Novel HIV-1 Gag-specific Exosome-targeted $CD8^+$ T cell-Based Therapeutic Vaccine Capable of Converting CTL Exhaustion in Chronic Infection

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) is the cause of acquired immune deficiency syndrome (AIDS). HIV-1 is a worldwide epidemic that currently affects over 35 million people worldwide, and continues to spread at an appalling rate. A universal HIV-1 preventive vaccine is considered to be the optimal solution in achieving the ultimate goal of AIDS eradication. Regretfully, most endeavors thus far of developing a prophylactic vaccine have been largely disappointing. Highly Active Anti-Retroviral Therapy (HAART) has been shown to reduce the plasma HIV-1 RNA level to below the detection limit of clinical assays (50 copies/ml); it combines three or more antiretroviral drugs which belong to at least two different classes – targeting distinct steps in the viral life cycle, and inhibiting viral replication. However, unless the infection is eradicated, strict adherence to a lifelong treatment regimen is required. HAART is limited by its high cost, drug availability, complicated administration schedules, serious side effects, and the potential that the virus will ultimately develop drug resistance. A more plausible approach lies in therapeutic vaccines that provide immunity to partially control viral replication postinfection – delaying or minimizing ART, and offering "drug holidays".

The primary goal of a therapeutic vaccine is to effectively induce HIV-1 specific cytotoxic T lymphocyte (CTL) responses, which plays a critical role in control of viral proliferation. Dendritic cells (DCs)-based therapeutic vaccines have been showing the most promising results. However, the therapeutic efficacy of DCs based vaccines is limited. This is partially due to the fact that DCs induced $CD8^+$ T cell responses are largely $CD4^+$ T cell dependent, while HIV-1 infection usually renders the immune system very "helpless" from $CD4^+$ T cells. In addition, infection, impaired function, and physical depletion of DCs are often reported during the early stage. Furthermore, DCs are often found to be inflammatory and immunosuppressive, which is mainly mediated by the interaction between HIV-1 Env gp120 and DC receptors. Thus, the search for a novel therapeutic vaccine strategy is warranted.

Using T-APC (T cells-antigen-presenting cells) as a novel T cell-based vaccine has emerged as a potential candidate for a HIV-1 therapeutic vaccine, which aims at boosting HIV-specific CTL responses. Our previous work demonstrated that $CD4^+$ and $CD8^+$ T cells derived from ovalbumin (OVA)-specific T cell receptor (TCR) transgenic OT II and OT I mice via co-culture with OVA-pulsed DCs (DC_{OVA}) can be activated, acquiring pMHC I, pMHC II, and costimulatory molecules, thus act as $CD4^+$ T helper-antigen-presenting cells (Th-APCs) and $CD8^+$ cytotoxic T-antigen-presenting cells (Tc-APCs). We also elucidated that DC-derived exosomes (EXO), which are 50- to 90-nm diameter vesicles containing antigen-presenting, tetraspan, adhesion, and costimulatory molecules, can transfer the antigen-presenting activity of DCs to activated $CD4^+$T cells through EXO uptake. EXO_{OVA}-targeted activated $CD4^+$T (aTexo) cells can (1) stimulate more efficient central memory $CD8^+$ CTL responses and T cell memory than EXO_{OVA} or DC_{OVA}, (2) activate $CD8^+$ CTL responses independent of $CD4^+$Th cells, and (3) counteract $CD4^+25^+$regulatory T (Tr) cell-mediated immune suppression. These results formed the new concept of novel EXO-targeted $CD4^+$ T cell vaccines.

In this study, we tailored EXO-targeted T cells vaccine by using polyclonal activated $CD8^+$ T cells instead of $CD4^+$ T cells, as $CD4^+$ T cells served as the primary target for HIV-1 infection. We showed that (1) OVA-specific exosome-targeted $CD8^+$ T cell-based vaccine (OVA-Texo) can stimulate efficient OVA-specific $CD8^+$ CTL and memory responses, inducing sufficient antitumor immunity against OVA-expressing tumor cells in mouse models. (2) This exosome-targeted $CD8^+$ T cell-based vaccine strategy could be applied to HIV-1-Gag protein, provoking effective Gag-specific $CD8^+$ CTL, T cell memory, and antitumor immunity against Gag-expressing tumor cells. (3) Engineering Gag-Texo with up-regulated 4-1BBL (APC derived costimulatory molecule) expression could improve the performance of Gag-Texo vaccine. (4) OVA-Texo is able to evoke a successful immune response in bystander chronic infection, converting $CD8^+$ T cell exhaustion, restoring effector functions of exhausted $CD8^+$ T cells. Moreover, combination of OVA-Texo vaccine with PD-L1 blockage in a dual treatment could

result in a synergistic effect in rescuing CTLs exhaustion in chronic infection. Those desired features make EXO-targeted $CD8^+$ T cells vaccine an appealing novel strategy in HIV-1 infection. The EXO-targeted $CD8^+$ T cells vaccine may be applicable to therapeutic HIV treatment through the use of autologous T cells with uptake of EXOs derived from engineered DCs.

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LIST OF ABBREVIATIONS

Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
Adv	Adenovirus
Ag	Antigen
AICD	Activation-induced cell death
AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen presenting cell
APOBEC3G(A3G)	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G
Bcl2l10	B-cell lymphoma 2 like 10
Bcl-$_{XL}$	B-cell lymphoma-extra large
BCR	B-cell receptor
BM	Bone marrow
BM-DC(s)	Bone marrow-derived dendritic cell(s)
BSA	Bovine serum albumin
BST-2	B cell stromal factor 2
BTLA	B- and T-lymphocyte attenuator
Casp12	Caspase 12
CCR	CC-chemokine receptor
CD	Cluster of differentiation
c-FLIP$_{short}$	Cellular FLICE-like inhibitory protein short
CFSE	Carboxyfluoroscein diacetate succinimidyl ester
CMV	Cytomegalovirus
ConA	Concanavalin A
CPE	Cytopathic effect

CTL(s)	Cytotoxic T lymphocyte(s)
CTLA-4	Cytotoxic T lymphocyte antigen 4
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DC(s)	Dendritic cell(s)
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DEX(s)	DC-derived exosome(s)
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
diAcH3	diacetylated histone H3
ds	Double stranded
DT	Diphtheria toxin
DTR	diphtheria toxin receptor
ECD	Electron coupled dye
EGFP	Enhanced green fluorescent protein
eIF4E	Eukaryotic translation initiation factor 4E
ELISA	Enzyme linked immunosorbent assay
EMEM	Eagle's minimal essential medium
Env	Envelope
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
EXO(s)	Exosomes
FADD	Fas-associated death domain
FasL	Fas ligand
FCS	Fetal cattle serum
FITC	Fluorescein isothiocyanate

Foxp3	Fork-head/winged helix transcription factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage-colony stimulating factor
gp	glycoprotein
Grail	gene related to anergy in lymphocytes
HAART	Highly active anti-retroviral therapy
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSP	Heat-shock protein
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
Itch	Itchy homologue E3 ubiquitin protein ligase
i.v.	Intravenous
Kb	Kilobase pair
kD	kilodalton
KO	Knockout
Lac Z	β-galactosidase
LAG-3	lymphocyte-activation gene 3
LCMV	lymphocytic choriomeningitis virus
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
LSP1	leukocyte-specific protein-1
LT-α	Lymphotoxin α

LTA	Lipoteichoic acid
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MIP-1	Macrophage inflammatory protein 1
mRNA	Messenger RNA
mTORC1	Mammalian target of rapamycin complex 1
MVA	Modified Vaccinia Virus Ankara
Naip1	NLR family, apoptosis inhibitory protein 1
Nef	Negative effector
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-κB
NK	Natural killer
NNRTIs	Non-nucleoside-analog reverse transcriptase inhibitors
NOL3	Nucleolar protein 3
NRTIs	Nucleoside-analog reverse transcriptase inhibitors
OVA	Ovalbumin
Pak7	p21 protein (Cdc42/Rac)-activated kinase 7
PAMP(s)	Pathogen-associated molecular pattern(s)
PBMC(s)	Peripheral blood monouclear cell(s)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L1	Programmed death ligand 1
PE	R-Phycoerythrin
PFU	Plaque forming unit
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase-B

pMHC	Peptide-MHC complex
PRRs	Pattern recognition receptors
RANTES	Regulated on activation normal T cell expressed and secreted
Rev	Regulator of expression of virion proteins
rLmOVA	OVA-expressing Listeria monocytogenes
RPMI	Roswell Park Memorial Institute medium
RT	Reverse transcriptase/reverse transcription
RT-PCR	Reverse transcription-polymerase chain reaction
SAMHD1	Sterile alpha motif (SAM) and histidine/aspartic acid (HD) domain-containing protein 1
SDF-1	Stromal cell-derived factor 1
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIV	Simian immunodeficiency virus
TAT	Trans-activator of transcription
TCR	T cell receptor
TET	tetramer
Tg	Transgenic
TGF-β	Transforming growth factor-beta
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor-alpha
TNFR	TNF receptor
Tnfrsf11b	Tumor necrosis factor receptor superfamily member 11b
TRAF	TNF receptor-associated factors

TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Treg(s)	Regulatory T cell(s)
TRP	Transformation related protein
Vif	Viral infectivity factor
Vpu	Virion protein unique to HIV-1
WT	Wild type
2-ME	2-mercaptoethanol

CHAPTER 1 LITERATURE REVIEW

1.1 Immune system

The immune system is a defense system that many organisms utilize to fight pathogenic infections mostly caused by virus/bacterial/fungi/parasites, as well as harmful matters, with the help of a variety of immune cells and the molecules they produce in particular organs. Ranging from very simple to highly complex, the immune system is comprised of the biological structures and processes that stave off disease. Humans have a very intricate immune system[1], which can effectively identify and protect against non-self pathogens from the massive number of objects we encounter daily; initially through innate immunity, then through adaptive immunity for more severe infections.

1.1.1 Innate immunity

The innate immune system is the first line of defense microorganisms would face if they made it through the physical barrier – such as skin for external invasions or mucous membranes for internal exposed surface (respiratory tract, alimentary tract and genitourinary tract)[2]. It is an ancestral and broad response characterized by non-specificity and instantaneity, and can be traced to many ancient life forms and found in all modern animals and plants[3].

Most cell components in the innate immune system are derived from the common myeloid progenitor, including granulocytes (neutrophil, eosinophil, and basophil), mast cells, macrophages, and majority of dendritic cells (DC). The exception lies with nature killer cells as they are derived from the common lymphoid progenitor. Nevertheless, their lack of antigen specificity makes them part of the innate immunity.

When an infection happens, an inflammatory response is triggered[4], during which macrophages at the site would recognize, engulf and kill microorganisms. In addition, cytokines are released at the site increasing blood vessel permeability causing more proteins and fluid to flow into the tissues. The chemokines released via this process attract neutrophils and more monocytes to leave bloodstream[5], migrating to the site. The collection of these processes working together is responsible for the characteristic symptoms of inflammation: redness, heat, swelling and pain.

Neutrophils, macrophage and dendritic cells are the three major phagocytes. They take up pathogens through phagocytosis and eliminate them via degradative enzymes and antimicrobial substances within the lysosome[6]. Mature dendritic cells are well known by another crucial function - the ability to process and present antigens in forms that can be recognized by T lymphocytes. Thus they carry another title as antigen-presenting cells (APCs) for their key role in activating T lymphocytes and initiating the adaptive immune response[7, 8]. Macrophages can also function as APCs in addition to their versatile jobs of activating other immune cells and signaling more of them to join the fight, and serving as general scavengers[9].

Eosinophils and basophils are involved in fighting parasites that are usually too large for phagocytosis, by the means of producing enzymes and antimicrobial chemicals upon activation by antibodies[10]. They are well known for their involvement in allergic reactions along with mast cells, where they actually do more harm than good. Mast cells express high-affinity IgE antibody receptors on the cell surface. Antigens binding to IgE activate mast cells to release inflammatory mediators such as histamine, triggering an allergic response[11]. Nature killer cells are important in early stage defense against intracellular infections caused by virus and killing abnormal cells such as tumor cells[12].

The complement system is a major humoral component in the innate immune response[13]. It is

an enzyme activated cascade reaction that involves serials of proteases. Each protease's activation depends on the cleavage of its precursor by the upstream enzyme. The active form can serve as a protease acting on the next one's precursor[14]. There are three different pathways to trigger a complement reaction: the classic pathway, lectin pathway, and alternative pathway[15]. Each pathway is initiated by a distinct set of proteins existing broadly in tissues and body fluids. These proteins trigger the cascade by either directly binding to pathogen surface components or binding to the antigen/antibody complexes, and thus link the innate and adaptive immune response. The three pathways joint at a same point which eventually lead to the generation of the same group of active effector molecules[16]. Complement effectors can opsonize pathogens – facilitating their uptake by phagocytes; attract inflammatory molecules – augmenting the immune response; and directly kill pathogens by forming holes in their membrane.

Innate immune cells recognize microorganisms via pattern recognition receptors (PRRs). These receptors can be expressed on both the cell surface or intracellular endosome membranes[17]. They are of limited diversity - each receptor can detect a pathogen structural component rather than one specific kind of pathogen, and every cell of the same cell type has the same group of receptors. Toll-like receptors (TLRs) are the most well studied family of PRRs because of their extensive involvement in the innate immune response[18]. Ten TLRs have been identified so far in humans[19]. The components recognized by PRRs are often repeated molecules or common patterns that are shared by a group of microbes but not self cells, collectively known as pathogen-associated molecular patterns (PAMPs). One such example of a PAMP is lipoteichoic acid (LTA) which constitutes the cell wall of Gram-positive bacteria can be detected by TLR2. Another example is the lipopolysaccharide (LPS) of Gram-negative bacteria detected by TLR4. TLR3 recognizes virus originated double-stranded RNA. Bacterial unmethylated CpG DNA is detected by TLR-9[20, 21].

1.1.2 Adaptive immunity

The role of adaptive immunity comes into play when the innate response is insufficient to eradicate an infection. It represents a more evolved mechanism that can only be found in humans and other vertebrates[1]. This response usually requires many days and is highly specific. Most importantly, it is capable of creating immunological memory that can last for years even a lifetime. Thus the foundation to vaccine design and application lies in understanding the adaptive immune response. Unlike the innate response which responds the same way to repetitive infections, the adaptive response adapts its form to different nature of infections, develops quicker and stronger response each successive time to the same pathogen.

Antigen specific lymphocytes including B cells and T cells are the core components of adaptive immunity. They are both derived from common lymphoid progenitors; differentiating at bone marrow and thymus, respectively[22]. These two sites are referred to as the primary lymphoid organs. Naïve lymphocytes that have not been stimiulated by their cognate antigens either circulate in the bloodstream or reside in the secondary lymphoid organs (spleen, lymph nodes, and mucosal lymphoid organs).

The most important feature of lymphocytes is that each of them bears a unique set of cell surface receptors specialized in recognizing a specific antigen. There are no identical receptors shared by any two lymphocytes when they are generated[23]. Since each lymphocyte only carries one kind of receptor, there has to be an immense lymphocyte pool to provide enormous variety required by the adaptive response so that at any time point a proper adaptive immune response could be provoked against any antigen a person is likely to encounter. This is made possible by an intricate genetic mechanism known as somatic gene segment rearrangements[24]. Briefly, the genes responsible for encoding the variable region of a B-cell or T-cell receptor exist as groups of gene segments. Randomly selected gene segment from each group undergo recombination to

form a DNA sequence that could be translated into a fragment of polypeptides, and this unique amino acid arrangement is corresponding to the antigen binding specificity. The diversity of receptors is further magnified by introducing changes at the joint point between segments, random pairing of heavy chain and light chain, and somatic hypermutation that only occurs for B-cell receptors[25]. In total, there are 10^{11} possibilities in the structure of each receptor. Adopting this strategy, the immune system focuses resources and energy on pathogens that manage to bypass innate immunity – only lymphocytes with the matching specificity being activated to proliferate and their daughter cells inheriting the same receptors forming a big army of effector T cells.

B-cell receptors can either be transmembrane or be secreted post activation as an antibody[26]. Each has a different immunological effector function regarding pathogen defense. T-cell receptors on the other hand are only transmembrane and their function is exclusively to bind to the matching antigen on APCs and targeting cells[27]. Another major difference between B-cell and T-cell receptors is that instead of recognizing epitopes (molecular features on an antigen surface) directly, T-cell receptors only respond to antigens presented in the form of peptide-fragments:MHC molecule complexes on APCs and infected cells[28]. The different mechanisms of the receptor's activation reflect the distinct effector functions of B-cells and T-cells. Activated B-cells differentiate into effector plasma cells, of which the sole mission is to secret antibodies that directly bind to extracellular antigens. In comparison, there are three major types of effector T-cells[29]. Cytotoxic T cells kill cells that are infected with intracellular pathogens (primarily viruses and intracellular bacteria); helper T cells offer assistance in B cell activation by providing additional signal; regulatory T cells are found responsible for immune regulation - limiting the damage when an immune response gets out of hand.

Immature dendritic cells consistently sample the peripheral environment through phagocytosis and macropinocytosis - engulfing large volume of fluid in a receptor independent manner.

Whenever dendritic cells uptake a pathogen, they migrate to secondary lymphoid tissue where they become mature dendritic cells able to display antigens in the specific manner as well as providing additional signals – all of which requisites for successful T cell activation[30]. The three key signals involved in T-cell activation are: 1.antigen-peptide:MHC molecule complex binding with TCR comprising the initial activation signal; 2.co-stimulatory molecules displayed on mature APCs binding with their ligands on T cells, driving clone expansion and survival; and 3.cytokines secreted by activated APCs, which often determine the fate of T cells[31]. Depending on the type, effector T cells could either migrate to the site of infection or stay in the secondary lymphoid organs to activate B cells. Upon activation, a small portion of plasma cells (activated B cells) stay in the secondary lymphoid organs, while majority of them migrate to the bone marrow, from where they send antibodies out to enter the blood stream[32].

In addition to dendritic cells and macrophages, mature naïve B cell could also be induced to express co-stimulatory molecules and thus act as APCs[33]. Once B-cell receptors (BCRs) on the surface of naïve B cells recognize and bind to a specific pathogen, the formed complexes will be internalized by B cells, split into peptides by lysosome, and then presented to the corresponding T cells.

After successfully eliminating an infection, most effector lymphocytes die through apoptosis. Roughly 5% of them survive and become memory cells. Memory T-cells and B-cells are the pillar of immune memory. Upon reencountering with the same antigen, memory cells are able to differentiate into effector cells in a much shorter amount of time, and elicit a stronger and more efficient secondary response to ensure the rapid elimination of invading pathogens.

1.1.2.1 Humoral immunity

Humoral and cellular immunity are the two arms of adaptive immune response. Humoral

immunity refers specifically to antibody mediated immune responses. The humoral response is named aptly due to its components exiting in blood plasma and bodily fluids. Antibodies comprise the immunoglobulin group of biochemistry molecules[26]. They structurally resemble the letter 'Y', composed of two identical heavy chains and two identical light chains[34]. Each arm of antibody carries one antigen epitope binding site, constituted by the top quarter of the heavy chain and the top half of the light chain. Those sections are the variable regions that bear almost infinite diversity. The remaining three quarters of the heavy chain and the bottom half of the light chain constitute the constant region of antibody[35]. The bottom half of the two heavy chain form the antibody stem. The constant regions are of very limited variety. One of five forms is chosen for an existing antibody - referred to as an isotype. Instead of determining the antigen recognizing specificity, the constant region control antibodies' functional properties[36].

Antibodies are engaged in three distinct immune mechanisms regarding host defense: neutralization, opsonization and complement activation[37]. Neutralization is the direct binding of small size extracellular pathogens by antibodies, preventing them from entering cells or causing further damage. It is mostly effective against viruses and bacterial toxins. However, for bigger size pathogens like bacteria and parasites, direct binding is not enough to weaken them. This is where opsonization comes into play. Opsonization is the coating of antibodies on pathogens. In this case, antibody binding facilitates the recognition and engulfing of pathogens by phagocytic cells. This is induced by the binding of surface receptors on phagocytotic cells to the stem part of antibody. Another immune mechanism that can be activated by antigen:antibody complexes is the complement system. Upstream protease members bind to the pathogen surface of the complexes, triggering downstream activities.

1.1.2.2 Cellular immunity

Pathogens that manage to invade host cells, or replicate inside infected cells are less vulnerable

to humoral immunity. The help from T lymphocyte mediated immune responses is required in this case, forming the concept of cellular immunity. The two major classes of effector T cells-cytotoxic T cells and helper T cells carry distinct surface function markers. These markers are CD8 and CD4 respectively. Naïve $CD4^+$ T cells can further differentiate into several main subtypes of helper T cells: T_H1, T_H2, T_H17 and regulatory T cells[38]. T_H 1 cells have dual functions. Aside from producing essential co-stimulation signals for B cell activation, T_H1 cells are also involved in helping macrophages with combating mycobacteria infection[39]. Certain intracellular bacteria reside inside sub-cellular vesicles of macrophages. They possess the ability to prevent dwelling vesicles from merging with lysosome, stopping further degradation. T_H1 cells bearing receptors for those bacteria activate infected macrophages enabling the fusion, thus clearing the infection with lysosomal enzymes and antimicrobial substances. T_H2 cells, on the other hand, are specialized in the process of B cell activation for antibodies production[40]. T_H17 cells are important in recruiting neutrophils for assisting defense against extracellular pathogens at early stage of adaptive immune responses[41].

1.1.2.2.1 Primary T cell response

The activation of $CD4^+$ and $CD8^+$ T cells require two kinds of MHC molecules along with the processed antigen peptide to interact with TCR as the first signal (signal one)[42]. The loading of the peptide occurs during the synthesis and assembly of the MHC molecule on the endoplasmic reticulum (ER) in the cytosol. The complexes are then transported and displayed on the cell surface. The two kinds of MHC molecules have very different preference for peptide origin, which in turn, tailor the targeting of $CD4^+$ and $CD8^+$ T cells[43]. MHC Class I molecules carry peptides derived from proteins that are synthesized in the host cells. The proteins are broken down by proteasome in the cytosol. Therefore all the self-peptides and foreign peptides that are made with the host cell's protein-making machinery will be presented. Self-peptides usually do not provoke an immune response, whereas all viruses replicate inside the host cells will be

exposed, inducing naïve $CD8^+$ T cell activation. These $CD8^+$ T cells then differentiate into effector cytotoxic T cells, destroying infected cells[44]. MHC Class II molecules bind to peptide originating from extracellular antigens internalized by phagocytic APCs. Those peptides are processed in intracellular vesicles, and then loaded on to MHC class II molecules[45]. The peptide: MHC II complex is detected by naïve $CD4^+$ T cells, stimulating their differentiation into effector helper T cells. These helper T cells go on to assist macrophage functions as well as B cell activation. Given this fine division of responsibility, it follows that MHC Class I molecules are found on virtually all nucleated cells, whereas MHC Class II molecules are normally expressed only on major APCs. The physical interactions between CD4/CD8 and MHC molecules are the first step in the activation of the first signal. They are thus known as co-receptors of T cells.

The three signals essential for the priming of T cells are generally provided by the same APC. The second signal (signal two) is indispensable for T cell activation. Without this signal, T cells migrate into an opposite fate; either being targeted for deletion or moving to a non-responsive state called anergy or tolerance[46]. The most common co-stimulators are the B7 molecules on DC, including B7.1 (CD80) and B7.2 (CD86). The ligand for it is CD28, which is present on the surface of all naïve T cells[47]. Several additional co-stimulators expressed by DCs and activated T cells are involved in modifying signal two. CD40 molecules on DC are members of the tumor necrosis factor receptor family (TNFR). The binding between CD40 and its ligand CD40L on T cells promote the activation of T cells, further enhancing the expression of B7 molecules[48]. Another pair of co-stimulators that belongs to the TNF receptor family is 4-1BB (CD137) on the T cells and 4-1BBL from APCs, which also sends mutual beneficial signals. However, some co-stimulators are found to induce the opposite effect. CTLA-4 (CD152) is a CD28 related surface protein. The binding of it with B7 delivers an inhibitory signal, hindering further expansion of T cells[49-51]. PD-1 on activated T cells binds PD-L1 from APCs leading to down-regulation of T cell response [52, 53]. Those inhibitory signals are of great importance for

preventing overstimulation of the immune system. The most crucial function of signal two is the induction of activated T cells to start synthesizing interleukin-2 (IL-2). IL-2 enables activated T cell to enter a robust proliferation phase, which generates thousands of progeny cells possessing the same specificity within 4 to 5 days. This process is known as clone expansion. Moreover, IL-2 promotes the activated progeny T cells to differentiate into effector T cells.

1.1.2.2.2 Effector $CD8^+$ T cells induce CTL responses

$CD8^+$ T cell mediated cytotoxic T-cell responses are highly important in combating virus induced intracellular infection[54]. Once $CD8^+$ T cells become cytotoxic lymphocytes (CTLs), they are able to deliver their killing effect on all virus infected target cells. They do so without the requirement for co-stimulator signals, inducing target cells to undergo apoptosis[55]. The killing effect of CLTs is achieved by releasing cytotoxic granules that contain effector proteins including perforin, granzymes and granulysin[56]. A well-ordered immunological synapse is built between CTLs and target cells, which helps direct the cytotoxic effector molecules towards the target cells in a highly polarized way[57]. CTLs can induce apoptosis of target cells in a cytotoxic granules-independent manner[58]. Fas, another TNF family member, is expressed by target cells including activated lymphocytes. Its corresponding ligand FasL is found on effector CTLs. The interaction of Fas and FasL leads to the recruitment of Fas-associated death domain (FADD), eventually activating the caspase pathway. This mechanism is commonly used for regulating lymphocyte proliferation after a successful clearance of pathogens, as well as promoting self-antigen tolerance[59]. CTLs also secrete effector cytokines like IFN-γ, TNF-α and LT-α. They can directly interfere with viral replication, increase the expression of MHC Class I molecules on infected cells, as well as enhance macrophage activation[60]. The quality and quantity of the CTL response is affected by a number of factors. These include the nature of the antigen, inflammatory stimuli, duration of the infection, and so on.

1.1.2.2.3 Memory T cell response

The most significant outcome of a primary response is the establishment of immunological memory. Once the infection is resolved, 90%-95% of effector CTLs are lost through apoptosis - named the contraction phase of the CTL response[61]. The small surviving population is composed of long-lived memory cells, which lay the foundation of potentially lifelong immune protection[62]. IL-7 and IL-15 are crucial for maintaining long-term survival of $CD8^+$ memory T cells[63]. There are two kinds of memory T cells – each with distinct activation characteristics[64]. Effector memory T cells express comparatively low levels of lymph node homing cytokine receptors CCR7 and CD62L, but are abundant in inflammatory chemokines receptors like CCR3, CCR5. They swiftly differentiate into effector T cells upon restimulation. On the contrary, central memory T cells are $CCR7^+CD62L^{high}$. They are often found resting in peripheral lymphoid tissues, taking longer to mature into effector T cells. The mechanism that drives the differentiation of effector cells into either one of those two memory cell phenotypes is still under exploration.

1.2 HIV-1 and AIDS

HIV stands for Human Immunodeficiency Virus. It has been identified as the pathogen responsible for Acquired Immune Deficiency Syndrome (AIDS)[65-67], which is arguably the most extreme case of immune dysfunction. Spread of HIV occurs by contact with bodily fluids - commonly through blood, semen, and breast milk. HIV-1 infection is characterized by a progressive loss of immune function, increased susceptibility to opportunistic infections and cancers. Most HIV-1 infected patients progress to AIDS within 10 years without treatment. After AIDS diagnosis, life-expectancy shortens to around 1 year. In 2013, it was estimated that around 35 million people were living with HIV globally – with 2.1 million new cases arising that year. Over 36 million people have died due to HIV-1 infection since its discovery in the 1980's.

1.2.1 HIV-1 virology

HIV-1 is categorized as a type of retrovirus. Retroviruses contain RNA as their genetic material and encode for a reverse transcriptase that enables the formation of a dsDNA provirus upon entry into a target cell. It falls into a subgroup known as lentiviruses, which are distinguished by their long and variable incubation periods[68] and the presence of additional regulatory genes.

1.2.1.1 Structure of HIV-1

The viral particle is roughly spherical in shape[69], with a diameter of approximately 100 nanometers. It is enclosed by a viral envelope comprised of two layers of phospholipids, taken from the host cell plasma membrane during the budding process. Protein Env, which plays a central role in the virus entering phase, is embedded in the envelope spiking through the surface. Three glycoproteins (gp120) compose the cap of Env, while three gp41 compose the stem part. Between the viral envelope and the viral core is matrix protein p17. Two single strands of positive sense HIV genomic RNA are surrounded by the conical-shaped viral core, which is made of viral capsid protein gag p24.

HIV-1 has a more complicated genome structure than most retroviruses. It contains 9 reading frames encoding three standard structure proteins including gag, pol and env, as well as six additional regulatory proteins: vif, vpr, tat, rev, vpu, and nef. The HIV-1 primary transcript is further spliced, forming an mRNA pool containing more than 25 mRNA. Those mRNA can be divided into three classes according to size: the full-length 9Kb mRNA, which will be translated into Gag and Gag-Pol proteins, the single-spliced 4Kb mRNA encoding Env, Vif, Vpr and Vpu proteins, and the double-spliced 2Kb mRNA generating Tat, Rev and Nef proteins.

Vif has been proven to be responsible for preventing antiviral host defense proteins from the APOBEC3 family (apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like)[70] from being incorporated in virions. APOBEC3 enzymes are DNA cytidine deaminases, which induce hypermutation on viral cDNA during reverse transcription to rendering HIV-1 non-viable[71]. Nef[71, 72] and Vpu[73] interact with the cytoplasmic tail of CD4 to decrease its surface expression, facilitating evasion of immune recognition[74]. Another important function of Vpu is counteracting B cell stromal factor 2 (BST-2)[75, 76], also known as tetherin – which blocks the releasing of mature viral particles from the host cell surface. Vpr[73] possesses the ability to arrest infected cells in the G2 phase of the cell cycle, possibly due to the fact that virus replication is more active in the G2 phase. Those four viral proteins are usually referred to as "accessory proteins". Tat proteins accelerate transcription and Rev proteins help shuttle unspliced viral mRNA from the nucleus to the cytoplasm. Reverse transcriptase, integrase and protease are three enzymes that exist in the viral core. They are crucial for HIV-1 replication after viral entry.

Based on the genetic heterogeneity, HIV-1 viruses are commonly divided into 3 phylogenetic groups: M (major), N (non-M/non-O), and O (outlier)[77]. Group M is predominate and responsible for over 90% of HIV-1 cases. There are at least 10 clades within the M group, according to their geographically distinct genomes[78]. Clade A is common in central and eastern Africa; clade B is found dominantly in Europe, North American, Australia and Thailand; clade C is mostly found in China, India, and some parts of Africa[78].

Recombination events between clades are frequently reported, termed as "circulation recombinant forms"[79]. Those hybrid forms are attributed to dual infection - individuals infected with more than one HIV-1 strain[80]. Two types of dual infections are described:

1. Coinfection. Infection of two distinct strains happens simultaneously, or within a short frame - the second infection happens during acute phase of initial infection.

2. Superinfection. Acquiring of the second strain takes place when infection with the initial strain is established, presumably after seroconversion but rarely in chronic phase[81].

It has been suggested by several studies that the immune responses elicited by the initial infection are not capable of preventing the second infection[82, 83]. Dual infection is associated with more severe disease progression, decrease in $CD4^+$ T cell count, and higher chance of drug resistance[84-87].

1.2.1.2 HIV-1 replication cycle

There are generally six steps in the replication cycle of HIV-1[88]. The first step is viral entry into the target host cells. The mechanism by which this occurs consists of the binding between viral gp120 molecules and CD4 molecules on the cell surface. This triggers a conformational change in gp120, enabling a secondary interaction between gp120 and cell surface co-receptors[89]. The viral envelope and the cell membrane then fuse, and the viral core is essentially injected into the cell[90]. In the next step, HIV-1 reverse transcriptase converts viral RNA into DNA using host nucleotides in the cytoplasm of the cell[91]. After that, viral integrase carries the newly synthesized dsDNA into nucleus, inserting the viral DNA into the host cell chromosome. From here on, viral DNA becomes part of the host genomic DNA. Using host cell machinery, both encoding mRNA as well as genomic RNA is transcribed. The encoding sequences are then translated into long chains of viral proteins and enzymes. Eventually, all the viral components are assembled at the inner surface of the cellular membrane and form an immature viral particle, which buds off the host cell. Viral proteases digest the poly-protein chain into individual parts. Thus a mature infectious virion is created. This last step marks the completion of the entire viral replication cycle for HIV.

1.2.2 HIV-1 pathology

HIV-1 targets the most crucial cells in the immune system such as $CD4^+$ helper T cells, dendritic cells, and macrophages [92]. This leads to the depletion of $CD4^+$ T lymphocytes and the dysfunction of DC, depriving the ability of immune system to fight natural infections – paving the way for life-threatening opportunistic infections and malignant tumors.

HIV-1's entry of the target cell requires the recognition of cell surface receptor CD4 by viral Env gp120 and co-receptor CXCR4 for T-tropic strains or CCR5 for M-tropic strains [93, 94]. These co-receptors naturally serve as targets for chemokines. CXCR4 is the receptor for the chemokine SDF-1 [95, 96], while CCR5 acts as a receptor for the chemokines MIP-1 α and β as well as RANTES [97]. The optimal replication of HIV-1 in $CD4^+$ helper T cells requires cell activation. An increased level of transcription factors such as NF-κB leads to more effective transcription, as well as a higher nucleotide pool in these activated cells allows for more efficient reverse transcription[98]. This offers one explanation as to why reverse transcription is not completed in quiescent cells[99].

At the early stage of an infection, DCs in mucosal tissues capture HIV-1 via DC-SIGN[100], carrying the virus from mucosal tissues to draining lymph nodes where DC-SIGN promotes HIV infection by delivering the virus to $CD4^+$ T cells as well as macrophage/monocyte cells. Thus HIV uses DCs as a Trojan horse. HIV-1 induces the killing of infected $CD4^+$ T cells in at least three manners: apoptosis of uninfected bystander cells[101], direct killing, and virus specific CTL responses of infected cells[102].

1.2.3 Courses of HIV-1 infection

HIV-1 infection can generally be broken down into four distinct stages. The acute infection stage consists of the first 2 to 4 weeks post infection. Most patients experience influenza-like symptoms. During this stage, an abundance of virus is generated, primarily in the peripheral

circulatory system. From there, it is spread throughout the body with an emphasis on the lymphoid organs. The number of $CD4^+$ cells drops profoundly in this phase. However, in the next phase, the count of $CD4^+$ T cells rebounds close to ordinary levels, owing to the adaptive immune response fighting back with cytotoxic T cells and humoral antibodies – drastically reducing viral titers. The infection then enters the clinical latency period. This stage lasts anywhere between 6 months to over 20 years and appears quiescent despite the fact that HIV-1 replicates persistently in the lymphoid organs and $CD4^+$ T cell count continually declines. The immune system is eventually damaged to such a degree that the human body is unable to fight off other infections. The HIV-1 load in the blood considerably increases while the number of $CD4^+$ T cells drops to dangerously low levels. HIV-1-infected patients are diagnosed with AIDS when they have one or more opportunistic infections and/or fewer than 200 $CD4^+$ T cells per milliliter of blood.

1.2.4 HIV-1 treatment

There is currently no universal effective vaccine or cure for HIV-1. Current therapies for the treatment of HIV-1 infection are aimed at developing reagents that interfere with distinct steps in the viral life cycle – inhibiting viral replication. Six mechanistic classes of drugs have been licensed so far for HIV-1 treatment.

1. Entry inhibitors (or fusion inhibitors) interfere with viral binding, fusion and entry[103] by blocking either gp41 of HIV to form an inactive bundle. An example of this is T20 (Enfuvirtide). Other drugs such as Maraviroc target co-receptor CCR5 on $CD4^+$ T cells.
2. Nucleoside-analog Reverse Transcriptase (RT) inhibitors (NRTIs)[104] act as competitive substrate inhibitors. They prevent other nucleosides from being incorporated because of they lack a 3'OH (hydroxyl) group – thus terminating viral DNA chain elongation. Examples of drugs in this class include zidovudine, dideoxyinosine, and dideoxycytidine.

3. Non-nucleoside-analog RT inhibitors (NNRTIs)[105] on the other hand act as non-competitive inhibitors. They bind to a hydrophobic cavity adjacent to the polymerase active site of RT, disturbing the handling of substrate nucleotides by the enzyme. Examples of drugs in this class are nevirapine, and rilpivirine.
4. Integrase inhibitors inhibit the enzyme activity of integrase. This is a relatively new class of drug and includes Elvitegravir, and Dolutegravir[106].
5. Protease inhibitors[107] block encapsidation of precursor proteins into mature viral capsids. Drugs in this class include Darunavir, and Atazanavir.
6. Maturation inhibitors[108-110] target the protease substrate Gag polyprotein precursors instead of the enzyme itself. An example of a drug in this category is Bevirimat [99].

Antiretroviral drugs are commonly used in combination of 3 or more drugs belonging to at least two different classes. This is referred to as Highly Active Anti-Retroviral Therapy (HAART)[111]. HAART is considered the greatest success in combating HIV[112, 113]. It virtually converts HIV infection from a "death sentence" to a long-term chronic infection. Unfortunately, drug resistance to most of these agents have been reported due to the rapid evolution of HIV *in vivo*[114, 115]. HARRT has been very effective in reducing viral titers to below detection limit and partial recovery of $CD4^+$ T cell levels. However, it is limited by its high cost, drug availability, complicated administration schedules, serious side effects, and the potential that the virus will ultimately develop drug resistance[116, 117]. It neither cures the patient nor does it completely eradicate HIV infection. HIV-1 viral loads rebound quickly if treatment ceases[112, 118].

1.2.5 HIV-1 latency and chronic infection

HARRT effectively reduces plasma HIV-1 RNA level from over 10,000 to below 50 copies/ml, which is well under the detection limit of clinical assays. However, a stable latent reservoir

composed of latently infected resting $CD4^+$ T cells persists despite decades of non-interrupted treatment[119, 120]. The latent reservoir is established during primary HIV-1 infection prior to the treatment[121]. Although less than one per million resting $CD4^+$ T cells harbor latent provirus, they represent the major barrier to the achievement of either a sterilizing cure or a functional cure for HIV-1. Naïve $CD4^+$ T cells and memory $CD4^+$ T cells are the two major latent reservoir hosts. Latency can be established in both naïve and memory $CD4^+$ T cells through direct infection[122]. This is inefficient due to the restrictive host environment (low CCR5 expression, limited nucleoside)[99, 123] and the presence of host restriction factors like APOBEC3G (low-molecular-mass complex, active form)[124], and SAMHD1 (sterile alpha motif (SAM) and histidine/aspartic acid (HD) domain-containing protein 1)[125]. As a result, viral DNA is generated in the cell without integration. This is known as pre-integration latency. It is unstable and decays rapidly[126, 127]. Post-integration latency mostly happens through infection of activated $CD4^+$ T cells, which allows integration of viral dsDNA in the host cell genome[128]. The small fraction of activated $CD4^+$ T cells becomes resting memory $CD4^+$ T cells, which carry stable provirus that can be reversed upon encountering their cognate antigen. Current strategies for purging viral latency are focused on reactivating of provirus without inducing global T cell activation. This alone does not eliminate latent reservoir owing to defective viral cytopathic effects (CPE) and the host CTL response[129]. Priming of effective HIV-1 specific CTL response becomes a pre-requisite[130]. However, CTL exhaustion induced by chronic HIV-1 infection complicates the situation[131].

Chronic infection related antigen-specific T cells exhaustion has been intensively studied since the early documentations more than a decade ago[132]. Chronic infection with HIV-1[131, 133], lymphocytic choriomeningitis virus (LCMV)[134], or adenovirus[135] does not lead to the generation of a highly polyfunctional memory T cell pool. Instead, CTLs adopt “exhausted phenotypes”, and display an array of dysfunctions in a gradual and orderly manner[136]. Early stage dysfunction compromises IL-2 secretion, proliferative capacity, and cytolytic ability. This

is followed by decrease in TNF-α production, and then IFN-γ production. In extreme circumstances, virus-specific CTLs could be physically deleted[137]. T cell exhaustion is also associated with increased and prolonged expression of surface inhibitory receptor molecules, such as PD-1, PD-L1, LAG-3, TIM-3, CTLA-4, 2B4 and many others[138]. An increased abundance of antigen, increased level of inhibitory receptor expression, lack of help from $CD4^+$ T cells, and a longer duration of infection correlate with the severity of exhaustion. The PD-1/PD-L1 pathway stands in the spotlight of research due to its great importance in exhaustion regulation[139]. *In vivo* blockage of PD-1/PD-L1 restores effector function of virus-specific $CD8^+$ T cells, enhancing virus control during chronic LCMV infection[140]. In HIV-specific CTLs, PD-1 expression is related to T cell dysfunction and disease progression[141-143]. *In vitro* targeting of the PD-1/PD-L1 pathway in HIV-infected cells enhanced effector function and proliferation[142, 144]. The maintenance of exhausted $CD8^+$ T cells in chronic infection is very distinct from that of memory $CD8^+$ T cells in acute infection. They show decreased level of homeostatic cytokine receptors CD62L and IL-7R[145], and rely on the presence of their cognate antigen for long-term survival[146]. This makes functional recovery of HIV-specific CTLs possible after efficient treatments or early epitope escape mutation[147]. Plenty of strategies have been exploited to rescue exhausted $CD8^+$ T cells. A dual *in vivo* treatment combining agonist anti-4-1BB Abs with PD-L1 blockage resulted in a vigorous boost in LCMV-specific CTL response, and enhanced viral control during chronic LCMV infection[148]. The co-stimulator CD40/CD40L pathway has been found to be indispensable for anti-PD-L1 treatment[149]. Moreover, agonistic anti-CD40 Abs could suppress PD-1 induction and restore CTL function[150].

1.2.6 HIV-1 vaccine and cure

A universal HIV-1 preventive vaccine is considered to be the optimal solution to achieve the ultimate goal of AIDS eradication. The ideal characteristics of a prophylactic vaccine should

evoke both overall and sterilizing immunity to block infection, prevent the establishment of a persistent infection, and finally impede its transmission. April 2015 marks the 31st anniversary of the discovery of HIV. The following obstacles are usually held responsible for preventing the development of an effective HIV vaccine:

1. Lack of broad neutralizing Abs: The conserved neutralizing epitopes of Env glycoprotein are masked by variable carbohydrates, and only transiently exposed during viral entry. The non-neutralizing domain stimulated B cell responses become immunodominant[151]. Furthermore, another factor hindering Ab induction is host tolerance deletion, as a result of autoreactivity with non-HIV antigens[152, 153].
2. Targeting the most essential immune cells: Activated $CD4^+$ T cells are fully permissive to viral replication. In other words, the key mediators of both humoral and cellular immunity that are currently fighting the infection are the ideal viral targets[154].
3. Establishment of viral latency: Once the retroviral genome is integrated into that of resting $CD4^+$ T cells, the provirus enters a stage where it is replication-competent but transcriptionally quiescent[155]. Latently infected memory $CD4^+$ T cells represent the biggest viral reservoir. They cannot be detected by the immune system and serve as the major source of viral rebound[156, 157].
4. High levels of genetic diversity: HIV is a rapidly evolving virus – it has an extremely high mutation rate. In contrast to the high fidelity of mammalian DNA polymerase α (typical error rate 10^{-4} to 10^{-5}), HIV-1 RT does not correct errors by exonucleolytic proofreading, shows relatively high efficiency both regarding misincorporation and subsequent extension of mispaired primers. The frequency of nucleotide-substitution introduced by HIV-1 RT could reach from 1/2000 to 1/4000[158], which could be interpreted as amaximum of 10 mutations per HIV-l genome each round of replication considering the process of RNA-ssDNA-dsDNA in the retroviral life cycle. In addition, the frequent recombination events and the fast replication cycle - approximately 10^{10} virions being produced every day, are contributing to HIV genetic variation[159-161].

5. Direct cell-to-cell transmission: Besides the classic cell-free transmission method, HIV infected cells can form virological synapses with uninfected cells to directly spread viral particles[162]. This may protect the virus from several biophysical, kinetic, and immune effector barriers[163, 164].

There are a handful of examples where a patient is cured of HIV. The "Berlin patient"[165] who suffered from both leukemia and HIV, received a bone marrow stem cell transplant from a donor homozygous for a 32-base pair deletion in the HIV-1 coreceptor CCR5, which is naturally resistant to HIV infection with R5 tropism[166]. Following transplantation, no persistent HIV could be detected – even after the cease of HAART treatment. This is the first and probably the only sterilizing cure of established HIV infection[167]. Another case is the "Mississippi baby", who was born infected in 2010, and received early, aggressive HAART treatment for 18 months[168]. The HIV level remained undetectable within the following one year after HAART interruption. The baby was believed to have been cured of HIV until rebound viremia was reported after an additional year of follow-up. For functional cure, which is defined as the establishment of effective host immunity that provides permanent suppression of HIV replication in the absence of ART, rather than a complete eradication, there are elite controllers[169], long-term nonprogressors[170], and post-treatment controllers[171]. Post-treatment controllers represent a small percentage of patients who were treated early during acute infection[172].

The above cure examples are considered invaluable for HIV-1 research; unfortunately, they cannot be applied to all 35 million HIV patients worldwide. A more realistic approach is to develop therapeutic vaccines that provide immunity to partially control viral replication post infection. Two major strategies represent the main principle of current HIV-1 therapeutic vaccine design: inducing protective neutralizing antibodies to interrupt viral entry via a humoral immune response – mostly targeted at Env gp120, and eliciting an HIV-1-specific CTL response via a cellular immune response – commonly tested in replication-incompetent viral

vectors or autologous DC.

1.3 Therapeutic vaccine for HIV-1 treatment

Therapeutic vaccines seem to be more feasible than prophylactic ones in terms of strategy execution and efficacy evaluation. Therapeutic vaccines impose less ethical issues, requiring much shorter and more affordable proof-of-concept efficacy assessments. In addition, they are more efficient at narrowing down pathogenic epitopes and relevant immune responses that should be examined. They could be used in combination with HAART as treatment intensification, helping with the purging of the viral reservoir, or as treatment substitution if desired efficacy is attained.

1.3.1 Overview of therapeutic vaccine strategies

Traditional approaches for vaccine development include the use of live-attenuated vaccines (oral polio, mumps and yellow fever), whole inactivated viruses (hepatitis A, influenza), and recombinant proteins (hepatitis B). These approaches have either been considered or tested for HIV-1 therapeutic vaccine design. Live-attenuated HIV-1 viruses are considered too risky due to the possibility of reverting to virulence via its high mutation rate[150]. Remune, a whole, inactivated HIV-1 immunogen lacking protein Env, was unable to induce an enhanced CTL response, increase $CD4^+$ T cell count, or diminish viral loads[173, 174]. Recombinant HIV-1 protein or peptide vaccines such as Env[175-177], Tat[178-180], and Gag p24-like peptides[181-183] were exhibiting promising outcomes including improvement of antigen related $CD4^+$ and $CD8^+$ T cell responses. Even so, they failed to deliver clinical efficacy or consistent immunogenicity. More innovative approaches are based on DNA vectors, recombinant viral vectors and DCs. DNA vaccines relying on bacterial plasmids encoding HIV-1 genes were able to evoke moderate level of HIV-specific CTL responses. However, there was little indication that

they restrained viral replication or had any effect on $CD4^+$ T cell counts[184-186]. Vaccines based on live recombinant viral or bacterial vectors have superior efficacy as they create natural intercellular infections that provoke an immune response against HIV-1 antigens. Their limitations are competition of antigen presentation between engineered HIV-1 proteins and the multitude of the vector's own proteins[187], and pre-existing immunity against the microbe from which the vectors are derived – either through endemic infection or vaccination[188]. Virtually every viral system, from which molecularly cloned vectors have been developed, has been probed for their potential usage as HIV-1 vaccine candidate. Poxvirus vectors are among the best characterized viral vectors[189]. The most commonly studied replication deficient poxvirus vectors are the Modified Vaccinia Virus Ankara (MVA), NYVAC, canarypox (ALVAC) and fowlpox (FPV). The QUEST study was based on ALVAC carrying HIV-1 gene sequence epitopes of env, gag, nef and pol[190]. The results were disappointing as there was no change in viral rebound, or reduction in viral replication[191]; it was found to be poorly immunogenic[192]. FPV vaccine expressing gag/pol and IFN-γ demonstrated improved control of viral replication, albeit with no difference regarding CTL responses[193]. MVA inserted with nef[194] or gag p24[195] maintained viral load post treatment interruption and increased effector cytokine IFN-γ production[196]. Replication deficient recombinant adenovirus type 5 (rAd5) is well known for its application in HIV-1 prophylactic vaccine clinical trials[197-199]. In a therapeutic context, patients with stable treatment were given rAd5 gag expressing vaccine. In these tests, a significant drop in plasma viral load was observed after treatment interruption, however, the impact on viral rebound kinetics was modest, and the level of significance was limited[200].

1.3.2 DC vaccine

DCs, as the most powerful APC initiating both innate and adaptive immune responses, have been heavily exploited for HIV-1 vaccine development[201]. The approaches for loading HIV-1 associated antigens to DC *in vitro* are quite versatile: pulsing with peptides, recombinant proteins

or apoptotic infected cells, or transfecting with antigen-encoding DNA, mRNA or viral vectors. Many groups have adequately reported their feasibility. Autologous DC pulsed with HIV-1 peptides and proteins were safe and brought a stronger HIV-1 specific immune response[202]. A recent study using DCs loaded with HIV-1 lipopeptides increased the breadth of immune response and effector cytokines, generating polyfunctional $CD4^+$ and $CD8^+$ T cells[203]. The drawbacks of this strategy are limited coverage over epitopes, and HLA polymorphism. Several DC immunotherapy clinical trials using autologous DC vaccines pulsed with autologous inactive HIV-1 virus exhibiting inconsistent levels of CTL response was able to partially reduce viral load in distinct scenarios[204-206]. A later study conducted in patients with high $CD4^+$ T cell counts with the same heat-inactivated HIV-1 pulsed DC vaccine significantly decreased plasma viral loads. This reduction in viral load is associated with an increased HIV-1 specific T cell response[207]. The shortcomings are potential risk of infection due to incomplete inactivation, and difficulty with standardization. DCs infected with ALVAC carrying HIV-1 genes prior to administration showed no difference in viral load rebound[208]. DCs electroporated with cDNA or mRNA enhanced antigen specific immunological response, CTL proliferation, and viral control[209-211]. Direct *in vivo* targeting of DCs, through HIV-1 antigens conjugated with antibodies of DC membrane molecules such as DC-SIGN or DC205, has been tested in animal models[212, 213]. Overall, only five out of thirteen DC based HIV-1 clinical trials elicited virological responses[214, 215]. The limited therapeutic efficacy of a DC based vaccine is partially due to the following reasons: DC induced $CD8^+$ T cell responses are largely $CD4^+$ T cell dependent (HIV-1 infection usually renders the immune system "helpless" from $CD4^+$ T cells)[216, 217]; infection, impaired function, and physical depletion of DCs are abundantly reported during early stage of HIV infection[218-220]; DCs are often inflammatory and immunosuppressive[221, 222], and these responses are mediated by the interaction between HIV-1 Env gp120 and DC receptors[223].

1.3.3 T-APC vaccine: a new concept

1.3.3.1 Forming of T-APC via immunological synapse

The concept of T cell-antigen-presenting cells (T-APC) was first raised by Xiang *et al.* through the examination of the transfer of APC associated membrane molecules from APCs to T cells during the process of T cell priming[224]. Antigen-peptide:MHC (pMHC) complexes (signal one) and co-stimulatory molecules (signal two) of mature APCs cluster in membrane lipid raft microdomains[225, 226]. In order to activate T cells, APCs connect with cognate T cell bearing TCRs and co-stimulatory molecule ligands. A stable APC-T cell junction forms via this interaction, known as an immunological synapse. Immunological synapse is a nanometer scale gap comprising two concentric rings. The central supramolecular activation cluster contains signal 1 and 2 molecules (pMHC complexes-TCR, APC CD80/86-T cell CD28), and the peripheral supramolecular activation cluster contains adhesion molecule ICAM-1 (CD54) of APC, which is a major ligand for the integrin leukocyte function-associated antigen-1 (LFA-1) on T cells[227, 228]. Consequently, APC-derived surface molecules are transferred to the T cells, and subsequently displayed on T cell surfaces by virtue of TCR-mediated internalization followed by recycling[229, 230]. This phenomenon is biologically significant – potentially changing T cell homing, effector functions, and above all, allowing T cells to act as APCs[231]. $CD4^+$ T cells acquire the synapse-composed antigen-presenting machinery (pMHC class I and II, costimulatory molecules CD54 and CD80) from APCs and act as $CD4^+$ Th-APCs capable of stimulating CTL responses *in vitro* and *in vivo*[224]. $CD8^+$ T cells are competent in the same manner - illustrating the concept of $CD8^+$ Tc-APC[232].

1.3.3.2 A novel exosome-targeted T cell vaccine

In addition to direct interaction, the membrane material of APCs can be transferred to T cells

through shedding vesicles. Exosomes are 50-90 nm membrane nanovesicles secreted by virtually all types of cells including DCs[233], and formed through the direct fusion between the endosome external membrane and plasma membrane. The biological function of exosome release was initially proposed as an obsolete mechanism for discarding cellular molecules[234, 235]. Later their essential role in intercellular communication drew growing attention since they can effectively transfer biological active molecules, proteins and RNAs[236]. Exosomes are enriched in MHC, costimulatory molecules, adhesion molecules, heat shock proteins (e.g., HSP70, HSP90) and tetraspanins. Transfer of exosomes to naïve DCs potentially amplifies cellular immune responses[237]. The potential immune function of exosomes derived from APCs has been confirmed in many studies[238-240]. DC derived exosomes (Dex) trigger $CD4^+$ and $CD8^+$ T cell activation, priming an *in vivo* CTL response, eradicating established murine tumor growth[241]. DC exosome based cancer therapeutic vaccines have been tested in phase I and II clinical trials[242]. Exosomes are highly involved in HIV-1 infection. Exosomes originating from HIV-1 infected cells contain viral Gag[243] and Nef[244, 245] proteins. The exosomal host intrinsic defense protein APOBEC3G confers HIV-1 resistance to exosome recipient cells[246]. Exosomes purified from $CD8^+$ T cells mediate noncytotoxic antiviral activity by suppressing HIV-1 transcription in both acute and chronic infection models[247].

Different approaches have been compared in order to maximize the immunogenicity of APC derived exosomes. Indirect loading of desired antigens via DC pulsing onto exosomes seems to increase their potency in inducing immune responses, compared to direct loading with peptides or proteins[248]. Mature DCs are superior to immature DCs since mature DCs release higher amount of exosomes. Such exosomes express increased level of immunostimulatory molecules pMHC class I and II, CD40, CD80, CD86, ICAM-1, and so on[249, 250]. Furthermore, in many studies, exosomes exhibit enhanced immunity when taken up by recipient cells – promoting T cell activation leading to tumor eradication[251, 252]. Hao *et al.* showed that DC acquiring exosomes derived from antigen pulsed DCs express high level of exosomal pMHC I,

costimulatory molecules, and stimulate antigen specific CTL responses and $CD8^+$ T cell memory[253]. T cells can take up Dex as well – although with lower efficiency compared to direct cell-to-cell interaction in terms of the amount of membrane immunostimulatory molecules transferred[254]. Therefore, Ag-presenting activity of DCs could be acquired by T cells through EXO[255]. Activated non-specific T cells adopting antigen-specific Dex are equipped with all the exosomal molecules, and become T-APC. EXO_{OVA}-targeted activated $CD4^+$T (aT_{EXO}) cells can (1) stimulate more efficient central memory $CD8^+$CTL responses and T cell memory than EXO_{OVA} or DC_{OVA}, (2) activate $CD8^+$CTL responses independent of $CD4^+$Th cells, and (3) counteract $CD4^+25^+$regulatory T (Tr) cell-mediated immune suppression. These results formed the new concept of a novel EXO-targeted $CD4^+$ T cell vaccine[256, 257].

1.4 Rationale, hypothesis and objectives

HIV-1 infection is a worldwide epidemic that is spreading at an appalling rate. It lays serious social and economical impact upon public health. Infection with HIV-1 fails to evoke protective immunity in the large majority of the population. The immune response is able to compromise the rate of replication but does not eliminate the infection. The acknowledgement that therapeutic vaccines can be a more plausible approach has been gaining growing attention in recent years. While the induction of broad neutralizing antibodies is typically a key element of prophylactic vaccines, therapeutic vaccines generally rely more on provoking cellular immunity. Consistent with this principle, Barouch *et al.* recently revealed their results of a SIV (simian immunodeficiency virus) prophylactic vaccine study, elucidating that protective immunity is mediated mostly by humoral anti-env antibodies, whereas post infection restraining of viral replication is mainly provided by cellular anti-gag response[258]. There exists an abundance of evidence supporting the critical role of HIV-1 specific CTLs in control of viral replication[259, 260]. Of note, up-regulated CTL activity mediated by $CD8^+$ effectors is associated with natural resistance to HIV-1 infection among a group of elite controllers[261]. Therefore, many attempts have been made or are ongoing for improving the HIV-specific CTL response.

T-APC as a novel T cell based vaccine has emerged as a potential candidate for HIV-1 therapeutic vaccines, aiming at boosting HIV-specific CTL responses. It provides distinct signals for $CD8^+$ T cells priming: exosomal pMHC I complex (signal 1), exosomal costimulatory CD80, CD40L (signal 2) and IL-2 cytokine secretion (signal 3)[232]. Exosome targeted T-APCs possess unique attributes, such as the stimulation of efficient antigen specific $CD8^+$ CTL responses in the absence of $CD4^+$ Th cells, and breaking $CD4^+25^+$ Tr cell-mediated immune tolerance, which makes EXO-targeted T cells vaccine an appealing novel strategy in HIV-1 infection. It is worth exploring if EXO-targeted T cells vaccine is applicable therapeutically for HIV patient treatment by using autologous T cells with uptake of EXOs derived from engineered

DCs[262, 263].

In this study, we tailored EXO-targeted T cells vaccine by using polyclonal activated $CD8^+$ T cells instead of $CD4^+$ T cells. $CD4^+$ T cells served as the primary target for HIV-1 infection[264]. Therefore, depletion of $CD4^+$ T cells is the hallmark of disease progression[265, 266]. We surmised that (1) OVA-specific exosome-targeted $CD8^+$ T cell-based vaccine (OVA-Texo) can stimulate efficient OVA-specific $CD8^+$ CTL and memory responses, inducing sufficient antitumor immunity against OVA-expressing tumor cells in mouse model. (2) This exosome-targeted $CD8^+$ T cell-based vaccine strategy could be applied to HIV-1- Gag protein, provoking effective Gag-specific $CD8^+$ CTL responses, T cell memory, and antitumor immunity against Gag-expressing tumor cells. (3) Engineering Gag-Texo with up-regulated 4-1BBL (APC derived costimulatory molecule) expression could improve the performance of Gag-Texo vaccine. (4) OVA-Texo is able to evoke a successful immune response in bystander chronic infection, converting $CD8^+$ T cell exhaustion, restoring effector functions of exhausted $CD8^+$ T cells.

In order to test the above hypotheses, the objectives of each corresponding study are as follows.
In part one:

1. Generation and characterization of $CD8^+$ OVA-Texo vaccine.
2. Assessing the ability of $CD8^+$ OVA-Texo vaccine to stimulate OVA-specific $CD4^+$ and $CD8^+$ T cell response.
3. Evaluation of $CD8^+$ OVA-Texo vaccine in stimulating of antitumor immunity and long-term memory against BL6-10_{OVA} in C57BL/6 mice.

In part two:

1. Generation and characterization of Gag-specific exosome-targeted $CD8^+$ T cell (Gag-Texo) vaccine.

2. Assessment of Gag-Texo vaccine in stimulating of Gag-specific $CD8^+$ CTL response and antitumor immunity against BL6-10_{Gag} in C57BL/6 mice.
3. Examination of Gag-Texo vaccine in stimulating of protective and therapeutic antitumor immunity against BL6-$10_{Gag/A2}$ in transgenic HLA-A2 mice.

In part three:

1. Generation and phenotype analysis of OVA-Texo vaccine with 4-1BBL transgene expression.
2. Investigate if OVA-Texo/$_{4\text{-}1BBL}$ vaccine can stimulate enhanced OVA-specific $CD8^+$ effector CTL responses and long-term recall memory responses.
3. Assessment of OVA-Texo/$_{4\text{-}1BBL}$ vaccine stimulated therapeutic and long-term antitumor immunity against BL6-10_{OVA} in C57BL/6 mice.
4. Evaluate if Gag-Texo/$_{4\text{-}1BBL}$ vaccine exhibits improved antitumor immunity against BL6-$10_{Gag/A2}$ in transgenic HLA-A2 mice.

In part four:

1. Assessment of CTL exhaustion caused by Adenovirus-OVA induced long-term chronic infection in C57BL/6 mice.
2. Evaluation of immune dysfunction induced by Adenovirus-LacZ long-term chronically infected C57BL/6 mice, and the counteraction ability of an OVA-Texo vaccine in bystander chronic infection.
3. Examining if OVA-Texo vaccine can convert exhausted CTLs in Adenovirus-OVA induced long-term chronic infected C57BL/6 mice.
4. Assessing if a dual treatment of OVA-Texo vaccine and PD-L1 blockage can result in a synergistic effect in rescuing CTLs from exhaustion in chronic infection.
5. Investigate if a Gag-Texo vaccine can stimulate antitumor immunity against BL6-$10_{Gag/A2}$ in chronic infected transgenic HLA-A2 mice.

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CHAPTER 2 GP120-SPECIFIC EXOSOME-TARGETED T CELL-BASED VACCINE CAPABLE OF STIMULATING DC- AND CD4+ T-INDEPENDENT CTL RESPONSES

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Running title: GP120-specific exosome-targeted T cell vaccine

Key words: T-cell vaccine, Gp120, exosome, $CD8^+$ CTL

[1]Note: Wang and Hebbandi Nanjundappa made the same contribution to the study

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2.1 Abstract

The limitations of highly active anti-retroviral therapy (HAART) have necessitated the development of alternative therapeutics. In this study, we generated ovalbumin (OVA)-pulsed and pcDNAgp120-transfected dendritic cell (DC)-released exosomes (EXOova and EXOgp120) and ConA-stimulated C57BL/6 $CD8^+$ T cells. OVA- and Gp120-Texo vaccines were generated from $CD8^+$ T cells with uptake of EXOova and EXOgp120, respectively. We demonstrate that OVA-Texo stimulates *in vitro* and in vivo OVA-specific $CD4^+$ and $CD8^+$ cytotoxic T lymphocyte (CTL) responses leading to long-term immunity against OVA-expressing BL6-10_{OVA} melanoma. Interestingly, $CD8^+$ T cell responses are DC and $CD4^+$ T cell independent. Importantly, Gp120-Texo also stimulates Gp120-specific CTL responses and long-term immunity against Gp120-expressing B16 melanoma. Therefore, this novel HIV-1-specific EXO-targeted Gp120-Texo vaccine may be useful in induction of efficient CTL responses in AIDS patients with DC dysfunction and $CD4^+$ T cell deficiency.

2.2 Introduction

Acquired immunodeficiency syndrome (AIDS) in human immunodeficiency virus type-1 (HIV-1)-infected patients is a rapidly expanding global pandemic, which causes millions of death each year. In AIDS patients, severe immune suppression and dysfuction have been found. For example, viral Gp120 causes $CD4^+$ T cell death and depletion [1] and dysfunctional dendritic cells (DCs) [2] through its binding to CD4 and C-type lectin DC-SIGN, respectively. Highly active antiviral therapy (HAART) suppresses viral replication and significantly improve the prognosis [3]. However, it does not restore immunity to HIV-1 [4]. Treatment in acute phase of human immunodeficiency virus type 1 (HIV-1) infection preserves $CD4^+$ T cells [5], but limits $CD8^+$ T cell responses [6], whereas treatment in chronic phase does not restore $CD4^+$ T cells [4, 5] and $CD8^+$ effector T cells decay with virus [7] leading to proviral latent reservoirs in AIDS patients with chronic infection [8, 9]. New immunotherapeutic strategies [10] are expected to be developed to enhance virus control, help to attenuate disease progression during treatment interruptions, and limit further usage of HAART, minimizing their toxicity and cost [11].

Neutralization antibodies (Abs) binding to the virus envelope can prevent a virus from entering cells. However, in the case of HIV-1 infections, the envelope proteins, which are the primary target for neutralization, have evolved multiple pathway to escape elimination by Abs [12]. As such, efforts to develop envelop-based HIV-1 vaccines that elicit high and sustained titers of broadly cross-neutralizing Abs have been unsuccessful [13, 14]. More and more evidence have shown that $CD8^+$ cytotoxic T lymphocytes (CTLs) play a critical role in control of HIV-1 proliferation [15-17]. Furthermore, $CD8^+$ CTLs appear to work against a wide array of variants, selectively targeting and destroying cells that the virus has managed to infect. Therefore, cellular immunity plays an important role in controlling the acute phase of infection and disease progression even in the absence of neutralizing Abs [18, 19]. Therefore, how to stimulate efficient $CD8^+$ CTL responses to clean the entire latent reservoir becomes one of the major

challenges in AIDS patients with HAART.

DCs are the most potent antigen-presenting cells (APCs) that can stimulate $CD4^+$ T cell-dependent $CD8^+$ CTL responses [20]. DC vaccine for induction of antitumor immune responses has been well documented [21]. Late structural proteins such as Gp120 are the major targets for HIV-1 vaccines. In the context of DC-based immunotherapy, DCs expressing HIV-1 antigens (Ags) have also been shown to stimulate HIV-1-specific $CD8^+$ CTL responses both *in vitro* [22, 23] and *in vivo* in animal models [24-27]. In addition, HIV-1-specific DC vaccine has also been applied to clinical trials in AIDS patients [28-31]. However, its therapeutic efficiency was very low, possibly due to lacking of functional $CD4^+$ T cells. DCs also process Ags in endosomal compartments which can fuse with plasma membrane, thereby releasing Ag presenting vesicles called "exosomes" (EXO) [32]. Recently, we have demonstrated that Con A-stimulated nonspecific $CD4^+$ T cells with uptake of ovalbumin (OVA) Ag-specific DC-released EXO can be used as vaccine capable of inducing $CD4^+$ T cell-independent OVA-specific $CD8^+$ CTL responses and antitumor immunity [33]. However, whether $CD8^+$ T cells with cytokine profile distinct from $CD4^+$ T cells can be used as vaccine to stimulate Ag-specific $CD8^+$ CTL responses after they uptake Ag-specific DC-released EXO is unknown.

In this study, we constructed an expression vector pcDNAgp120 expressing HIV-1 Gp120. We generated DCgp120 and BL6-10gp120 cell lines expressing gp120 by transfection of DC cell line (DC2.4) and B16 melanoma cell line (BL6-10) with pcDNAgp120, respectively. We then generated OVA-pulsed DC (DCova)- and DCgp120-released EXO (EXOova and EXOgp120) and ConA-activated polyclonal $CD8^+$ T (ConA T) cells of C57BL/6 mice. By incubation of $CD8^+$ ConA T cells with EXOova and EXOgp120, we further generated EXO-targeted T (OVA-Texo and Gp120-Texo) cells with uptake of exosomal molecules of EXOova and EXOgp120 for vaccination. We demonstrated that OVA-Texo vaccine is capable of stimulating DC- and $CD4^+$ T cell-independent OVA-specific $CD8^+$ CTL responses leading to long-term

antitumor immunity against OVA-expressing B16 melanoma (BL6-10ova). Importantly, Gp120-Texo vaccine also stimulates efficient Gp120-specific CTL responses and long-term immunity against Gp120-expressing B16 melanoma (BL6-10gp120).

2.3 Materials and methods

2.3.1 Reagents, cell lines and animals.

Ovalbumin (OVA) was obtained from Sigma (St. Louis, MO). OVAI (SIINFEKL) specific for H-2K^b and Mut1 (FEQNTAQP) peptide specific for H-2K^b of an irrelevant 3LL lung carcinoma were synthesized by Multiple Peptide Systems (San Diego, CA). Biotin-labeled or fluorenscein isothiocyanate (FITC)-labeled antibodies (Abs) specific for H-2K^b, Ia^b, CD8, CD11c, CD25, CD40, CD54, CD80, LFA-1, LAMP-1, FITC-conjugated avidin, PE-anti-IFN-γ and ECD-anti-CD44 Abs were all obtained from BD Biosciences (Mississauga, Ontario, Canada). The goat anti-gp120 polyclonal Ab was obtained from Cedarlane Lab Ltd, Burlington, Ontario, Canada. The anti-H-2K^b/OVA I complex (pMHC I) Ab was obtained from Dr. Germain (National Institute of Health, Bethesda, MD) [33]. The 5-carboxy-fluorescein diacetate succinimidyl ester (CFSE) was obtained from Molecular Probes, Eugene, OR. Expression vector pcDNAgp120 expressing HIV-1 Gp120 was constructed by cloning Gp120 gene from PConBgp120 vector (NIH AIDS Research and Reference Reagent Program, Bethesda, MD) into pcDNA (Invitrogene, Carlsbad, CA) by recombinant DNA technology. OVA gene-transfected BL6-10 (BL6-10$_{OVA}$) melanoma cell line was previously generated in our laboratory [34]. BL6-10 tumor cell line was also transfected with pcDNAgp120 or pcDNA to form HIV-1 Gp120-expressing BL6-10gp120 or BL6-10null by Lipofectamine 2000 (Invitrogene). Female C57BL/6 and OVA-specific T cell receptor (TCR)-transgenic OT I and OT II mice and diphtheria toxin receptor (DTR)-CD11c transgenic (Tg) mice on a C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, MA). Dendritic cells of DTR-CD11c Tg mice expressing DTR under CD11c promoter can be completely depleted by a single dose of diphtheria toxin (DT) treatment (1.5 μg/kg mouse body weight) [35, 36]. All mice were treated according to animal care committee guidelines of the University of Saskatchewan.

2.3.2 Dendritic cell and exosome preparation.

The wild-type C57BL/6 mouse dendritic cell (DC) line DC2.4 obtained from Dr. K. Rock (Dana-Farber Cancer Institute, Boston, Mass) was cultured in DMEM plus 10% fetal calf serum (FCS) and IFN-γ (20 ng/ml) and pulsed with OVA (0.5 mg/ml) for overnight. OVA-pulsed DC2.4 (DC_{OVA})-released exosomes (EXO_{OVA}) were purified from its culture supernatants by differential ultracentrifugation [33]. To generate CFSE-labeled EXOs, DC2.4 cells were stained with 0.5 μM CFSE at 37°C for 20 minutes [37] and then CFSE-labeled EXO (EXO_{CFSE}) were harvested and purified from its culture supernatants by differential ultracentrifugation [34]. The pcDNAgp120- and the control pcDNA-transfected DC2.4 cells were termed DCgp120 and DCnull, respectively. EXOs released by DCgp120 were termed EXOgp120.

2.3.3 RT-PCR analysis

Total RNA was obtained from DCnull and DCgp120 cells or BL6-10null and BL6-10gp120. The first-strand cDNA synthesis was performed as described. Two sets of PCR primers were used, including the sense (5' tgatg cactc cttca actgc 3') and anti-sense primer (5' tcctg ccaca tgttg atgat 3') for gp120 gene, and the sense (5' ttcgt tgccg gtcca ca 3') and anti-sense primer (5' accag ggcag cgata tcg 3') for the control gene β-actin. The protocol employed for amplification of both mRNA species comprised: 1 cycle of 94°C (5 min), 52°C (1 min) and 72°C (1 min); and 25 cycles of 94°C (1 min), 52°C (1 min) and 72°C (1 min). All PCR reaction products were resolved using ethidium bromide stained 1% agarose gels[38].

2.3.4 Electron microscopic analysis.

EXOs were fixed in 4% paraformaldehyde. The pellets were then loaded onto carbon-coated formvar grids. After incubation in a moist atmosphere for 20 min, the samples were washed

twice in PBS and then fixed for 5 min in 1% glutaraldehyde. After washes for three times, the EXO sample-loaded grids were stained for 10 min with saturated aqueous uranyl. EXO samples were then examined with a JEOL 1200EX electron microscope at 60kV[39].

2.3.5 Western blot analysis.

EXO samples (10 μg/lane) were loaded onto 12% acrylamide gels, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred onto nitrocellulose membrane (Millipore, Bedford, MA). The membrane was blocked by incubation for 2 hrs at room temperature with ODYSSEY blocking buffer (LI-COR Bioscience, Lincoln, NE) and immunoblotted with a panel of Abs specific for gp120, CD54, CD80, LAMP-1 and Calnexin at 4 °C for overnight. After washes for three times with PBS containing 0.05% (V/V) Tween 20, the membrane was further incubated with goat anti-rat/mouse IRDyeR800CW and scanned using ODYSSEY instrument according to manufacture's instruction (LI-COR Bioscience)[39].

2.3.6 T cell preparation.

Naïve $CD8^+$ or $CD4^+$ T cells were isolated from OVA-specific TCR transgenic OT I or OT II mouse spleens, enriched by passage through nylon wool columns (C&A Scientific, Manassas, VA), and then purified by negative selection using anti-mouse CD4 or CD8 paramagnetic beads (DYNAL Inc, Lake Success, NY) to yield populations that were >98% $CD4^+/V\alpha2V\beta5^+$ or $CD8^+/V\alpha2V\beta5^+$ [40]. To generate active $CD8^+$ T cells, the spleen cells from naïve C57BL/6 mice were cultured in RPMI1640 medium containing IL-2 (20 U/ml) and ConA (1 μg/ml) for 3 days[41]. ConA-activated $CD8^+$ T cells were then purified from these activated T cells as described above to yield T cell populations that were >98% $CD8^+$ T (ConA T) cells. The culture supernatant of ConA $CD8^+$ T cells was used for measurement of cytokine secretion using

cytokine ELISA kits (R&D Systems Inc, Minneapolis, MN).

2.3.7 Uptake of EXO by $CD8^+$ T cells.

For assessment of EXO uptake, $CD8^+$ ConA T cells were incubated with different amounts of EXO_{CFSE} at 37°C for 1-6 hrs and then analyzed for CFSE expression by flow cytometry and confocal microscopy [33]. In another set of experiments, $CD8^+$ T cells were co-cultured with EXO_{CFSE} in presence or absence of blocking reagents such as anti-LFA-1 and anti-CD28 Ab (20 μg/ml), and then analyzed by confocal microscopy[39].

2.3.8 *In vitro* T cell proliferation assay.

To assess the functional effect of OVA-Texo cells, we performed T cell proliferation assay. The irradiated (3,000 rad) OVA-Texo or ConA T (0.6×10^5 cells/well) cells, DC_{OVA} (0.3×10^5 cells/well), and their 2-fold dilutions were cultured with a constant number of naïve OT I $CD8^+$ or OT II $CD4^+$ T cells (1×10^5 cells/well) in a 96-well plate. After culturing for 3 days, ^{3}H-thymidine incorporation was determined by liquid scintillation counting [33].

2.3.9 Flow cytometric analysis.

Six days after the immunization, one hundred microliter of blood was taken from the mice with i.v. immunization of irradiated (4,000 rad) DCova (1×10^6 cells/mouse), ConA T cells, OVA-Texo (1×10^6 cells/mouse), respectively. The blood samples harvested from immunized mice at different times after the immunization were then stained with FITC-conjugated anti-CD4 (FITC-CD4) or anti-CD8 Ab (FITC-CD8) and PE-conjugated Ia^b/OVAII ($OVA_{265\text{-}280}$) tetramer (NIH tetramer facility, Bethesda, MD) and PE-conjugated H-$2K^b$/OVAI ($OVA_{257\text{-}264}$) tetramer (Beckman Coulter, Mississauga, Ontario, Canada), respectively, for 30 min at room temperature

and analyzed by flow cytometry [37]. To assess Gp120-Texo-stimulated CTL responses, the blood samples harvested from mice immunized with irradiated DCgp120, ConA T and Gp120-Texo (1×10^6 cells/mouse) 6 days after the immunization were stained with PE-conjugated anti-CD8 Ab (PE-CD8) and ECD-conjugated anti-CD44 Ab (ECD-CD44) and analyzed by flow cytometry. In another set of experiments, the blood samples were first stained with PE-anti-CD8 Ab and the T cells were then fixed and cell membranes were permeabilized with Cytofix/Cytoperm solution (BD Biosciences) and stained with ECD-conjugated anti-IFN-γ Ab and then analyzed by flow cytometry[42].

2.3.10 Cytotoxicity assay.

The *in vivo* cytotoxicity assay was performed as previously described. Briefly, splenocytes were harvested from naive mouse spleens and incubated with either high (3.0 μM, $CFSE^{high}$) or low (0.6 μM, $CFSE^{low}$) concentrations of CFSE, to generate differentially labeled target cells. The $CFSE^{high}$ cells were pulsed with OVAI peptide, whereas the $CFSE^{low}$ cells were pulsed with Mut1 peptide and served as internal controls. These peptide-pulsed target cells were washed extensively to remove free peptides, and then i.v. co-injected at 1:1 ratio into OVA-Texo immunized mice six days after the immunization. Sixteen hrs later, the residual $CFSE^{high}$ and $CFSE^{low}$ target cells remaining in the recipients' spleens were analyzed by flow cytometry.

2.3.11 Animal studies.

To examine the antitumor protective immunity conferred by OVA-Texo or Gp120-Texo vaccine, wild-type C57BL/6 mice (n=8) or $Ia^{b-/-}$ or $H\text{-}2K^{b-/-}$ KO mice were injected i.v. with DCova or DCgp120 (1×10^6 cells/mouse), the control ConA-stimulated $CD8^+$ T (ConA T) and OVA-Texo or Gp120-Texo (1×10^6 cells/mouse) cells, respectively. The mice injected with PBS were used as another control. The immunized mice were challenged i.v. with 0.5×10^6 BL6-10ova or

BL6-10gp120 cells six days or two months subsequent to the immunization. The mice were sacrificed 3 weeks after tumor cell injection, and the lung metastatic tumor colonies were counted in a blind fashion. 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 >100[33].

2.4 Results

2.4.1 Characterization of DC-released exosomes

C57BL/6 mouse bone-marrow cell-derived DC cell line DC2.4 was *in vitro* cultured in DMEM plus 10% FCS and IFN-γ (20 ng/ml) and pulsed overnight with OVA (0.5 mg/ml) and subjected to flow cytometric analysis. As shown in **Figure 2.1A**, OVA-pulsed DCs (DCova) expressed CD11c, MHC II (Ia^b), CD40 and CD80, indicating that they are mature DC. In addition, they also displayed MHC class I ($H\text{-}2K^b$), adhesion molecule CD54 and pMHC I complexes. DCova-released EXO (EXOova) were purified from DCova culture supernatants by differential ultracentrifugation and subjected to electron microscopic, flow cytometric and Western blotting analysis. As shown in **Figure 2.1B**, EXO_{OVA} had a typical exosomal characteristic of "saucer" or round shape with a diameter between 5- to 90 nm. EXOova were then stained with a panel of antibodies (Abs) and analyzed by flow cytometry calibrated with microbeads (3.8 μm in diameter) (**Figure 2.1C**). As shown in **Figure 2.1D**, EXOova displayed expression of all DCova molecules including CD80 and pMHC I complexes, but at a lower extent than DC_{OVA}. EXOova also displayed EXO-associated proteins such as LAMP-1, CD54 and CD80, but not apoptotic body marker Calnexin [43] by Western blot analysis (**Figure 2.1E**).

Fig 2.1

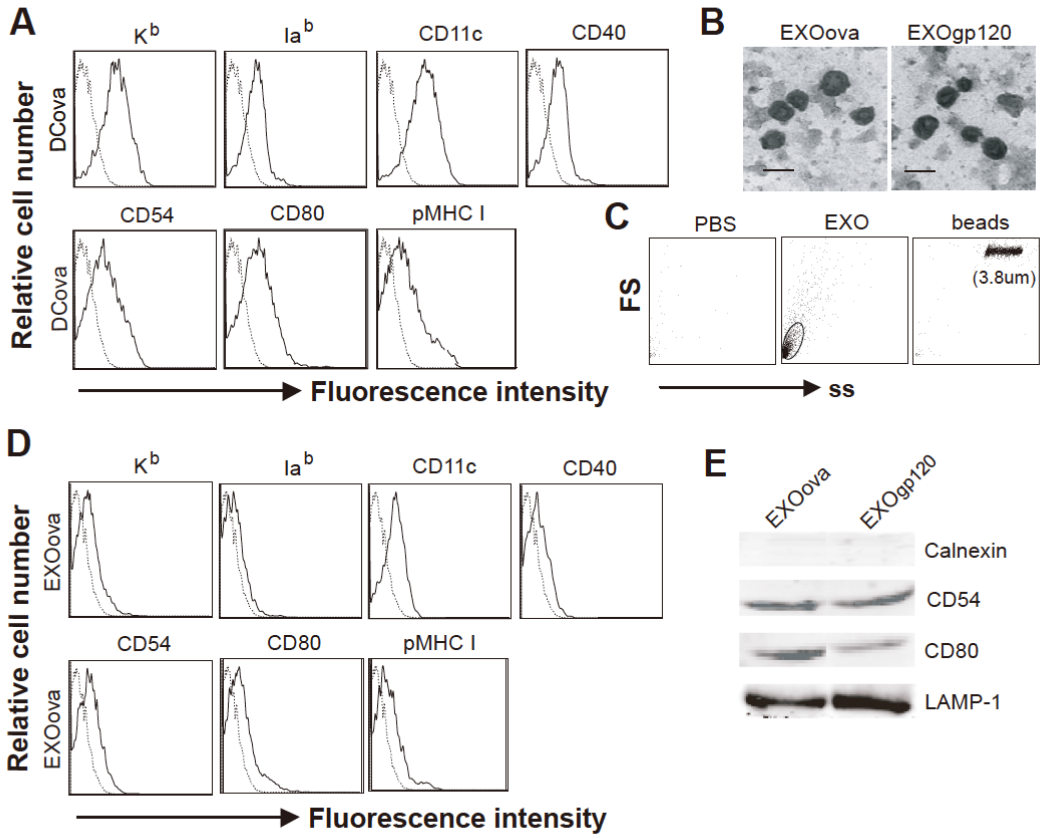

Figure 2.1 Phenotypic analysis of DCova and DCova-released EXOova.

(A) DC_{OVA} were stained with a panel of Abs (solid lines) or isotype-matched irrelevant Abs (dotted lines) and analyzed by flow cytometry. (B) Electron micrograph of EXO. Bar, 100 nm. (C & D) Flow cytometric analysis of EXOs stained with a panel of Abs and microbeads (3.8 μm in diameter). EXOs stained with a panel of FITC-Abs (solid lines) or isotype-matched irrelevant FITC-Abs (dotted lines) were sorted (in circle) and analyzed by flow cytometry. (E) Western blot analysis of EXOova and EXOgp120 using a panel of Abs. One representative experiment of two is shown.

2.4.2 Acquisition of exosomal molecules by $CD8^+$ T cells via LFA-1

Active $CD8^+$ T cells were in vitro generated from naïve $CD8^+$ T cells purified from C57BL/6 mouse spleen T cells, which were then cultured with ConA for activation and expansion.

Activated T cells were then subjected to flow cytometric analysis. As shown in **Figure 2.2A**, these ConA-stimulated T cells expressed CD8, CD25 (T cell activation marker), CD54 and LFA-1, indicating that they are active $CD8^+$ T cells. In addition, these $CD8^+$ T cells secreted IL-2 (2.4 ng/ml/$l0^6$ cells/24 hr) and IFN-y (2.6 ng/ml/$l0^6$ cells/24 hr), but no IL-4 and IL-l 0 in their culture supernatants**,** indicating that they are type 1 $CD8^+$ T cells. To explore the potential uptake of EXOs by T cells, these ConA-stimulated $CD8^+$ T cells were incubated with CFSE-labelled EXOs (EXO_{CFSE}) derived from CFSE-labelled DCs (DC_{CFSE}), and then analyzed by flow cytometry. CFSE dye was detectable on EXO_{CFSE} (panel a, **Figure 2.2B**) and $CD8^+$ T cells with incubation of EXO_{CFSE} (panel b, **Figure 2.2B**), indicating that EXO_{CFSE} could be uptaken by $CD8^+$ T cells. The uptake of EXO_{CFSE} by $CD8^+$ T cells increased with the amount of EXO_{CFSE} (**Figure 2.2C**) and with the length of the incubation time (**Figure 2.2D**). A maximal level of EXO_{CFSE} uptake was reached when 1×10^6 T cells were incubated with 10 μg EXO_{CFSE} for 4 hrs which was visualized by confocal microscopy (**Figure 2.2E**). T cells can absorb DC EXO via TCR or CD28 [44] and LFA-l [1, 20]. To assess the molecular mechanism for EXO uptake by $CD8^+$ T cells, we added blocking reagents to the above mixture of T cells and EXO_{CFSE}. We found that anti-LFA-l Ab, but not anti-CD28 Ab significantly inhibited the uptake of EXO_{CFSE} by $CD8^+$ T cells ($p<0.05$) (**Figure 2.2F**), indicating that the CD54/LFA-l interaction mediates $CD8^+$ T cell's absorption of DC EXO. To assess uptake of exosomal molecules, we performed flow cytometric analysis. Similar to CFSE dye transfer, some exosomal molecules such as CD80 and pMHC I (the critical component in stimulation of OVA-specific $CD8^+$ CTL responses) [40] were also transferred onto $CD8^+$ T cells (**Figure 2.2A**).

Fig 2.2

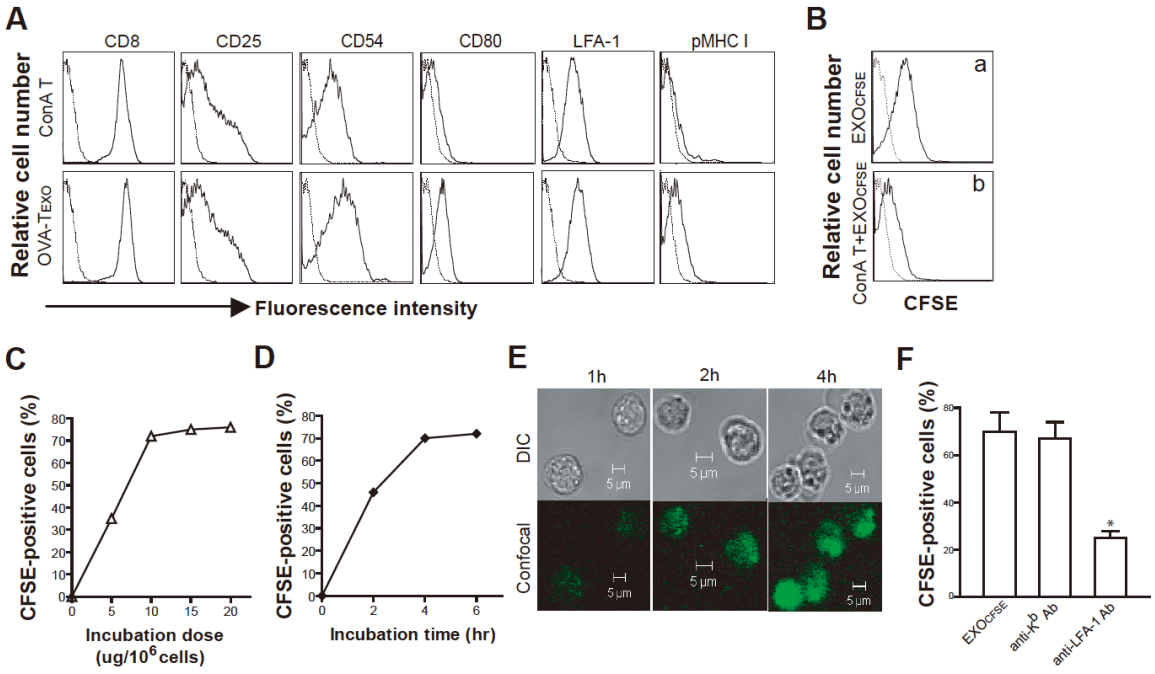

Figure 2.2 Exosome uptake by ConA-stimulated CD8+ T cells.

(A) ConA-stimulated $CD8^+$ T cells and OVA-Texo were stained with a panel of Abs (solid lines) or isotype-matched irrelevant Abs (dotted lines), and then analyzed by flow cytometry. (B) EXO_{CFSE} (solid line) and EXO (dotted line) (a) or ConA T cells after (solid line) and before (dotted line) incubation with EXO_{CFSE} (b) were analyzed by flow cytometry. (C) ConA-stimulated $CD8^+$ T (ConA T) cells were incubated with different doses of EXO_{CFSE} for 4 hrs. CFSE-positive T cells were detected by confocal microscopy. (D) ConA T cells were incubated with EXO_{CFSE} (10 μg/1×10^6 T cells) for different times. (E) CFSE-positive T cells were detected under differential interference contrast (DIC) by confocal microscopy. (F) The blocking reagents (anti-H-$2K^b$ or anti-LFA-1 Ab, 20 μg/ml) were added to the mixture of ConA T cells and EXO_{CFSE}. After 4 hrs of incubation, CFSE-positive T cells were detected by confocal microscopy. One representative experiment of two is shown.

2.4.3 OVA-Texo stimulates *in vitro* OVA-specific $CD4^+$ and $CD8^+$ T cell proliferation

Since EXO harbor many immune molecules including pMHC complexes and costimulatory molecules, $CD8^+$ aTexo with acquired exosomal molecules may thus have potent effect in stimulation of T cell responses. We performed *in vitro* OVA-specific $CD4^+$ and $CD8^+$ T cell proliferation assays. We demonstrated that OVA-Texo stimulated *in vitro* OVA-specific OT II

$CD4^+$ (**Figure 2.3A**) and OT I $CD8^+$ (**Figure 2.4A**) T cell proliferation by ^3H-thymidine incorporation.

Fig 2.3

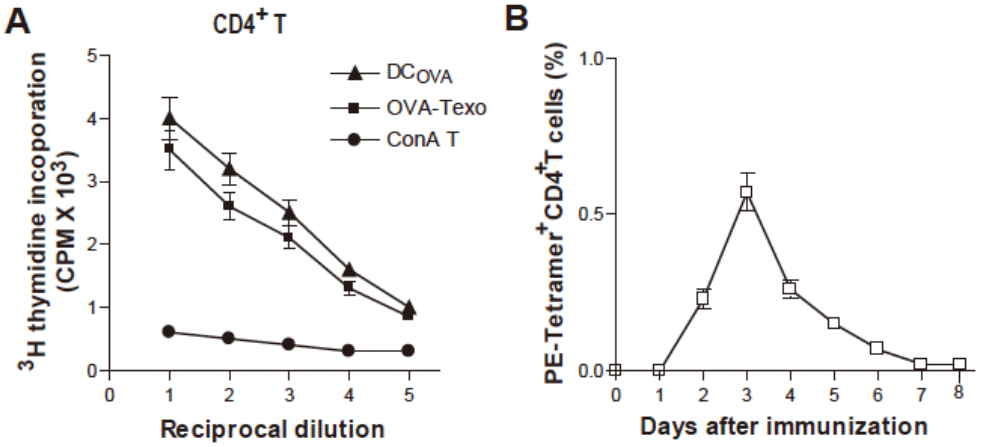

Figure 2.3 OVA-Texo induces OVA-specific $CD4^+$ T cell responses.

(A) Naive OT II mouse CD4+ T cells (0.5×10^5 cells/well) were incubated with irradiated DC_{OVA} and OVA-Texo (0.25×10^5 cells/well) and their 2 fold dilutions in a 3H-thymidine uptake assay. The ConA-stimulated C57BL/6 CD8+ T (ConA T) cells were used as a control. (B) The immunized mouse tail blood samples harvested at different days after the immunization of OVA-Texo were stained with PE-Ia^b/OVA II peptide tetramer and FITC-anti-CD4 Ab, and then analyzed by flow cytometry. The value in each panel represents the percentage of OVA-specific (tetramer-positive) $CD4^+$ T cells vs the total $CD4^+$ T cell population. The value in parenthesis represents the standard deviation (SD). One representative experiment of three is shown.

2.4.4 OVA-Texo stimulates *in vivo* functional OVA-specific $CD4^+$ and $CD8^+$ T cell responses

To assess whether OVA-Texo can also stimulate T cell proliferation *in vivo*, we performed tetramer staining assays. As shown in **Figure 2.3B**, OVA-Texo stimulated PE-Ia^b/$OVA_{265-280}$ tetramer-positive $CD4^+$ T cell responses with a peak time at day 3 subsequent to the immunization, which accounted for 0.56% of the total $CD4^+$ T populations in the mouse blood. In addition, OVA-Texo also stimulated H-$2K^b$/$OVA_{257-264}$ tetramer-positive $CD8^+$ T cell

responses with a peak time at day 6 subsequent to immunization (**Figure 2.4B**), which accounted for 1.13% of the total $CD8^+$ T cell populations in the mouse blood (**Figure 2.4C**). Next, we assessed the ability of OVA-Texo to induce the differentiation of stimulated $CD8^+$ T cells into CTL effectors. We adoptively transferred OVAI peptide-pulsed splenocytes that had been strongly labeled with CFSE ($CFSE^{high}$), as well as the control peptide Mut1-pulsed splenocytes that had been weakly labeled with CFSE ($CFSE^{low}$), into recipient mice that had been vaccinated with OVA-Texo cells. As expected, there was substantial loss (75%) of the $CFSE^{high}$ (OVAI peptide-pulsed) cells in the OVA-Texo-immunized mice, whereas no cytotoxicity was induced in mice immunized with ConA-stimulated $CD8^+$ T (ConA T) cells without acquired exosomal molecules (**Figure 2.4D**)**,** indicating that OVA-Texo vaccine efficiently stimulates $CD8^+$ T cell differentiation into functional CTL effectors. In the above experiments, a positive control DCova vaccine similarly stimulated $CD8^+$ effector CTL response as OVA-Texo.

Fig 2.4

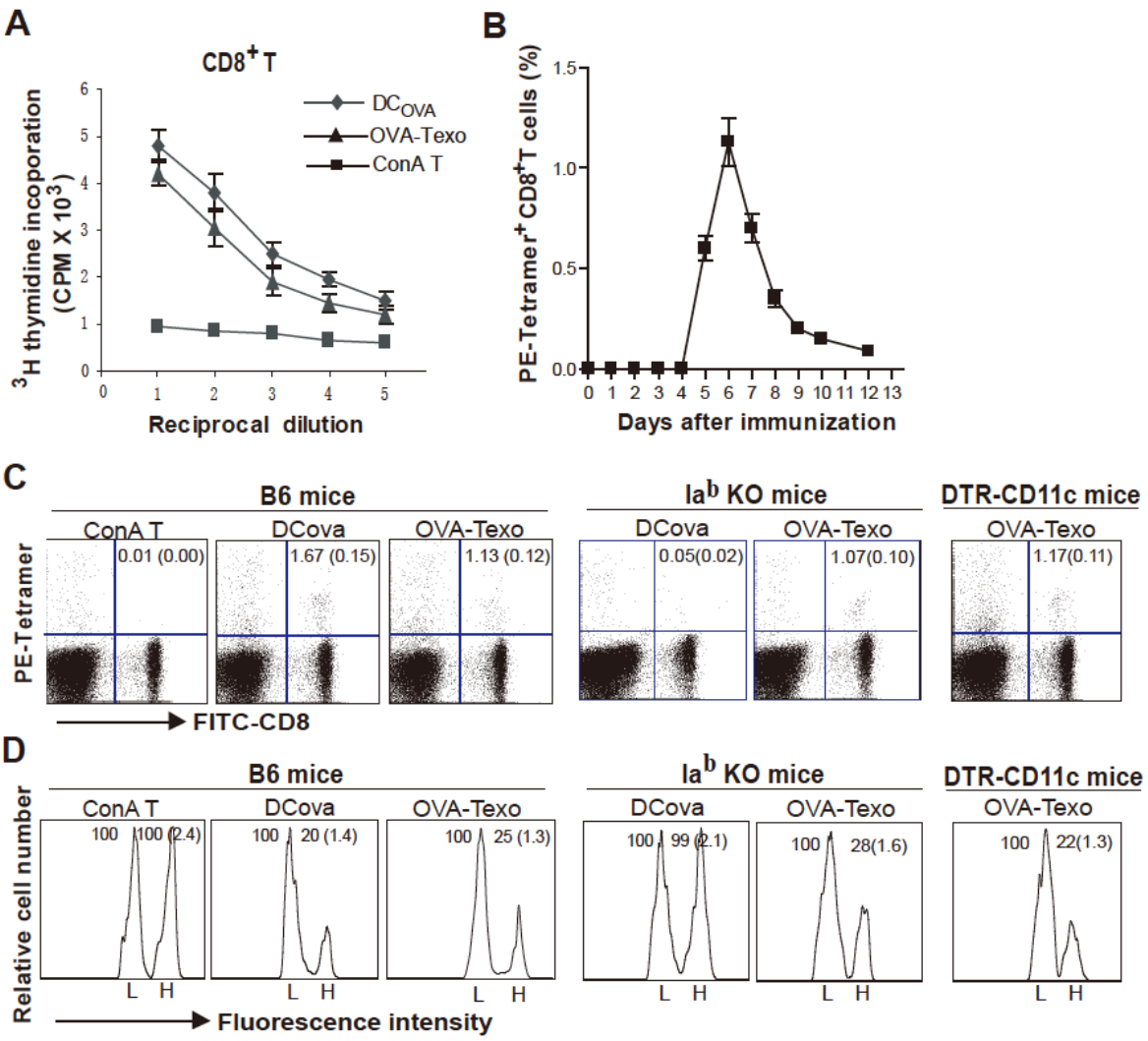

Figure 2.4 OVA-Texo induces OVA-specific CD8$^+$ T cell responses.

(A) Naive OTI mouse CD8$^+$ T cells (0.5×10^5 cells/well) were incubated with irradiated DC_{OVA} and OVA-Texo (0.25×10^5 cells/well) and their 2 fold dilutions in a 3H-thymidine uptake assay. The ConA-stimulated C57BL/6 CD8$^+$ T (ConA T) cells were used as a control. (B) The immunized C57BL/6 mouse tail blood samples harvested at different days after the immunization of OVA-Texo were stained with PE-H-2Kb/OVAI peptide tetramer and FITC-anti-CD8 Ab, and then analyzed by flow cytometry. (C) The tail blood samples harvested at day 6 after the immunization of Iab-/- KO mice or DTR-CD11c transgenic mice (with a single dose treatment of DT) with OVA-Texo were stained with PE-H-2Kb/OVAI peptide tetramer and FITC-anti-CD8 Ab, and then analyzed by flow cytometry. The value in each panel represents the percentage of OVA-specific (tetramer-positive) CD8$^+$ T cells vs the total CD8$^+$ T cell population. The value in parenthesis represents the standard deviation (SD). (D) In vivo

cytotoxicity assay. Six days after immunization, the immunized mice were i.v. injected with 2×10^6 cells containing a 1:1 mixture of $CFSE^{high}$ and $CFSE^{low}$ -labeled splenocytes that had been pulsed with OVAI or Mut1 peptides, respectively. After 16 hrs, the spleens of immunized mice were removed and the percentages of the residual $CFSE^{high}$ (H) and $CFSE^{low}$ (L) target cells remaining in the recipients' spleens were analyzed by flow cytometry. The value in each panel represents the percentage of $CFSE^{high}$ vs $CFSE^{low}$ target cells remaining in the spleen. The value in parenthesis represents the standard deviation (SD). One representative experiment of three is shown.

2.4.5 OVA-Texo-stimulated $CD8^+$ T cell responses are DC and $CD4^+$ T cell independent

To assess the potential involvement of the host DC and $CD4^+$ T cells in OVA-Texo-induced $CD8^+$ CTL responses, we performed OVA-Texo vaccination in both $Ia^{b-/-}$ gene KO mice lacking the host $CD4^+$ T cells and transgenic diphtheria toxin receptor (DTR)-CD11c mice with treatment of diphtheria toxin (DT) for depletion of the host DCs. Interesting, we found that the efficiency of OVA-Texo-stimulated CTL responses (1.17%) in DT-treated DTR-CD11c mice lacking the host DCs was maintained at the same level, whereas OVA-Texo-stimulated CTL responses (1.07%) in $Ia^{b-/-}$ gene KO mice lacking the host $CD4^+$ T cells declined slightly compared to those (1.13%) in wild-type C57BL/6 mice (**Figure 2.4C**), indicating that OVA-Texo vaccine stimulates $CD8^+$ CTL responses which are DC and $CD4^+$ T cell independent. In comparison, DCova failed in stimulation of CTL responses in $Ia^{b-/-}$ KO mice lacking $CD4^+$ T cells, indicating that DCova stimulated $CD4^+$ T cell-dependent CTL responses. In addition, OVA-Texo-stimulated CTL in $Ia^{b-/-}$ gene KO mice also displayed efficient killing activity to OVAI-pulsed $CFSE^{high}$ target cells *in vivo* (**Figure 2.4D**), indicating that they are also functional.

2.4.6 Induction of $CD8^+$ CTL-mediated antitumor immunity and long-term T cell memory by OVA-Texo

Next, we assessed the potential antitumor immunity derived from OVA-Texo vaccine against OVA-expressing B16 melonoma. As shown in Exp I of **Table 2.1**, all the mice injected with PBS or $CD8^+$ T cells without uptake of exosomal molecules had large numbers (> 100) of lung

metastatic BL6-10ova tumor colonies. OVA-Texo vaccine, however, induced a complete immune protection against BL6-10ova tumor cell challenge in all 8/8 (100%) mice, indicating that OVA-Texo induces efficient antitumor immunity. To study the immune mechanism, la^b and H-$2K^b$ gene KO mice were used for immunization. As shown in Exp 11 of **Table 2.1**, most (7/8, 88%) of la^b gene KO mice lacking $CD4^+$ T cells were still tumor free, whereas all H-$2K^b$ gene KO mice (8/8) lacking $CD8^+$ T cells had numerous lung tumor metastases, indicating that OVA-Texo-induced antitumor immunity is relatively $CD4^+$ T-cell independent, but completely mediated by $CD8^+$ CTLs. To assess whether these OVA-Texo-stimulated $CD8^+$ T cells can become long-lived memory $CD8^+$ T (Tm) cells leading to long-term immunity. One month after the immunization, we challenged the immunized mice with BL6-10_{OVA} tumor cells. As shown in Exp III of **Table 2.1**, all mice immunized with OVA-Texo were free of tumor, indicating that $CD8^+$ aTexo vaccine can induce OVA-specific $CD8^+$ memory T cell development leading to a long-term T cell memory.

Table 2.1 OVA-Texo vaccine protects against lung tumor metastases

Vaccines[A]	Tumor cell challenge	Tumor growth incidence (%)	Median number of lung tumor colonies
Exp. I.			
DC_{OVA}	BL6-10_{OVA}	0/8 (0)	0
OVA-T_{EXO}	BL6-10_{OVA}	0/8 (0)	0
Con A T	BL6-10_{OVA}	8/8 (100)	>100
PBS	BL6-10_{OVA}	8/8 (100)	>100
OVA-T_{EXO}	BL6-10	8/8 (100)	>100
PBS	BL6-10	8/8 (100)	>100
Exp. II.			
OVA-T_{EXO} (B6)	BL6-10_{OVA}	0/8 (0)	0
OVA-T_{EXO} (Ia^b KO)	BL6-10_{OVA}	0/8 (0)	0
OVA-T_{EXO} (K^b KO)	BL6-10_{OVA}	8/8 (100)	>100
Exp. III			
OVA-T_{EXO}	BL6-10_{OVA}	0/8 (0)	0
DC_{OVA}	BL6-10_{OVA}	0/8 (0)	0

PBS	BL6-10_{OVA}	8/8 (100)	>100

A. In experiment I, wild-type C57BL/6 (B6) mice were immunized with DC_{OVA}, ConA-stimulated $CD8^+$ T (ConA T) and OVA-T_{EXO} cells or PBS. In experiment II, B6, Ia^b or K^b KO mice were immunized with aT_{EXO} cells. Six days after the immunization, each mouse was challenged with BL6-10_{OVA} or BL6-10 tumor cells. In experiment III, B6 mice were immunized with OVA-T_{EXO}. One month later, mice were challenged with BL6-10_{OVA} tumor cells. The mice were sacrificed 3 weeks after tumor cell challenge and the numbers of lung metastatic tumor colonies were counted. One representative experiment of two is shown.

2.4.7 Stimulation of gp120-specific $CD8^+$ CTL responses and long-term immunity against gp120-expressing tumor by Gp120- Texo

To assess whether HIV-1 gp120-specific EXO-targeted Gp120-Texo vaccine can stimulate $CD8^+$ CTL responses leading to long-term immunity, we first transfected DC cell line DC2.4 with pcDNAgp120 by lipofectamin to form DCgp120. We demonstrated that DCgp120 displayed expression of CD11c, CD40, CD54, CD80 and Ia^b by flow cytometry (**Figure 2.5A**), indicating that they are mature DCs. To confirm expression of gp120, we performed RT-PCR and Western blotting analysis. We demonstrated that DCgp120, but not the control DCnull transfected with the control pcDNA vector expressed Gp120 molecule by RT-PCR (**Figure 2.5B**) and Western blotting (**Figure 2.5C**) analysis. We then purified EXOgp120 derived from culture supernatants of DCgp120 by differential ultracentrifugation. EXOgp120 were then subjected to electron microscopic, Western blot and flow cytometric analysis. As shown in **Figure 2.1B**, EXOgp120 also had a typical exosomal characteristic of "saucer" or round shape with a diameter between 50 to 90 nm. We also confirmed that EXO-associated proteins including CD54, CD80 and LAMP-1 were abundant in EXOgp120 preparation by Western blot analysis (**Figure 2.1E**)**.** EXOgp120 displayed expression of all DCgp120 cell-surface molecules, but at a lower extent than DCgp120 (**Figure 2.5A**). To assess the stimulatory effect on $CD8^+$ T cell responses, we immunized C57BL/6 mice with Gp120-Texo and then performed flow cytometric analysis. Since no H-$2K^b$ epitope of Gp120 was defined, we could not use Gp120-specific tetramer staining to detect Gp120-specific $CD8^+$ T cells. Instead, we used FITC-anti-CD8 and ECD-anti-CD44 Ab as well

as PE-anti-IFN-γ Ab for double staining to assess Gp120-Texo-stimulated $CD8^+$ T cell responses by flow cytometry as described by Shedlock et al [45]. We demonstrated that Gp120-Texo more efficiently stimulated upregulation of T cell activation marker CD44 on $CD8^+$ T cells than ConA T cells ($p<0.05$) (**Figure 2.5D**), indicating that Gp120-Texo stimulates efficient Ag-specific $CD8^+$ CTL responses. In addition, we also quantified the Ag-specific $CD8^+$ T cell responses in Gp120-Texo-immunized mice by intracellular IFN-γ staining. We found that 3.21% $CD8^+$ T cells from Gp120-Texo-immunized mice produced IFN-γ, which is significantly more than ConA T cells ($p<0.05$), indicating that Gp120-Texo stimulates efficient Ag-specific effector $CD8^+$ CTL responses. In the above experiments, a positive control DCgp120 vaccine similarly stimulated $CD8^+$ effector CTL response as OVA-Texo. To assess the short- and long-term immunity, we immunized C57BL/6 mice with Gp120-Texo and challenged the mice with Gp120-expressing BL6-10gp120 tumor cells (**Figure 2.5B & 2.5C**) at day 6 and 30 subsequent to the immunization, respectively. We found that all (8/8) of mice were tumor free in both Exp I and II of **Table 2.2**, indicating that Gp120-Texo vaccine can stimulate both efficient short- and long-term immunity against Gp120-expressing BL6-10gp120 tumor cells. In the above experiments, we used a positive control (DCgp120) vaccine showing a similar short- and long-term antitumor immunity as Gp120-Texo.

Fig 2.5

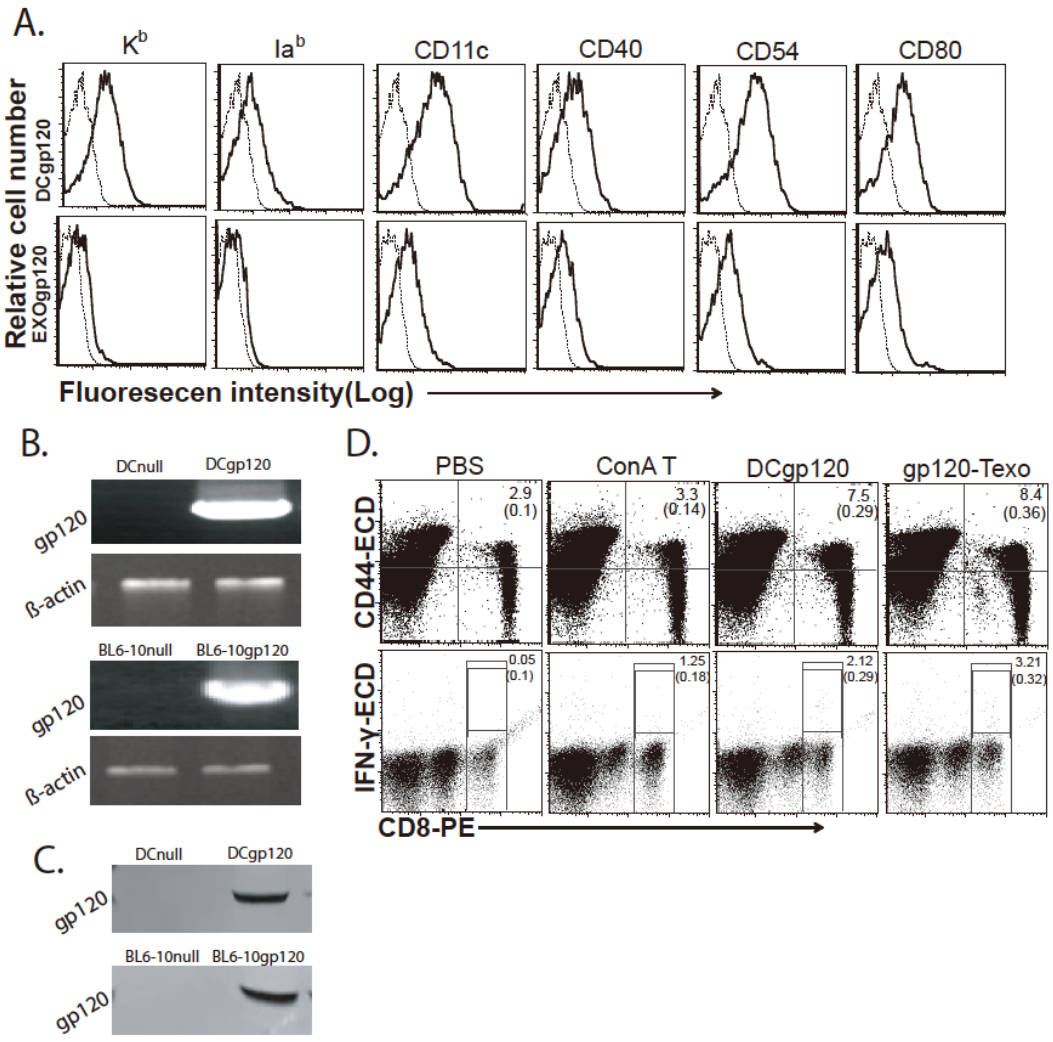

Figure 2.5 Phenotypic and functional analysis of Gp120-Texo.

(A) DCgp120 and DCgp120-released EXOgp120 were stained with a panel of Abs (solid lines) or isotype-matched irrelevant Abs (dotted lines) and analyzed by flow cytometry. (B) RT-PCR and (C) Western blotting analysis of DCgp120 and BL6-10gp120 tumor cells. (D) C57BL/6 mice were i.v. immunized with DCgp120 and Gp120-Texo. Six days after the immunization, tail blood samples of the immunized mice were stained with ECD-anti-CD44 Ab or PE-anti-IFN-γ Ab and FITC-anti-CD8 Ab, and then analyzed by flow cytometry. The value in each panel represents the percentage of CD8+CD44+ T cells in the total cells analyzed or the percentage of $CD8^+$ T cells producing IFN-γ in the total $CD8^+$ T cell population. The value in parenthesis represents the standard deviation (SD). *P<0.05 versus cohorts of ConA T cells (Student t test). One representative experiment of two is shown.

Table 2.2 Gp120-Texo vaccine protects against lung tumor metastases

Vaccines[A]	Tumor cell challenge	Tumor growth incidence (%)	Median number of lung tumor colonies
Exp. I.			
DCgp120	BL6-10gp120	0/8 (0)	0
Gp120-T_{EXO}	BL6-10gp120	0/8 (0)	0
ConA T	BL6-10gp120	8/8 (100)	>100
PBS	BL6-10gp120	8/8 (100)	>100
Exp. II			
aT_{EXO}	BL6-10gp120	0/8 (0)	0
DCgp120	BL6-10gp120	0/8 (0)	0
PBS	BL6-10gp120	8/8 (100)	>100

A. In experiment I, wild-type C57BL/6 (B6) mice were immunized with DCgp120, ConA-stimulated T (ConA T) and Gp120-T_{EXO} cells or PBS. In experiment II, B6 mice were immunized with DCgp120 and Gp120-T_{EXO}. Two months later, mice were challenged with BL6-10gp120 tumor cells. The mice were sacrificed 3 weeks after tumor cell challenge and the numbers of lung metastatic tumor colonies were counted. One representative experiment of two is shown.

2.5 Discussion

Antigen (Ag)-specific naive T cells can absorb dendritic cell (DC) exosomes (EXO) via either TCR or CD28 [44] and LFA-1 [20]. Activation of specific G protein receptors, cytokine stimulation motility and TCR-mediated signals all induce a transient conformational change in LFA-1, thereby highly increasing T cell LFA-1 avidity for CD54 [46]. We previously demonstrated that Con A-stimulated polyclonal $CD4^+$ T cells acquired OVA-specific DCova-released EXOova via LFA-1 [33]. Recently, it has been also shown that T cells recruit DC EXO by LFA-1 [1], indicating that CD54/LFA-1 interaction plays an important role in not only intercellular cell-to-cell interactions but also DC EXO uptake by T cells. Since the uptake of EXO by T cells was a saturable process that required close cell proximity, actin polymerization and a permissive temperature [47], internalization/recycling and the fusion of DC EXO onto $CD8^+$ T cells may be the two major pathways of exosomal molecule acquisition[48]. In this study, we demonstrate that CD54 expressing DC can uptake active T cell-released EXO expressing LFA-1 and the anti-LFA-1 Ab can block the uptake of T cell EXO by DC, indicating that active T cells recruit DC EXO via exosomal LFA-1.

In AIDS patients, severe immune suppression and dysfunction have been found [46] For example, HIV-1 viral cytopathic effect causes $CD4^+$ T cell depletion after HIV-1 gp120 binds to CD4 molecule of the T cells [46]. Individuals with high viral loads had increased frequencies of $CD4^+$ T cells producing only IFN-γ, but not IL-2, resulting $CD4^+$ T cell death and depletion and disease progression [44, 46, 49]. The $CD4^+$ Th cell depletion due to the viral cytopathic effect [1] also quantitatively and qualitatively decreases $CD8^+$ CTL responses [50]. $CD4^+$ T cells are required for the generation and maintenance of cytotoxic $CD8^+$ T cells[51]. Clinically, the association between peripheral blood $CD4^+$ Th cell counts and risk of disease becomes well established [4]. The loss of non-helped $CD8^+$ T cells is particularly apparent during chronic HIV-1 infection [52, 53]. It has recently been demonstrated that the primary $CD8^+$ T cell responses and short-term memory to HIV-1 Env and vesicular stomatitis virus (VSV) nucleocapsid protein were largely intact [54]. However, their long-term memory was largely or

completely lost in CD4-deficient mice. Importantly, the qualitative nature of $CD8^+$ T cell responses is key to the control of HIV-1 infection [55]. DCs expressing C-type lectin DC-SIGN, the target protein for Gp120 have also been found to be impaired in individuals with progressive HIV-1 infection [56, 57]. Infected DC-released EXO-associated virons are also likely to be transmitted to $CD4^+$ T cells through membrane binding and fusion, either within the infectious synapse or over longer distances [58].

Administration of attenuated T cells to animals has been shown to stimulate immune suppression and to prevent the development of experimental autoimmune diseases. For example, vaccination using myelin-basic-protein autologous T cells has also been applied preventing the development of experimental autoimmune disease [59] and to clinical trial in multiple sclerosis [60]. Recently, we have shown that ConA-stimulated nonspecific polyclonal $CD4^+$ T cells with uptake of Ag-specific DC-released EXO can stimulate Ag-specific $CD8^+$ CTL responses and antitumor immunity [33]. $CD8^+$ T cells have been originally defined as effector T cells with cytotoxic activity to tumor or virally infected cells [37]. Interestingly, it has also been demonstrated that $CD8^+$ T cells have helper effects as $CD4^+$ Th cells [61, 62]. In this study, we further show that non-specific active $CD8^+$ T cells can uptake OVA- and Gp120-specific DC-released EXO via LFA-1 to form OVA- and Gp120-Texo vaccines. Antigen-presenting cells (APCs) stimulate $CD8^+$ T cell responses via 3 signaling including peptide major histocompatibility complex (pMHC) I/TCR (signal 1), CD80/CD28 (signal 2) and IL-12 (signal 3) [50]. Our OVA- and Gp120-Texo vaccines stimulate OVA- and Gp120-specific CD8+ CTL responses also via 3 signaling such as exosomal pMHC I/TCR (signal 1), CD40L/CD40 and exosomal CD80/CD28 (signal 2) and IL-2 (signal 3) [50]. We demonstrate that OVA-Texo can stimulate DC- and $CD4^+$ T cell-independent OVA-specific $CD8^+$ CTL responses leading to antitumor immunity and $CD8^+$ T cell long-term memory against OVA-expressing B16 melanoma. Importantly, Gp120-Texo vaccine can also stimulate Gp120-specific effector $CD8^+$ CTL responses and long-term memory against Gpl20-expressing B16 melanoma.

Although DCs have also been found to be dysfunctional in HIV-1 patients [56, 57, 63], administration of monocyte-derived and Ag-loaded DCs from HIV-1 patients could still generate cellular immune responses leading to reduction in viremia [29] and partial control of viral replication [30]. Mature DCs can still be obtained by culturing peripheral blood monocytes from AIDS patients or HLA-A-matched donors in culture medium with GM-CSF, IL-4 and TNF-α [64] and transfected with a recombinant Gp120-expressing adenovirus (AdVgp120) to form Gp120-expressing DCgp120. Therefore, it is feasible to generate functional Gp120-Texo vaccine by using ConA-stimulated patient's peripheral blood $CD8^+$ T cells with uptake of purified Gp120-specific EXOgp120 released from DCgp120.

Taken together, this novel HIV-1 gp120-specific EXO-targeted $CD8^+$ Gp120-Texo vaccine capable of stimulating efficient HIV-1-specific $CD8^+$ CTL responses in absence of DC and $CD4^+$ T cell help may represent a new strategy for immunotherapeutic vaccine in treatment of AIDS patients with DC dysfunction and $CD4^+$ T cell deficiency.

2.6 References

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2.7 Acknowledgement

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CHAPTER 3 HIV-1 GAG-SPECIFIC EXOSOME-TARGETED T CELL-BASED VACCINE STIMULATES EFFECTOR CTL RESPONSES LEADING TO THERAPEUTIC AND LONG-TERM IMMUNITY AGAINST GAG/HLA-A2-EXPRESSING B16 MELANOMA IN TRANSGENIC HLA-A2 MICE

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3.1 Abstract

Human immunodeficiency virus type-1 (HIV-1)-specific dendritic cell (DC) vaccines have been applied to clinical trials that show only induction of some degree of immune responses, warranting the search of other more efficient vaccine strategies. Since HIV-1-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) have been found to recognize some HIV-1 structural protein Gag conserved and cross-strain epitopes, Gag has become one of the most attractive target candidates for HIV-1 vaccine development. In this study, we generated HIV-1 Gag-specific Gag-Texo vaccine by using ConA-stimulated polyclonal $CD8^+$ T-cells with uptake of Gag-expressing adenoviral vector AdV_{Gag}-transfected DC (DC_{Gag})-released exosomes (EXOs), and assessed its stimulation of Gag-specific $CD8^+$ CTL responses and antitumor immunity. We demonstrate that Gag-Texo and DC_{Gag} vaccines comparably stimulate Gag-specific effector $CD8^+$ CTL responses. Gag-Texo-stimulated CTL responses result in protective immunity against Gag-expressing BL6-10_{Gag} melanoma in 8/8 wild-type C57BL/6 mice. In addition, we show that Gag-Texo vaccine also induces CTL responses leading to protective and long-term immunity against Gag/HLA-A2-expressing BL6-$10_{Gag/A2}$ melanoma in 8/8 and 2/8 transgenic HLA-A2 mice, respectively. The average number of lung tumor colonies in mice with 30-days post-immunization is only 23, which is significantly less than that (>300) in control ConA-T-immunized HLA-A2 mice. Furthermore, Gag-Texo vaccine also induces some degree of therapeutic immunity. The average number (50) and size (1.02 mm in diameter) of lung tumor colonies in Gag-Texo-immunized HLA-A2 mice bearing 6-day-established lung BL6-$10_{Gag/A2}$ melanoma metastasis are significantly less than the average number (>300) and size (0.23 mm in diameter) in control ConA-T-immunized HLA-A2 mice. Taken together, HIV-1 Gag-Texo vaccine capable of stimulating Gag-specific CTL responses and therapeutic immunity may be useful as a new immunotherapeutic vaccine for viral control in HIV-1 patients.

3.2 Introduction

Immune surveillance by $CD8^+$ cytotoxic T lymphocytes (CTLs) represents a major mechanism for the detection and elimination of cells infected with intracellular pathogens, especially viruses. CTLs are also essential for effective immunity against human immunodeficiency virus type 1 (HIV-1), and the induction of such responses using vaccines has become a major objective in the strategy to halt the pandemic [1]. HIV-1 structural proteins such as Gp120 and Gag are the major targets in vaccine development [2, 3]. Dendritic cells (DCs), the most potent antigen presenting cells (APCs), play an important role in inducing $CD4^+$ T-cell dependent CTL responses [4]. DCs expressing HIV-1 Gp120 and Gag have been used as vaccines to stimulate HIV-1-specific CTL responses in animal models [5-8]. HIV-1-specific DC vaccines have been also applied to clinical trials [9-12]. However, they have been found to only induce some degree of immune responses in these clinical trials [13], warranting the search of other more efficient vaccine strategies.

We previously developed a novel $CD4^+$ T cell-based vaccine (OVA-Texo) using $CD4^+$ T-cells with uptake of ovalbumin (OVA)-specific DC-released exosomes (EXO) [14-16]. We demonstrated that $CD4^+$ OVA-Texo vaccine is capable of stimulating potent OVA-specific CTL responses and long term immunity via IL-2/CD80 and CD40L signaling and counteracting regulatory T cell-mediated immune suppression, and is also capable of inducing more efficient immunity than DC vaccine and [14-16]. In addition, we have recently demonstrated that ConA-stimulated mouse $CD8^+$ T cells with uptake of HIV-1 Gp120-specific DC-released EXO (EXO_{Gp120}) can also be used as $CD8^+$ Gp120-Texo vaccine capable of stimulating Gp120-specific CTL responses leading to therapeutic and long-term immunity against Gp120-expressing B16 melanoma in both wild-type C57BL/6 and transgenic HLA-A2 mice [17, 18].

$CD8^+$ CTLs capable of recognizing some conserved and cross-strain epitopes [19-21] play a

critical role in control of HIV-1 proliferation [22, 23], acute phase of infection and disease progression even in the absence of neutralizing Abs [3, 24]. Monkeys immunized with viral vectors developed cellular immune responses that did slow disease progression [25, 26]. Thus, CTLs become essential for effective immunity against HIV-1 infection [1]. Compared to HIV-1 structural protein Gp120, HIV-1 Gag has the following advantages as a target antigen for developing HIV-1-specific vaccines. Gag as a viral antigen can enter the defective ribosomal product (Drip) pathway [27], leading to enhanced major histocompatibility complex (MHC)-1 antigen presentation and $CD8^+$ T-cell activation [28]. Gag vaccine thus stimulated persistent and broader CTL responses against conserved Gag epitopes in animal models [21, 29, 30]. HLA-B57 HIV-1-infected individuals have also been found to have autologous CTL responses against four conserved Gag epitopes, leading to reducing virus replication and viral control [31]. In addition, effective CTL responses against Gag, but not other viral antigens, have been found to correlate with suppression of HIV-1 replication in HIV-1 patients [32-35]. Therefore, Gag has become one of the most attractive target candidates for HIV-1 vaccine development.

In this study, we generated Gag-Texo vaccine by using ConA-stimulated mouse $CD8^+$ T cells with uptake of Gag-specific DC-released EXOs, and assessed its stimulation of Gag-specific CTL responses and immunity against Gag-expressing B16 melanoma in both wild-type C57BL/6 and transgenic HLA-A2 mice.

3.3 Materials and methods

3.3.1 Reagents, cell lines and animals

Biotin-labeled or fluorenscein isothiocyanate (FITC)-labeled antibodies (Abs) specific for H-2K^b, Ia^b, CD8, CD11c, CD40, CD54, CD80, CD86, 4-1BBL, OX40L and HLA-A2 antibodies (Abs), FITC-conjugated avidin and PE-anti-IFN-r and PE-anti-CD44 Abs were all obtained from BD Biosciences (Mississauga, Ontario, Canada). The rabbit anti-Gag polyclonal Ab was obtained from Fisher Scientific Inc, Waltham, MA. The $Gag_{76\text{-}84}$ peptide (SLYNTVATL) and the irrelevant control human epidermal growth factor receptor-2 (HER2) peptide (ILHNGAYSL) were synthesized by Multiple Peptide Systems (San Diego, CA). Adenoviral vector AdV_{Gag} expressing HIV-1 Gag was constructed by insertion of Gag cDNA fragment of $pcDNA_{Gag}$ into pShuttle vector (Stratagene, La Jolla, CA) by recombinant technology. The recombinant AdV_{Gag} vector was linealized by PacI digestion, and then transfected into 293 cells using Lipofectamine 2000 (Invitrogene, Carlsbad, CA) to generate AdV_{Gag} expressing transgene Gag (**Figure 3.1A**). AdV_{Gag} was amplified in 293 cells, and purified by a series of cesium chloride ultracentrifugation gradients [36]. Vectors $pcDNA_{HLA\text{-}A2}$ expressing α1 and α2 domains of HLA-A2 and α3 domain of H-2K^b and vector pcDNA-Gag/GFP expressing a fused gene Gag/GFP were previously constructed in our laboratory [15]. B16 melanoma cell line BL6-10 was transfected with pcDNA-Gag/GFP by Lipofectamine 2000 (Invitrogene) to generate $BL6\text{-}10_{Gag/GFP}$ cells, which were then further transfected with $pcDNA_{HLA\text{-}A2}$ to form Gag/HLA-A2-expressing $BL6\text{-}10_{Gag/A2}$. Female C57BL/6 and transgenic (Tg) HLA-A2 mice (#3584) were obtained from the Jackson Laboratory (Bar Harbor, MA). All mice were treated according to animal care committee guidelines of the University of Saskatchewan.

3.3.2 Dendritic cell and exosome preparations

Bone marrow-derived dendritic cells (DCs) were obtained by culturing the wild-type C57BL/6 or transgenic HLA-A2 mouse bone marrow cells in culture medium containing GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) for six days as previously described [15]. DCs were infected with AdVGag and termed DC_{Gag}. DC_{Gag}-released exosomes (EXO_{Gag}) were then purified from DC culture supernatants by differential ultracentrifugation [15].

3.3.3 Gag-Texo preparation

The wild-type C57BL/6 or transgenic HLA-A2 mouse splenocytes were cultured in RPMI1640 medium containing IL-2 (20 U/ml) and ConA (1 μg/ml) for 3 days [37]. ConA-activated $CD8^+$ T (ConA-T) cells were enriched by passage through nylon wool columns (C&A Scientific, Manassas, VA), and then purified by negative selection using anti-mouse CD8 paramagnetic beads (DYNAL Inc, Lake Success, NY) [38]. Gag-Texo vaccine was generated by incubation of $CD8^+$ ConA-T cells with EXO_{Gag} as previously described [17].

3.3.4 Electron microscopic analysis

EXOs were fixed in 4% paraformaldehyde. The pellets were then loaded onto carbon-coated formvar grids. The EXO sample-loaded grids were stained with saturated aqueous uranyl, and then examined with a JEOL 1200EX electron microscope at 60 kV [39].

3.3.5 Western blot analysis

Cell lysates (10 μg/well) were loaded onto 12% acrylamide gels, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred onto nitrocellulose membrane (Millipore, Bedford, MA). The membrane was blocked with ODYSSEY blocking buffer (LI-COR Bioscience, Lincoln, NE), immunoblotted with anti-gag

Ab, incubated with anti-goat IRDyeR800CW Ab, and then scanned using ODYSSEY instrument according to manufacture's instruction (LI-COR Bioscience) [39].

3.3.6 Flow cytometric analysis

Cells and EXOs were stained with a panel of Abs and analyzed by flow cytometry [17]. To assess Gag-Texo stimulated CTL responses, the blood samples harvested from immunized mice were stained with FITC-conjugated anti-CD8 Ab (FITC-CD8) and PE-conjugated anti-CD44 Ab (PE-CD44), and analyzed by flow cytometry. In another set of experiments, the spleen cells were harvested from immunized mice, and depleted of erythrocytes. The splenocytes were cultured for 4 hrs with 2 μM Gag peptide (SLYNTVATL), and then stained with FITC-anti-CD8 Ab. Subsequently, the T cells were fixed, and cell membranes were permeabilized with Cytofix/Cytoperm solution (BD Biosciences). The cells were stained with PE-conjugated anti-IFN-r Ab, and then analyzed by flow cytometry [40].

3.3.7 Cytotoxicity assay

The *in vivo* cytotoxicity assay was performed as previously described [41]. Briefly, splenocytes were harvested from naive C57BL/6 mouse spleens and incubated with either high (3.0 μM, $CFSE^{high}$) or low (0.6 μM, $CFSE^{low}$) concentrations of CFSE, to generate differentially labeled target cells. The $CFSE^{high}$ cells were pulsed with Gag peptide, whereas the $CFSE^{low}$ cells were pulsed with irrelevant HER2 peptide, and served as internal controls. These peptide-pulsed target cells (1×10^6 cells/mouse) were i.v. co-injected at 1:1 ratio into immunized mice six days after the immunization. Sixteen hrs later, the residual Gag-specific $CFSE^{high}$ and irrelevant control $CFSE^{low}$ target cells remaining in the recipients' spleens were analyzed by flow cytometry.

3.3.8 Animal studies

To examine the protective antitumor immunity conferred by Gag-Texo vaccine, the wild-type C57BL/6 or transgenic HLA-A2 mice (n=8) were injected i.v. with DC_{Gag}, Gag-Texo and the control ConA-stimulated $CD8^+$ T (ConA-T) cells (2×10^6 cells/mouse) respectively. The immunized mice were challenged i.v. with 0.5×10^6 BL6-$10_{Gag/GFP}$ or HLA-A2-expressing BL6-$10_{Gag/A2}$ cells 6 or 30 days subsequent to the immunization. To examine the therapeutic antitumor immunity conferred by Gag-Texo vaccine, the transgenic HLA-A2 mice (n=8) were first injected i.v. with 0.5×10^6 BL6-$10_{Gag/A2}$ cells. Six days after tumor cell inoculation, HLA-A2 mice were then injected i.v. with Gag-Texo cells (2×10^6 cells/mouse). The mice were sacrificed 3 weeks after tumor cell injection, and the lung metastatic tumor colonies were counted in a blind fashion. 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 [15].

3.3.9 Statistic analyses

Statistical analyses were performed using the Student *t* test or the Mann-Whitney *U* test for comparison of variables from different groups. A value of $p<0.05$ was considered to be statistically significant [16].

3.4 Results

3.4.1 Generation of Gag-Texo vaccine

We previously prepared Gp120-specific $CD8^+$ T cell-based vaccine (Gp120-Texo) by using ConA-stimulated $CD8^+$ T cells with uptake of AdV_{Gp120}-infected DC (DC_{Gp120})-released exosomes (EXO_{Gp120}), and showed that Gp120-Texo vaccine stimulated Gp120-specific CTL responses and therapeutic immunity [17, 18]. To assess whether HIV-1 Gag-specific T cell-based vaccine can also stimulate CTL responses and therapeutic immunity, we first constructed a recombinant adenoviral vector AdV_{Gag} expressing HIV-1 Gag (**Figure 3.1A**), and generated bone marrow-derived DCs which express CD11c, DC maturation markers such as Ia^b, CD40, CD80 and CD86 and other costimulatory molecules such as 41BBL and OX40L (**Figure 3.1B**), indicating that they are mature DCs. We then infected DCs with AdV_{Gag} to form DC_{Gag}. We showed that DC_{Gag} expressed HIV-1 Gag (55 KDa) assessed by Western Blotting analysis (**Figure 3.1C**). We also purified DC_{Gag}-released EXO (EXO_{Gag}) from DC_{Gag} culture supernatants by ultracentrifugation. We demonstrated that EXO_{Gag} showed a typical exosomal characteristic of "saucer" or round shape with a diameter between 50-90 nm (**Figure 3.1D**), and similarly expressed the above DC's molecules, but at much less content than DCs (**Figure 3.1E**).

Fig 3.1

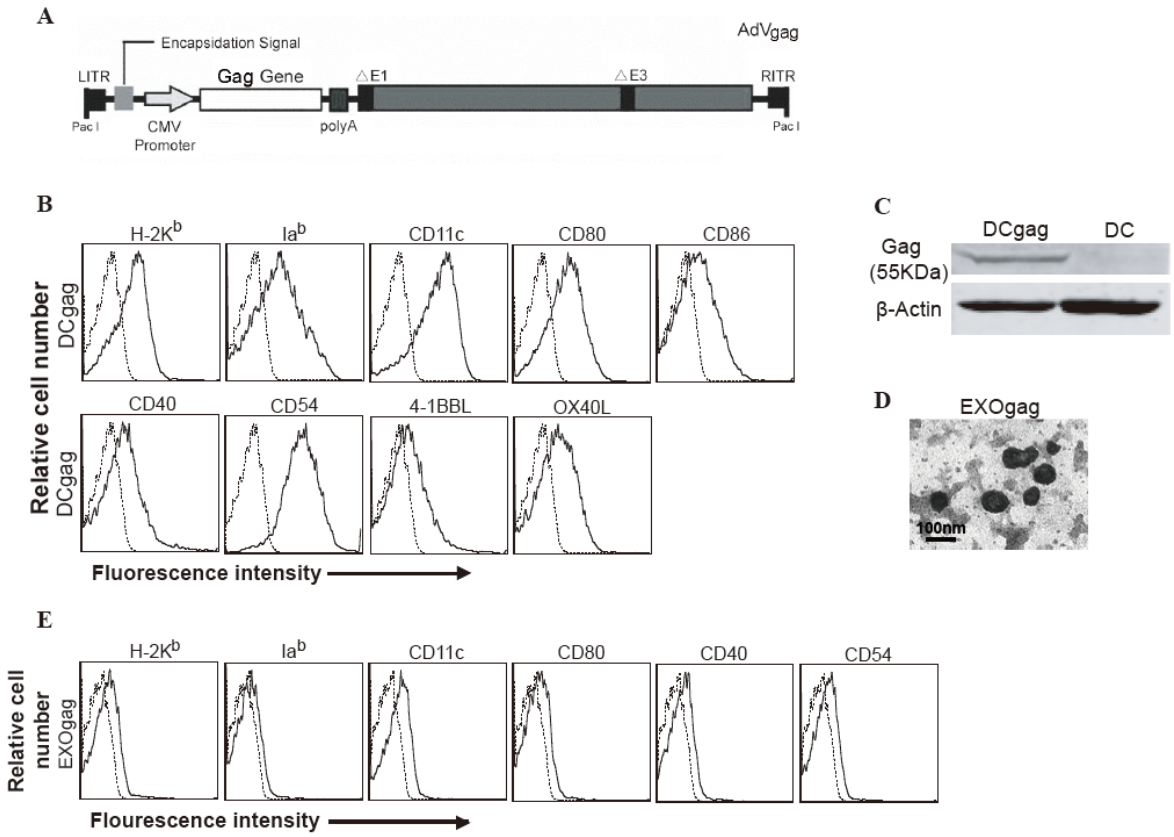


Figure 3.1 Phenotypic analysis of Gag-Texo.

(A) Schematic representation of adenoviral (AdV) vector AdV_{Gag}. The E1/E3 depleted replication-deficient AdV is under the regulation of the cytomegalovirus (CMV) early/immediate promoter/enhancer. ITR, inverted terminal repeat. (B) DC_{Gag} were stained with a panel of Abs (solid lines) or isotype-matched irrelevant Abs (dotted lines) and analyzed by flow cytometry. (C) Western blot analysis of lysates of DC_{Gag} and DCs using the anti-gag Ab. (D) Electron micrograph of EXO_{Gag}. Bar, 100 nm. (E) EXO_{Gag} were stained with a panel of Abs (solid lines) or isotype-matched irrelevant Abs (dotted lines) and analyzed by flow cytometry. One representative experiment of two is shown.

3.4.2 Gag-Texo vaccine stimulates Gag-specific effector CTL responses in C57BL/6 mice

We then immunized C57BL/6 mice with Gag-Texo and DC_{Gag}, and then assessed CTL responses by flow cytometry using FITC-anti-CD8 and PE-anti-CD44 antibodies for double staining 6 days

after immunization [17, 18]. We demonstrated that both Gag-Texo and DC_{Gag} vaccines were capable of more efficiently stimulating proliferation of CTLs expressing T cell activation marker CD44 than ConA-T cells ($p<0.05$) (**Figure 3.2A**). In addition, we also quantified Gag-specific CTL responses in Gag-Texo- and DC_{Gag}-immunized mice by flow cytometry using FITC-anti-CD8 and PE-anti-IFN-γ antibodies for double staining after *in vitro* Gag peptide stimulation of splenocytes purified from the immunized mice. We found that 3.76% and 5.61% of $CD8^+$ T-cells from Gag-Texo- and DC_{Gag}-immunized mice produced IFN-γ, which is significantly more than $CD8^+$ T-cells (1.31%) from ConA-T-immunized mice ($p<0.05$) (**Figure 3.2B**), indicating that both Gag-Texo and DC_{Gag} stimulate efficient Gag-specific CTL responses. Next, we assessed the ability of Gag-Texo to induce the differentiation of stimulated $CD8^+$ T-cells into effector CTLs. We adoptively transferred Gag_{76-84} peptide-pulsed splenocytes that had been strongly labeled with CSFE ($CFSE^{high}$), as well as the control HER2 peptide-pulsed splenocytes that had been weakly labeled with CFSE ($CFSE^{low}$) at 1:1 ratio, into recipient mice that had been vaccinated with either Gag-Texo or DC_{Gag} cells 7 days after immunization. Sixteen hours after the cell transfer, mouse splenocytes were analyzed by flow cytometry. Thus, the loss of Gag-specific $CFSE^{high}$ target cells represents the Gag-specific killing activity of CTLs in immunized mice. As expected, there was substantial loss (72% and 80%) of the $CFSE^{high}$ cells in the Gag-Texo- and DC_{Gag}-immunized mice, respectively, whereas little cytotoxicity (3%) was induced in control ConA-T-immunized mice (**Figure 3.2C**), indicating that Gag-Texo- and DC_{Gag} -stimulated CTLs are effector CTLs with Gag-specific cellular cytotoxicity.

Fig 3.2

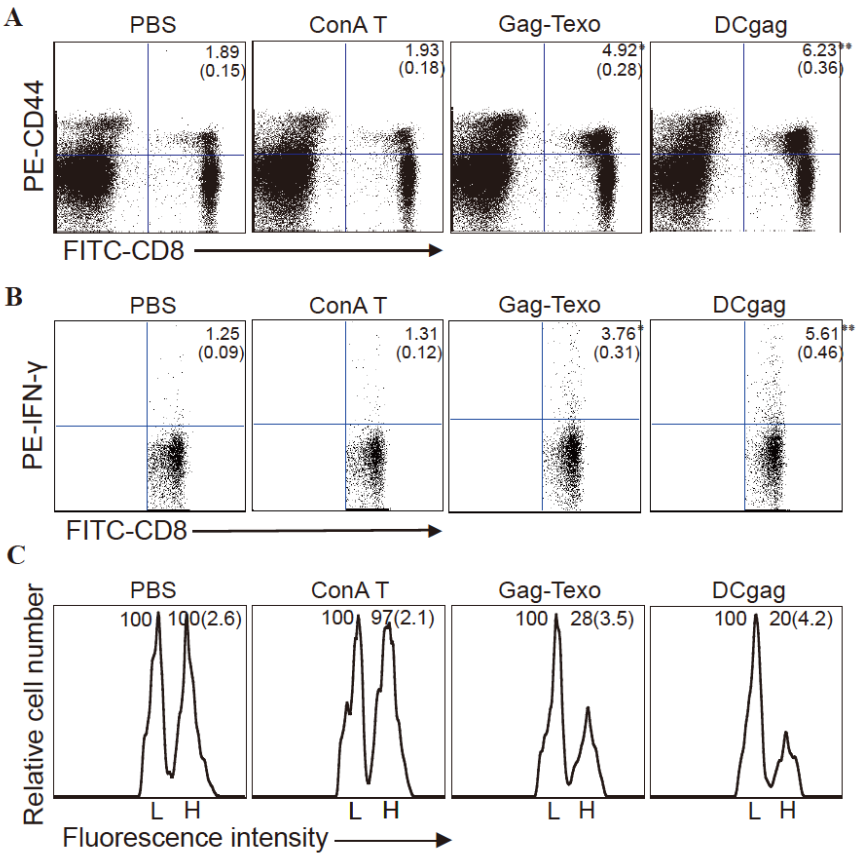


Figure 3.2 Gag-Texo stimulates Gag-specific functional effector CTL responses.

(A, B) C57BL/6 mice were i.v. immunized with DC_{Gag}, Gag-Texo or active $CD8^+$T cell (ConA T) without uptaking EXOGag. Six days after the immunization, tail blood samples of the immunized mice were stained with PE-anti-CD44 Ab and FITC-anti-CD8 Ab, and analyzed by flow cytometry. In another set of experiments, the splenocytes of immunized mice were first stimulated by Gag peptide in culture, and then stained with FITC-anti-CD8 Ab. After cell membrane permeabilization, the cells were stained with PE-anti-IFN-r Ab, and then analyzed by flow cytometry. The value in each panel represents the percentage of $CD8^+CD44^+$ T cells in the total cells analyzed or the percentage of $CD8^+$ T cells producing IFN-r in the total $CD8^+$ T cell population. (C) *In vivo* cytotoxicity assay. Six days after the immunization, the immunized mice were i.v. injected with a mixture of $CFSE^{high}$ and $CFSE^{low}$ -labeled splenocytes (at 1:1 ratio) that had been pulsed with $Gag_{76\text{-}84}$ or irrelevant Mut1 peptide, respectively. After 16 hrs, the spleens of immunized mice were removed and the percentages of the residual $CFSE^{high}$ (H) and $CFSE^{low}$ (L) target cells remaining in the recipients' spleens were analyzed by flow cytometry. The value in each panel represents the percentage of $CFSE^{high}$ vs $CFSE^{low}$ target cells remaining in the spleen. The value in parenthesis represents the standard deviation (SD). *$p < 0.05$ versus cohorts of ConA- T cells (Student *t* test). One representative experiment of two is shown.

3.4.3 Gag-Texo vaccine stimulates protective antitumor immunity in C57BL/6 mice

Next, we transfected BL6-10 cells with pcDNA-Gag/GFP expression vector to form BL6-10_{Gag} melanoma cells expressing a fluorescent fusion protein Gag/GFP (**Figure 3.3A,** panel a), but not HLA-A2 (**Figure 3.3A,** panel c) by flow cytometric analysis. Expression of Gag portion of Gag/GFP (82 KDa) was confirmed using anti-Gag antibody by Western blotting analysis (**Figure 3.3B**). We then assessed the protective immunity derived from Gag-Texo vaccine against B16 melanoma BL6-10_{Gag} by i.v. injection of BL6-10_{Gag} cells into immunized C57BL/6 mice 6 days after immunization. Three weeks after tumor cell injection, mice were sacrificed, and mouse lungs were checked for tumor metastasis colonies. As shown in **Figure 3.3C**, all mice injected with ConA-T cells without uptake of EXO_{Gag} had large numbers (>300) of lung metastatic BL6-10_{Gag} tumor colonies. Gag-Texo or DC_{Gag} vaccine, however, induced a complete immune protection against BL6-10_{Gag} tumor cell challenge in all 8/8 (100%) mice, indicating that Gag-Texo induces efficient protective immunity in wild-type C57BL/6 mice.

Fig 3.3

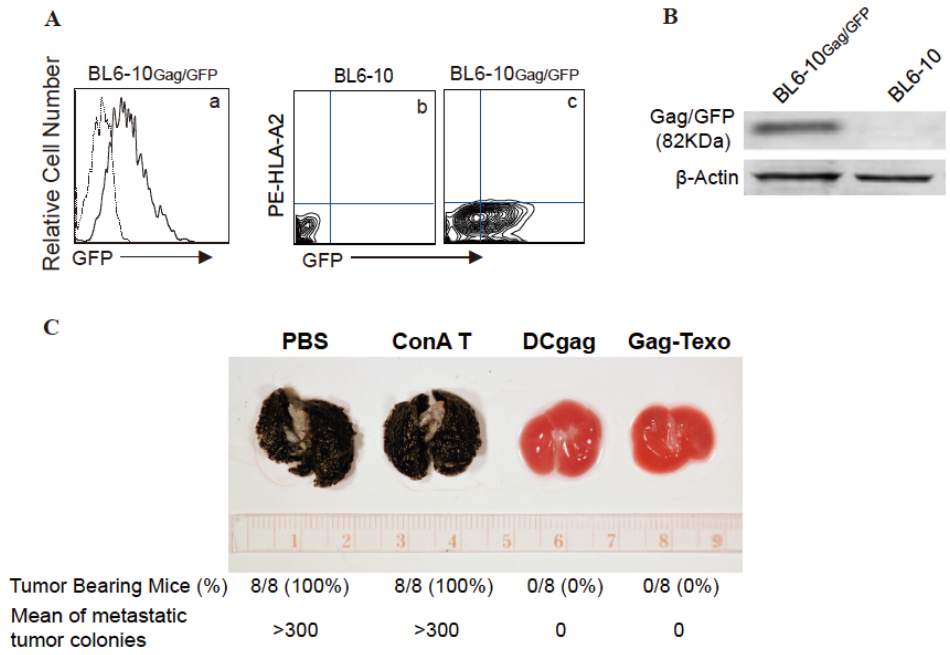


Figure 3.3 Gag-Texo stimulates preventive anti-tumor immunity.

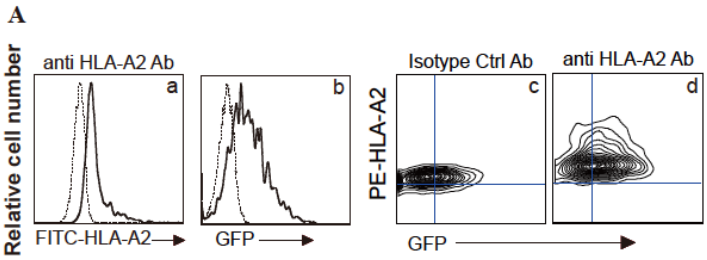
(A) Gag/GFP expression in BL6-10_{Gag} (solid lines) or BL6-10 cells (dotted lines) was analyzed by flow cytometry (panel a). BL6-10_{Gag} and BL6-10 cells were stained with the anti-HLA-A2 Ab and analyzed by flow cytometry (panels b and c). (B) Western blot analysis of lysates of BL6-10_{Gag} and BL6-10 cells using the anti-Gag Ab. (C) C57BL/6 mice were immunized with PBS, ConA T, or Gag-Texo or DC_{Gag}. 6 days after the immunization, the immunized mice were i.v. injected with BL6-10_{Gag} cells, then sacrificed 3 weeks subsequent to tumor challenge. The lung samples were collected and metastatic tumor colonies were counted. One representative experiment of two is shown.

3.4.4 Gag-Texo vaccine induces protective and long-term antitumor immunity in transgenic HLA-A2 mice

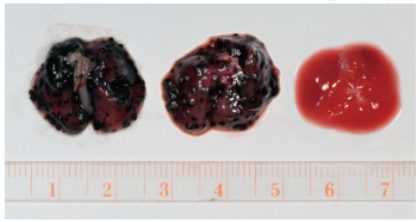
Next, we generated BL6-$10_{Gag/A2}$ melanoma cells by transfection of BL6-$10_{Gag/GFP}$ cells with pcDNA-HLA-A expression vector, and showed that transfected BL6-$10_{Gag/A2}$ melanoma cells expressed both HLA-A2 and Gag (**Figure 3.4A,** panels a/d and b/d). We then assessed the protective (6 days after immunization) and long-term immunity (30 days after immunization) derived from Gag-Texo vaccine against B16 melanoma BL6-$10_{Gag/A2}$. As shown in **Figure 3.4B**,

all mice injected with ConA-T cells without uptake of EXO_{Gag} had a large number (>300) of lung metastatic BL6-$10_{Gag/A2}$ tumor colonies. Gag-Texo vaccine, however, induced immune protection against BL6-$10_{Gag/A2}$ tumor-cell challenge in 8/8 (100%) and 2/8 (25%) HLA-A2 mice with 6 and 30 days post immunization, respectively. The average number of lung tumor colonies in mice with 30 days post immunization is only 23, which is significantly less than that (>300) in control ConA-T-immunized mice ($p<0.05$). Our data indicate that Gag-Texo induces both protective and long-term immunity in HLA-A2 mice.

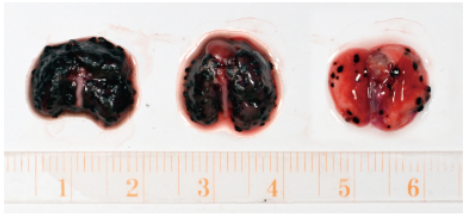
Fig 3.4



B

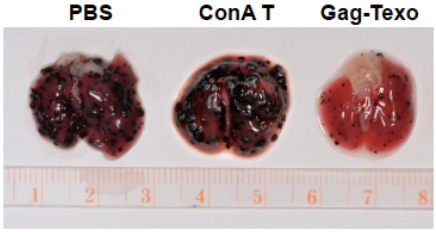


	PBS	ConA T	Gag-Texo
Tumor Bearing Mice (%)	8/8 (100%)	8/8 (100%)	0/8 (0%)
Mean of metastatic tumor colonies	>300	>300	0



	PBS	ConA T	Gag-Texo
Tumor Bearing Mice (%)	8/8 (100%)	8/8 (100%)	6/8 (75%)
Mean of metastatic tumor colonies	>300	>300	23 (13-32)*

C



	PBS	ConA T	Gag-Texo
Tumor Bearing Mice (%)	8/8 (100%)	8/8 (100%)	8/8 (100%)
Mean of metastatic tumor colonies	>300	>300	50 (34-65)*
Average diamater of metastatic tumor colonies	0.91mm (0.41-2.10)	1.02mm (0.52-2.23)	0.23mm** (0.10-0.45)

Figure 3.4 Gag-Texo induces preventive and therapeutic anti-tumor immunity in HLA-A2 mice.

(A) BL6-$10_{Gag/A2}$ cells were stained with FITC-anti-HLA-A2 Ab (solid lines) or isotype-matched irrelevant Ab (dotted lines) and analyzed by flow cytometry (panel a). In addition, Gag/GFP expression in BL6-$10_{Gag/A2}$ (solid lines) or BL6-10 cells (dotted lines) was analyzed with flow cytometry (panel b). BL6-$10_{Gag/A2}$ cells were also stained with PE-anti-HLA-A2 Ab, and the cells were then analyzed for PE-HLA-A2 and GFP-Gag expression by flow cytometry (panels c and d). (B) To assess protective and long-term immunity, HLA-A2 mice were i.v. injected with BL6-$10_{Gag/A2}$ 6 or 30 days after PBS, ConA-T, or Gag-Texo immunization. (C) To assess therapeutic immunity, HLA-A2 mice were i.v. injected with BL6-$10_{Gag/A2}$. Six days after tumor challenge, mice were then vaccinated with after ConA-T, or Gag-Texo. The mice were sacrificed 3 weeks after tumor cell challenge. The average number and diameter of lung metastatic tumor colonies were counted and measured using a caliper, respectively. *$p < 0.05$ versus cohorts of ConA- T cells (Mann-Whitney U test) and **$p < 0.05$ versus cohorts of ConA- T cells (Student t test). One representative experiment of two is shown.

3.4.5 Gag-Texo vaccine induces therapeutic immunity against Gag/HLA-A2-expressing tumor in transgenic HLA-A2 mice

To assess the potential therapeutic effect of Gag-Texo, we first challenged HLA-A2 mice with BL6-$10_{Gag/A2}$ tumor cells. Six days after tumor cell challenge, mice were then immunized with Gag-Texo. We found that, though all Gag-Texo-immunized mice (8/8) post 6-day tumor-cell challenge still carried lung tumor metastasis, the average number (50) and size (1.02 mm in diameter) of lung tumor colonies in Gag-Texo-immunized mice are significantly less than the average number (>300) and size (0.23 mm in diameter) in control ConA-T-immunized mice ($p<0.05$) (**Figure 3.4C**), indicating that Gag-Texo vaccine also induces some degree of therapeutic immunity against 6-day-established lung tumor metastasis in transgenic HLA-A2 mice.

3.5 Discussion

We previously generated Gp120-Texo vaccine using ConA-T-stimulated mouse polyclonal $CD8^+$ T cells with uptake of HIV-1 Gp120-specific DC-released EXO (EXO_{Gp120}), and demonstrated that $CD8^+$ Gp120-Texo vaccine is capable of stimulating $CD4^+$ T cell-independent Gp120-specific CTL responses leading to protective and long-term immunity against Gp120/HLA-A2-expressing B16 melanoma ($BL6\text{-}10_{Gp120/A2}$) in 8/8 transgenic HLA-A2 mice [17, 18]. In addition, $CD8^+$ Gp120-Texo vaccine also induced complete therapeutic immunity against 6-day-established lung $BL6\text{-}10_{Gp120/A2}$ melanoma metastasis in 2/8 HLA-A2 mice [17, 18]. Since Gag has become one of the most attractive target candidates for HIV-1 vaccine development, we generated Gag-Texo vaccine in this study. We demonstrate that both Gag-Texo and DC_{Gag} vaccines are capable of comparably stimulating Gag-specific effector $CD8^+$ CTL responses. We also show that Gag-Texo-stimulated CTL response results in protective immunity against Gag-expressing $BL6\text{-}10_{Gag}$ melanoma in 8/8 wild-type C57BL/6 mice. In addition, we show that Gag-Texo vaccine also induces Gag-specific CTL responses leading to protective and long-term immunity against Gag/HLA-A2-expressing $BL6\text{-}10_{Gag/A2}$ melanoma in 8/8 and 2/8 transgenic HLA-A2 mice, respectively. However, the average number of lung tumor colonies in the remaining 6 mice is only 23, which is significantly less than that (>300) in control ConA-T-immunized mice. In addition, Gag-Texo vaccine also induces some degree of therapeutic immunity against 6-day-established lung tumor metastasis in transgenic HLA-A2 mice. Although all Gag-Texo-immunized mice (8/8) post 6-day tumor-cell challenge still carried lung tumor metastasis, the average number (50) and size (1.02 mm in diameter) of lung tumor colonies in Gag-Texo-immunized mice is significantly less than the average number (>300) and size (0.23 mm in diameter) in control ConA-T-immunized mice. Compared to Gp120-Texo vaccine inducing therapeutic immunity against 6-day-established lung $BL6\text{-}10_{Gp120/A2}$ melanoma metastasis in 2/8 HLA-A2 mice, Gag-Texo vaccine induces a relatively less efficient therapeutic immunity in HLA-A2 mice bearing 6-day-established lung $BL6\text{-}10_{Gag/A2}$ melanoma. It was

demonstrated that B16 melanoma expressing a low density of tumor antigen-specific pMHC-I complexes became less sensitive to CTL killing [42]. The less effectiveness of therapeutic immunity in Gag-Texo-immunized mice may be due to the lower density of Gag-specific peptide/MHC-I (pMHC-I) complexes presented on target BL6-$10_{Gag/A2}$ cells expressing both Gag and irrelevant GFP, compared to the higher density of Gp120-specific pMHC-I complexes presented on BL6-$10_{Gp120/A2}$ cells carrying only Gp120. Costimulatory molecule 41BBL has been shown to play important role in CTL priming and memory development, and in rescuing functionally impaired HIV-1-specific CTLs [43, 44]. To improve effectiveness of Gag-Texo vaccine, we are currently engineering Gag-Texo vaccine to express transgene-encoded 41BBL, and assessing its enhanced stimulatory effect on Gag-specific CTL responses and antitumor immunity in transgenic HLA-A2 mice.

HIV-1 patients often have $CD4^+$ T cell deficiency derived from the binding of HIV-1 gp120 to T cell CD4 molecules leading to T cell death by viral cytopathic effect [45]. Since $CD4^+$ T cells are required for $CD8^+$ CTL response and memory development [46], the quantitative and qualitative nature of HIV-1-specific $CD8^+$ T cells has been greatly affected in HIV-1 patients [47]. HIV-1-specific DC vaccines have been also applied to clinical trials [9-12]. However, these DC vaccines which are capable of HIV-1-specific CTL responses in the presence of $CD4^+$ T cells have been found to only induce some degree of immune responses in these studies [13]. Therefore, the quality of CTL responses may be the key to HIV-1 control [48]. Since HIV-1-specific $CD8^+$ CTLs have been found to recognize some Gag conserved and cross-strain epitopes [21, 29, 30], Gag, but not Gp120 becomes one of the most attractive target candidates for HIV-1 vaccine development. Our HIV-1 Gag-specific DC-released exosome-targeted $CD8^+$ T cell-based Gag-Texo vaccine may thus bypass the high mutation problem faced by preventive vaccines for induction of Abs [49], and become more efficient HIV-1 vaccine than our previously reported Gp120-Texo one [17, 18]. Our previous data showed that HIV-1 Gp120-Texo, but not DC_{Gp120} vaccine stimulated Gp120-specific $CD8^+$ T cells in the absence of

$CD4^+$ T cells [17, 18], indicating that our Gag-Texo vaccine may similarly stimulate $CD4^+$ T cell-independent Gag-specific CTL responses. Although both Gag-Texo and DC_{Gag} vaccines stimulate comparable CTL responses, Gag-Texo vaccine may become more useful for treatment of HIV-1 patients with $CD4^+$ T cell deficiency than DC_{Gag}. The assessment of Gag-specific CTL responses in patient's blood samples, which are stimulated *in vitro* by Gag-Texo cells derived from patient's autologous T cells with uptake of AdVGag-transfected autologous DC-released exosomes, is currently underway in our laboratory.

Taken together, our novel $CD8^+$ Gag-Texo vaccine capable of stimulating Gag-specific effector CTL responses and therapeutic immunity in transgenic HLA-A2 mice may be useful as a new immunotherapeutic vaccine for viral control in HIV-1 patients.

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CHAPTER 4 TRANSGENE 4-1BBL-ENGINEERED VACCINE STIMULATES POTENT GAG-SPECIFIC THERAPEUTIC AND LONG-TERM IMMUNITY VIA PRIMING INCREASED $CD44^+CD62L^{high}IL\text{-}7R^+$ CTLs WITH UP- AND DOWN-REGULATION OF ANTI- AND PRO-APOPTOSIS GENES

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Running title: 4-1BBL signaling enhances HIV-1 vaccine-stimulated therapeutic immunity

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The manuscript "Transgene 4-1BBL-engineered vaccine stimulates potent Gag-specific therapeutic and long-term immunity via priming increased $CD44^+CD62L^{high}IL\text{-}7R^+$ CTLs with up- and down-regulation of anti- and pro-apoptosis genes" has been published in Cellular & Molecular Immunology by Wang R *et al.* 2015. 12: 456-465. It is reproduced here with the permission of the copyright owner.

4.1 Abstract

Human immunodeficiency virus type-1 (HIV-1)-specific dendritic cell (DC) vaccines have been applied to clinical trials. However, they have been found to only induce some degree of immune responses in clinical trial studies. We previously demonstrated HIV-1 Gag-specific Gag-Texo vaccine stimulated Gag-specific effector $CD8^+$ cytotoxic T lymphocyte (CTL) responses leading to complete protective, but very limited therapeutic immunity. In this study, we constructed a recombinant adenoviral vector $AdV_{4\text{-}1BBL}$ expressing mouse 4-1BBL, and generated transgene 4-1BBL-engineered OVA-$Texo_{/4\text{-}1BBL}$ and Gag-$Texo_{/4\text{-}1BBL}$ vaccines by transfection of ovalbumin (OVA)-Texo and Gag-Texo cells with $AdV_{4\text{-}1BBL}$, respectively. We demonstrate that OVA-specific OVA-$Texo_{/4\text{-}1BBL}$ vaccine stimulates more efficient OVA-specific CTL responses (3.26%) compared to OVA-Texo-activated ones (1.98%) in wild-type C57BL/6 mice and the control OVA-$Texo_{/Null}$ vaccine without transgene 4-1BBL expression, leading to enhanced therapeutic immunity against 6-day established OVA-expressing B16 melanoma BL6-10_{OVA}. OVA-$Texo_{/4\text{-}1BBL}$-stimulated CTLs which show $CD44^+CD62L^{high}IL\text{-}7R^+$ phenotype are likely memory CTL precursors demonstrating prolonged survival and enhanced differentiation into memory CTLs with functional recall responses and long-term immunity against BL6-10_{OVA} melanoma. In addition, we demonstrate that OVA-$Texo_{/4\text{-}1BBL}$-stimulated CTLs up- and down-regulate expression of anti-apoptosis (*Bcl2l10*, *Naip1*, *Nol3*, *Pak7* and *Tnfrsf11b*) and pro-apoptosis (*Casp12*, *Trp63* and *Trp73*) genes, respectively, by RT^2 Profiler™ PCR array analysis. Importantly, Gag-specific Gag-$Texo_{/4\text{-}1BBL}$ vaccine also stimulates more efficient Gag-specific therapeutic and long-term immunity against HLA-A2/Gag-expressing B16 melanoma BL6-$10_{Gag/A2}$ than the control Gag-$Texo_{/Null}$ vaccine in transgenic HLA-A2 mice. Taken together, our novel Gag-$Texo_{/4\text{-}1BBL}$ vaccine capable of stimulating potent Gag-specific therapeutic and long-term immunity may represent a new immunotherapeutic vaccine for controlling HIV-1 infection.

Key words: 4-1BBL, Gag, HLA-A2 mice, T-cell-based vaccine, Therapeutic immunity.

4.2 Introduction

$CD8^+$ cytotoxic T lymphocytes (CTLs) are considered to be an important immune component for effective immunity against human immunodeficiency virus type 1 (HIV-1), and the induction of such responses using vaccines has become a major objective in the strategy to halt the pandemic [1, 2]. Dendritic cells (DCs), the most potent antigen presenting cells (APCs), expressing HIV-1 structure proteins Gp120 and Gag have been used as vaccines and shown to stimulate HIV-1-specific CTL responses in animal models [3-6]. However, HIV-1-specific DC vaccines have been found to only induce some degree of immune responses in clinical trials [7], warranting the search of other more efficient vaccine strategies.

We previously developed a novel T cell-based vaccine (OVA-Texo) using ConA-stimulated $CD4^+$ T cells with uptake of ovalbumin (OVA)-specific DC-released exosomes (EXOs) [8-10]. We demonstrated that OVA-Texo vaccine induces more efficient immunity than DC vaccine, and is capable of stimulating potent $CD4^+$ T-cell independent CTL responses and long-term antitumor immunity via IL-2/CD80 and CD40L signaling, and counteracting regulatory T cell-mediated immune suppression [8-10]. In addition, we also developed HIV-1 Gp120- and Gag-specific T cell-based (Gp120-Texo and Gag-Texo) vaccines using ConA-stimulated mouse $CD8^+$ T cells with uptake of Gp120- and Gag-specific DC-released EXO [11, 12][13]. We demonstrated that Gp120-Texo and Gag-Texo stimulated Gp120- or Gag-specific CTL responses in transgenic HLA-A2 mice [11, 12][13]. However, their therapeutic efficacy was still very limited. For example, Gp120-Texo vaccine only cured 2/8 transgenic HLA-A2 mice bearing 6 day-established HLA-A2/Gp120-expressing BL6-$10_{Gp120/A2}$ B16 melanoma，though the vaccine cured 8/8 HLA-A2 mice bearing 3 day-established tumor [11, 12][13]. Gag-Texo vaccine could only induce some degree of therapeutic immunity by showing a decreased number and size of 6 day-established HLA-A2/Gag-expressing BL6-$10_{Gag/A2}$ B16 melanoma in transgenic HLA-A2 mice [13].

4-1BB ligand (4-1BBL) is a member of the tumor necrosis factor family [14]. It is inducible on activated APCs, and can provide a CD28-independent signal leading to cell division, induction of effector function and enhancement of $CD8^+$ T cell survival and memory development [15-19]. It synergizes with CD80 and PD-1 blockade for re-activation of anergic T cells [20], and for augmentation of CTL responses during chronic viral infection [21]. In addition, 4-1BBL signaling is also a critical component in the costimulation-dependent rescue of exhausted HIV-specific CTLs [22], and the combination of 4-1BBL and CD40L signaling enhances stimulation of HIV-1-specific CTLs [23]. Therefore, 4-1BBL becomes an attractive candidate signaling to improve efficacy of immunotherapy [19]. We assume that incorporation of 4-1BBL into our Gag-Texo vaccine may enhance its therapeutic effect against Gag-expressing tumor cell challenge.

In this study, we generated transgene 4-1BBL-engineered OVA-Texo$_{/4\text{-}1BBL}$ and Gag-Texo$_{/4\text{-}1BBL}$ vaccines and assessed OVA- and Gag-specific CTL responses, therapeutic and long-term immunity against OVA- and Gag-expressing B16 melanoma in vaccinated-wild-type C57BL/6 and -transgenic HLA-A2 mice, respectively.

4.3 Materials and methods

4.3.1 Reagents, cell lines and animals

Biotin-labeled or fluorescein isothiocyanate (FITC)-labeled antibodies (Abs) were obtained from BD Biosciences (Mississauga, Ontario, Canada). Gag/HLA-A2-expressing BL6-$10_{Gag/A2}$ tumor cell line was generated by transfection of BL6-10 tumor cells with two expression vectors $pcDNA_{Neo}$Gag and $pcDNA_{Hygro}$HLA-A2 expressing Gag and HLA-A2, respectively, in our lab [13]. Female C57BL/6 and transgenic (Tg) HLA-A2 mice (#003475) carrying the transgene Tg(HLA-A2.1)1Enge were obtained from the Jackson Laboratory (Bar Harbor, MA). All mice were treated according to animal care committee guidelines of the University of Saskatchewan.

4.3.2 Recombinant adenovirus construction

The construction of recombinant adenovirus (AdV) expressing 4-1BBL ($AdV_{4\text{-}1BBL}$) was performed by insertion of mouse 4-1BBL gene cloned from bone marrow-derived DCs into pShuttle vector (Stratagene Inc, La Jolla, CA) using the cloned 4-1BBL cDNA to form $pLpA_{4\text{-}1BBL}$ expressing 4-1BBL gene. The PmeI-digested shuttle vector was then co-transformed into BJ5183 E. coli cells that already containing the backbone vector for homologous recombination to form the recombinant vector $AdV_{4\text{-}1BBL}$ as described previously (**Figure 4.1A**) [13]. Adenoviral vector AdV_{Gag} expressing HIV-1 Gag and the control AdV_{Null} without any transgene expression were previously constructed in our laboratory (**Figure 4.1A**) [13].

4.3.3 Preparation of engineered OVA-Texo$_{/4\text{-}1BBL}$ and Gag-Texo$_{/4\text{-}1BBL}$ vaccines

Ovalbumin (OVA)- and Gag-specific OVA-Texo and Gag-Texo vaccines were generated by incubation of ConA-activated $CD8^+$ T (ConA-T) cells with OVA protein-pulsed bone

marrow-derived dendritic cell (DC_{OVA})- and AdV_{Gag}-transfected DC (DC_{Gag})-released exosomes (EXO_{OVA} and EXO_{Gag}) as previously described [13]. $CD8^+$ OVA-$Texo_{/4\text{-}1BBL}$ and Gag-$Texo_{/4\text{-}1BBL}$ or the control $CD8^+$ OVA-$Texo_{/Null}$ and Gag-$Texo_{/Null}$ vaccines were then generated by transfection of $CD8^+$ OVA-Texo and Gag-Texo cells with $AdV_{4\text{-}1BBL}$ or the control AdV_{Null} as previously described [13].

4.3.4 Flow cytometric analysis

To assess CTL responses, blood samples of C57BL/6 mice i.v. immunized with OVA-$Texo_{/4\text{-}1BBL}$ or OVA-$Texo_{/Null}$ cells (2×10^6 cells/mouse) 6 or 10 days after the immunization were doubly stained with FITC-conjugated anti-CD8 Ab (FITC-CD8) and PE-conjugated H-$2K^b$/$OVA_{257\text{-}264}$ tetramer (PE-Tetramer) or triply stained with FITC-CD8 Ab, PE-tetramer and PE-Cy5-conjugated anti-CD44, CD62L and IL-7R Abs, respectively, and then analyzed by flow cytometry [8-10]. To assess intracellular expression of granzyme B and IFN-γ, spenocytes of immunized mice were stimulated with 2 μM OVAI (SIINFEKL) peptide *in vitro* in presence of 2 μM monensin (GolgiStop) for four hours, and stained with FITC-anti-CD8 antibody. The cells were then fixed, and the cell membranes were permeabilized in Cytofix/Cytoperm solution (BD Biosciences) and stained with PE-anti-granzyme B antibody or PE-anti-IFN-γ antibody for flow cytometric analysis [24]. To assess recall responses, immunized mice were i.v. boosted with DC_{OVA} (1×10^6 cells/mouse) 30 days after immunization. CTL responses were analyzed by flow cytometry. The absolute numbers of OVA-specific tetramer-positive $CD8^+$ T cells in each spleen of immunized mice in primary and recall responses were calculated as we previously described [24].

4.3.5 Cytotoxicity assay

The *in vivo* cytotoxicity assay was performed in immunized mice with transfer of both

OVA-specific $CFSE^{high}$-labeled (H) and the control irrelevant $CFSE^{low}$-labeled (L) target splenocytes at ratio of 1:1 (each 1×10^6 cells) as previously described [13]. Sixteen hrs after cell transfer the residual $CFSE^{high}$ and $CFSE^{low}$ target cells remaining in the recipients' spleens were analyzed by flow cytometry.

4.3.6 RT^2 profiler PCR array analysis

T cells were enriched from splenocytes of C57BL/6 mice immunized with OVA-$Texo_{/4\text{-}1BBL}$ and OVA-$Texo_{/Null}$ vaccines by using nylon wool-column [8-10]. OVA-specific CTLs were then purified from enriched T cell population by using PE-tetramer staining followed by using anti-PE microbeads (Miltenyi Biotec) [25]. A highly purified population of OVA-specific CTLs was obtained by positive selection through passing in 2 separate MACS columns sequentially. The expression of pathway-focused panel of 84 genes related to apoptosis in the above two groups of CTLs was examined using RT^2 Profiler^{TM} PCR Array Mouse Apoptosis (SuperArray Bioscience) [25].

4.3.7 Animal studies

To examine the therapeutic antitumor immunity conferred by Gag-$Texo_{/4\text{-}1BBL}$ vaccine, the wild-type C57BL/6 and transgenic HLA-A2 mice (n=8) were first injected i.v. with 0.5×10^6 BL6-10_{OVA} and BL6-$10_{Gag/A2}$ cells. Six days after tumor cell inoculation, C57BL/6 and HLA-A2 mice were then injected i.v. with OVA-$Texo_{/4\text{-}1BBL}$ and Gag-$Texo_{/4\text{-}1BBL}$ cells (2×10^6 cells/mouse), respectively. OVA-$Texo_{/Null}$ and Gag-$Texo_{/Null}$ cells (2×10^6 cells/mouse) were used as vaccine controls. To assess the long-term antitumor immunity, C57BL/6 mice immunized with OVA-$Texo_{/4\text{-}1BBL}$ vaccine and HLA-A2 mice immunized with Gag-$Texo_{/4\text{-}1BBL}$ vaccine were i.v. challenged with a large dose of BL6-10_{OVA} cells (1×10^6 cells/mouse) and a regular dose of BL6-$10_{Gag/A2}$ cells (0.5×10^6 cells/mouse), respectively, 30 days after immunization. The mice

were sacrificed 3 weeks after tumor cell injection, and the lung metastatic tumor colonies were counted in a blind fashion. 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 [9].

4.3.8 Statistic analyses

Statistical analyses were performed using the Student *t* test or the Mann-Whitney *U* test for comparison of variables from different groups. A value of $p<0.05$ was considered to be statistically significant [10].

4.4 Results

4.4.1 Generation of $AdV_{4\text{-}1BBL}$-infected OVA-Texo ($OVA\text{-}Texo_{/4\text{-}1BBL}$) vaccine expressing transgene 4-1BBL

We first constructed a recombinant 4-1BBL-expressing adenoviral vector $AdV_{4\text{-}1BBL}$ by recombinant DNA technology (**Figure 4.1A**). We generated OVA-Texo vaccine by using ConA-stimulated $CD8^+$ T cells with uptake of OVA-pulsed DC (DC_{OVA})-released EXO (EXO_{OVA}) [11]. We then generated transgene 4-1BBL-engineered and the control OVA-Texo vaccines ($OVA\text{-}Texo_{/4\text{-}1BBL}$ and $OVA\text{-}Texo_{/Null}$) by transfection of OVA-Texo cells with $AdV_{4\text{-}1BBL}$ and the control AdV_{Null}, respectively, followed by phenotypical assessment of $OVA\text{-}Texo_{/4\text{-}1BBL}$ and $OVA\text{-}Texo_{/Null}$ by flow cytometry. We demonstrated that $OVA\text{-}Texo_{/4\text{-}1BBL}$ expressed comparable amount of T cell CD8, CD25, CD28, CD44, CD40L, CD62L, IL-7R, inhibitory PD-1 and exosomal costimulatory CD80, but enhanced amount of costimulatory 4-1BBL compared to the control $OVA\text{-}Texo_{/Null}$ and OVA-Texo (**Figure 4.1B**), indicating that $OVA\text{-}Texo_{/4\text{-}1BBL}$ expresses transgene-encoded cell-surface 4-1BBL.

Fig 4.1

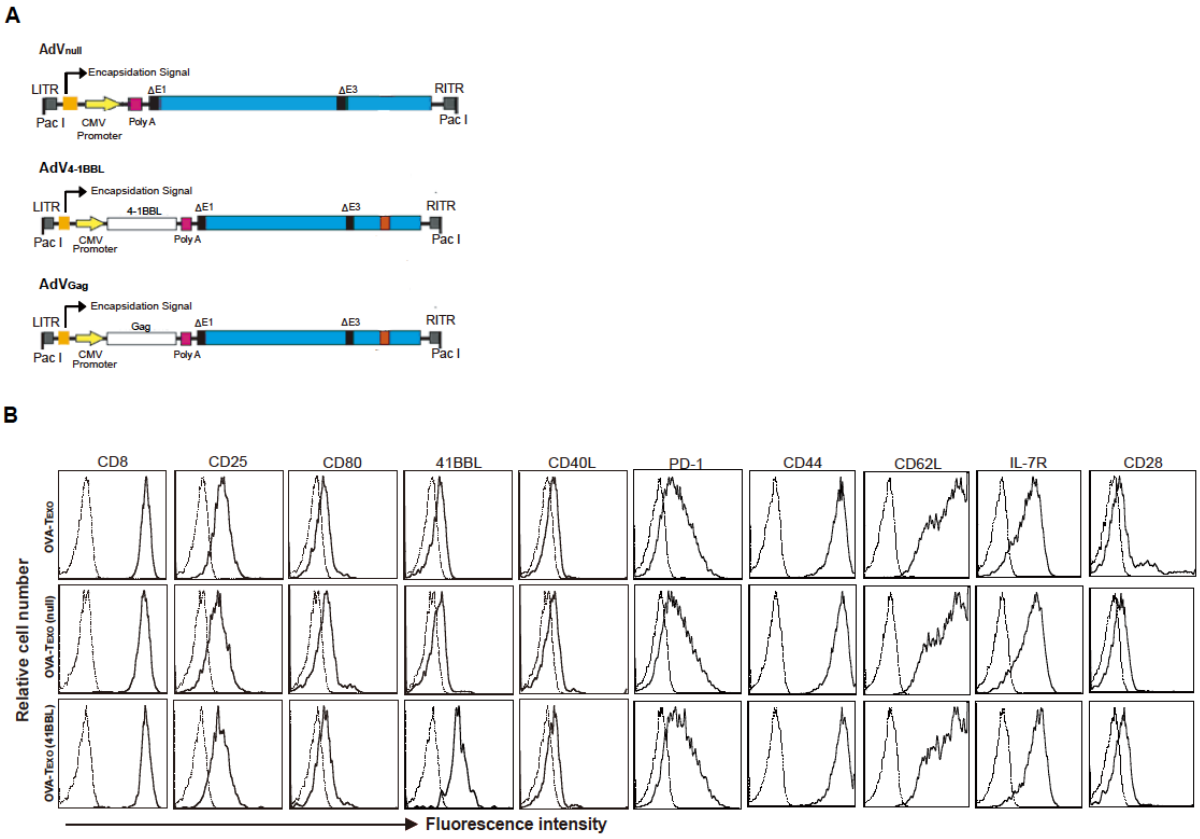

Figure 4.1 Phenotypic analysis of OVA-Texo/$_{4\text{-}1BBL}$.

(A) Schematic representation of adenoviral (AdV) vectors AdVNull, AdVGag and AdV$_{4\text{-}1BBL}$. The E1/E3 depleted replication-deficient AdV is under the regulation of the cytomegalovirus (CMV) early/immediate promoter/enhancer. ITR, inverted terminal repeat. (B) The engineered OVA-Texo/$_{4\text{-}1BBL}$ and the control OVA-Texo/Null and OVA-Texo cells were stained with a panel of Abs (solid lines) or isotype-matched irrelevant Abs (dotted lines), and then analyzed by flow cytometry. One representative experiment of two is shown.

4.4.2 OVA-Texo$_{/4\text{-}1BBL}$ stimulates enhanced OVA-specific CD8$^+$ effector CTL responses in wild-type C57BL/6 mice

To assess whether OVA-Texo$_{/4\text{-}1BBL}$ vaccine stimulates enhanced CTL responses, we immunized wild-type C57BL/6 mice with OVA-Texo$_{/4\text{-}1BBL}$, and then assessed OVA-specific CTL responses using FITC-CD8 and PE-tetramer antibody staining by flow cytometry. We demonstrated that OVA-Texo$_{/4\text{-}1BBL}$ vaccine was able to more efficiently stimulate OVA-specific CTL responses

[3.26%, equivalent to 0.33×10^6 cells per spleen [24]] than OVA-Texo vaccine (1.98%, equivalent to 0.20×10^6 cells per spleen) ($p<0.05$) (**Figure 4.2A**). To assess their phenotypes, the splenocytes of immunized mice were in vitro stimulated with OVAI peptide and $CD8^+$ T cells were examined for their intracellular expression of INF-γ and Granzyme B by flow cytometry. We demonstrated that 2.69% and 1.98% of IFN-γ- and Granzyme B-expressing $CD8^+$ T cells were found in OVA-$Texo_{/4\text{-}1BBL}$-immunized mice (**Figure 4.2B**), which are more than those in either OVA-Texo- or OVA-$Texo_{/Null}$-immunized mice. Next, we assessed the ability of OVA-$Texo_{/4\text{-}1BBL}$ vaccine to induce the differentiation of stimulated $CD8^+$ T cells into effector CTLs. We adoptively transferred $OVA_{257\text{-}264}$ peptide-pulsed splenocytes that had been strongly labeled with CSFE ($CFSE^{high}$), as well as the control peptide Mut1-pulsed splenocytes that had been weakly labeled with CFSE ($CFSE^{low}$), into recipient mice that had been vaccinated with OVA-$Texo_{/4\text{-}1BBL}$ or OVA-$Texo_{/Null}$. Thus, the loss of $CFSE^{high}$ target cells represents the killing activity of CTLs in immunized mice. As expected, there was substantial loss (90%) of the $CFSE^{high}$ (OVA peptide-pulsed) cells in the OVA-$Texo_{/4\text{-}1BBL}$-immunized mice, whereas significantly less cytotoxicity (77%) was induced in mice immunized with the control OVA-$Texo_{/Null}$ vaccine ($p<0.05$) (**Figure 4.2C**), indicating that OVA-$Texo_{/4\text{-}1BBL}$ efficiently stimulates OVA-specific $CD8^+$ T cell differentiation into functional effector CTLs with OVA-specific cytotoxicity.

Fig 4.2

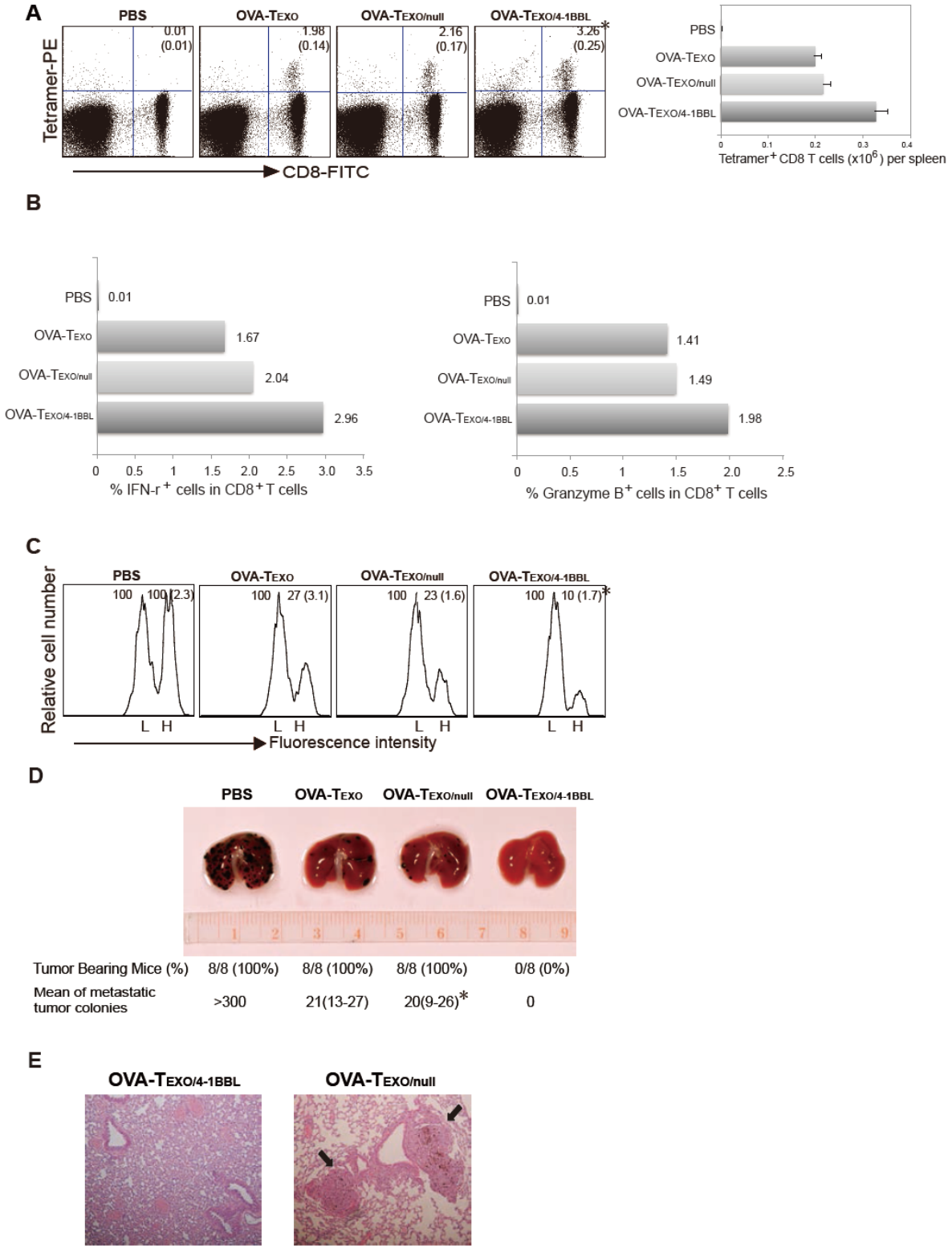
A
PBS
OVA-TEXO
OVA-TEXO/null
OVA-TEXO/4-1BBL
0.01 (0.01)
1.98 (0.14)
2.16 (0.17)
3.26 (0.25)*
Tetramer-PE
CD8-FITC
PBS
OVA-TEXO
OVA-TEXO/null
OVA-TEXO/4-1BBL
Tetramer+ CD8 T cells (x10^6) per spleen
B
PBS 0.01
OVA-TEXO 1.67
OVA-TEXO/null 2.04
OVA-TEXO/4-1BBL 2.96
% IFN-r+ cells in CD8+ T cells
PBS 0.01
OVA-TEXO 1.41
OVA-TEXO/null 1.49
OVA-TEXO/4-1BBL 1.98
% Granzyme B+ cells in CD8+ T cells
C
PBS
OVA-TEXO
OVA-TEXO/null
OVA-TEXO/4-1BBL
100 100 (2.3)
100 27 (3.1)
100 23 (1.6)
100 10 (1.7)*
Relative cell number
L H
Fluorescence intensity
D
PBS
OVA-TEXO
OVA-TEXO/null
OVA-TEXO/4-1BBL
Tumor Bearing Mice (%) 8/8 (100%) 8/8 (100%) 8/8 (100%) 0/8 (0%)
Mean of metastatic tumor colonies >300 21(13-27) 20(9-26)* 0
E
OVA-TEXO/4-1BBL
OVA-TEXO/null

Figure 4.2 OVA-Texo$_{/4\text{-}1BBL}$ stimulates potent OVA-specific effector CTL responses and therapeutic immunity.

(A) C57BL/6 mice (3-4 per group) were immunized with OVA-Texo$_{/4\text{-}1BBL}$, OVA-Texo$_{/Null}$ and OVA-Texo. Six days after the immunization, tail blood samples of the immunized mice were stained with FITC-anti-CD8 Ab and PE-tetramer, and then analyzed by flow cytometry. The value in each panel represents the percentage of OVA-specific $CD8^+$ T cells in the total $CD8^+$ T cell population. The value in parenthesis represents the standard deviation (SD). *p<0.05 versus cohorts of the control OVA-Texo$_{/Null}$ and OVA-Texo groups (Student t test). (B) Intracellular staining. To assess intracellular expression of granzyme B and IFN-γ, spenocytes of immunized mice were stimulated with OVAI peptide *in vitro* in presence of monensin, followed by FITC-anti-CD8 antibody staining. After permeabilization, cells were further stained with PE-anti-granzyme B antibody or PE-anti-IFN-γ antibody. CD8 positive T cells were gated for assessment of granzyme B and IFN-γ expression by flow cytometry. (C) *In vivo* cytotoxicity assay. Six days after the immunization, the immunized mice (3-4 per group) were i.v. injected with a mixture of $CFSE^{high}$- and $CFSE^{low}$-labeled splenocytes (at 1:1 ratio) that had been pulsed with $OVA_{257\text{-}264}$ (SIINFEKL) peptide and the control Mut1 (FEQNTAQP) peptide of an irrelevant 3LL lung carcinoma antigen, respectively. After 16 hrs, the spleens of immunized mice were removed and the percentages of the residual $CFSE^{high}$ (H) and $CFSE^{low}$ (L) target cells remaining in the recipients' spleens were analyzed by flow cytometry. The value in each panel represents the percentage of $CFSE^{high}$ vs $CFSE^{low}$ target cells remaining in the spleen. The value in parenthesis represents the standard deviation (SD). *p<0.05 versus cohorts of the control OVA-Texo$_{/Null}$ and OVA-Texo groups (Student t test). (C) C57BL/6 mice (8 per group) were first i.v. injected with BL6-10_{OVA} tumor cells (0.5×10^6 cells/mouse) followed by immunization of the mice with OVA-Texo$_{/Null}$ and OVA-Texo$_{/4\text{-}1BBL}$ (2×10^6 cells/mouse) for assessment of the therapeutic immunity against 6 day-established tumor. The mice were sacrificed 3 weeks after tumor cell challenge. The numbers of black lung metastatic tumor colonies were counted. *p<0.05 versus cohorts of OVA-Texo$_{/4\text{-}1BBL}$ group (Mann-Whitney U test). (D) H&E staining of lung tissues. 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. Tumor colonies were marked with arrows. Magnification × 150. One representative experiment of two is shown.

4.4.3 OVA-Texo$_{/4\text{-}1BBL}$ vaccine stimulates enhanced therapeutic immunity against OVA-expressing BL6-10_{OVA} melanoma in wild-type C57BL/6 mice

To assess the potential therapeutic effect of OVA-Texo$_{/4\text{-}1BBL}$, we first challenged wild-type C57BL/6 mice with BL6-10_{OVA} tumor cells. Six days after tumor cell challenge, mice bearing 6 day-established tumor were then immunized with OVA-Texo$_{/4\text{-}1BBL}$. We demonstrated that both OVA-Texo$_{/Null}$ and OVA-Texo vaccines were able to significantly reduce average number of

lung tumor metastatic colonies (p<0.05) compared to the control mice with PBS injection, though they failed in curing any mice bearing 6 day-established tumor (**Figure 4.2D**). However, OVA-Texo$_{/4\text{-}1BBL}$ vaccine was able to cure 8/8 mice (**Figure 4.2D**), indicating that OVA-Texo$_{/4\text{-}1BBL}$ vaccine induces an excellent therapeutic immunity against 6-day established lung tumor metastasis in C57BL/6 mice. The presence and absence of lung tumor metastatic colonies in OVA-Texo$_{/Null}$- and OVA-Texo$_{/4\text{-}1BBL}$-immunized mouse lungs were confirmed by histopathologic analysis (**Figure 4.2E**).

4.4.4 OVA-Texo$_{/4\text{-}1BBL}$-stimulated CD8$^+$ T cells are likely CD62L^{high}IL-7R$^+$ memory CTL precursors

To assess the phenotypic characteristics of OVA-Texo$_{/4\text{-}1BBL}$-stimulated CD8$^+$ T cells, we first gated OVA-Texo$_{/4\text{-}1BBL}$- or OVA-Texo$_{/Null}$- or OVA-Texo-stimulated CD8$^+$ T cells for further analysis of the expression of CD44, CD62L and IL-7R 10 days after immunization. We demonstrated that all vaccine-stimulated CD8$^+$ T cells expressed these molecules 10 days after immunization (**Figure 4.3A**). However, OVA-Texo$_{/4\text{-}1BBL}$-stimulated CD8$^+$ T cells showed a higher amount of CD62L and IL-7R expression than OVA-Texo$_{/Null}$- or OVA-Texo-stimulated CD8$^+$ T cells, indicating that they are likely CD62L^{high}IL-7R$^+$ memory CTL precursors [26].

Fig 4.3

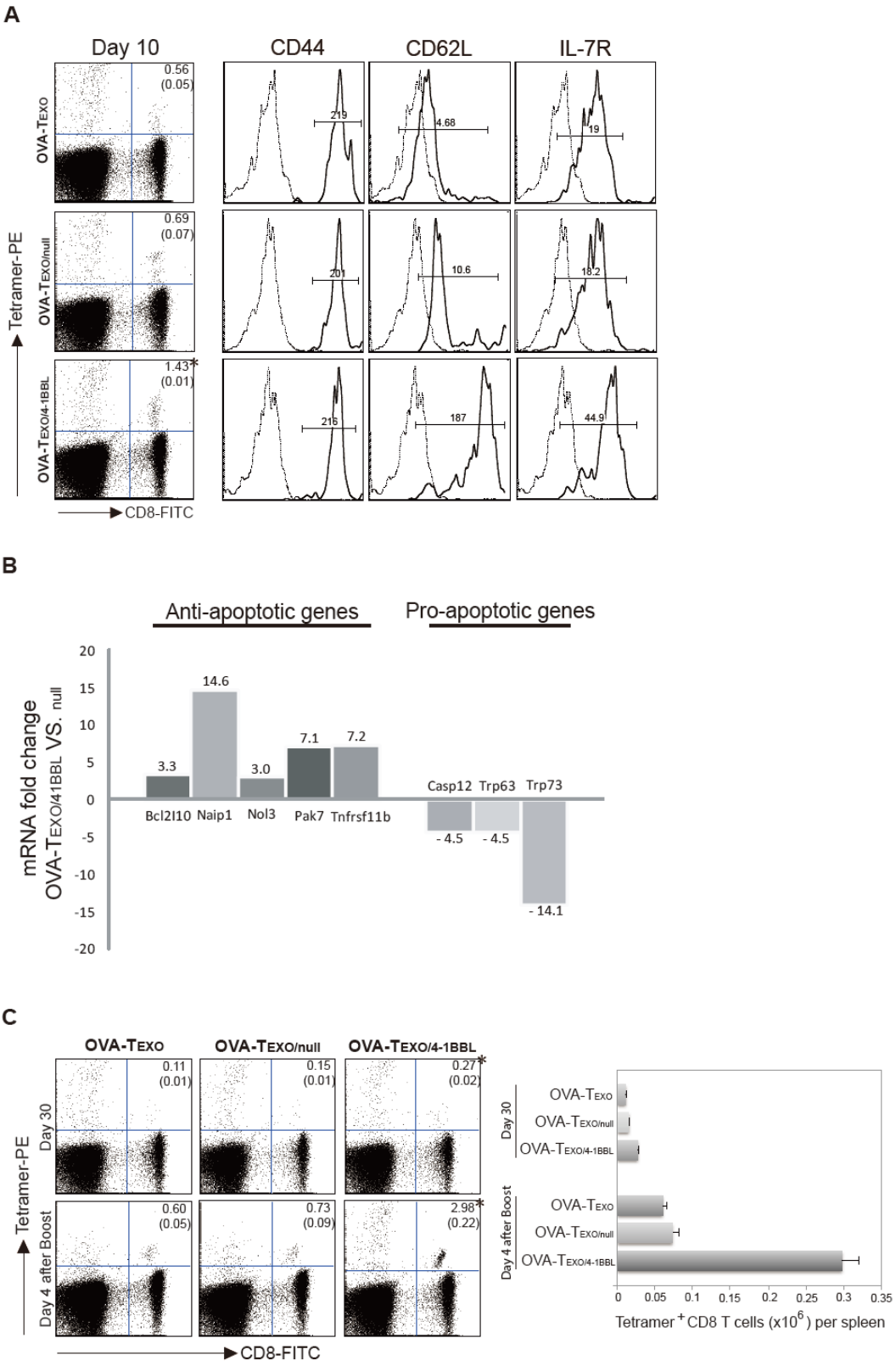
A
Day 10
CD44
CD62L
IL-7R
OVA-TEXO
OVA-TEXO/null
OVA-TEXO/4-1BBL
Tetramer-PE
CD8-FITC
0.56 (0.05)
0.69 (0.07)
1.43* (0.01)
219
4.68
19
201
10.6
18.2
216
187
44.9
B
Anti-apoptotic genes
Pro-apoptotic genes
mRNA fold change OVA-TEXO/41BBL VS. null
Bcl2l10 Naip1 Nol3 Pak7 Tnfrsf11b Casp12 Trp63 Trp73
3.3
14.6
3.0
7.1
7.2
- 4.5
- 4.5
- 14.1
C
OVA-TEXO
OVA-TEXO/null
OVA-TEXO/4-1BBL
Day 30
Day 4 after Boost
0.11 (0.01)
0.15 (0.01)
0.27* (0.02)
0.60 (0.05)
0.73 (0.09)
2.98* (0.22)
Tetramer+CD8 T cells (x10^6) per spleen

Figure 4.3 Characterization of OVA-Texo$_{/4\text{-}1BBL}$-stimulated CTLs.

(**A**) The tail blood samples were harvested from the immunized C57BL/6 mice 10 days after immunization and triply stained with FITC-anti-CD8 and PE-tetramer and PE-Cy5-anti-CD44, CD62L and IL-7R Abs, respectively, for flow cytometric analysis. The value in each panel represents the percentage of OVA-specific $CD8^+$ T cells in the total $CD8^+$ T cell population. The value in parenthesis represents the standard deviation (SD). *p<0.05 versus cohorts of the control OVA-Texo$_{/Null}$ and OVA-Texo groups (Student t test). OVA-specific $CD8^+$tetramer$^+$ CTLs were sorted for assessment of expression of CD44, CD62L and IL-7R by flow cytometry (solid lines). Irrelevant isotype-matched Abs were used as controls (dotted lines). (**B**) Apoptosis real-time PCR (RT-PCR) array were performed to compare apoptosis-related gene expression differences between OVA-Texo$_{/4\text{-}1BBL}$-stimulated and OVA-Texo$_{/Null}$-stimulated CTLs. Total RNA was isolated from purified OVA-specific CTLs using RNeasy extraction kit (Qiagen) and reversely transcribed using RT^2 First Strand kit (SuperArray Bioscience). The mRNA expression of each gene in array system was performed using StepOnePlus thermocycler (Applied Biosystem) and analyzed using *Hprt1*, *Gapdh* and *β-actin* as interal controls in web-based software as per manufacturer's instructions. Only genes with mRNA fold changes of more than 3 are shown. (**C**) Recall responses. The tail blood samples were harvested from the immunized C57BL/6 mice 30 days after immunization or 4 days after the boost, and then doubly stained with FITC-anti-CD8 and PE-tetramer for flow cytometric analysis. The value in each panel represents the percentage of OVA-specific $CD8^+$ T cells in the total $CD8^+$ T cell population. The value in parenthesis represents the standard deviation (SD). *p<0.05 versus cohorts of the control OVA-Texo$_{/Null}$ and OVA-Texo groups on day 30 after immunization or day 4 after the boost (Student t test). One representative experiment of two is shown.

4.4.5 OVA-Texo$_{/4\text{-}1BBL}$-stimulated $CD8^+$ T cells show up- and down-regulation of anti- and pro-apoptosis genes

To assess expression of anti- and pro-apoptosis genes, we extracted RNA from mouse OVA-specific CTLs purified from immunized mouse splenic T cell population 10 days after immunization using RNeasy extraction kit. Apoptosis real-time PCR (RT-PCR) arrays were then performed to compare apoptosis-related gene expression between OVA-Texo$_{/4\text{-}1BBL}$-stimulated and OVA-Texo$_{/Null}$-stimulated CTLs by using their RNA samples and RT^2 Profiler^{TM} PCR Array Mouse Apoptosis. We demonstrated that anti-apoptosis-related genes such as *Bcl2l10* (3.3-fold), *Naip1* (14.6-fold), *Nol3* (3.0-fold), *Pak7* (7.1-fold) and *Tnfrsf11b* (7.2-fold) were found to be up-regulated in OVA-Texo$_{/4\text{-}1BBL}$-stimulated CTLs compared to OVA-Texo$_{/Null}$-stimulated CTLs

(**Figure 4.3B**). In contrast, pro-apoptosis-related genes such as *Casp12* (-4.5-fold), *Trp63* (-4.5-fold) and *Trp73* (-14.1-fold) were found to be down-regulated in OVA-Texo$_{/4\text{-}1BBL}$-stimulated CTLs (**Figure 4.3B**). Our results indicate that compared to OVA-Texo$_{/Null}$-stimulated CTLs, OVA-Texo$_{/4\text{-}1BBL}$-stimulated CTLs up- and down-regulate expression of anti- and pro-apoptosis related genes, respectively.

4.4.6 OVA-Texo$_{/4\text{-}1BBL}$ vaccine stimulates enhanced long-term immunity against OVA-expressing BL6-10$_{OVA}$ melanoma in wild-type C57BL/6 mice

To assess whether OVA-Texo$_{/4\text{-}1BBL}$-stimulated CD8$^+$ T cells differentiate into memory CD8$^+$ T cells, we harvested blood samples from immunized C57BL/6 mice 30 days after the primary immunization, when OVA-Texo$_{/4\text{-}1BBL}$-stimulated effector CD8$^+$ T cells should become memory CD8$^+$ T cells. We then stained the blood samples with FITC-conjugated anti-CD8 Ab and PE-conjugated H-2K^b/OVA$_{257\text{-}264}$ tetramer, and analyzed them by flow cytometry. We demonstrated that CD8$^+$ T cells (0.27%) in OVA-Texo$_{/4\text{-}1BBL}$-vaccinated group became memory CD8$^+$ T cells, which are significantly more than that (0.15%) in OVA-Texo$_{/Null}$-vaccinated group (p<0.05) (**Figure 4.3C**). To assess whether these memory CD8$^+$ T cells are functional, we then i.v. boosted the immunized mice with DC$_{OVA}$ cells 30 days after the primary immunization. Four days after the boost, we assessed OVA-specific CTL responses by flow cytometry. We demonstrated that more recall responses (2.98%, equivalent to 0.30×10^6 cells per spleen) were seen in OVA-Texo$_{/4\text{-}1BBL}$-vaccinated group than OVA-Texo$_{/Null}$-immunized group (0.73%, equivalent to 0.07×10^6 cells per spleen) (p<0.05) (**Figure 4.3C**), indicating that OVA-specific CTLs in OVA-Texo$_{/4\text{-}1BBL}$-vaccinated group become 4-fold more than those in OVA-Texo$_{/Null}$-vaccinated group after the boost stimulation. To assess the potential long-term protective immunity, we also i.v. challenged the immunized mice with BL6-10$_{OVA}$ tumor cells 30 days after the primary immunization, when vaccine-stimulated CTLs should have become memory CTLs. We demonstrated that OVA-Texo$_{/4\text{-}1BBL}$-immunized, but not OVA-Texo$_{/Null}$- or

OVA-Texo-immunized C57BL/6 mice obtained long-term immunity efficiently against challenge with a large dose of BL6-10$_{OVA}$ tumor cells (1×10^6/mouse) in all 8/8 mice (**Figure 4.4**), indicating that OVA-Texo$_{/4\text{-}1BBL}$ vaccine also induces enhanced long-term immunity against OVA-expressing BL6-10$_{OVA}$ tumor in C57BL/6 mice.

Fig 4.4

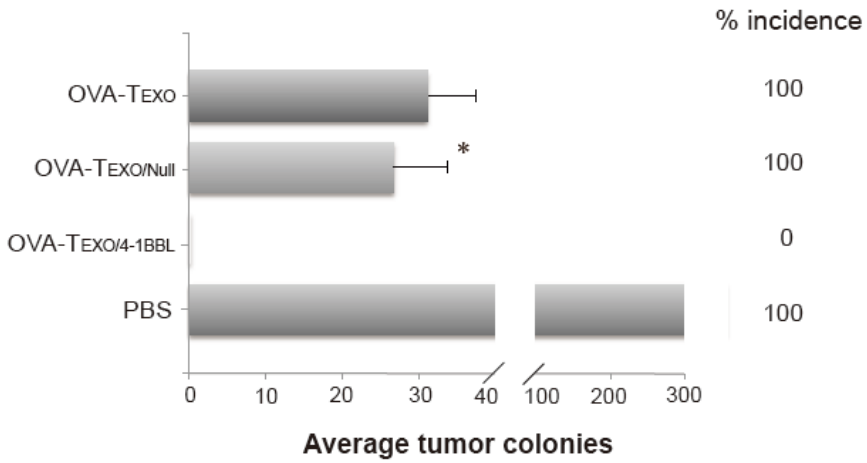

Figure 4.4 OVA-Texo$_{/4\text{-}1BBL}$ vaccine stimulates efficient long-term immunity in C57BL/6 mice.

To assess long-term immunity, C57BL/6 mice (8 per group) were first i.v. immunized with OVA-Texo, OVA-Texo$_{/Null}$ and OVA-Texo$_{/4\text{-}1BBL}$ (2×10^6 cells/mouse), 30 days after vaccination, mice were then i.v. injected with a large dose of OVA-expressing BL6-10$_{OVA}$ tumor cells (1×10^6 cells/mouse). The mice were sacrificed 3 weeks after tumor cell challenge. The numbers of black lung metastatic tumor colonies were counted. *p<0.05 versus cohorts of the control PBS group (Mann-Whitney U test). One representative experiment of two is shown.

4.4.7 Gag-Texo$_{/4\text{-}1BBL}$ vaccine induces enhanced preventive, therapeutic and long-term immunity against Gag/HLA-A2-expressing BL6-10$_{Gag/A2}$ melanoma in transgenic HLA-A2 mice

To assess preventive immunity, we first immunized transgenic HLA-A2 mice with Gag-Texo$_{/4\text{-}1BBL}$ or Gag-Texo$_{/Null}$ followed by challenging the immunized mice with HLA-A2/Gag-expressing BL6-10$_{Gag/A2}$ tumor cells 6 days after immunization. We demonstrated that both Gag-Texo$_{/4\text{-}1BBL}$ and Gag-Texo$_{/Null}$ vaccines were able to protect all 8/8 mice from

tumor challenge (**Figure 4.5A**), indicating that Gag-Texo$_{/4\text{-}1BBL}$ vaccine stimulates potent preventive immunity. To assess the potential therapeutic effect of Gag-Texo$_{/4\text{-}1BBL}$, we first challenged transgenic HLA-A2 mice with BL6-10$_{Gag/A2}$ tumor cells. Six days after tumor cell challenge, mice were then immunized with Gag-Texo$_{/4\text{-}1BBL}$ or Gag-Texo$_{/Null}$. We demonstrated that Gag-Texo$_{/Null}$ vaccine was able to significantly reduce the average number of lung tumor metastatic colonies ($p<0.05$) compared to the control mice with PBS injection, but they failed in curing any mice bearing 6 day-established tumors (**Figure 4.5B**). However, Gag-Texo$_{/4\text{-}1BBL}$ vaccine was still able to cure all 8/8 mice (**Figure 4.5B**), indicating that Gag-Texo$_{/4\text{-}1BBL}$ vaccine stimulates potent therapeutic immunity. Next, we assessed long-term immunity by challenging immunized mice with a regular dose of BL6-10$_{Gag/A2}$ tumor cells (0.5×10^6/mouse) 30 days after immunization. We demonstrated that Gag-Texo$_{/4\text{-}1BBL}$, but not Gag-Texo$_{/Null}$ vaccine was able to protect all 8/8 mice from tumor challenge 30 days after immunization, though Gag-Texo$_{/Null}$ vaccine significantly reduced the average number of lung tumor metastatic colonies ($p<0.05$) compared to the control mice with PBS injection (**Figure 4.5C**), indicating that Gag-Texo$_{/4\text{-}1BBL}$ vaccine also stimulates efficient long-term immunity in HLA-A2 mice.

Fig 4.5

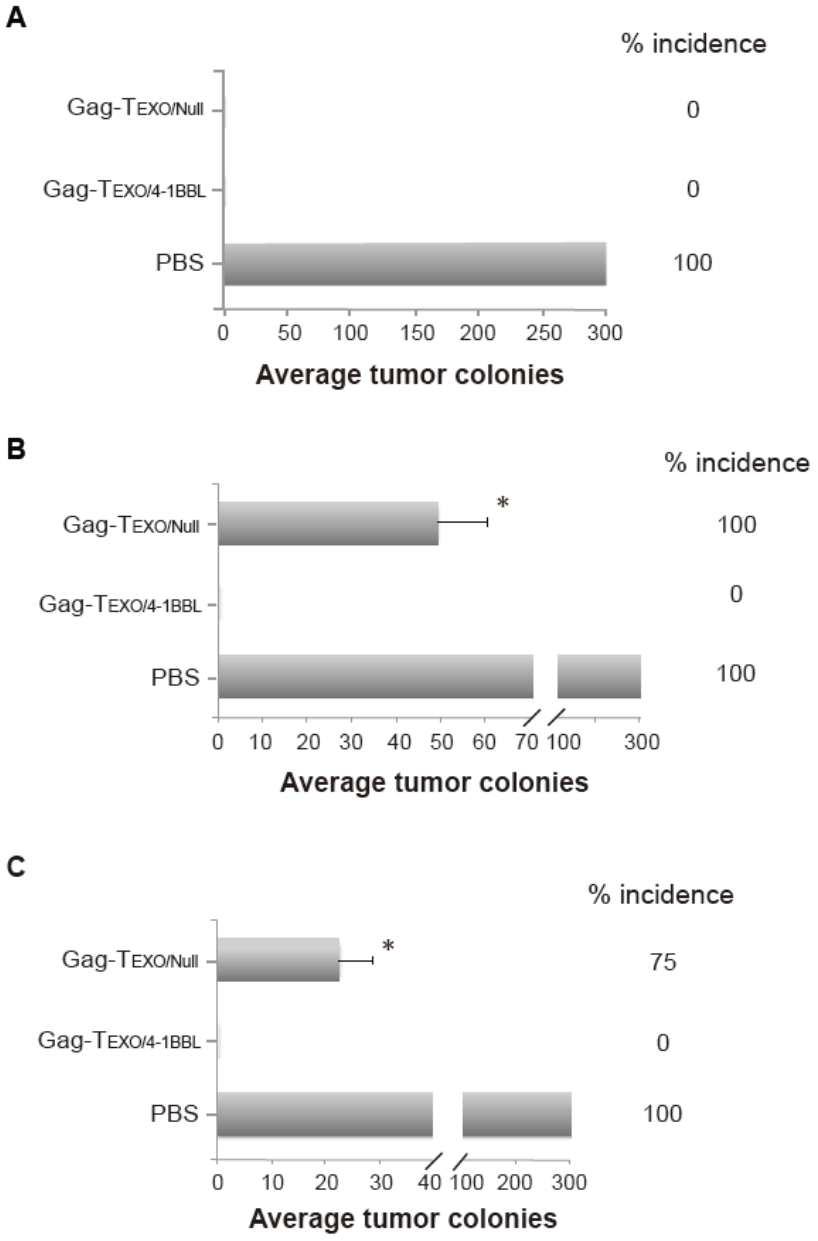

Figure 4.5 Gag-Texo$_{/4\text{-}1BBL}$ vaccine stimulates potent immunity in transgenic HLA-A2 mice.

(A) Transgenic HLA-A2 mice (8 per group) were first i.v. immunized with Gag-Texo/$_{Null}$ and Gag-Texo/$_{4\text{-}1BBL}$ (2×10^6 cells/mouse) followed by i.v. injection of HLA-A2/Gag-expressing BL6-10$_{Gag/A2}$ tumor cells (0.5×10^6 cells/mouse) 6 days after immunization for assessment of the preventive immunity. (B) Transgenic HLA-A2 mice (8 per group) were first i.v. injected with BL6-10$_{Gag/A2}$ tumor cells (0.5×10^6 cells/mouse) followed by immunization of the mice with Gag-Texo/$_{Null}$ and Gag-Texo/$_{4\text{-}1BBL}$ (2×10^6 cells/mouse) for assessment of the therapeutic immunity

against 6 day-established tumor. (C) HLA-A2 mice (8 per group) were first i.v. immunized with Gag-Texo/$_{Null}$ and Gag-Texo/$_{4\text{-}1BBL}$ (2×10^6 cells/mouse) followed by i.v. injection of BL6-10$_{Gag/A2}$ tumor cells (0.5×10^6 cells/mouse) 30 days after immunization for assessment of the long-term immunity. The mice were sacrificed 3 weeks after tumor cell challenge. The numbers of black lung metastatic tumor colonies were counted. *$p<0.05$ versus cohorts of the control PBS group (Mann-Whitney U test). One representative experiment of two is shown.

4.5 Discussion

We previously developed a novel OVA-specific T cell-based vaccine (OVA-Texo) capable of stimulating efficient CTL responses and protective immunity via counteracting regulatory T cell-mediated immune suppression, and inducing long-term antitumor immunity via IL-2/CD80 and CD40L signaling [8-10]. To assess its therapeutic effect, C57BL/6 mice bearing 6 day-established OVA-expressing B16 melanoma BL6-10_{OVA} were immunized with OVA-Texo in this study. We demonstrate that OVA-Texo vaccine is unable to cure any mice bearing 6 day tumor though OVA-Texo vaccination significantly reduces the average number of lung tumor colonies compared to the control PBS. To improve its therapeutic efficacy, we generated an engineered OVA-$Texo_{/4\text{-}1BBL}$ vaccine expressing transgene 4-1BBL. We demonstrate that OVA-$Texo_{/4\text{-}1BBL}$ vaccine stimulates more efficient OVA-specific effector CTL responses leading to potent therapeutic immunity curing 8/8 C57BL/6 mice bearing 6 day-established BL6-10_{OVA} melanoma. In addition, we also demonstrate that OVA-$Texo_{/4\text{-}1BBL}$-stimulated CTLs are more likely the memory CTL precursors with preferential differentiation into $CD44^{+}CD62L^{high}IL\text{-}7R^{+}$ central memory CTLs [26] since OVA-$Texo_{/4\text{-}1BBL}$-stimulated mice demonstrated enhanced recall responses and more efficient long-term immunity against challenge of a large dose of OVA-expressing B16 melanoma BL6-10_{OVA} cells.

Several apoptosis-related molecules that play key roles in regulating apoptosis in T cells have been identified [27, 28], including the Bcl-2 family with either anti-apoptotic or pro-apoptotic effects. It has been reported that 4-1BBL signaling promotes the survival of $CD8^{+}$ T cells via 4-1BB-mediated NF-κB activation leading to up-regulation of *Bcl-$_{XL}$* and *Bfl-1* [17], and inhibits T cell apoptosis via phosphatidylinositol 3-kinase (P13K)-AKT/protein kinase-B (PKB)-mediated up-regulation of *Bcl-$_{XL}$* and *c-$FLIP_{short}$* [29]. Recently, it has been shown that 41BB signaling enhances T cell survival via cooperation of TNF receptor-1 (TNFR1) and leukocyte-specific protein-1 (LSP1) leading to activation of ERK and Bcl-2-interacting mediator

of cell-death (Bim) [30]. In this study, we demonstrated that 4-1BBL signaling up- and down-regulates expression of anti-apoptosis (*Bcl2l10*, *Naip1*, *Nol3*, *Pak7* and *Tnfrsf11b*) and pro-apoptosis (*Casp12*, *Trp63* and *Trp73*) genes, respectively, in $CD8^+$ CTLs with prolonged survival. It has also been reported that 4-1BBL can interact with Toll-like receptors (TLRs) to sustain tumor necrosis factor (TNF) production [31]. Active $CD8^+$ T cells express TLRs, and TLR signaling has been shown to play a role in T cell survival independently of APCs [32-34]. TNF receptor-2 (TNFR2) is also present on active $CD8^+$ T cells and TNF/TNFR2 interaction is important in sustaining their survival [35, 36]. Therefore, it is possible that the transgene 4-1BBL expression on OVA-$Texo_{/4\text{-}1BBL}$ cells interacts with TLRs of $CD8^+$ T cells leading to enhanced TNF production and prolonged survival of activated $CD8^+$ T cells via TLR signaling. Enhanced TNF production may also induce prolonged survival of activated $CD8^+$ T cells via its auto-crime TNF/TNFR2 loop [35, 36].

Gag vaccine has been shown to stimulate persistent and broader HIV-1-specific CTL responses against conserved Gag epitopes in animal models [37-40]. HLA-B57 HIV-1-infected individuals have been found to have autologous CTL responses against four conserved Gag epitopes, leading to reducing virus replication and viral control [41]. In addition, clinically, effective CTL responses against Gag, but not other viral antigens, have been found to correlate with a significant suppression of HIV-1 replication in patients [42-45]. We have recently generated Gag-specific exosome-targeted Gag-Texo vaccine and demonstrated that Gag-Texo could stimulate Gag-specific CTL responses, but only induce some degree of therapeutic immunity against Gag/HLA-A2-expressing B16 melanoma BL6-$10_{Gag/A2}$ in transgenic HLA-A2 mice [13]. To improve its vaccination efficacy, we incorporated 4-1BBL into Gag-Texo vaccine to generate an engineered Gag-$Texo_{/4\text{-}1BBL}$ vaccine expressing transgene 4-1BBL. We demonstrate that Gag-$Texo_{/4\text{-}1BBL}$ vaccine stimulates potent preventive immunity leading to protection of 8/8 transgenic HLA-A2 mice from challenge of BL6-$10_{Gag/A2,}$ and induces potent therapeutic immunity leading to curing 8/8 transgenic HLA-A2 mice bearing 6 day-established BL6-$10_{Gag/A2}$.

In addition, Gag-Texo$_{/4\text{-}1BBL}$ vaccine also induces an enhanced long-term immunity against BL6-10$_{Gag/A2}$ challenge in transgenic HLA-A2 mice. Since HIV-1-specific CTLs are essential for effective immunity against HIV-1 [1, 2], Gag-Texo-stimulated Gag-specific CTLs which showed their killing effects to Gag-expressing tumor cells in HLA-A2 mice in this study should also play important roles in controlling HIV-1 infection by their cytolytic effects to virus-infected T cells or DCs in HIV-1 patients. Assessment of Gag-Texo$_{/4\text{-}1BBL}$ vaccination efficacy in controlling Gag-specific viral infection in wild-type C57BL/6 mice infected by a recombinant replication-competent poxvirus rTTV-luc-gag expressing Gag [46] is underway in our laboratory.

In this study, we also demonstrated that Gag-Texo$_{/4\text{-}1BBL}$ vaccine cells express PD-1 which was originally reported to be a T cell activation antigen [47], and later found to be an inhibitory regulator promoting immune exhaustion [48] and suppressing T cell responses [49, 50]. PD-1 blockade with its antibody could restore T cell's function [51]. It has been reported that the HIV-1 early regulatory protein Tat can broaden T cell responses to HIV-1 envelope proteins [52, 53]. To enhance the therapeutic efficacy of Gag-Texo$_{/4\text{-}1BBL}$ vaccine, the construction and the assessment of Gag-Tat-Texo$_{/4\text{-}1BBL}$ vaccine expressing both late HIV-1 Gag and early HIV-1 Tat proteins, and the examination of Gag-Texo$_{/4\text{-}1BBL}$ vaccine cells with its PD-1 blocked by anti-PD-1 antibody in our transgenic HLA-A2 mouse model are also underway in our laboratory.

Taken together, our novel engineered Gag-Texo$_{/4\text{-}1BBL}$ vaccine capable of stimulating potent therapeutic and long-term immunity may represent a new immunotherapeutic vaccine for controlling HIV-1 infection.

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CHAPTER 5 Novel exosome-targeted T cell-based vaccine counteracts T cell anergy/tolerance and converts CTL exhaustion via CD40L signaling the mTORC1 pathway in chronic infection

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Running Title：Conversion of CTL exhaustion via CD40L signaling the mTORC1 pathway in chronic infection

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Abbreviations used in this article: OVA, ovalbumin; CTL, $CD8^+$ cytotoxic T lymphocytes; HIV-1, human immunodeficient virus type 1; AIDS, acquired immunodeficiency syndrome; LCMV, lymphocytic choriomeningitis virus; rLmOVA, OVA-expressing Listeria monocytogenes; AdV, adenovirus; Ags, antigens; DC, dendritic cells, APC, antigen-presenting cells; Treg, The regulatory T cell; AICD, activation-induced cell death; EXO, exosome; Abs,

antibodies; Tet, Tetramer; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; LAG-3, lymphocyte-activation gene 3; BTLA, B- and T-lymphocyte attenuator; diAcH3, diacetylated histone H3; IFN-γ, interferon gamma; IL-2, Interleukin 2; IL-6, Interleukin 6; TNF-α, tumor necrosis factor alpha; TNFR, tumor necrosis factor receptor; TRAF, TNF receptor-associated factors; Grail, gene related to anergy in lymphocytes; Itch, itchy homologue E3 ubiquitin protein ligase; mTORC1, mammalian target of rapamycin complex 1; eIF4E, eukaryotic translation initiation factor 4E; NFAT, nuclear factor of activated T-cells; PE, phycoerythrin; FITC, fluorenscein isothiocyanate; CFSE, carboxyfluorescein succinimidyl; GM-CSF, Granulocyte-macrophage colony-stimulating factor; HLA-A2, human leukocyte antigen-A2; WT, wild-type; TCR, T cell receptor; Tg, transgenic; KO, gene knockout.

5.1 Abstract

$CD8^+$ cytotoxic T lymphocytes (CTL) are important effector cells providing protection against various viral infections, while T cell exhaustion is probably a chief reason for the ineffective virus elimination in chronic infectious diseases, such as human immunodeficient virus type 1 (HIV-1) infection. We have recently generated novel HIV-1 Gag-specific exosome (EXO)-targeted T cell-based vaccine (Gag-Texo) capable of activation of Gag-specific CTL responses and immunity in transgenic HLA-A2 mice. To assess the potential therapeutic immunity derived from Gag-Texo vaccination in chronic infection, we developed a chronic infection model by injecting of C57BL/6 mice with an ovalbumin (OVA)-expressing recombinant adenoviral vector AdVova. We found that OVA-specific memory CTLs expressing inhibitory PD-1 and LAG-3 are inflated and functionally exhausted (defective in T cell proliferation, IFN-γ expression, cytotoxicity and recall responses, and in tumor suppression) in mice with AdVova-induced chronic infection. In addition, we also demonstrate that during chronic infection, naïve $CD8^+$ T cells increase expression of inhibitory PD-L1 and BTLA molecules, and of T cell anergy-associated genes (Grail and Itch), and reduce *in vitro* T cell proliferation. Remarkably, OVA-specific OVA-Texo vaccine efficiently counteracts T cell anergy/tolerance and converts CTL exhaustion during chronic infection. Its effect on converting memory CTL exhaustion is also synergistic with PD-L1 blockade. Furthermore, our work reveals that CD40L signaling of OVA-Texo vaccine plays a critical role in counteracting T cell anergy/tolerance and CTL exhaustion, and that *in vivo* OVA-Texo-treated CTLs up-regulate mTORC1 pathway-related molecules including Akt, S6, elF4E and T-bet. Importantly, Gag-Texo vaccine is capable of inducing a strong protective and therapeutic immune responses in the course of AdV-induced chronic infection. Therefore, this study is likely to have a serious impact on the development of therapeutic vaccines for treatment of HIV-1 and other chronic infectious diseases.

5.2 Introduction

$CD8^+$ cytotoxic T lymphocytes (CTLs) are important effector cells actively involved in the immune response against various viral infections. After acute viral infection, CTL immunity develops in the three phases of expansion, contraction and memory [1]. In brief, initial encounter with viral antigens (Ags) derived from an infectious agent triggers activation and proliferation of Ag-specific CTLs [2, 3]. CTL responses often peak 7 days after an infection followed by the contraction-phase when majority (>90%) of effector CTLs (eCTLs) die of activation-induced cell death (AICD) [4, 5]. Remaining 5-10% of 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 same viral Ags. In contrast, during chronic viral infections with lymphocytic choriomeningitis virus (LCMV) and human immunodeficiency virus (HIV), Ag-specific $CD8^+$ T cells initially acquire effector function, but gradually become less functional as the infection progresses. This loss of function, known as "exhaustion", is hierarchical, with properties such as proliferative potential and production of IL-2 affected early and IFN-γ later in the course [7, 8]. T cell exhaustion is probably a chief issue leading to the ineffective virus control in these chronic infectious diseases.

Dendritic cells (DCs) are the most potent antigen-presenting cells (APC). They have been used as vaccines to stimulate HIV-specific CTL responses *in vitro* and in animal models [9-11], as well as in clinical trials in acquired immunodeficiency syndrome (AIDS) patients [12-16]. In these trials, the safety profile has been excellent with minor local side-effects found. However, dendritic cell vaccine-induced HIV-specific CTL responses and immunotherapeutic efficacy were relatively poor in AIDS patients and usually were associated with a deficiency in helper $CD4^+$ T cell. Nevertheless, some inhibitory effects on viral titers and disease progression have been found [14-16], warranting a search for more potent 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 [17]. Based upon this concept, we also developed ovalbumin (OVA)-specific DC (DC_{OVA})-released exosome (EXO)-targeted T cell-based (OVA-Texo) vaccine, while demonstrating that non-specific or Ag-specific T cells were able to uptake Ag-specific DC-released EXO *via* CD54/LFA-1 interaction [18, 19]. The OVA-Texo vaccination is of more potent immunogenicity than the DC_{OVA} approach, inducing $CD4^+$ T cell-dependent CTL responses, and capable of directly stimulating potent OVA-specific CTL responses and memory even in the absence of the host $CD4^+$ T cell help, and counteracting $CD4^+25^+FoxP3^+$ regulatory T (Treg) cell suppression [18, 19]. To develop an HIV-specific T cell-based vaccine, we constructed recombinant HIV Gp120-expressing adenoviral vectors (AdV_{Gp120}), and generated a Gp120-specific Gp120-Texo vaccine by using AdV_{Gp120} [20]. We found that Gp120-Texo vaccine stimulated Gp120-specific CTL responses leading to therapeutic immunity in both wild-type (WT) C57BL/6 and transgenic HLA-A2 mice [20, 21]. Since HIV-1 Gag is more suitable [22], we thus developed an HIV Gag-specific EXO-targeted T cell-based (Gag-Texo) vaccine by using AdV_{Gag}, and showed that the Gag-Texo vaccine was capable of activation of Gag-specific CTL responses and immunity [23]. To enhance its immunogenicity, we generated a 4-1BBL-expressing Gag-Texo vaccine, and demonstrated that it assured more efficient Gag-specific CTL responses and therapeutic immunity against Gag-expressing tumor challenges than Gag-Texo vaccine, while also inducing enhanced long-term Gag-specific CTL memory in WT C57BL/6 and transgenic HLA-A2 mice [24].

In this study, we established a mouse AdV-induced chronic infection model by i.v. inoculation of WT C57BL/6 mice with OVA- or beta galactosidase-expressing adenoviruses (AdVova or AdV_{LacZ}) [18, 25, 26]. We found OVA-specific memory CTLs expressing inhibitory programmed cell death protein-1 (PD-1) and lymphocyte-activation gene 3 (LAG3) molecules were inflated and became functionally exhausted, while naïve $CD8^+$ T cells expressed inhibitory

programmed death-ligand 1 (PD-L1) and B- and T-lymphocyte attenuator (BTLA) molecules [27] and became anergic in mice with the AdVova-induced chronic infection. Interestingly, our work also revealed that the OVA-Texo vaccine stimulated potent CTL responses and immunity in the AdV_{LacZ}- and AdVova-induced chronic infection models by counteracting naïve $CD8^+$ T cell anergy/tolerance and converting CTL exhaustion *via* the CD40L-activated mammalian target of rapamycin complex-1 (mTORC1) pathway. To examine whether the Gag-Texo vaccine is capable of effectively stimulating Gag-specific CTL immunity in chronic infection, we performed animal studies by immunizing C57BL/6 mice in the AdVova-induced chronic infection model with the HIV-1-specific Gag-Texo vaccine, and this vaccination induced therapeutic immunity against Gag-expressing tumors.

5.3 Materials and Methods

5.3.1 Reagents, cell lines and animals

Ovalbumin (OVA) was obtained from Sigma (St. Louis, MO). OVAI ($OVA_{257-264}$, SIINFEKL) peptide specific for H-$2K^b$ and Mut1 (FEQNTAQP) peptide specific for H-$2K^b$ of an irrelevant 3LL lung carcinoma were synthesized by Multiple Peptide Systems (San Diego, CA). Biotin-labeled antibodies (Abs) specific for CD44 (IM7) and CD40 (3/23) and Phycoerythrin (PE)-IFN-γ (XMG1.2) and PE-CD45RA (14.8) Abs were obtained from BD Biosciences (Mississauga, ON, Canada). Biotin anti-CD62L (MEL-14), IL-7R (SB/199), lymphocyte-activation gene 3 (LAG-3) (C9B7W) Abs, purified anti-Histone H3 (Poly6019), T-bet (Poly6235) Abs, phycoerythrin/Cy5 (PE/Cy5)-conjugated streptavidin were obtained from Biolegend (San Diego, CA, USA). PE/Cy5-CD8 (53-6.7), biotin-programmed cell death protein 1 (PD-1) (J43), programmed death-ligand 1 (PD-L1) (1-111A), B- and T-lymphocyte attenuator (BTLA) (8F4), and Ki67 (SolA15) Abs were obtained from eBioscience (San Diego, CA). Purified anti-pAkt 1/2/3 (Ser 473) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). Purified anti-phospho-eukaryotic translation initiation factor 4E (eIF4E) (Ser209), and anti-phospho-S6 ribosomal protein (Ser235/236) (D57.2.2E) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). PE-labeled H-$2K^b$/$OVA_{257-264}$ tetramer (YTS 169.4) was obtained from Beckman Coulter (Miami, FL). Fluorenscein isothiocyanate (FITC)-labeled CD8 (53-6.7) was obtained from Caltag (Burlingame, CA). FITC-conjugated AffiniPure goat anti-rabbit IgG (H+L) was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Carboxyfluorescein succinimidyl (CFSE) was obtained from Molecular Probes (Eugene, OR). The depleting anti-CD4 antibody was purified from ascites of hybridoma cell lines GK1.5. Rat anti-mouse PD-L1 (10F.9G2) was obtained from BioXCell Inc (West Lebanon, NH) [28]. Adenoviral vector AdVova, $AdV_{Lac\ Z,}$ and AdV_{Gag} expressing OVA, β-galactosidase, HIV-1 Gag respectively were previously constructed in our laboratory [23][24,

26]. Recombinant *Listeria mono-cytogenes* rLmOVA [29] expressing OVA was obtained from Dr. Hao Shen, University of Pennsylvania, Philadelphia, PA. The highly lung metastatic OVA-expression BL6-10_{OVA} and Gag-expressing BL6-10_{Gag} tumor cell lines were generated in our lab [17, 26]. Female WT C57BL/6 (B6), OVA-specific T cell receptor (TCR)-transgenic (Tg) OTI or OTII mice and various gene knockout (KO) mice were obtained from the Jackson Laboratory (Bar Harbor, MA). All mice were treated according to animal care committee guidelines of the University of Saskatchewan.

5.3.2 Dendritic cell and exosome preparations

Bone marrow-derived dendritic cells (DCs) were obtained by culturing bone marrow cells of WT B6 in culture medium containing GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) for six days as previously described [19]. DCs were pulsed with OVA (0.5 mg/ml) for overnight or infected with AdV_{Gag} and termed DC_{OVA} or DC_{Gag}, respectively. DC_{OVA} or DC_{Gag}-released exosomes (EXO_{OVA} or EXO_{Gag}) were then purified from DC culture supernatants by differential ultracentrifugation [19].

5.3.3 T cell preparation

Naïve or memory $CD8^+$ T cells were isolated from naïve or AdV_{LacZ}-infected WT B6 and OVA-specific TCR transgenic OT I mouse spleens, enriched by passage through nylon wool columns (C&A Scientific, Manassas, VA), and then purified by negative selection using anti-mouse CD4 paramagnetic beads (DYNAL Inc, Lake Success, NY). To generate active $CD8^+$ T cells, the spleen cells from naïve C57BL/6 mice were cultured in RPMI1640 medium containing IL-2 (20 U/ml) and ConA (1 μg/ml) for 3 days [30]. $CD8^+$ T cells were then purified from ConA-activated T cells using MACS anti-CD8 microbeads (Miltenyi Biotech GmBH, Germany) to yield T cell populations that were >98% $CD8^+$ T (ConA-T) cells [17].

ConA-T cells derived from IL-2, IL-6, TNF-α and CD40L KO mice were termed (IL-$2^{-/-}$), (IL-$6^{-/-}$), (TNF-$\alpha^{-/-}$) and (CD40$L^{-/-}$) ConA T cells, respectively.

5.3.4 Preparation of OVA-Texo and Gag-Texo vaccines

OVA- and Gag-specific OVA-Texo and Gag-Texo vaccines were generated by incubation of $CD8^+$ ConA-T cells with EXO_{OVA} and EXO_{Gag} [23]. OVA-Texo and Gag-Texo cells were then transfected with $AdV_{4\text{-}1BBL}$ as previously described [24]. OVA-Texo derived from (IL-$2^{-/-}$), (IL-$6^{-/-}$), (TNF-$\alpha^{-/-}$) and (CD40$L^{-/-}$)ConA-T cells were termed OVA-Texo(IL-$2^{-/-}$), OVA-Texo(IL-$6^{-/-}$), OVA-Texo(TNF-$\alpha^{-/-}$) and OVA-Texo(CD40$L^{-/-}$), respectively.

5.3.5 Chronic and acute infection animal model

B6 mice and OTI mice were i.v. injected with AdVova (2.5×10^6 pfu) or AdV_{LacZ} (2.5×10^6 pfu) for over 60 days to establish an chronic infection. B6 mice were i.p. injected with anti-CD4 Ab (GK1.5; 400 μg) to deplete endogenous $CD4^+$ T cells one day before adenoviral infection. For acute infection, B6 mice were i.v. injected with rLmOVA (2,000 cfu). To generate long-lasting $CD8^+$ memory T cells during the acute infection, naive $CD8^+$ and $CD4^+$ T (0.25×10^6) cells derived from OT I and OTII mice were i.v. injected into B6 mice, one day after transfer, mice were i.v. immunized with rLmOVA (2,000 cfu) [29]. Seven or nine, fourteen, thirty and sixty days after immunization, mice tail blood was harvested for detecting OVA-specific $CD8^+$ T cells by flow cytometry.

5.3.6 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 the control irrelevant Mut1-pulsed CFSE (0.6 μM $CFSE^{low}$)-labeled (L) target splenocytes at ratio of 1:1 (each 4×10^6 cells) as previously described [17]. Sixteen hrs after cell transfer, the residual $CFSE^{high}$ (H) and $CFSE^{low}$ (L) target

cells remaining in the recipients' spleens were analyzed by flow cytometry.

5.3.7 T cell proliferation assays

In *in vitro* T-cell proliferation assay, the purified $CD8^+$ T cells from naïve and chronically AdV_{LacZ}-infected B6 mice were labeled with CFSE (3 mM). CFSE-labeled $CD8^+$ T cells were then incubated with CD3/CD28-microbeads in presence of IL-2 (40 U/mL) and beta-mercaptoethanol (2-ME; 50 μM). Three days after incubation, active $CD8^+$ T cells were harvested, and analyzed for determination of the number of CFSE-labeled T cell divisions by flow cytometry. In *in vivo* T-cell proliferation assay, naïve $CD8^+$ T cells were purified from naïve OTI mice or OTI mice chronically infected with AdV_{LacZ}, labeled with CFSE (4 mM), and then adoptively transferred (1×10^6/mouse) into naïve B6 recipients. DCova or OVA-Texo vaccines were injected on the following day to active OTI-$CD8^+$ T cells in vivo. Seventy two hours later, splenocytes were collected and analyzed by flow cytometry.

5.3.8 RNA array analysis

Naïve $CD8^+$ T cells from naïve or chronically infected WT B6 mice spleens were purified by using EasySep™ Mouse $CD8^+$ T Cell Isolation Kit (STEMCELL Technologies Inc, Vancouver, BC, Canada), followed by Biotin-CD45RA (BD Biosciences, Mississauga, ON, Canada) and MACS anti-biotin microbeads (Miltenyi Biotech GmBH, Germany). RNA isolation was performed using RNeasy Plus Mini Kit (QIAGEN Inc, Toronto, ON, Canada), cDNA was synthesized immediately using RT² First Strand Kit (QIAGEN Inc.). Real-time PCR was carried out with RT² SYBR Green qPCR Mastermix (QIAGEN Inc.) using a StepOnePlus Real-time PCR system (Applied Biosystems, Burlington, ON, CANADA). A panel of primers specific for T-cell anergy-associated genes were synthesized [31]. Expression of each gene was normalized to β-actin. The primers were as follows: β-actin: F-GTGAC GTTGA CATCC GTAAA GA; R-GCCGG ACTCA TCGTA CTCC; Grail: F-GCGCA GTCAG CAAAT GAA; R-TGTCA ACATG GGGAA CAACA; Ikaros: F-GCTGG CTCTC GGAGG AG; R-CGCAC TTGTA

CACCT TCAGC; Casp3: F-ACGCG CACAA GCTAG AATTT; R-CTTTG CGTGG AAAGT GGAGT; EGR2: F-TCAGT GGTTT TATGC ACCAG C; R-GAAGC TACTC GGATA CGGGA G; Grg4 F-TCACT CAAGT TTGCC CACTG; R-CACAG CTAAG CACCG ATGAG; Itch: F-GTGTG GAGTC ACCAG ACCCT; R-GCTTC TACTT GCAGC CCATC. The fold induction represents the ratio of mRNA expression in $CD8^+$ T cells from chronically infected mice spleens to that in naive mice spleens.

5.3.9 Conversion of CTL exhaustion by PD-L1 blockade and OVA-Texo vaccine

To assess the conversion of memory CTL (mCTL) exhaustion by OVA-Texo vaccine, chronically AdVova-infected WT B6 mice with CTL exhaustion were given i.v. OVA-Texo vaccine (2×10^6 cells/mouse) or OVA-Texo vaccines with defect of various molecules such as IL-2, IL-6, TNF-α and CD40L. Four days after vaccination, OVA-specific $CD8^+$ T cell proliferation was assessed at day 4 post vaccination by cytometry. For *in vivo* PD-L1 blockade, chronically AdVova-infected WT B6 mice with CTL exhaustion were given two hundred micrograms of rat anti-mouse PD-L1 (10F.9G2) or rat IgG2b isotype control (BioXCell) every third day intraperitoneally, five times in total [32]. OVA-specific $CD8^+$ T cell proliferation was analyzed on the following day after the last treatment by flow cytometry. To assess the synergistic effect on conversion of CTL exhaustion, OVA-Texo (2×10^6 cells/mouse) was given intravenously on day 14 (one day post the last PD-L1 blockade treatment), and OVA-specific $CD8^+$ T cell proliferation was assessed at day 4 post vaccination by flow cytometry.

5.3.10 Flow cytometric analysis

For OVA-specific $CD8^+$ T cell response kinetic study, periphery blood samples of B6 mice i.v. infected with AdVova (1×10^8 pfu/mouse) or rLmOVA (2,000 cfu/mouse) were collected at different time points. Mouse blood samples were then stained with FITC-anti-CD8 antibody

(FITC-CD8) and PE-H-2K^b/OVA$_{257-264}$ tetramer (PE-tetramer), and then analyzed by flow cytometry. To phenotypically analyze mCTLs or naïve CD8$^+$ T cells, mouse blood samples of B6 mice i.v. infected with AdVova (1×10^8 pfu/mouse) or rLmOVA (2,000 cfu/mouse) were triply stained with FITC-CD8, PE-tetramer or PE-CD45RA and biotin-CD44, CD62L, IL-7R, PD-1, PD-L1, LAG-3, CD40, and BTLA, respectively, followed by staining of PE/Cy5-streptavidin, and then analyzed by flow cytometry. To assess CTL recall responses, mouse splenic T cell populations containing mCTLs from B6 mice with rLmOVA-induced acute infection or AdVova-induced chronic infection were adoptively transferred into naïve B6 mice, and then the mice 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. To assess OVA-specific CD8$^+$ T cell responses, B6 mice were i.v. infected chronically with AdV$_{LacZ}$ (2.5×10^6 pfu/mouse), or AdVova (2.5×10^6 pfu/mouse) were i.v. immunized with DCova, OVA-Texo and OVA-Texo cells (2×10^6 cells/mouse) derived from various gene KO mice, respectively. Mouse blood samples or cell suspensions prepared from mouse spleens and lungs were then stained with FITC-CD8 and PE-tetramer, and then analyzed by flow cytometry [18, 19, 33]. The chronically AdV$_{LacZ}$-infected B6 mice were first i.v. immunized with DCova or OVA-Texo (2×10^6 cells/mouse), and the vaccinated mice were then i.v. boosted with rLmOVA (1,000 cfu) 30 days after immunization. To assess conversion of CTL exhaustion, chronically AdVova-infected B6 mice were i.v. vaccinated with OVA-Texo (2×10^6 cells/mouse). Four days after the immunization, mouse splenocyte samples were intracellularly stained with PE/Cy5-CD8, PE-tetramer and polyclonal rabbit anti-pAKT, pelF4E, pS6, NFAT, T-bet, Histone H3 Abs, and biotin-Ki67, respectively, followed by FITC-anti-rabbit IgG and FITC-streptavidin, and then analyzed by flow cytometry. To analyze OVA-specific CD8$^+$ T cell proliferation inanti-PD-L1 Ab or rat IgG2b isotype control-treated, OVA-Texo-vaccinated B6 mice with AdVova chronic infection, mouse blood samples were stained with FITC-CD8 and PE-tetramer, and then analyzed by flow cytometry. For intracellular staining, the splenocytes samples were collected, re-stimulated with 2 μM OVAI peptide and subjected to intracellular staining (BD Biosciences)

of IFN-γ, as described previously [34].

5.3.11 Animal studies

To assess functional effect of mCTLs in chronic infection, splenocytes from chronically AdVova-infected mice or rLmova-infected mice 60 days after primary infection were transferred into naive B6, the recipient mice (n=4) were injected with 0.5×10^6 BL6-10_{OVA} cells on the following day. The mice were sacrificed 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 Gag-Texo vaccine, B6 mice (n=8) 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 Gag-Texo vaccine, B6 mice (n=8) with chronic infection were first i.v. injected with 0.5×10^6 BL6-10_{Gag} cells. Six days after tumor cell inoculation, B6 mice were then i.v. injected with Gag-Texo or DC_{Gag} cells (2×10^6 cells/mouse). The mice were sacrificed 3 weeks after tumor cell challenge, and the lung metastatic tumor colonies were counted in a blind fashion. Metastases on freshly isolated lungs appeared as discrete black-pigmented foci 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 [19].

5.3.12 Statistic analyses

Unless stated otherwise, data are expressed as mean (with SD). Statistical analyses were performed using the Student *t* test or the Mann-Whitney *U* test for comparison of variables from different groups. Probability values of $p > 0.05$ and $p < 0.01$ are considered statistically not significant and very significant, respectively [33].

5.4 Results

5.4.1 OVA-expressing adenoviral vector induces mouse chronic infection:

Distinctive $CD8^+$ T cell responses ranging from immunogenic CTL responses to CTL exhaustion or depletion were reported to be elicited post vaccination with adenoviral vectors (AdVs) expressing different antigens and after immunization with AdVs at various doses or through different routes [35-41]. For example, mice i.v. injected with β-galactosidase-expressing AdV_{LacZ} induced mouse chronic infection with CTL exhaustion and deletion [36]. To assess whether a replication-deficient transgene OVA-expressing AdVova [26] also induces mouse chronic infection, C57BL/6 (B6) mice were i.v. injected with AdVova. Our further analysis demonstrated that 60 days later, AdVova injection resulted in a dose-dependent OVA-specific memory $CD8^+$ T cell inflation (**Figure 5.1A** and **sFig 5.1**). Inflated memory CTLs down-regulated their cell surface markers for T cell memory, CD62L and IL-7R, compared to memory CTLs developed in rLmOVA-immunized mice (**Fig 5.1B**). Interestingly, we found that inflated CTLs expressed CD40. Importantly, we also demonstrated that inflated CTLs up-regulated inhibitory molecules, such as PD-1, PD-L1 and LAG-3 (**Fig 5.1B**), indicating that these CTLs may be exhausted. To assess the functional traits (capacity for Ag-specific cell killing and CTL recall responses) of these potentially "exhausted" CTLs, we performed flow cytometric analyses to measure cellular IFN-γ expression, cytolytic effectiveness and CTL recall responses following antigen boost, and performed animal studies to analyze protective immunity against OVA-expressing tumor ($BL6\text{-}10_{OVA}$) challenge. We found that only 17% of these CTLs possessed intracellular IFN-γ in comparison to nearly 75% of of IFN-γ-positive CTLs observed in rLmOVA-immunized control (**Fig 5.1C**). This observation clearly indicates that CTLs in chronic infection display a significant reduction in the expression of an effector cytokine, IFN-γ. In addition, these CTLs also showed defects in the cytolytic response against OVA-specific highly CFSE-labeled (H) target cells (**Fig 5.1D**), in CTL recall responses upon DC_{OVA} boost (**Fig 5.1E**), and in the

immunity against OVA-expressing BL6-10_{OVA} tumors (**Fig 5.1F**), indicating that the OVA-specific inflated CTLs are functionally exhausted in AdVova-induced chronic infection model. Taken together, our observations implicate that OVA-expressing AdVova-infected mice represent a new chronic infection mouse model with memory CTL inflation and exhaustion.

Fig 5.1

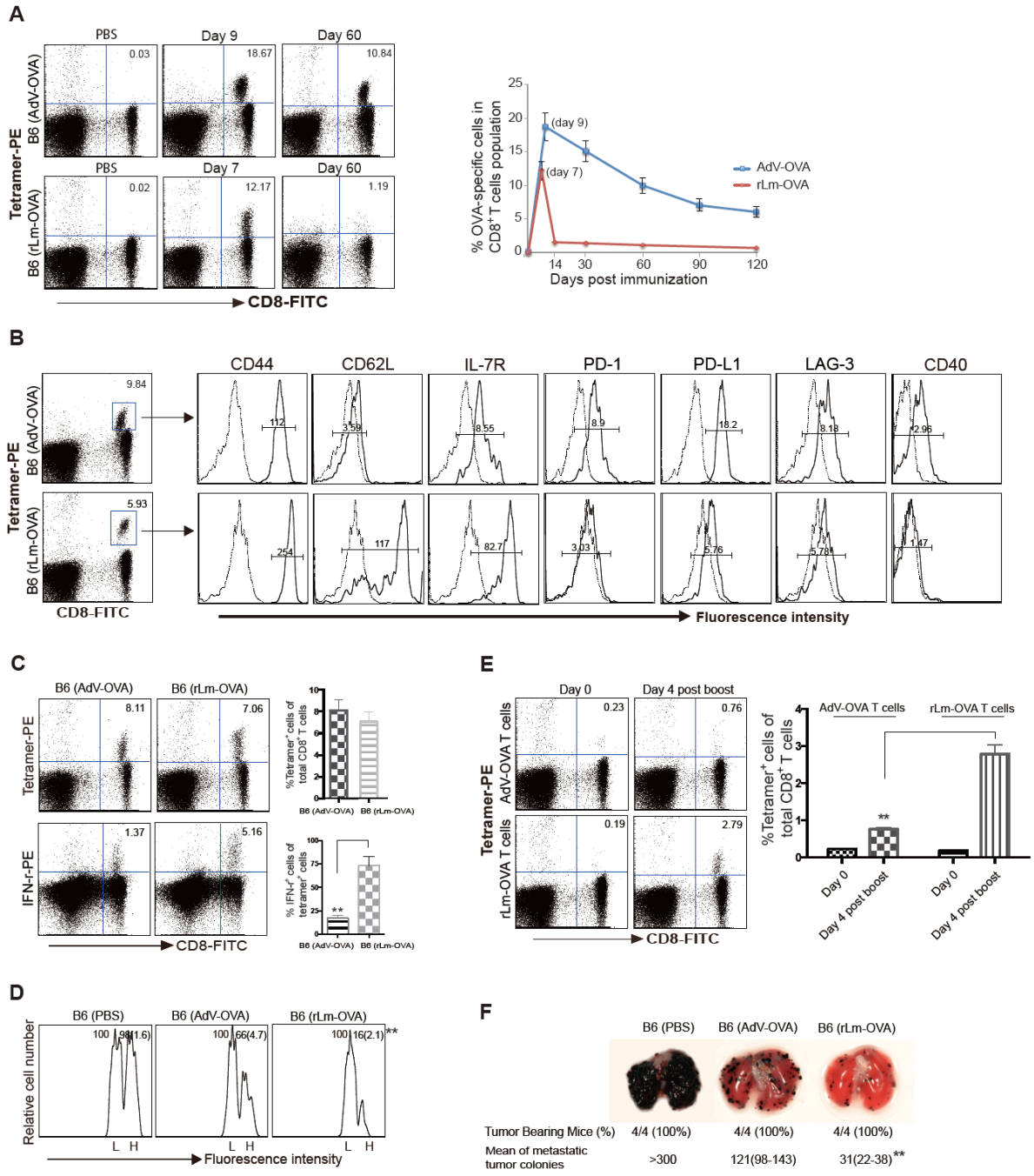

Figure 5.1 Figure 1. AdVova induces chronic infection in mice.

(A) Periphery blood samples harvested at peak days (Day 9 and Day 7) and up to Day 120 after i.v. injection of C57BL/6 mice with AdVova (2.5×10^6 pfu) or rLmOVA (2,000 cfu) were stained with the PE-H-$2K^b$/OVAI peptide

tetramer (PE-tetramer) and FITC-anti-CD8 Ab (FITC-CD8), and analyzed by flow cytometry. The value in each panel represents the percentage of OVA-specific (tetramer-positive) $CD8^+$ T cells vs total $CD8^+$ T cell population. Sixty days after the infection, mouse periphery blood samples from each group were stained with the PE-tetramer, FITC-CD8, and PE-Cy5-labeled Abs, and analyzed by flow cytometry. (B, C) Assessment of phenotype and intracellular IFN-γ of CTLs in chronic infection. OVA-specific $CD8^+$ T cells with positive PE-tetramer and FITC-CD8 staining (B) were gated, and assessed for expression of the indicated molecules (solid lines). Dotted lines represent isotype-matched controls. In (C), cells were permeabilized for assessment of intracellular IFN-γ by flow cytometry. The value in each panel represents the percentage of $CD8^+$ T cells producing IFN-γ. (D) *In vivo* cytotoxicity assay. Sixty days after the infection, mice were i.v. injected with a mixture of $CFSE^{high}$ and $CFSE^{low}$ -labeled splenocytes (at 1:1 ratio) that had been pulsed with OVAI or irrelevant Mut1 peptide, respectively. After 16 hrs, the residual $CFSE^{high}$ (H) and $CFSE^{low}$ (L) target cells remaining in the recipients' spleens were analyzed by flow cytometry. The value in each panel represents the percentage of $CFSE^{high}$ vs $CFSE^{low}$ target cells remaining in the spleen. (E) Sixty days after the infection, splenocytes of AdVova- or rLmOVA-infected mice were adoptively transferred into naïve C57BL/6 mice, and 1 day after the transfer, the mice were boosted with DCova. Four days after the boost, their periphery blood samples were analyzed for OVA-specific memory CTL recall responses by flow cytometry. The value in each panel represents the percentage of PE-tetramer-positive $CD8^+$ T cells vs total peripheral $CD8^+$ T cells. (F) One day after the transfer, the mice were i.v. injected with BL6-10_{OVA} tumor cells, and were euthanized 3 weeks later. The numbers of lung metastatic tumor colonies were counted. **$P < 0.01$. Error bars represent standard deviation (SD). One representative experiment of two to three is shown.

5.4.2 $CD8^+$ T cell anergy/tolerance in chronic infection:

The bystander chronic infection associated with some level of immune tolerance often enhances susceptibility of chronically infected hosts to a variety of co-infections [42]. To assess a potential immune tolerance in our chronic infection model, we assessed OVA-specific CTL responses derived from vaccination of DCova and OVA-Texo, respectively, in WT B6 and chronically AdV_{LacZ}-infected B6 mice. This analysis demonstrated that DCova stimulated significantly less OVA-specific CTL and memory CTL recall responses in mice with chronic infection, compared to WT B6 mice (**Fig 5.2A**), indicating that some immune tolerance occurs in the presence of the AdV_{LacZ}-induced chronic infection. In contrast, only slightly decreased CTL and memory CTL recall responses were found in OVA-Texo-immunized mice with chronic infection (**Fig 5.2A**), indicating that OVA-Texo vaccine can counteract T cell tolerance caused by chronic infection.

To assess whether the immune tolerance involves $CD8^+$ T cells, we performed an *in vitro* T cell proliferation assay. In the assay, naïve $CD8^+$ T cells purified from chronically infected-B6 mice or WT B6 mice were labeled with CFSE, and were activated with anti-CD3/CD28 beads, and IL-2 *in vitro*. Three days later, these T cells were analyzed by flow cytometry. We demonstrated that naïve $CD8^+$ T cells derived from chronically AdV_{LacZ}-infected B6 mice underwent less cell divisions (**Fig 5.2B**, panel c) than those from WT B6 mice (**Fig 5.2B,** panel b), indicating that naïve $CD8^+$ T cells in chronic infection are tolerogenic. In addition, we also performed an *in vivo* T cell proliferation assay. In the assay, naïve $CD8^+$ T cells purified from OTI mice or AdV_{LacZ}-infected OTI mice were labeled with CFSE and then, transferred into WT B6 mice. One day after the cell transfer, the mice were immunized with DCova, and mouse splenocytes were analyzed by flow cytometry. We demonstrated that CFSE-labeled OTI $CD8^+$ T cells derived from AdV_{LacZ}-infected OTI mice passed through less cell divisions (**Fig 5.2C**, panel e) than those derived from uninfected OTI mice (**Fig 5.2C,** panel b), thus confirming that naïve $CD8^+$ T cells with chronic infection background are indeed tolerogenic. It has been shown that naïve splenocytes representing B and T cells and DCs up-regulated inhibitory PD-L1 in the course of chronic infection [32]. To assess the phenotype of the tolerogenic $CD8^+$ T cells, we performed flow cytometric analysis. Interestingly, we found that naïve $CD8^+$ T cells in AdV-induced chronic infection up-regulated inhibitory PD-L1 and BTLA, compared to cells derived from rLmOVA-infected mice (**Fig 5.2D**), suggesting that the inhibitory PD-L1 and BTLA may be responsible for $CD8^+$ T cell tolerance. In addition, we also performed RT-PCR analysis, and showed that naïve $CD8^+$ T cells in AdV-induced chronic infection model up-regulated T cell anergy-associated genes Grail and Itch (**Fig 5.2E**), implicating that naive $CD8^+$ T cell tolerance in chronic infection is associated with T cell anergy. Interestingly, OVA-Texo vaccine induced proliferation of $CD8^+$ T cells transferred from AdV_{LacZ}-infected OTI mice at a level similar to proliferation of cells transferred from WT OTI mice (**Fig 5.2C**), thus further confirming that OVA-Texo vaccine is capable of counteracting $CD8^+$ T cell tolerance in chronic infection.

Fig 5.2

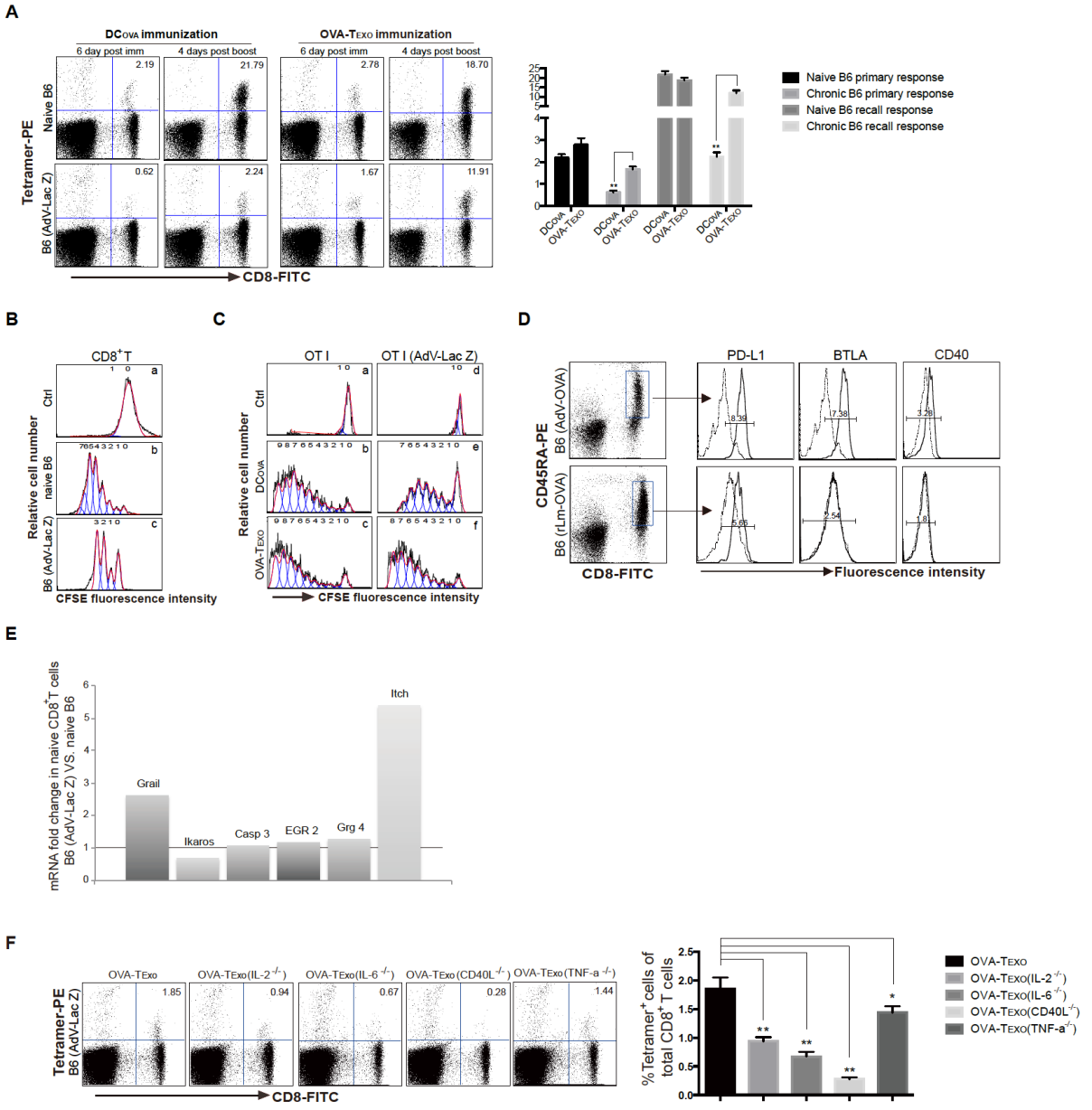

Figure 5.2 T cell anergy/tolerance in chronic infection.

(A) Naive C57BL/6 mice or C57BL/6 mice chronically infected with AdV_{LacZ} were immunized with DCova or OVA-Texo. Six days after the immunization, mouse periphery blood samples were stained with the PE-tetramer and FITC-CD8, and analyzed by flow cytometry. Thirty days after immunization, mice were boosted. The periphery blood samples were analyzed 4 days after the boost for OVA-specific memory CTL recall responses by flow cytometry. (B, C) Cell division was monitored by levels of CFSE dilution. Histograms show the FACS profiles of (B) $CD8^+CFSE^+$ T cells from the *in vitro* proliferation assay or (C) PE-tetramer$^+$ (Tet^+) $CFSE^+$ T cells from the *in vivo* proliferation assay. Numbers of cell division (n) are indicated on top of each peak. (D) Periphery blood samples

from mice with rLmOVA-induced acute infection or mice with AdVova-induced chronic infection were stained with PE-CD45RA, FITC-CD8, and PE-Cy5 labeled Abs, respectively, and then analyzed by flow cytometry. Naive $CD8^+$ T cells with positive PE-CD45RA and FITC-CD8 staining were gated, and assessed for expression of co-stimulatory or inhibitory molecules (solid lines). Dotted lines represent isotype-matched controls. (E) Expression of anergy-associated genes was assessed by RT-PCR using mRNA purified from naïve $CD8^+$ T cells derived from naïve or chronically infected mice. (F) C57BL/6 mice chronically infected with AdV_{LacZ} were immunized with OVA-Texo or with OVA-Texo with indicated molecule defects. Six days after the immunization, cells from periphery blood samples were stained with PE-tetramer and FITC-CD8, and analyzed by flow cytometry. The value in each panel represents the percentage of PE-tetramer-positive $CD8^+$ T cells vs total peripheral $CD8^+$ T cells. *$p < 0.05$, **$P < 0.01$. Error bars represent SD. One representative experiment of two to three is shown.

5.4.3 OVA-Texo-induced counteraction of $CD8^+$ T cell anergy/tolerance depends upon CD40L signal:

It has been demonstrated that IL-2, IL-6, TNF-α as well as CD40L counteract T cell anergy and tolerance [43-45]. To assess a potential molecular mechanism for OVA-Texo-mediated counteraction of $CD8^+$ T cell tolerance, we generated a panel of OVA-Texo vaccines with various gene defects, such as OVA-Texo(IL-$2^{-/-}$), OVA-Texo(IL-$6^{-/-}$), OVA-Texo(TNF-$\alpha^{-/-}$) and OVA-Texo(CD40L$^{-/-}$) lacking expression of IL-2, IL-6, TNF-α and CD40L, respectively. Mice with AdV_{LacZ}-induced chronic infection were then immunized with each of these vaccines, respectively, followed by assessing by flow cytometry OVA-specific CTL responses at day 6 after immunization. This approach demonstrated that CD40L deficiency alone reduced OVA-Texo-stimulated OVA-specific CTL responses in chronic infection by 85%, though CTL responses were also slightly to moderately down-regulated when using OVA-Texo(TNF-$\alpha^{-/-}$), OVA-Texo(IL-$2^{-/-}$) and OVA-Texo(IL-$6^{-/-}$) vaccines (**Fig 5.2F**), indicating that OVA-Texo-induced counteraction of $CD8^+$ T cell anergy/tolerance in chronic infection is mediated mainly by CD40L signaling.

5.4.4 OVA-Texo vaccine converts T cell exhaustion in chronic infection:

In our further work, we examined whether OVA-Texo vaccine would enhance responses of $CD8^+$ T cells primed in the absence of $CD4^+$ T cell help. This is a more stringent model of chronic infection, in which the 'helpless' $CD8^+$ T cells demonstrated stronger functional defects [46]. Mice with AdVova-induced chronic infection were boosted with the OVA-Texo vaccine. Four days after the boost, the proliferation, IFN-γ expression and cytolytic avtivity of OVA-specific CTLs were analyzed by flow cytometry. These experiments demonstrated that, though no significant change in expression of inhibitory molecules (PD-1 and PD-L1) on CTLs could be observed, there were around 3-fold more of OVA-specific CTLs in OVA-Texo-boosted mouse peripheral blood (**Fig 5.3A**), spleen (**Fig 5.3B**), and non-lymphoid tissues, including lungs (**Fig 5.3B**). We next measured by intracellular staining the effect of the OVA-Texo vaccine on the stimulation-induced expression of the effector cytokine IFN-γ in exhausted CTLs on a 'per-cell' basis. We determined that there was nearly 4.5-fold increase in IFN-γ-producing OVA-specific CTLs (nearly 90% of IFN-γ^+ cells) in OVA-Texo-treated mice, compared to untreated (PBS) control mice (only ~20% of IFN-γ^+ cells) (**Fig 5.3C**). Since in chronic infection, exhausted CTLs have a functional defect in their ability to lyse target cells (**Fig 5.1D**) [7], we attemted to determine whether OVA-Texo-converted CTLs resume their 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 1:1 ratio into OVA-Texo-immunized mice, and monitoring the lysis of OVA-specific (H) target cells by flow cytometry. This showed a significant increase in OVA-specific cytolytic response (93.5% lysis) in OVA-Texo-treated mice, compared to weak (13.5%) target cell lysis in untreated mice (**Fig 5.3D**).

Fig 5.3

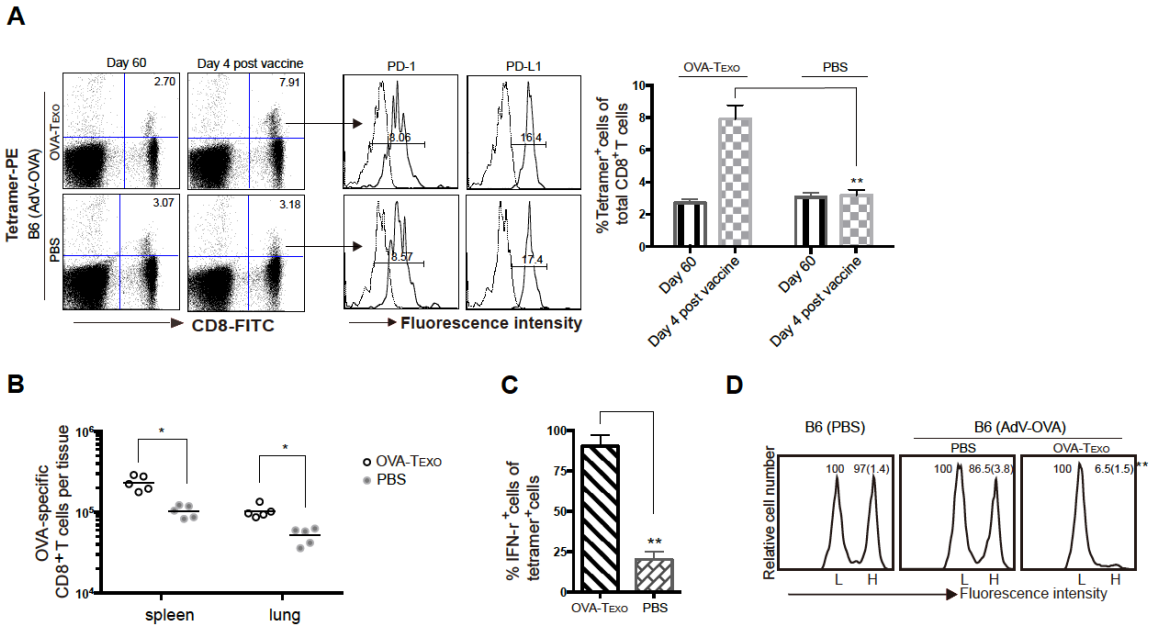

Figure 5.3 OVA-Texo converts OVA-specific exhausted CD8$^+$ T cells.

(A) AdVova-infected C57BL/6 mice were immunized with OVA-Texo. Before the immunization and 4 days after the immunization, cells from periphery blood samples were stained with PE-tetramer, FITC-CD8, and PE-Cy5-labeled Abs, and analyzed by flow cytometry. The value in each panel represents the percentage of PE-tetramer-positive CD8$^+$ T cells vs total peripheral CD8$^+$ T cells. PBS was used as control treatment. The OVA-specific CD8$^+$ T cells with positive PE-tetramer and FITC-CD8 staining were gated, and assessed for expression of inhibitory molecules (solid lines). Dotted lines represent isotype-matched controls. (B) Total numbers of PE-tetramer$^+$/FITC-CD8$^+$ T cells in spleens and lungs of immunized mice were measured by flow cytometry. (C) Percentage of IFN-γ producing cells in PE-tetramer$^+$/FITC-CD8$^+$ T cell population was analyzed. (D) *In vivo* cytotoxicity assay was performed in OVA-Texo-treated mice with AdVova-induced chronic infection 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. *p < 0.05, **P < 0.01. Error bars represent SD. One representative experiment of two is shown.

5.4.5 OVA-Texo-induced conversion of CTL exhaustion is triggered through the CD40L-induced activation of the PI3K/Akt/mTORC1 pathway:

Since CD40L signaling of OVA-Texo vaccine was found to be important for the counteraction of CD8$^+$ T cell tolerance, we examined whether CD40L plays any 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, lacking CD40L signaling. Interestingly, CD40L deficiency alone caused a significant decrease in its activity to enhance T cell proliferation (**Fig 5.4A**), indicating that OVA-Texo-induced conversion of CTL exhaustion occurs mainly via its CD40L signaling. In addition, there was also no increase in OVA-specific CTLs in spleens and lungs in OVA-Texo($CD40L^{-/-}$)-stimulated mice with chronic infection (**Fig 5.4B**). It has been shown that CD40L signaling assures T cell activation via the PI3K/Akt/mTORC1 pathway [47]. To assess whether OVA-Texo vaccine activates the PI3K/Akt/mTORC1 pathway, we analyzed CTLs by flow cytometry for the phosphorylation status and intracellular expression of a panel of molecules associated with the PI3K/Akt/mTORC1 pathway, including Akt, mTORC1-regulated S6, elF4E and T-bet. We determined that OVA-specific CTLs up-regulated levels of pAkt, pS6, pelF4E and T-bet as well as T cell proliferation marker Ki67 in B6 mice with the AdVova-induced chronic infection following the OVA-Texo vaccination (**Fig 5.4C**). This indicates that the OVA-Texo vaccine stimulates proliferation of exhausted CTLs at least in part by activating the PI3K/Akt/mTORC1 pathway. In addition, we also measured expression of diacetylated histone H3 (diAcH3) molecule, which is a useful marker for memory T cell activation and functionality [48]. These experiments revealed that OVA-specific CTLs up-regulated expression of diAcH3 after the OVA-Texo vaccination (**Fig 5.4C**).

Fig 5.4

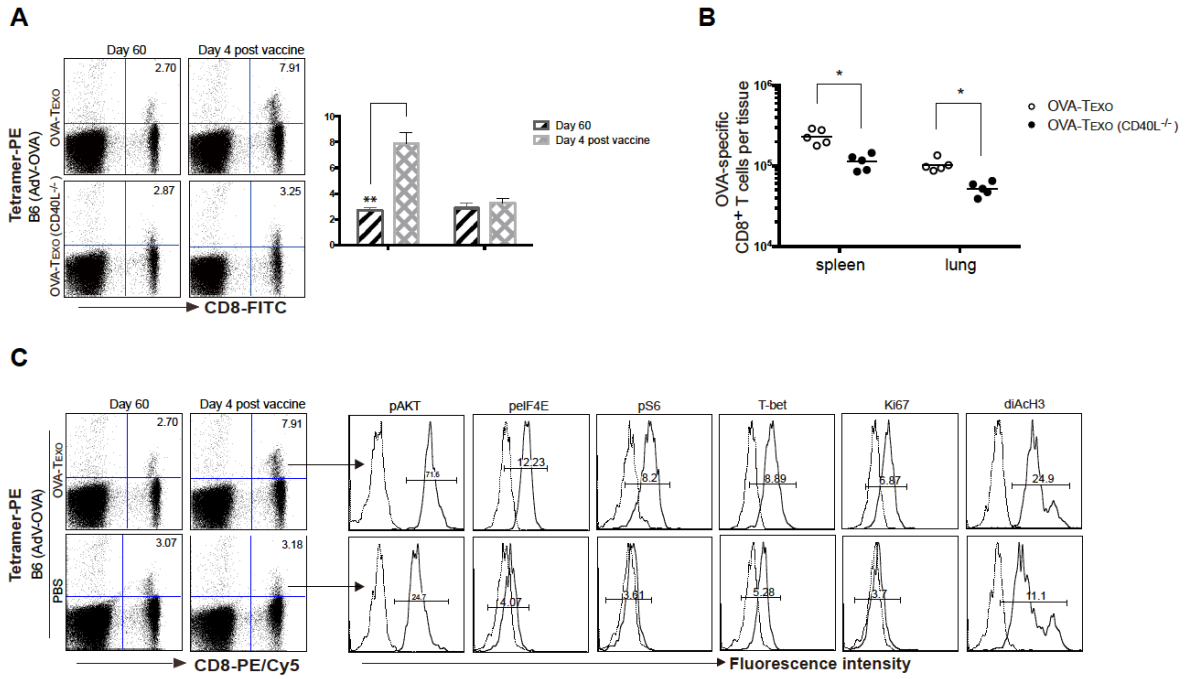

Figure 5.4 OVA-Texo vaccine converts T cell exhaustion via CD40L signaling.

(A) AdVova-infected C57BL/6 mice were immunized with OVA-Texo or OVA-Texo with CD40L deficiency 60 days after primary infection. Prior to 4 days post-immunization, periphery blood samples were analyzed for OVA-specific CTL responses by flow cytometry. The value in each panel represents the percentage of PE-tetramer-positive $CD8^+$ T cells vs the total peripheral $CD8^+$ T cell population. (B) Total number of PE-tetramer$^+$/FITC-$CD8^+$ T cells in spleens and lungs following OVA-Texo or OVA-Texo($CD40L^{-/-}$) immunization. (C) Four days after immunization, mouse splenocytes were triply stained with PE-tetramer, PE/Cy5-CD8, and FITC-labeled Abs, and analyzed by flow cytometry. The OVA-specific $CD8^+$ T cells with positive PE-tetramer and PE/Cy5-CD8 staining were gated, and assessed for the presence of various markers (solid lines). Dotted lines represent isotype-matched controls. *$p < 0.05$, **$P < 0.01$. Error bars represent SD. One representative experiment of two to three is shown.

5.4.6 OVA-Texo vaccine and PD-L1 blockade synergistically converts CTL exhaustion:

To assess a potential synergistic effect of the OVA-Texo vaccine on the PD-L1 blockade, we initially measured OVA-specific CTL proliferation in mice with chronic infection treated anti-PD-L1 antibody (**Fig 5.5A**), and then, assessed OVA-specific CTL proliferation in mice

with anti-PD-L1 antibody treatment followed by the OVA-Texo vaccination (**Fig 5.5B**). In this experiment, OVA-specific CTLs slightly and gradually proliferated with each individual treatment, and OVA-specific CTL population increased nearly 2.5-fold after the fifth treatment (**Fig 5.5A**). Remarkably, a dual (both anti-PD-L1 antibody and OVA-Texo vaccine) treatment resulted in almost 6-fold CTL increase, compared to the untreated exhausted CTLs (1.90%) in the chronic infection model (**Fig 5.5B**). To assess whether CTLs were functional, we measured intracellular production of IFN-γ by flow cytometry. This showed that around 50% of CTLs in mice with the PDL-1 blockade and nearly 90% of CTLs in mice with the dual treatment expressed the IFN-γ effector cytokine, indicating a synergistic effect of the PD-L1 blockade and the OVA-Texo vaccine in converting CTL exhaustion.

Fig 5.5

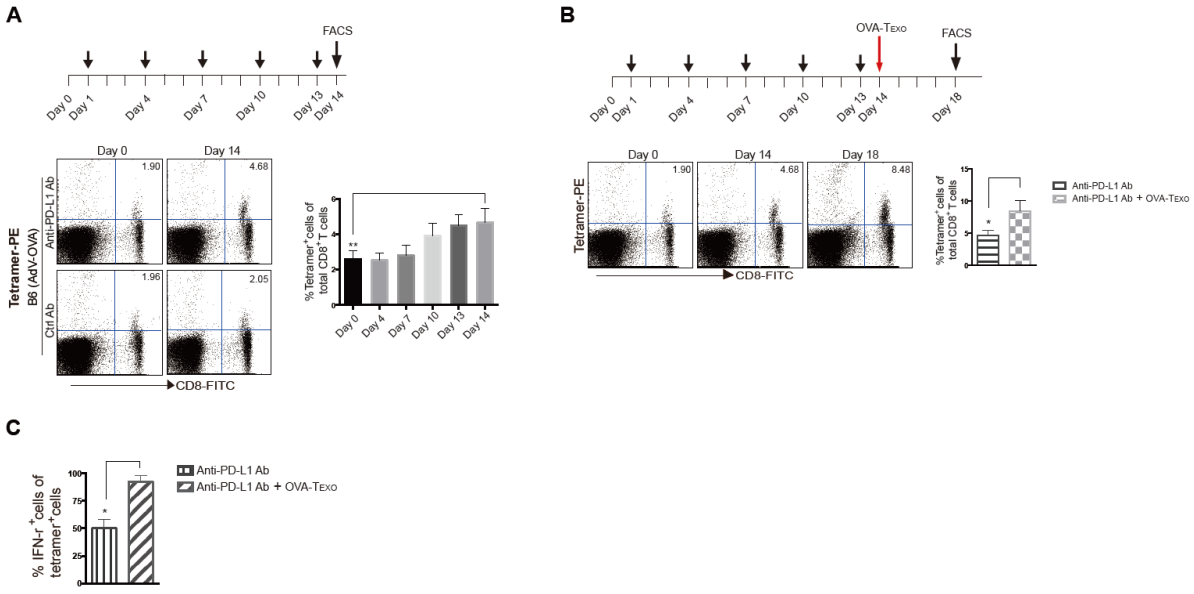

Figure 5.5 OVA-Texo vaccine synergizes with the PD-L1 blockade.

(A) AdVova-infected C57BL/6 mice were treated with anti-PD-L1 Ab five times in total at three day intervals. (B) OVA-Texo was administrated one day subsequent to the last anti-PD-L1 Ab treatment. Peripheral blood samples were collected at the indicated days, stained with the PE-tetramer and FITC-CD8, and then analyzed by flow cytometry. The value in each panel represents the percentage of PE-tetramer-positive $CD8^+$ T cells vs total peripheral $CD8^+$ T cells. (C) Percentage of IFN-γ producing cells in PE-tetramer$^+$ and FITC-$CD8^+$ T cell population was analyzed in each treatment group. *p < 0.05, **P < 0.01. Error bars represent SD. One representative experiment of two is shown.

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

To assess the preventive immunity, chronically AdVova-infected B6 mice were immunized with Gag-Texo and DC_{Gag} vaccines, respectively, and six days after immunization, the mice were challenged with Gag-expressing BL6-10_{Gag} melanoma cells. Our analysis showed that the Gag-Texo, but not the DC_{Gag} vaccine completely protected all challenged mice from tumor growth even in the presence of chronic infection (**Fig 5.6A**). To assess a potential therapeutic immunity in this model, mice with chronic infection were initially inoculated with

Gag-expressing BL6-10$_{Gag}$ melanoma cells, and six days later, were immunized with the Gag-Texo or the DC$_{Gag}$ vaccine. Excitingly, we observed significantly less BL6-10$_{Gag}$ lung tumor colonies in mice immunized with Gag-Texo than in experimental animals immunized with DC$_{Gag}$ (**Fig 5.6B**), indicating that our Gag-Texo vaccine is capable of inducing some degree of therapeutic immunity against established tumors even in the presence of chronic infection.

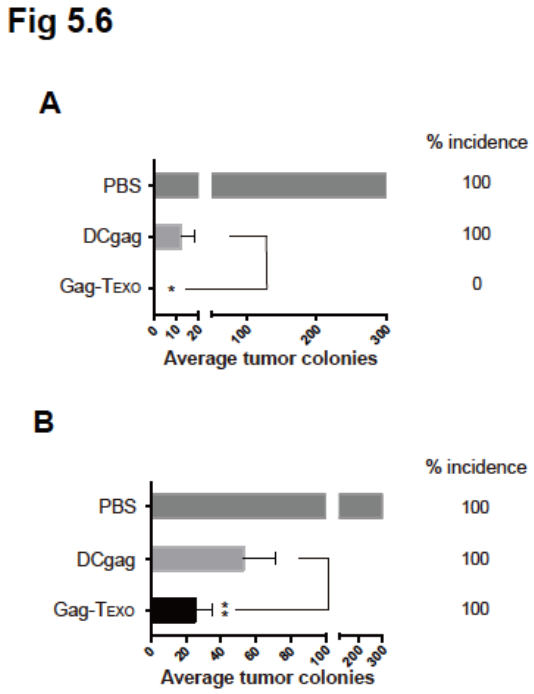

Figure 5.6 Gag-Texo vaccine induces protective, and therapeutic antitumor immunity in chronically infected mice.

AdVova-infected mice were i.v. injected with BL6-10$_{Gag}$ cells at day 6 post Gag-Texo or DC$_{Gag}$ immunization (A), or 6 days prior to immunization (B). The mice were sacrificed 3 weeks after tumor cell challenge, and lung-metastatic tumor colonies were counted in lungs. *p < 0.05, **P < 0.01. Error bars represent SD. One representative experiment of two is shown.

5.5 Discussion

Vaccines based on the recombinant human E1/E3-depleted adenovirus (AdV)-5 vector are well characterized and proved to be effective in inducing $CD8^+$ CTL responses due to their strong immunogenicity [49]. Adenoviral vectors which are easy to manipulate infect different types of cells, and their safety is well defined since vector genome does not integrate into cellular DNA [50]. However, transgene expression could persist in a host at low levels for a long time post AdV infection [35, 37]. Depending upon a nature of a transgene (Gag, LacZ and Luc) used, the dose of vector, the route of immunization, and the persistency of transgene expression, distinctive memory CTL responses ranging from effective CTL immunity to CTL exhaustion or depletion were reported [35-41]. Chicken ovalbumin (OVA)-expressing adenoviral vector (AdVova) has been assessed for AdVova-stimulated OVA-specific CTL trafficking *in vivo*, but not for CTL's phenotype and functional effect [39]. Exhausting CTLs, resulting from excessive or persistent Ag stimulation, often poses a significant barrier to the immune responses and managing chronic infection [51]. 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 [8, 52]. In this study, we i.v. immunized C57BL/6 mice with our OVA-expressing AdVova, and phenotypically and functionally characterized OVA-specific CTLs 60 days following immunization. In line with previous reports, we observed inflated memory CTLs with defects in proliferation, IFN-γ expression and cytotoxicity in the presence of AdVova-induced chronic infection, indicating that these OVA-specific mCTLs are functionally exhausted. In addition, these exhausted mCTLs up-regulated expression of inhibitory PD-1, PD-L1 and LAG-3. Therefore, our data implicate that AdVova-immunized mice represent an OVA-specific mouse model of chronic infection.

A chronic infection with persisting pathogens can impair immune responses to unrelated pathogens and vaccines, and a dysfunctional immunity (a bystander immunity in chronic infection) might be responsible for increased susceptibility to various coinfections [42]. However,

our current understanding of the bystander chronic infection is mainly based upon epidermiological evidence with limited insights into molecular mechanisms. DC defects in maturation, cytokine production and antigen-presentation have been reported in chronic infection models [53-55]. Defects in T cell proliferation have also been demonstrated in both animal models and humans with chronic infection [56-58]. However, it is not clear whether T cell proliferation defect is intrinsic to T cells themselves or is derived from the functional deficiency of DCs. The inhibitory PD-1 molecule was previously found on naive $CD4^+$ T cells in chronic HCV infection [58], whereas up-regulation of inhibitory PD-L1 was originally observed on splenocytes of chronically LCMV-infected mice [32]. Later, reports confirmed that PD-L1 expression was high in spleen tissues, especially on spleen $CD11b^+F4/80^+$ macrophages and $B220^+CD19^+$ B cells, but low in bone marrows [59]. In our work, we demonstrate that naïve $CD8^+$ T lymphocytes up-regulate expression of inhibitory PD-L1 and immunoglobulin-like co-inhibitory receptor BTLA [60], and overexpress T cell anergy-associated genes Grail and Itch [61], indicating that they may become anergic in the presence of chronic infection. Anergy of naïve CD8 T cells in our chronic infection model is further confirmed by showing that these naïve $CD8^+$ T cells have some degree of proliferation defect both in *in vitro* and *in vivo*. Interestingly, our OVA-Texo, but not DCova vaccine is capable of counteracting T cell anergy/tolerance associated with chronic infection. It has been demonstrated that IL-2, IL-6, TNF-α as well as CD40L counteract T cell anergy and tolerance [45]. To assess a potential molecular mechanism of counteraction of T cell tolerance by OVA-Texoe, we prepared a panel of OVA-Texo vaccines, OVA-Texo(IL-$2^{-/-}$), OVA-Texo(IL-$6^{-/-}$), OVA-Texo(TNF-$\alpha^{-/-}$) and OVA-Texo($CD40L^{-/-}$), deficient in IL-2 or IL-6 or TNF-α or CD40L, and compared OVA-specific CTL responses in mice with chronic infection vaccinated with these preparations. We demonstrate here, that the deficiency in CD40L alone, but not in other signaling components of the OVA-Texo vaccine almost completely abolishes its stimulatory effect on CTL responses in our model of chronic infection, indicating that CD40L signaling plays a central role in counteracting $CD8^+$ T cell anergy/tolerance induced by chronic infection conditions.

A member in the CD28 superfamily, PD-1 is a negative regulator of activated T cells [62, 63]. It is markedly up-regulated on the surface of exhausted CTLs in mice with chronic viral infection [64]. Blocking the PD-L1-PD-1 pathway *in vivo* by using anti-PD-L1 antibody converts CTL exhaustion, increases virus-specific CTL responses and decreases the viral load [32]. Apart from cytokine milieu, one of the most critical factors regulating CTL responses is the balance between positive and negative co-stimulations [63]. While the PD-1 negative co-stimulatory molecule is over-expressed on exhausted CTLs, the positive co-stimulatory proteins, members of the TNF-α receptor (TNFR) superfamily, including OX40, 4-1BB and CD27, have been demonstrated to suppress CTL exhaustion [65-67]. In this study, we demonstrate that during chronic infection, exhausted CTLs express CD40, and the OVA-Texo vaccine is capable of converting CTL exhaustion by enhancing their proliferation, cytokine production and cytolytic responses. In addition, we also measured expression of a conventional marker of memory T cell activation and functionality, diacetylated histone H3 (diAcH3) [48]. Interestingly, the OVA-specific CTLs appeared to up-regulate expression of diAcH3 in response to the OVA-Texo vaccination, thus confirming that OVA-Texo efficiently stimulates exhausted CTLs leading to not only the cell proliferation but also the resume of cell functionality.

To assess a potential role of CD40L-initiated signaling in OVA-Texo-induced conversion of CTL exhaustion, we have immunized mice with chronic infection with the OVA-Texo($CD40L^{-/-}$) lacking CD40L expressiong, and we demonstrate that CD40L absence strongly decreases its stimulatory effect on CTL proliferation, indicating that CD40L signaling by the OVA-Texo vaccine also plays a critical role in converting CTL exhaustion. CD40L signaling has been reported to play an important role in suppressing CTL exhaustion via the up-regulation of T-bet and IL-21 and the down-regulation of PD-1 [68-70]. However, our study is the first report demonstrating that CD40L signaling by the OVA-Texo vaccine is directly involved in converting CTL exhaustion in chronic infection. It has been shown that CD40L stimulates T cell activation

by inducing recruitment of the adaptor proteins known TRAF, leading to the activation of the PI3K/Akt/mTORC1 pathway [47]. 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 infected mice with the OVA-Texo vaccine, the OVA-specific CTLs up-regulate expression or phosphorylation status of pI3K, mTORC1-regulated pS6, pelF4E and T-bet that controls T cell activation, and of a protein associated with cell cycle progression, Ki67, indicating that the conversion of CTL exhaustion by the OVA-Texo vaccine may occur via the activation of the PI3K/Akt/mTORC1 pathway.

LAG-3 is known to negatively regulate T cell activation and proliferation [71-73]. The blockade of LAG-3 by its antagonistic antibody suppressed CTL exhaustion, and the dual blockade of PD-1 and LAG-3 resulted in a synergistic effect on conversion of CTL exhaustion in chronic infection [8]. In addition, the dual blockade of PD-L1 and TNFR superfamily members also exerts similar synergistic effect [65, 67]. Interesting, our data show that the OVA-Texo vaccine also synergizes with the PD-L1 blockade in converting CTL exhaustion, indicating that mechanisms derived from the OVA-Texo vaccine and PD-L1 blockade most likely impart distinct regulatory effects on exhausted CTLs. The exhausted CTLs expressed both PD-1 and LAG-3 in the AdVova-induced chronic infection. We, therefore, speculate that a triple therapy using the OVA-Texo vaccine and a dual blockade of PD-1 and LAG-3 with anti-PD-L1 and anti-LAG-3 antibodies may assure even more efficient conversion of CTL exhaustion than the dual blockade of PD-1 and LAG-3.

Taken together, our data demonstrate that our novel exosome-targeted T cell-based vaccine is capable of counteracting $CD8^+$ T cell anergy/tolerance and converting CTL exhaustion by inducing CD40L-triggered signaling through the mTORC1 pathway, thus efficiently inducing 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.

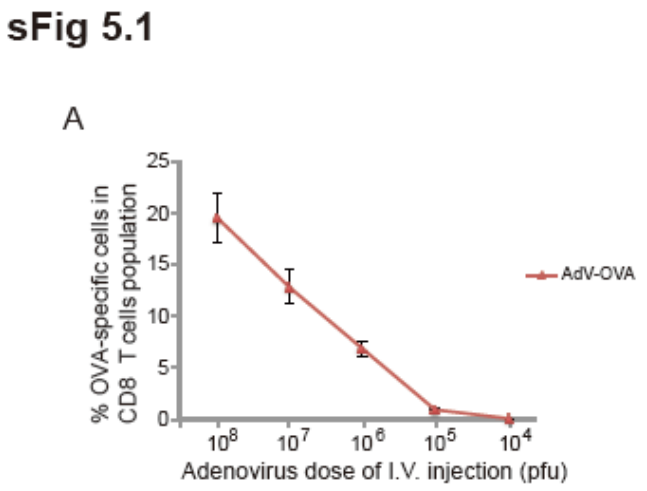

Figure s5.1 AdVova injection resulted in a dose-dependent OVA-specific memory CD8$^+$ T cell response.

Periphery blood samples harvested at Day 60 after i.v. injection of C57BL/6 mice with different dose of AdVova were stained with the PE-H-2K^b/OVAI peptide tetramer (PE-tetramer) and FITC-anti-CD8 Ab (FITC-CD8), and analyzed by flow cytometry.

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CHAPTER 6 GENERAL DISCUSSION AND CONCLUSIONS

Combined antiretroviral therapy (cART) dramatically improves HIV-1 patient lifespan and life quality. It has also been confirmed that ART significantly reduces the risk of viral transmission due to the considerably low level of HIV copies in the blood and genital fluid. This is termed "treatment as prevention"[1, 2]. Despite all the remarkable success of ART, it is not the means to the end. Without eradicating the infection, strict adherence to lifetime treatment is required. In addition, the persistent inflammation and drug toxicity are associated with progressive immune dysregulation and impaired B and T cell restoration. This results in higher risk of many non-AIDS related diseases such as neurocognitive abnormalities, bone disorders, cardiovascular disease and so on[3]. Facing the reality of approximately 35 million people infected with HIV-1 worldwide, the need for finding a cure is greater now than even before.

No "sterilizing cure" has been discovered yet with antiretroviral drugs. Development of a universal prophylactic vaccine seems to be extremely unlikely at this time. The RV144 Thai trial, using a heterologous prime/boost protocol with a canarypox vector encoding HIV-1 Gag, Pol, Gp120, followed by boosting with Gp120 protein from clade B and C is so far the only HIV-1 vaccine efficacy trial in which some level of protection (31%) from acquisition of infection was achieved[4].

"Functional cures" are most likely to be achieved by incorporating therapeutic strategies to restore HIV-1-specific CTL responses and restrain viral replication. This has been the focus of numerous research endeavors in recent years. Elite controllers, who are HIV-1 seropositive, but able to maintain HIV-1 RNA level under the detect limit without ART treatment, represent a natural functional cure model of HIV-1 infection[5]. Many studies have been focused on probing into unique host factors that contribute to viral control. Two protective MHC class I molecule alleles HLA-B27 and 57 are found to be highly overrepresented among elite controllers[6-8]. Of note, the superior HIV-1 $CD8^+$ T cell responses mediated by these MHC I alleles are restricted to immunodominant conserved HIV-1 Gag epitopes[9, 10]. Viral mutations mapping to conserved regions are associated with a major fitness cost. Therefore, in the rapid expanding field of therapeutic vaccines, finding relevant epitopes with superior immunogenicity is of equal

importance as finding an effective therapeutic vaccine strategy.

Early studies employed classic approaches, including whole inactivated virus[11] and recombinant protein subunits[12]. These exhibited limited capacity in evoking HIV-specific T cell responses and reducing viral load. More innovational approaches such as recombinant viral vectors and DC-based therapeutic vaccines have had more promising results[13, 14]. Although the stimulation of viral related immune response was observed, their impacts over viral replication were weak and inconsistent, necessitating the exploration of alterative novel vaccine strategies.

Immature DCs residing at the mucosal surfaces are one of the earliest immune cells to meet invading HIV-1 viruses. The initial encounter could either serve as an effective priming of HIV-specific T cell responses, or on the contrary, facilitating the viral dissemination to $CD4^+$ T cells, especially HIV-specific ones[15]. Furthermore, impaired DC function is largely associated with chronic HIV infections[16, 17]. Most DC vaccines adopt *in vitro* generation methods to avoid these issues – loading autologous DCs with various forms of HIV-related antigens. However, immune-suppressive factors released by HIV chronic infection can still compromise the fitness and immunogenicity of *in vitro* generated DCs, resulting in suppression, deletion, and failure in stimulating an ideal immune response. One intriguing alternative approach is to use DC-derived exosomes. As inner vesicles, exosomes are much more resistant to surrounding milieu. Hence, in the present work, autologous bone marrow DCs from C57BL/6 mice were culture and stimulated to become mature DCs *in vitro* and then pulsed by OVA protein, or infected with recombinant adenovirus vector encoding HIV-1 Gag. These two methods are well documented to be effective and well-tolerated in loading DCs with desired antigens[18-21]. Next, exosomes derived from those antigen-loaded DCs carrying pMHC I and II, costimulatory molecules and adhesion molecules were purified from culture supernatants. Non-specific polyclonal $CD8^+$ T cells capturing exosomes via ICAM-1 and LFA-1 interaction, display DC originated antigen-presenting machinery, and become the exosome-targeted activated $CD8^+$ T cell (aTexo) vaccine.

Our work in Chapters 2 and 3 demonstrated that: (1) Non-specific active $CD8^+$ T cells are able to

uptake DC-derived exosomes carrying DC molecules and exogenous antigens. (2) OVA-Texo and Gag-Texo vaccines stimulate *in vitro* antigen-specific $CD8^+$ T cell proliferation. (3) They are competent at inducing *in vivo* DC and $CD4^+$ T cell independent, functional antigen-specific $CD8^+$ T cell responses. (4) These vaccines efficiently promote $CD8^+$ T cell differentiation into functional CTL effectors. (5) They induce $CD8^+$ CTL-mediated antitumor immunity and long-term T cell memory in wild-type C57BL/6 and transgenic HLA-A2 mice. Those encouraging results provide early evidence of this novel T cell-based vaccine in stimulating HIV Gag-specific CTL responses and inducing some degree of therapeutic immunity against tumor cells bearing the Gag immunogen. Moreover, the desirable feature of priming CTL responses in the absence of assistance from DC and $CD4^+$ Th cells make the $CD8^+$ Gag-Texo vaccine an appealing immunotherapeutic strategy.

In Chapter 4, experiments were performed to elucidate if 4-1BBL overexpression could improve the outcome of the $CD8^+$ Texo vaccine. Many groups have reported positive adjuvant effect of costimulatory molecule 4-1BBL in HIV-specific T cell responses. 4-1BBL enhanced Gag-specific cellular immune responses of a HIV-1 Gag DNA vaccine[22]. A recombinant replication-defective adenovirus expressing 4-1BBL converted autologous monocytes into efficient APCs, and led to an expansion of antigen-specific $CD8^+$ effector cells, elevating effector cytokine generation in both acute and chronic viral infections[23]. Co-expression of 4-1BBL with a recombinant fowlpox virus vector expressing HIV Gag significantly boosted Gag-specific $CD8^+$ T cell memory responses[24]. Loading of mRNA encoding 4-1BBL in addition to HIV antigen to HIV-1 infected patient derived DCs reinforced HIV-specific $CD8^+$ T cell responses by promoting proliferation, effector functions, survival, and resistance to Treg-mediated immune suppression[25]. In line with those studies, our work in Chapter 4 illustrated: (1) OVA-Texo/$_{4\text{-}1BBL}$ vaccine induces enhanced *in vivo* OVA-specific effector $CD8^+$ CTL response. (2) OVA-Texo/$_{4\text{-}1BBL}$ vaccine stimulates efficient protective and therapeutic immunity against OVA-expressing tumor cells in C57BL/6 mice. (3) OVA-Texo/$_{4\text{-}1BBL}$ vaccine is capable of inducing $CD44^+62L^{high}IL\text{-}7R^+$ central memory CTLs formation. (4) OVA-Texo/$_{4\text{-}1BBL}$ vaccine evokes functional $CD8^+$ memory CTLs and long-term antitumor immunity. (5) OVA-Texo/$_{4\text{-}1BBL}$ vaccine improves CTLs survival via regulating apoptosis pathway. (6) Gag-Texo/$_{4\text{-}1BBL}$ vaccine exhibits improved therapeutic and long-term antitumor

immunity in transgenic HLA-A2 mice. These improvements mediated by 4-1BBL are particularly meaningful for the Gag-Texo/$_{4\text{-}1BBL}$ vaccine, in consideration of the profoundly impaired expression of CD28 on defective T cells during HIV-1 infection. 4-1BBL may serve as a compensative costimulation signal pathway. Additionally, it has been proposed that strong $CD8^+$ T cell responses are critical for control and eradication of HIV infection[26]. Furthermore, 4-1BBL can efficiently promote the generation of more mature $CD27^-$ effectors and induce effector T cell expansion. This is of great importance as HIV-specific effector T cells are usually difficult to mature with down-regulated perforin expression[27, 28].

During chronic infection with HIV-1, HIV-1-specific $CD8^+$ T cells show progressive impairment of effector functions[29]. Recent studies showed that 4-1BBL could increase HIV-1-specific T cells expansion with restored functions in chronically HIV-infected subjects[30]. Current work in Chapter 5 shows that: (1) adenovirus is able to induce a long lasting chronic infection with high percentage of antigen-specific CTL persistency. (2) Those CTLs expressing a variety of up-regulated inhibitory molecules are functionally exhausted with impaired proliferation, cytotoxic effect, effector cytokine production as well as defective memory response. (3) Naïve T cells exhibit increased inhibitory markers and compromised proliferation. (4) OVA-Texo vaccine is able to stimulate antigen specific primary and memory recall CTL responses, inducing CTL proliferation during bystander chronic infection. (5) OVA-Texo vaccine is able to convert exhausted OVA-specific $CD8^+$ T cells during chronic infection, reversing CTL dysfunction. (6) Blockage of inhibitory PD-1-PD-L1 pathways is a novel "non-specific" therapeutic approach that has shown encouraging results[31, 32]. In this study, a dual treatment combining OVA-Texo/$_{4\text{-}1BBL}$ vaccine with PD-L1 blockage resulted in a synergistic effect – rescuing CTLs exhaustion in chronic infection. (7) Gag-Texo vaccine could stimulate effective antitumor immunity in chronic infected transgenic HLA-A2 mice. A number of molecular mechanism studies are conducted in this part as well – via the usage of T cells derived from gene knockout mouse, and detection of expression level changes of molecules in certain pathways. Those molecular mechanisms provide some valuable insight into understanding our novel T cell-based vaccine. It highlights key molecules that are indispensable, and offer ideas regarding how we can further engineer or manipulate the vaccine for desired outcomes.

The two primary goals of a therapeutic vaccine is to provoke *de novo* HIV-specific $CD8^+$ T cell responses from naïve T cells, as well as restore functionality of existing HIV-specific $CD8^+$ T cells - ideally suppressing viral replication to below the detection level, and thus delay or minimize ART providing "drug holidays". Despite all the exciting and inspiring results achieved so far, there is still a long way ahead on optimizing the exosome-target activated $CD8^+$ T cell aTexo vaccine before it is at our disposal as an effective "cellular adjuvant".

It is a consensus that the selection of relevant epitopes plays a critical part in vaccine design. We have constructed adenovirus vector expressing Gp120 and/or Tat in addition to Gag. HIV Tat protein as a key HIV virulence factor has been proposed as an ideal candidate to target. It is referred as "pathogenic-driven" intervention since Tat promotes the spreading of HIV to DCs and T cells. Recent evidence also indicates that Tat protein displays immunomodulatory activities which can be exploited for the development of combined subunit vaccines[33-35]. More importantly, Tat protein can enhance T cell responses against cryptic OVA epitopes and broaden T cell responses to HIV-1 envelope proteins (Gag and Env)[36]. This is possibly due to the cationic region of Tat being capable of increasing Env and Gag epitope expression on DCs with uptake of DNA plasmids expressing Env and Gag[37]. In addition, Tat vaccine strategies have also proven to be safe and immunogenic in mice, in non-human primates, and in humans. In addition, they have been shown to be effective in protecting from pathogenic challenge in monkeys[38-43]. It will be interesting to assess if Gp120 and/or Tat incorporation would enhance the magnitude and breadth of HIV-specific immunity in the future.

Lymphocytic choriomeningitis virus (LCMV) induced chronic infection in adult mice is a well-characterized model system[44, 45]. Abundant studies on altered immune responses and the underlying molecular mechanisms have been conducted in LCMV persistent infection established by Clone 13 strain[46-48]. To further explore the potential converting of exhausted CTLs by this novel T cell-based vaccine, we constructed LCMV glycoprotein (gp) 33- specific GP-Texo. Influence on effector functions of CTLs and viral control post our therapeutic GP-Texo vaccine will be evaluated in a LCMV chronic infection.

Although Clone 13 of LCMV represents an ideal mouse model that largely recapitulates T cell

dysfunction and immune tolerance seen in human persistent viral infection, more direct and convicting studies are achieving through Simian immunodeficiency viruses (SIVs) infected non-human primates. Strong evidence suggest HIV-1, HIV-2 evolved from chimpanzees infecting SIVcpz and sooty mangabeys infecting SIVsmm via cross-species infections[49, 50]. SIV-infected Chinese rhesus macaques develop simian AIDS[51], which is remarkably similar to HIV-1 infection in human. Viral load and severity of infection after SIVmac239 challenge in Chinese rhesus macaques were closer to that observed in untreated human HIV-1 infection, serving as a more suitable AIDS model[52]. Thus, it is considered as one of the most valuable animal models for HIV research over the last two decades[53, 54]. One prophylactic vaccine study in rhesus monkeys showed large protection against SIV_{MAC251} infection using SIV_{SME543} Gag, Pol and Env antigens expressing, adenovirus/poxvirus vector-based vaccines[55]. In another report, SIV protein-expressing rhesus cytomegalovirus vectors (RhCMV) vaccine elicited SIV-specific effector memory T-cell responses, 50% of rhesus macaques exhibited durable viral control after a highly pathogenic strain SIVmac239 infection[56]. There is no doubt that performance and mechanism evaluation of SIV Gag-specific Gag-Texo vaccine in Chinese rhesus macaques will provide more integrated, insightful knowledge of exosome-target $CD8^+$ T cell-based Texo vaccine.

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