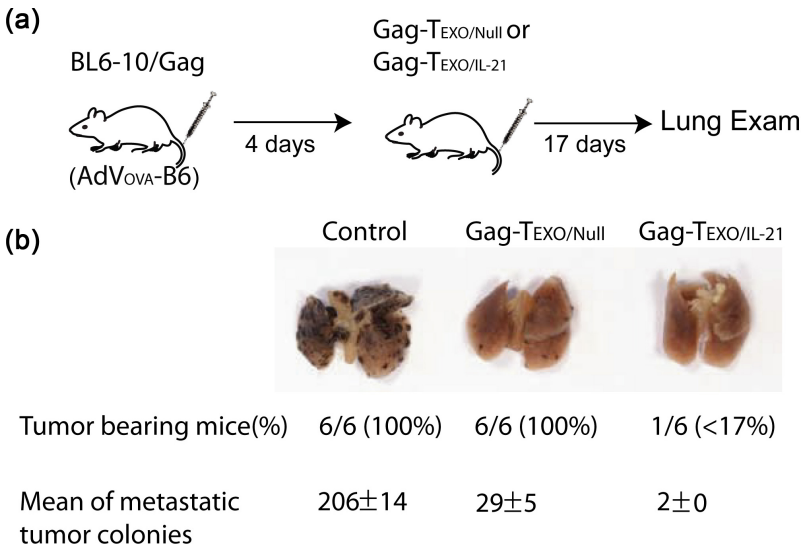


**Figure 5.** Gag-$T_{EXO/IL-21}$ vaccine stimulates enhanced antitumor immunity in chronic infection mice. (a) Experimental set-up to examine the therapeutic antitumor immunity of $T_{EXO}$ vaccines. Chronic AdVova-B6 mice (n = 6) were i.v. injected with BL6-$10_{OVA}$ cells. Four days after tumor challenge, mice were vaccinated with Gag-$T_{EXO/Null}$, Gag-$T_{EXO/IL-21}$ or control ConA-T. The mice were sacrificed 3 weeks after tumor cell challenge. (b) The average number of lung metastatic tumor colonies was counted. *$p < 0.05$ versus cohorts of ConA-T cells. One representative experiment of three is shown.

cell functions through inhibiting TCR signaling by recruiting phosphatases [40] [41] and modulating the mTORC1 pathway responsible for T cell proliferation and effector function [38] [39]. Later, expression of other inhibitory molecules such as LAG-3 and TIM-3 has also been found on exhausted CTLs [42]. It has been demonstrated that PD-1 blockade rescued exhausted CTLs in chronic viral infection [17]. An enhanced effect on conversion of CTL exhaustion was observed upon combination treatments with antagonists of various inhibitory molecules, such as PD-1, LAG-3 and Tim-3 [42] [43]. In addition to blockade therapies, costimulatory signaling, such as 41BB-, OX40- and CD27-mediated costimulation also synergized with PD-L1 blockade by forcing exhausted CTLs to exit quiescence [4] [44] [45]. Moreover, costimulating CD40L signaling has been shown to assure T cell activation by recruiting tumor necrosis factor (TNF) receptor-associated factor (TRAF), which leads to the activation of the mTORC1 pathway [46]. Consistent with this, it has recently been demonstrated that CD40 agonist enhances PD-1 blockade's effect in rescuing exhausted CTLs in chronic infection [19].

IL-21 belongs to the common $\gamma$-chain cytokine family and is closely related to another family member, IL-2, which is encoded upstream of IL-21 on chromosome 3 [47]. IL-21 binds to a heterodimeric receptor CD123 encoded on chromosome 7 [48], which is widely expressed by B cells, natural killer cells, DCs, macrophages and T cells [47] [48]. The IL-21 cytokine was originally found to be produced by $CD4^+$ T cells [20] and to serve as a "third" signal, functioning in cooperation with TCR ligation and costimulation, to trigger $CD8^+$ T cell responses [21]. IL-21 promotes CTL activation and survival by inducing signaling through the phosphatidylinositol-3 kinase (PI3K) and the mTORC1-regulated T-bet pathway [49] [50]. In addition, IL-21 also upregulates granzyme-B expression in CTLs [51] and triggers proliferation of B cells and NK cells [52]. Recently, it has been shown that IL-21 plays a significant role in controlling chronic LCMV infection by rescuing exhausted CTL via diminishing their exhaustion phenotype and maintaining their ability to proliferate [22] [23] [24]. IL-21 also restricts virus-driven regulatory T cell expansion in chronic LCMV infection [53], enhances cytolytic and virus-controlling abilities of HIV-specific CTLs *in vitro* [25] [26], and enhances virus control in immunodeficiency virus -infected rhesus macaques or in HCV- and HIV-1-infected patients [4] [7] [28] [54] [55].

We previously developed a novel OVA-specific EXO-targeted T cell-based OVA-$T_{EXO}$ vaccine by using non-specific polyclonal T cells with the uptake of OVA-specific DC-released EXO [29]. We found that the OVA-Texo vaccine was able to directly stimulate potent OVA-specific CTL responses by counteracting $CD4^+25^+FoxP3^+$ Treg-induced CTL suppression [29] [30]. We also demonstrated that the 4-1BBL-expressing OVA-$T_{EXO}$ vaccine triggered an enhanced therapeutic immunity in WT B6 mice [32] and induced an efficient conversion of CTL exhaustion in chronic infection model via the CD40L-initiated signaling through the mTORC1 pathway [18]. In this study, we generated new OVA-$T_{EXO/IL\text{-}21}$ and

Gag-$T_{EXO/IL-21}$ vaccines, expressing IL-21, by infecting the above 41BBL-expressing OVA-$T_{EXO}$ and Gag-$T_{EXO}$ cells with $AdV_{IL-21}$, as previously described [32]. We assessed the effectiveness of the OVA-$T_{EXO/IL-21}$ vaccine in the conversion of CTL exhaustion and examined the effectiveness of the Gag-$T_{EXO/IL-21}$ vaccine in therapeutic immunity against Gag-expressing tumor in chronic infection. We discovered that in chronic infection, the OVA-$T_{EXO/IL-21}$ vaccination more strongly rescued CTL exhaustion than the OVA-$T_{EXO/Null}$ vaccine. In addition, we also found that OVA-$T_{EXO/IL-21}$-boosted CTLs strongly up-regulated phosphorylation of mTORC1-controlled EIF4E. OVA-$T_{EXO/IL-21}$ also very effectively enhanced mTORC1-controlled expression of T-bet that regulates T cell activation. These OVA-$T_{EXO/IL-21}$-induced responses were significantly stronger than OVA-$T_{EXO}$-boosted ones. It has been shown that IL-21 promotes CTL survival via activation of the PI3K signaling cascade [49]. Our data showing that OVA-$T_{EXO/IL-21}$ vaccination more strongly rescued CTL exhaustion than the OVA-$T_{EXO}$ one indicates that the IL-21 signaling of OVA-$T_{EXO/IL-21}$ vaccine plays an important role in conversion of CTL exhaustion *via* the activation of the mTORC1 pathway in chronic infection. Our data thus provide the first evidence that our novel T cell-based vaccine is capable of converting CTL exhaustion in chronic infection *via* its CD40L and IL-21 signaling through the mTORC1 pathway.

$CD8^+$ CTLs are important effector T cells capable of directly destroying HIV-1-infected cells, and their activity correlates with acute viral control and long-term nonprogression [56] [57] [58] [59]. $CD8^+$ CTLs play a critical role in controlling HIV-1 proliferation and disease progression even in the absence of neutralizing antibodies [60] [61]. Stimulation of HIV-1-specific CTLs has been also reported to facilitate elimination of latent viral reservoirs [62] [63]. We originally generated HIV-1 Gag-specific Gag-$T_{EXO}$ vaccine by using polyclonal ConA-T cells with the uptake of Gag-specific DC-released EXO, and demonstrated that Gag-$T_{EXO}$ stimulated Gag-specific CTL responses in transgenic HLA-A2 mice [31]. We also generated a 4-1BBL-expressing Gag-$T_{EXO}$ vaccine capable of triggering more efficient CTL responses and therapeutic immunity against Gag-expressing tumor challenges than the original Gag-$T_{EXO}$ vaccine [32]. In this study, we have generated a new Gag-$T_{EXO/IL-21}$ vaccine engineered to express IL-21 by infection of the 41BBL-expressing Gag-$T_{EXO}$ cells with $AdV_{IL-21}$ as [32], and demonstrated that the Gag-$T_{EXO/IL-21}$ vaccine triggered more effective therapeutic immunity against established Gag-specific BL6-$10_{Gag}$ melanoma lung metastases in chronic infection. We expect that combinations of similarly designed vaccines with blockades against various inhibitory molecules, such as PD-1, TIM-3 and LAG-3, may become new startegies for combined immunotherapies to covert CTL exhaustion in chronic infections such as HIV-1.

Taken together, our data demonstrate that our novel transgene IL-21-engineered T cell-based vaccine OVA-$T_{EXO/IL-21}$ is capable of strongly converting the exhaustion of CD40-expressing CTLs in chronic infection *via* the activation of the mTORC1 pathway caused by endogenous CD40L- and transgene IL-21-triggered

signals. Therefore, this study is likely to have a strong impact on developing new therapeutic vaccines that might be used as monotherapies or in combination with other HIV-1 blockades for treating immune deficiency syndrome patients.

## Acknowledgements

We thank Mark Boyd for help in flow cytometric analyses. This work was supported by a Canadian Institute of Health Research (CIHR) grant (OCH 126276).

## Conflicts of Interest

The authors declare no conflict of interest.

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