TRANSGENE IL-21-ENGINEERED ANTIGEN-SPECIFIC EXOSOME TARGETED T CELL-BASED VACCINE POTENTLY CONVERTS CTL EXHAUSTION IN CHRONIC INFECTION

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Saskatoon

By

Xueying Zhang

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Vaccinology and Immunotherapeutics Program

School of Public Health

Health Science Building 107 Wiggins Road

University of Saskatchewan

Saskatoon, Saskatchewan S7N 5E5

</center>

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ABSTRACT

CD8^{+} cytotoxic T lymphocytes (CTLs), the potent effector T cells, capable of directly destroying virus-infected cells, correlate with acute viral control and long-term non-progression in virus-mediated infectious diseases, play an important role in controlling viral infections. However, CD8^{+} CTLs due to persistent viral stimulation showed functional exhaustion in virally induced chronic infections, which expressed inhibitory molecules such as inhibitory programmed death (PD)-1, programmed death ligand (PDL)-1, T-cell Ig and mucin protein-3 (TIM3) and lymphocyte-activation gene 3 (LAG-3), and were functionally exhausted such as defect in effector cytokine IFN-γ production, lack of cytolytic effect and reduction of recall responses upon the pathogen reencounter. Therefore, CTL exhaustion has become one of the major obstacles for the ineffectiveness of viral control in chronic infectious diseases such as human immunodeficiency virus (HIV)-1. We previously generated novel ovalbumin (OVA)-specific 41BBL-expressing OVA-T_{EXO} and HIV-1 Gag-specific Gag-T_{EXO} vaccines inducing therapeutic immunity in B6 mice and converting CTL exhaustion via its CD40L signaling activation of the PI3K-Akt-mTORC1 pathway in recombinant OVA-specific adenovirus AdV$_{OVA}$-infected B6 (AdV$_{OVA}$-B6) mice with chronic infection. In AdV$_{OVA}$-B6 mice, OVA-specific CTLs expressing IL-7R, IL-21R and inhibitory PD-1, PDL-1 and LAG-3 were inflated and functionally exhausted. Cytokine IL-21, a member of the common γ-chain cytokine family, produced by CD4^{+} helper T cells, plays an important role in controlling chronic infections. IL-21 promotes CTL activation and survival by activation of the phosphatidylinositol-3 kinase (PI3K) and the mTORC1-regulated T-bet pathway. In this study, we constructed recombinant transgene IL-21-expressing AdV$_{IL-21}$ by recombinant DNA technology, generated IL-21-expressing OVA-$T_{EXO/IL-21}$ and Gag-$T_{EXO/IL21}$ vaccines or the control OVA-$T_{EXO/Null}$ and Gag-$T_{EXO/Null}$ vaccines by infection of OVA-T_{EXO} and Gag-T_{EXO} cells with AdV$_{IL-21}$ or the control AdV$_{Null}$ without transgene, and assessed their stimulatory immunogenicity in wild-type B6 or AdV$_{OVA}$-B6 mice, respectively. We demonstrate that both OVA-$T_{EXO/IL-21}$ and the control OVA-$T_{EXO/Null}$ vaccines are capable of converting CTL exhaustion in chronic infection. However, IL-21-expressing OVA-$T_{EXO/IL-21}$ vaccine more efficiently rescues exhausted CTLs through increasing CTL proliferation and effector cytokine IFN-ɤ expression by 6-fold than the 3-fold in OVA-$T_{EXO/Null}$-vaccinated AdV$_{OVA}$-B6 mice, though these two vaccines stimulated comparable OVA-specific responses and

immunity against OVA-expressing BL6-10$_{OVA}$ melanoma in B6 mice. *In vivo* OVA-T$_{EXO/IL-21}$-stimulated CTLs more efficiently up-regulate phosphorylation of mTORC1-regulated EIF4E and expression of mTORC1-controlled T-bet molecules as well as Ki67 (a protein associated with cell-cycle progression) than the control OVA-T$_{EXO/Null}$-stimulated CTLs, indicating that enhancement of converting CTL exhaustion in chronic infection by OVA-T$_{EXO/IL-21}$ vaccination is mostly through the stronger activation of the PI3K-Akt-mTORC1 pathway derived from both its endogenous CD40L and transgenic IL-21 signaling. Importantly, Gag-T$_{EXO/IL21}$ vaccine also induces stronger Gag-specific therapeutic immunity against established Gag-expressing BL6-10$_{Gag}$ melanoma lung metastases than Gag-T$_{EXO/Null}$ vaccine in chronic infection. Therefore, this study should have a strong impact on developing new therapeutic vaccines for chronic infectious diseases such as HIV-1 infection.

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LIST OF ABBREVIATION

Ab	antibody
AdVs	Adenovirus
Ag	antigen
AIDS	Acquired Immune Deficiency Syndrome
APC	antigen presenting cell
ART	antiretroviral drug therapy
CCL4	chemokine ligand 4
CCR5	C-C chemokine receptor type 5
CD	cluster of differentiation
CTLs	cytotoxic lymphocytes
ConA	Concanavalin A
CXCR4	CXC-chemokine receptor type 4
DC	dendritic cell
pDC	Plasmid DC
cDC	conventional DC
Envs	envelope glycoproteins
EXO	exosome
GALT	gut-associated lymphoid tissue
HAART	highly active antiretroviral therapy
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IDO	indoleamine dioxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kDa	kilodalton

LAG-3	lymphocyte-activation gene 3
LCMV	lymphocytic choriomeningitis virus
MHC	major histocompatibility complex
mTORC1	mammalian target of rapamycin complex 1
MVB	multivesicular bodies
NK	natural killer
NRTIs	nucleoside-analog reverse transcriptase inhibitors
NNRTIs	non-nucleoside-analog reverse transcriptase inhibitors
OH	hydroxyl
OVA	ovalbumin
PAMPs	pathogen-associated molecular patterns
PD-1	programmed death-1
PD-L1	programmed death-ligand 1
PMNs	polymorphonuclear leukocytes
PR	precursor proteins
PRRs	pattern recognition receptors
RT	reverse transcriptase
Tregs	regulatory T cells
SIV	simian immunodeficiency virus
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TLRs	toll-like receptors
TNF	tumor necrosis factor

CHAPTER 1 LITERATURE REVIEW

1.1   Immune system

The host relies on its immune system that provides protection against pathogens such as bacteria, viruses, fungi, and parasites. The body's defense mechanism against pathogens is mediated by a collection of immune cells, tissues, and molecules. The coordinated action of these immune cells, tissues, and molecules is critical in an attempt to prevent diseases from occuring [1]. The most remarkable feature of the immune system is to distinguish "non-self" from "self," which is crucial to human health and life [2]. The body's immune system coexist peacefully with its own molecules with "self" markers, while it launches an attack when coming across "foreign" molecules [2]. The innate immunity and adaptive immunity are two critical arms of the immune system. Innate immunity mediates early protection against infections, whereas acquired immunity develops slowly and it is more specific and provides the long-term immunity [3].

The immune system consists of lymphoid organs that are located throughout the body [2]. Primary lymphoid organs include bone marrow and thymus, where B and T lymphocytes become mature, respectively. Mature B and T lymphocytes migrate to secondary lymphoid organs (spleen, lymph nodes, and mucosal lymphoid organs) and bloodstream. Naïve lymphocytes that have not been stimulated reside in secondary lymphoid organs and circulate in the bloodstream [4]. The antigen (Ag)-specific lymphocyte activation occurs in the secondary lymphoid organs, and activated lymphocytes circulate in the bloodstream and migrate to peripheral tissues [4].

1.1.1 Innate immunity

Innate immunity is also known as intrinsic immunity or natural immunity that remains present from the birth. The innate immunity is the first line of defense and is characterized by instant broad immune responses. The skin and mucous membrane forms a tough, impenetrable physical

and chemical barriers against infections and constitutes an integral part of innate immunity [5]. Most foreign organisms cannot penetrate intact skin, but some can enter the body through hair follicles and sebaceous glands. On internal surfaces (mucosal surfaces/ mucosae) of respiratory, gastrointestinal, and urogenital tracts, mucus is secreted to protect the epithelial cells from damage and infections.

Once the immune system detects an infection, the innate immune responses are turned on, and they become fully functional within a few minutes to hours. The specificity of the innate immunity is different from the acquired immunity. The innate immune cells express several receptors such as pattern recognition receptors (PRRs) and toll-like receptors (TLRs) that help in recognizing specific pathogen-associated molecular patterns (PAMPs). Subsequently, the recruitment of destructive mechanisms kills and eliminates the pathogen. These mechanisms are mediated mainly by leukocytes, phagocytes, mast cells, natural killer cells, several plasma proteins, including complement proteins which recognize and react against pathogens and cytokines (interferons (IFNs), chemokines, interleukins (ILs)) [6].

1.1.1.1 Components of innate immunity

- Cellular components in innate immunity
a) Phagocytes

   Phagocytes are white blood cells circulating in the body; they are recruited to infection sites to ingest and kill microbes through phagocytosis [1]. Phagocytosis is the ingestion of foreign substances followed by the fusion with lysosomes, which destroys infectious microorganisms by the toxic peroxide and superoxide radicals. There are mainly two kinds of phagocytes: neutrophils and monocytes. Neutrophils, also known as polymorphonuclear leukocytes (PMNs), which is the first immune cell to respond to most infections, and they are important against acute infections caused by bacteria and fungi. Neutrophils survive for a few hours, while monocytes, on the other hand, live for long periods. Monocytes differentiate into macrophages when they enter extravascular tissues. Macrophages have PRRs so that pathogens coated with these can be recognized and

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captured [4]. Macrophages produce cytokines that initiate and regulate inflammation, digest and destroy microbes, they can also repair tissues and clear dead tissues.

b) Mast cells

Mast cells that contain granules are derived from bone marrow and remain present in the skin and mucosal epithelium. Microbes or antibodies (Abs) binding to TLRs can activate them. Mast cells express receptors with high affinity to immunoglobulin (Ig) E [7] Abs on cell surfaces, they release inflammatory mediators such as histamine when antigens bind to IgE Abs [8].

c) Natural killer (NK) Cells

NK cells are large granular lymphocytes, they are cytotoxic for abnormal cells and stressed cells such as virus-infected or cancer cells by secreting cytokine IFN-γ. NK cells play an important role in fighting against pathogens and cancer cells in early stages of viral-infection and tumorigenesis.

d) Dendritic cells (DCs)

In innate immunity, DCs are activated when coming across pathogens with certain molecular patterns and initiate inflammation [4]. DCs are the most significant antigen-presenting cells (APCs) in the immune system, and DCs serve as a bridge between innate and adaptive immunity.

e) Eosinophils and basophils are involved in the fight against parasites and allergic reactions [9].

• Soluble components in innate immunity

a) The complement system is an important humoral element in innate immune system. It is an enzyme activated cascade reaction where a series of proteases are involved. The activation of each protease in the cascade reaction depends on the cleavage of its precursor [10]. Complement effectors facilitate pathogens' uptake by phagocytes, increase immune responses by attracting inflammatory molecules or kill pathogens directly by forming holes in their membrane.

b) Cytokines and some plasma proteins

Innate immune cell secreted cytokines play critical roles in regulation of immune responses. These cytokines act as intercellular messengers and soluble regulatory signals,

that initiate and limit inflammatory responses to tissue injury due to trauma or pathogens [11]. Innate immune cells release a plethora of cytokines and chemokines such as tumor necrosis factor (TNF), IFN-γ, IL-1β, IL-4, IL-6, IL-10, IL-12, IL-18, chemokine ligand 4 (CCL4/RANTES), and transforming growth factor (TGF)-β to orchestrate immune responses [11]. IFN-α/β are the most effective agents against viral infections; they are induced in high levels to mediate and regulate antiviral immune responses [12]. Components of innate immune immunity are also involved in acquired immunity. Innate and acquired immunity are interrelated in lots of conditions through signals sent by cytokines and cell adhesion molecules [13].

1.1.1.2   Principal responses in innate immunity

Inflammation is a body's defense mechanism, wherein the body reacts to external factors such as injuries or biological infections and internal factors such as tissue necrosis or bone fracture [3]. Inflammation induces immune responses, that attempt to bring the injury or tissue damage back to normal. In the situation of pathogenic insults, phagocytes with PRRs recognize microorganisms that penetrate the epithelial surfaces, and subsequently, triggering the inflammation process. Inflammation can also be initiated by complement components coated on bacterial surfaces [13]. Circulating leukocytes migrate to the site of infection and release inflammation mediators such as cytokines and chemokines. During inflammation, the affected area shows heat and redness, increased blood flow and the permeability of blood vessels. The inflammatory response can occur at localized positions as well as systemically (fever, chronic inflammation) [13].

1.1.2   Acquired immunity

The innate immunity provides the first line of host defense in the immune system. The second line of host defense is mediated by acquired immunity, which is also called adaptive immunity or specific immunity [3]. Acquired immunity comes into play when innate immunity fails to eliminate pathogens. Although acquired immunity is slow to mobilize, it is more powerful than innate immunity and provides long-lasting protection from further infections by the pathogen.

Main effector cells responsible for acquired immunity are B and T lymphocytes. B cell-mediated immunity is known as humoral immunity, whereas the immunity mediated by T cells is called cellular immunity [3]. Acquired immunity requires expansion and differentiation of lymphocytes. Cells included in the mechanisms of innate immunity recognize structures (PAMPs) shared by classes of microbes, while the cells in acquired immunity express receptors and recognize much more specific substances on foreign microbes and noninfectious substances. The adaptive immune system specificity is the ability to distinguish between many different antigens, it means that total lymphocytes consist many different clones (each clone made up of one cell and its progeny) and lymphocytes are extremely diverse [1]. The clonal selection hypothesis was formulated in the 1950s; it predicted that different clones of lymphocytes developed before they encounter with antigens, and immune responses were elicited by selecting and activating a specific clone of lymphocytes. The main features of an adaptive immune system are 1) remarkable expansion of the pool of lymphocytes specific for an antigen, 2) unique selection mechanisms that preserve the most useful lymphocytes, 3) positive feedback pathways that augment the immune responses.

1.1.2.1 Humoral immunity

Humoral (B-cell mediated) immunity and cellular (T-cell mediated) immunity are two arms of acquired/ adaptive immunity. After the binding of one specific antigen to B cell receptor, the B cell receiving the signal makes a specific form of Immunoglobulin. Immunoglobulins share common structural features. Each immunoglobulin molecule is composed of two identical heavy (H) chains and two identical light (L) chains. There are five major classes of H chains (γ, μ, α, ε and δ) [14]. Based on differences in H chains, immunoglobulin molecules are divided into five major classes: IgG, IgM, IgA, IgM, IgD, and each class has its unique biological properties. Besides neutralization of antigens, Abs have functional effect against pathogens via activation of complement components and antibody-dependent cell-mediated cytotoxicity (ADCC) [4].

1.1.2.2 T cell immunity

Two major T cell components, cluster of differentiation (CD)4$^+$ and CD8$^+$ T cells, are involved in T cell immunity. Based upon secretion of different cytokines, CD4$^+$ T cells are divided into different subsets of CD4$^+$ T cells, including T helper 1 (Th1), T helper 2 (Th2) and regulatory T (Treg) cells [15]. For example, Th1 cells secrete IFN-γ, IL-2 and TNF-α, help CD8$^+$ T cell responses and modulate B cells to produce IgG2a, while Th2 cells secrete IL-4 and IL-5, IL-6 and IL-10, and modulate B cells to produce IgE. CD4$^+$ Treg cells, on the other hand, secrete immune suppressive IL-10 and TGF-β, and inhibit CD4$^+$ and CD8$^+$ T cell responses [15]. CD8$^+$ T cells also called CD8$^+$ cytotoxic T lymphocytes (CTLs) secreting IFN-γ and TNF-α cytokines and producing cytolytic granules granzyme-B play a critical role in immunity against pathogen infected cells and cancer cells [16]. Antigen-specific T cell activation needs at least two signals: Signal one is derived from binding of antigen-specific T-cell receptor (TCR) to antigen peptide-loaded major histocompatibility complex-I (pMHC-I) on APCs, while signal two is delivered via engagement of co-stimulation B7-1 (CD80)/B7-2 (CD86) on APCs with CD28 on CD8$^+$ T cells [16-18]. It might have the third signal comes from help cytokines such as IL-2 secreted by CD4$^+$ T cells, which further enhance, modify, and skew the responding effector CTLs [19].

Humoral and cellular immunity are fundamentally interconnected at many levels, any particular pathogen may involve a complex interaction between them. Along with innate immunity, they work together to destroy and eliminate the invading microorganism and its products.

1.2 HIV-1 and AIDS

Human immunodeficiency virus (HIV) as the causative agent of Acquired Immune Deficiency Syndrome (AIDS) was discovered in 1983. Although, it was documented as a new disease in 1981 when many homosexual men became susceptible to unusual and rare malignancies and opportunistic infections (Origins of HIV and the AIDS pandemic. [21, 22]. In 1986, a morphologically related but antigenically different virus, now termed HIV-2, was found which was closely related to a simian virus causing immunodeficiency in captive macaques [23]. HIV-2 was found to cause AIDS in patients in western Africa [24]. HIV-1 strains have been classified into four divergent groups: M (main), O (outlier), N (non M or O) and P (putative) [25], in which, Group M is the major group, which is responsible for more than 95% of HIV-1 infections in the world [26]. The prevalence of other groups is extremely low and mostly restricted to West Central Africa [27]. AIDS caused about 1.1 million deaths in 2016 around the world [28], and it is a rapidly expanding global pandemic. HIV-1 enters the body by crossing mucosal barrier or transfer of blood, semen, vaginal fluid, pre-ejaculate or breast milk and infecting cells bearing a CD4 receptors [29]. The virus-infected cells are the main source of HIV-1 transmission, and up to 50-100 times more infected cells than the free virus can be found in body fluids [30]. An HIV-1 virus infects CD4$^+$ T lymphocytes and DCs and replicates inside of these cells by integrating viral DNA into host genome. Studies have shown a cell-to-cell transfer of HIV from lymphocytes or macrophages to mucosal cells in culture [31]. With the protective mechanisms of integration, reverse transcription, and extensive mutation of viral DNA, the immune system cannot deplete HIV-1 virus completely, and HIV-1 infection suppresses the function of the immune system [32, 33]. Without treatment, it may take 10-15 years for the immune system to be severely damaged and susceptible to cancers and other infections. However, this progress also depends on patient's age, health, and background. Highly Active Antiretroviral Therapy (HAART) is an effective treatment against HIV-1 infection on the market. HAART controls HIV-1 infection progression during the early stage of infection. However, HAART can't cure the infection, and there is no cure for HIV infection/ AIDS currently [34]. HAART treatment is very expensive, and it has several side effects such as mitochondrial toxicity, liver toxicity, pancreatitis, and predisposition to coronary heart disease [35]. Therefore, it is urgent to develop new therapeutic strategies to enhance virus control during treatment interruptions, limit usage of HAART, and stimulating

function of the immune system [36]. Current HIV-1 vaccine strategies, including using viral proteins, peptides, plasmid DNA or recombinant viruses.

1.2.1 HIV-1 virology

HIV is a retrovirus and retroviruses were classified into the family *Retroviridae.* The *Retroviridae* is divided into three main groups, (i) *Oncoviridae* or oncoviruses (tumor-causing virus) [37], (ii) *Spumaviridae* or spuma- or foamy viruses and (iii) *Lentiviridae* or lentiviruses. The name lentivirus was derived from the Latin word "lentus" meaning slow, based on the slowly progressive nature of the infection [38]. Retroviruses are a family of single-strand (+) RNA viruses that encode a reverse transcriptase enzyme which reverse transcribes its genome (7-12 kb in length) into double-stranded DNA, and integrate viral DNA into the genome of the host cell. All retroviruses contain two linked copies of genomic RNA (gRNA) in the mature virion [39, 40].

1.2.1.1 Genome organization of HIV-1

The HIV-1 genome is a 9.2 kb positive-stranded RNA molecule. HIV-1 contains nine genes that encode fifteen viral proteins. Three of the open reading frames encode Gag, Pol and Env polyproteins, which are proteolytically processed into individual proteins [39]. Protease [41], cleaves Gag (p55) to generate structural proteins Matrix (p17), Capsid (p24), Nucleocapsid (p7), and p6 protein. The Gag-pol precursor is proteolytically processed into protease, reverse transcriptase and integrase (IN). Host endoprotease furin cleaves the Env precursor gp160 into gp120 and gp41 [42]. There are also two regulatory protein genes, transactivator [43] and regulator of expression of virion protein (Rev) and accessory proteins which are virion infectivity factor (Vif), viral proteins R (Vpr), viral proteins U (Vpu) and negative factor (Nef).

1.2.1.2 HIV-1 structure

The HIV-1 particle is roughly spherical in shape and is approximately 100 to 120 nanometers in diameter. The structure of HIV-1 is more complicated than most retrovirus. The virion is coated with a lipid envelope, which contains viral envelope glycoproteins (Envs) and host cell-derived lipid bilayers [44]. HIV-1 is composed of two copies of single-stranded RNA, which are enclosed by a conical capsid made up of a viral protein p24 and tightly bound to a viral protein p7, a matrix composed of a viral protein p17 surrounds the capsid. "Gag" proteins are composed of p24, p7, p17 viral proteins in HIV-1 [44]. On the surface of the mature virion, Envs are displayed in knobbed spike structure, formed from surface protein gp120 and transmembrane protein gp41. These two glycoproteins noncovalently bind to each other and form the heterodimers GP 160 complexes [45]. One Gp160 complex composed of three Gp120 subunits and three Gp41 subunits [46]. The central core of the virion is constituted of the Gag structural proteins: matrix (p17), capsid (p24), and nucleocapsid (p7). The nucleocapsid [37] protein p7 is located within the core, as a binding partner of viral RNA [47]. Inside the viral core, there are two identical copies of single-stranded RNA genome, which is associated with viral enzymes and accessory proteins [48].

1.2.1.3 Viral proteins

1.2.1.3.1 Viral structural proteins

Gag is a 55 kilodalton (kDa) precursor protein that is cleaved by PR to produce the mature proteins: MA (P17), CA (P24), p2, NC (P7) [42]. This protein is essential to the reproduction of HIV-1 virus and constitutes a necessary element for the HIV-1 virus particle construction. Also, Gag protein is involved in the virus assembly and budding process; binding between Gag and molecules in the host cell directs the accumulation of HIV components in multivesicular bodies (MVB) [50].

MA is a 17 kDa structural protein that is myristoylated at N-terminus as post-translationally modification [51].

NC is a 7 kDa nucleic acid chaperone protein that is able to chaperon nucleic acid rearrangement during viral assembly, by binding to genomic RNA [52].

1.2.1.3.2 Viral enzyme proteins

The Pol protein of HIV-1 is composed of three viral enzymes; PR, RT, and IN [53]. HIV-1 PR functions as a homodimer. PR (aspartic protease) is known for cleaving viral precursor proteins. HIV-1 RT (reverse transcriptase) is one of the unique features of HIV-1. This enzyme is highly error-prone, which results in a high rate of non-specific mutations in HIV-1 genome during viral replication. Since mutations arise in antigenic epitopes often present a novel epitope, these viruses will not be recognized by the initial immune responses [54]. HIV-1 integrase functions in a tetrameric form and facilitates the integration of viral DNA into the host genome.

1.2.2 HIV-1 pathogenesis

HIV-1 preferentially infects T cells, mainly CD4$^+$ T cells and those subsets of T cells that express C-C chemokine receptor type 5 (CCR5), particularly memory T cells [55]. Gp120 binds the CD4 receptor, which causes a conformational change in Gp120, leading to chemokine-binding of either CCR5 or CXC-chemokine receptor type 4 (CXCR4) co-receptor [56, 57]. Gp41 pulls target cell towards the virus, thus facilitating HIV-1 attach and fuse into target cells and subsequently release capsid components into the host cell. With the onset of immunodeficiency, the tropism switch which involves switching from using CCR5 to CXCR4 and infects naïve CD4$^+$ T cells also [55]. HIV-1 infection also resultant into the development of dysfunctional or tolerogenic DCs because it continuously exposed to viral proteins, such as Gp120. Progressive CD4$^+$ lymphocytes depletion, dysfunctional dendritic cells (DCs) and tolerogenic cytotoxic CD8$^+$ lymphocytes are some features of HIV-1 pathogenesis [58], which made HIV-1 distinct from other viruses. The progression of the HIV-1 virus is discussed below.

1.2.2.1 HIV-1 transmission

The initial phases of virus infection include three major steps: attachment, fusion, and entry. HIV-1 and simian immunodeficiency virus (SIV) can be transmitted as a free or cell-bound virus [59, 60]. In most cases, the initial HIV-1 infects through the genital tract and rectal mucosa [38]. Entry of HIV-1 to target cell requires CD4^{+} receptor to bind viral Env gp120 and co-receptor CCR5 or CXCR4 for further binding serving as targets for chemokine released from the virus, for example CXCR4 is the co-receptor for chemokine SDF-1 released following the infection with T-tropic strains of HIV-1 virus, while CCR5 is the co-receptor for the chemokine MIP-1 α and β as well as RANTES released following the infection with M-tropic strains of HIV-1 virus [61]. Subsequently, HIV-1 enters into lymphocytes or macrophages into local draining lymph nodes and then spreads all over the body through bloodstream [62]. However, the mechanism of how HIV-1 cross the mucosal epithelium is not known, and the virus may cross the mucosal barrier by transcytosis or directly contact with CD4^{+} T cells and DCs [62]. If an HIV-1 virus enters the human body through the bloodstream, infection would first take place in lymph nodes, where activated T cells and differentiated macrophages reside. Naïve T cells and undifferentiated monocytes circulating in blood would not be susceptible to the virus in early infection stage.

1.2.2.2 Courses of HIV-1 infection

Eclipse Phase
Following transmission of the virus, early-stage infection is an acute-like infection, and replication peaks between 2-4 weeks post infection [63]. In this stage, an abundance of the virus is generated in the peripheral circulatory system, and studies showed infection initiated by a single virus in ~80% of mucosal transmitted HIV-1 infection [64]. CD4^{+} T cells are found more susceptible to viral infection when they interact with infected DCs than other CD4^{+} T cells [65]. Thus, presentation of HIV-1 Ag by DCs is both advantageous and disadvantageous.

Peak viremia
In addition to other lymphoid tissues, HIV-1 preferentially spreads to gut-associated lymphoid tissues, which contain large numbers of activated CD4^{+}CCR5^{+} memory T cells. 20% of CD4^{+} T cells in GALT (Gut-Associated Lymphoid Tissue) are infected with HIV-1, and ~60% of uninfected CD4^{+} T cells become activated and subsequently become dead due to apoptosis,

resulting in the depletion of up to 80% of CD4$^+$ T cells in GALT during this period. Large numbers of CD4$^+$ T cells loss and releasing of apoptosis micro particles would suppress immune function [66, 67]. Subsequently, the HIV-1 infection spreads into the blood and other target cells, reaching peak viremia, usually more than a million RNA copies per ml of blood and blood CD4$^+$ T cell counts drop to <200-400 cells/µl.

Viral set point

The Human body responds to HIV-1 infection by developing both humoral and cellular immune responses, leading to a drastic decrease in the viremia. Viral load decreases over 12-20 weeks to reach a more stable level, known as the viral set point. CD4$^+$ T cells number returns to a normal level in the blood but not in the GALT [68, 69]. The viral set point is maintained by the balance between virus turnover and immune responses in the absence of antiretroviral drugs. Under the pressure of adaptive immune responses, virus diversification occurs, and multiple escape mutants are selected during this period.

Symptomatic stage and AIDS

HIV-1 mutants generated in the asymptomatic stage are capable of avoiding previously generated immune responses and continuously replicate in CD4$^+$ T helper cells. CD4$^+$ T helper cells are master regulators of the adaptive immune system, and without functional T helper cells, the immune system of the HIV-1 patient become immune-compromised. Subsequently, the HIV-1 patient becomes not only less able to fight against HIV-1 infection but also develop susceptibility to other infections such as opportunistic microbial infections, herpes viruses and more susceptible to the development of cancers [38]. Symptoms of HIV-1 infected patients in this stage are commonly weight loss, repeated respiratory tract infections and skin rashes [70]. Patients exhibit complex syndrome associated with HIV-1 are called AIDS patients.

1.2.3 Immune responses in HIV-1 infection

1.2.3.1 Innate immune responses in HIV-1 infection

It was shown, multiple TLR7/8 ligands encoded by HIV-1 ssRNA can mediate direct activation of the immune system *in vitro [71]*. The plasmacytoid DC (pDC) in contrast to the conventional DC (cDC), constitutively expresses TLR7 and TLR9. The pDC endocytoses HIV-1 virions via Env interaction with CD4, and the virion genomic RNA is detected by TLR7 within the endocytic compartment [72]. Stimulation of TLR7/8 induces the production of several antiviral and immunomodulatory cytokines such as IFNα. A variety of immune cells contribute to innate immune responses against HIV-1 infection, such as DCs, NK and NKT cells [73]. First detectable innate immune responses are the increase in the level of proteins, such as serum amyloid A, and cytokines. As the infection progress, levels of IL-1, IL-15. Type-I IFNs and CXC-chemokines increase quickly but transiently, while IL-18, TNF, IFN-γ and IL-22 sustain at a high level [74]. Lapenta C, *et al.* showed that Type I IFNs inhibit HIV-1 replication in vivo [75]. Also, type I IFNs, IL-15 and IL-18 showed antiviral activity to enhance innate and adaptive immune responses, however, over-secretion of these cytokines may promote viral replication [75].

DCs play a multifaceted role in HIV-1 infection. DC express high amounts of the HIV entry receptors CCR5 and CXCR4, as well as relatively low levels of CD4, allowing gp120 binding and attachment of HIV virions. Unlike other infections, DCs are not fully activated in HIV-1 infection, and the number of DCs is reduced markedly in circulating DCs but not in lymph nodes. In HIV-1 infection DCs secrete lower levels of IL-12 [74]. IFNα secreted by DCs retains at a normal level, enhancing adaptive immune responses. However, DCs produce indoleamine 2, 3-dioxygenase (IDO), and IDO might suppress HIV-1 specific immune responses by inducing the differentiation of T cells to T_{reg} cells. Furthermore, DCs might transfer HIV-1 to T cells through 1) DC and T cell contact in Ag-presentation with viral binding particles on DC surface; 2) exocytic pathway, where HIV-1 associated exosomes released by DCs contact with CD4^{+} T cells and taken up by CD4^{+} T cells through membrane binding and fusion [76, 77].

NK cells and NKT cells become activated during an early stage of HIV-1 infection. Alter GD *et al.* showed that NK cells proliferate and enhance immune responses prior to viremia peak period *in vivo* [78]. NK and NKT cells have been demonstrated to control HIV-1 replication through the release of antiviral cytokines and chemokines, leading to cytolysis of virally infected cells. NK cells involved in the control of HIV-1 infection by an expansion of KIR3DS1 and/or KIR3DL1

expressing cells, which have a definite role in determining the viral set point [79]. The timing of NK cell-mediated antiviral activity remains uncertain. However, some studies suggested that NK cells might have more influence at later time points in HIV-1 infection [73]. NK cells do not select viral escape mutants, but they may account for some viral mutants, which are selected by early T cell responses at viral set point [80].

1.2.3.2 Adaptive immune responses in HIV-1 infection

Cellular immune responses

Upon exposure of HIV-1, the primary immune responses are initiated. Ag-presenting cells (such as DCs) engulf the viral particles, and internalize, process and present viral peptides on the cell surface through its MHC class-I and-II molecules. DCs then activate both CD4$^+$ T cells and CD8$^+$ T cells, triggering viral-specific cell-mediated immune responses. Activated HIV-1-specific CD8$^+$ CTLs kill virally infected cells, including infected CD4$^+$ T cells. CD8$^+$ CTLs also suppress HIV-1 replication and prevent viral spread by secreting different types of cytokines (IFN-γ and TNF-α) and chemokines [81].

Several studies showed that HIV-1 specific CD8$^+$ T cell responses were developed during an early stage of HIV-1 infection before Abs were developed [82]. As viremia peaks, the first CD8$^+$ T cell responses to HIV-1 infection also approaches to its peak, and viremia declines ~1-2 weeks after CD8$^+$ T cell responses peak [83, 84]. T cell responses specific to Env and Nef usually occur at first, and T cell responses to other viral proteins, such as Gag p24 and Pol, tend to occur later [85].

The sequence of viral genome starts to change due to error-prone RT of HIV-1, and mutations occur following the CD8$^+$ T cell responses peak, and a viremia declines to a viral set point. Neutralizing antibodies-selected viral mutations occur later [80]. T cell- and antibody-mediated viral escape mutations involve mostly multiple amino acid changes until the fittest type of mutation is selected. T cell selected mutants could replace the original HIV-1 virus within ten days, followed by replacement of mutations at different epitopes and this mutation mode

continues throughout the whole course of HIV-1 infection[80]. The rapid loss of founder viral sequence indicated the effectiveness of early T cell responses.

Mutations generated during HIV-1 replication (due to error-prone nature of HIV-1 RT) result in new epitopes being presented on MHC-I and MHC-II complexes, which inhibits development and maturation of HIV-1 specific CD4^{+} and CD8^{+} T cells because of mutant variant of epitopes presented on the APCs [86, 87]. Continuous T cell activation against HIV-1 viral escape mutants lead to T cell exhaustion, and high levels of microbial components and inflammatory molecules circulating in blood of HIV-1 infected patient, thus causing increased expression of programmed death-1 marker on monocytes, which is involved in apoptosis and negative regulation of T cells activation, leading to T cell dysfunction or tolerated T cells [88]. However, strong HIV virus-specific CD8^{+} CTL responses are important in controlling infection and its progression [89, 90].

HIV-1 infects CD4^{+} T cells and depletes memory CD4^{+} T cells significantly [91], and CD4^{+} T cell responses to HIV proteins have been difficult to show, but several epitopes, in Gag particularly, for CD4^{+} T cells have been identified [92]. The first CD8^{+} T cell responses could be strong with even suboptimal help from weakened CD4^{+} T cell repertoire. In the long term, the function of memory T cells could be impaired as CD4^{+} T cells are depleted [93].

Humoral immune responses

The antibody-mediated humoral response is crucial in neutralizing cell-free viruses and for activating cell-mediated Ab-dependent cytotoxic effect thus suppressing the spread of infection. Non-neutralizing antibodies are first produced against the structural proteins, such as p24, p17 and Gag are usually detected ~ 4-6 weeks post HIV-1 transmission, followed by neutralizing Abs against the envelope proteins (such as Gp120 and Gp41) develop even slower, ~12 weeks or longer against autologous virus and may take up to years to develop Abs against heterologous virus [94]. Antibodies show neutralization against heterologous virus were observed in ~20% of patients post infection [94, 95], and the production of these rare, late, broad-specificity neutralizing antibodies might relate to genetic factor and maturation of the antibody responses to HIV-1. Based on the study of Tomaras GD. et al. [96], the initial gp41-specific IgG and IgM responses did not affect the viral load significantly in the early stage of HIV-1 infection. During

this stage, IgA, IgG, and IgM Abs are produced, but these antibodies exhibit narrow specificity, and they did not select escape viral mutations, indicating that these antibodies were not efficient against HIV-1.

Another level of difficulties that immune system encounters, regarding the generation of Abs against mutated forms of the initial HIV-1 strain, is that the outnumbered $CD4^+$ T cell that prevents efficient helper activity for maturation of new B cell populations. HIV-1 highly replicates in mucosal microenvironments, such as Peyer's patches leading to apoptosis and loss of more than 50% B cells at germinal centers in early-stage infection [97]. The loss of germinal centers may lead to defects in generation of high-affinity HIV-1 antibodies and result in neutralizing antibodies production delay as well [97]. Existing neutralizing Abs fail to recognize viral progeny because of continuous changes in the glycosylation sites and structure of Gp120 epitopes [43]. Due to all these factors, humoral immune response against HIV-1 is inefficient.

1.2.4 HIV-1 treatment

Currently, there is no cure for HIV-1. The treatment for HIV-1 in the market aim at developing regiments that interfere with distinct steps of the viral replication process. Antiretroviral drugs therapies (ARTs) that have been licensed so far are divided into six classes [98]:

a) Entry inhibitors interfere with the viral binding, fusion, and entry of HIV-1 to the host cell;

b) Nucleoside-analog Reverse Transcriptase Inhibitors (NRTIs) prevent other nucleosides from being incorporated as an integrated viral DNA chain because they lack a 3 hydroxyl (3'OH) group;

c) Non-nucleoside-analog RT inhibitors (NNRTIs) interfere with viral DNA incorporation by attaching to polymerase active site;

 d) Integrase inhibitors: these drugs decrease the activity of a viral enzyme, which is responsible for the integration of viral DNA into the DNA of infected cell;

 e) Protease inhibitors block viral protease enzyme to produce mature virions upon budding from the host membrane.

HAART consists of two NRTIs and either one of the following inhibitors: protease inhibitors, integrase inhibitors and non-nucleoside reverse transcriptase inhibitors, this is known as

combination therapy [99]. HAART is currently being used as an HIV-1 treatment regimen; it has been reported effective in reducing viral titers to a level below detection limit and recovery of partial CD4$^+$ T cells. However, HAART is limited by its high cost, drug unavailability, side effects, and drug resistance will be developed ultimately [98, 100].

1.2.5 HIV-1 latency

HAART reduces plasma HIV-1 RNA from 10,000 copies/ml to 50 copies/ml, which is below the detection level. However, a stable latent reservoir consisting of infected resting CD4$^+$ T cells is developed during the early stage of HIV-1 infection [100]. The ability of a pathogenic virus to reside within a cell, as part of the viral life cycle is known as viral latency [101]. The result of the viral latency is that the virus can be reactivated and produce a large amount of viral progeny without the host being infected with the new virus. The viral latency presents a major barrier to the treatment and functional cure/ sterilizing cure for HIV-1 although there is less than one per million resting CD4$^+$ T cells harbor latent virus. Hosts of the latent reservoir can be naïve CD4$^+$ T cells and memory CD4$^+$ T cells. Integration is a vital step in HIV-1 viral replication. Pre-integration latency is generated in the cell without integration, and it is unstable and decays rapidly [102]. Post-integration latency happens mostly through the infection of activated CD4$^+$ T cells, and it is integrated but transcriptional silent. A small part of activated CD4$^+$ T cells become resting memory CD4$^+$ T cells, which can be reversed upon encountering their cognate antigen or another stimulus [103].

Therefore, it is difficult to achieve the goal of complete viral eradiation because of HIV-1 latency properties and the nature of host cells. However, with a better understanding of the mechanism of HIV-1 latency, it gives us an idea focusing on latent HIV-1 reservoirs attack. Current strategies for purging viral latency focus on reactivating provirus and inducing global T cell activation, however, the effect is limited due to CTL exhaustion.

1.2.6 Chronic infections

CTL exhaustion has been studied intensively since a decade ago, and it is defined by poor effector function, sustained expression of inhibitory molecules and memory T cells inflation. It is a state that can be observed in many chronic infections and cancer [104]. CTL exhaustion occurs mostly in conditions like antigen-persistence, and infections with high viral replication, although some precise features may vary during different infections. CTL exhaustion develops during infections such as HIV, lymphocytic choriomeningitis virus (LCMV), hepatitis C virus (HCV), hepatitis B virus (HBV), and tumor outgrowth [104].

HIV-1 infection does not lead to highly functional memory T cell pool and resulting memory T cells display defects in phenotypes and functions gradually and in a stepwise manner [105]. The decrease in T-cell proliferation, cytolytic ability, and IL-2 secretion, is usually the first sign of T cell exhaustion (CD8$^+$ T cells), followed decrease in TNF-α production but IFN-γ remains unaffected [105]. IL-2 is a type of interleukin regulates and activates white blood cells, and it is critical against microbial infection. TNF-α is involved in systemic inflammation, and the macrophage mainly secretes it. IFN-γ is a cytokine important against viral, bacterial infections in innate and acquired immunity. Exhausted T cells express increased and prolonged inhibitory molecules such as programmed death-1 (PD-1), programmed death ligand-1 (PD-L1), and lymphocyte-activation gene 3 (LAG-3). Severe exhaustion might result in complete function loss of virus-specific cells (loss of the ability to produce IFN-γ and β-chemokines), and physical deletion of virus-specific T cells. In HIV-1 infection, CD4$^+$ T cells loose effector functions like CD8$^+$ T cells but less is known about CD4$^+$ T cell dysfunction [106]. CD4$^+$ T cells are the source of IL-21 and also produce IL-10. Given that IL-21 has pleiotropic effects therefore it is probable that CD4$^+$ T cell defects affecting IL-21 availability may potentially impact several facets of the immune response in chronically infected patients [105]. Several recent reports have demonstrated a pivotal role for IL-21, which is mainly produced by CD4 T cells, in sustaining CD8 T-cell responses to chronic infection. A defect in effector CD4$^+$ T cells might lead to the defect in sustain antiviral CD8$^+$ T cells and more diverse functional properties than its effect on CD8$^+$ T cells [107]. PD-1, a member of the CD28 family, plays a role in inhibiting T cell proliferation and builds peripheral tolerance. Based on several studies, PD-1/PD-L1 pathway constitutes a topic of great interest in exhaustion research [108, 109]. The level and frequency of PD-1 expression positively correlate with viral loads and negatively correlate with CD4$^+$ T cell quantity. PD-1/PD-

L1 pathway blockade with anti-PD-1 antibody would enhance proliferation and functionality of $CD4^+$ and $CD8^+$ T cells, thereby promoting cytokine production, and reducing viral loads [110]. In contrast to immunosuppressive cytokines noted above, positive regulators of T cell responses can enhance immunity during chronic infection [111]. IL-2 and IL-7 treatment have provided a role in rescuing T cell exhaustion as well as other aspects of immune responses during chronic viral infections [112, 113]. IL-2 and IL-7 have been tested as effective candidates for treatment of chronic infection in animal challenges.

1.3 HIV Vaccines

1.3.1 Traditional and novel vaccine approaches

An immune response can be not only induced by infection but also by immunization [2]. The last century has seen significant accomplishment in the development of vaccines against many pathogens such as smallpox, polio, rabies, measles and hepatitis B, and those related diseases have been successfully controlled. Inactivated and live-attenuated viruses have shown their effectiveness in past vaccines; however, safety concerns were raised against using live-attenuated vaccines. The reversion of non-pathogenic virus to the virulent form occurred in the case of Simian Immunodeficiency Virus (SIV)-live-attenuated vaccine and HIV-1-live-attenuated vaccine [114]. Inactivated or killed viral vaccines are considered to be safer and effective in controlling diseases such as polio and influenza. Nevertheless, HIV-1 inactivated vaccine studies failed to induce protective or therapeutic immunity [115].

Novel vaccine approaches include live vector-based, plasmid DNA-based, and genetically modified vaccine vectors have been developed [116]. Recombinant vaccines were found to induce cellular and humoral immune responses to both the original virus and the inserted viral gene segment. Enveloped glycoprotein gp120 is a 120 kDa glycoprotein, forming the spikes exposed on the surface of the HIV particle. Because of its central role in mediating virus binding to cellular CD4 and facilitating co-receptor interaction, the gpl20 of Env has been used as a principle HIV-I vaccine immunogen [117, 118] , such vaccines exhibit antigen-specific CD4$^+$ and CD8$^+$ T cell responses. Even so, they failed to deliver clinical efficacy or show consistent protection.

1.3.2 Dendritic Cell (DC) vaccine

DCs are the most powerful APCs, often referred as "professional" APCs or "sentinels," And are involved in both innate and adaptive immune responses. DCs play a crucial role in identifying invading pathogens and sensitizing Ag-specific T cells [4]. Immature DCs internalize pathogens such as virus, bacteria and other microbial pathogens through receptor-mediated endocytosis and

toll-like receptors-mediate phagocytosis or C-type lectins. Upon exposure to antigens, DCs are activated and move to lymph nodes to stimulate adaptive immune responses. Then DCs present antigenic peptides on its MHC class-I and -II molecules through which Ag-specific CD8^{+} and CD4^{+} T cells are activated (signal 1) respectively. DCs also provide co-stimulatory (signal 2) and cytokine (signal 3) signals [119]. Mature DCs express elevated levels of co-stimulatory molecules, such as CD86, CD80, peptide MHC class-I and -II complexes and adhesion molecules result in enhanced secretion of IL-12 and IL-23 cytokines and reduced expression of endocytic or phagocytic receptors facilitating T cell response. Because of their immune-regulatory effect, vaccinations with tumor antigen-presenting DCs have been proposed as a treatment modality for chronic infections.

Vaccination with whole DCs

DCs bearing specific tumor peptides DCs loaded with total tumor lysate antigens, and tumor mRNA- pulsed/ transfected DCs have been studied for their therapeutic potential [120]. These studies found that administration of tumor peptides with DCs can lead to dramatic immune-stimulation. DC vaccines have shown to break disease-associated tolerance, which is beneficial in the treatment of cancer or chronic infection [121]. DCs cause the stimulation of both naive and memory T cells. Autologous DC vaccine has been used for the treatment of prostate cancer, increasing the survival rates for patients [122]. According to Levy Y et al., DCs loaded with HIV-1 lipo-peptides lead to increased levels of effector cytokines, and they generate HIV-1 specific poly-functional immune responses [123]. It was shown that DC vaccines pulsed with autologous inactive HIV-1 decrease viral load in distinct conditions [124, 125].

Although DC vaccines have obtained highest success rate among other vaccines, the efficiency of which is still limited when it comes to clinical trials. DC-induced CTL responses are dependent on CD4^{+} T cells help, notwithstanding, CD4^{+} T cells are dysfunctional or even depleted in HIV-1 infected patients. Therefore, DC-based vaccines need additional improvement before they could be considered as practical alternatives to HIV-1 treatment [66].

1.3.3.1 Adenovirus

Adenovirus (AdVs) is a non-enveloped virus, and it contains a linear double-stranded DNA genome [126]. Adenoviral transcripts are classified as early genes (E1, E2, E3, and E4), intermediate genes, and late genes. Early genes are important for viral replication, intermediate genes are involved in viral encapsidation and virus packaging, while late genes are crucial for the assembly of virion before releasing. Till now, there are over 50 AdV serotypes have been identified in human, human serotype 5 is most commonly used as recombinant AdV strains in laboratory [126]. AdVs are divided into several species, species A-G. AdV-caused infections can be self-limiting, resulting in respiratory diseases (species B and C), gastroenteritis (species F and G). Thus, infections caused by AdVs vector-based pathogens might be influenced by the immune responses against the AdV vector. Due to its high efficiency, broad infection range, and a remarkable capacity to insert a gene, AdVs are very handy tools for the expression of transgenes, and they are the most commonly used gene therapy vehicle in transgene clinical trials [127].
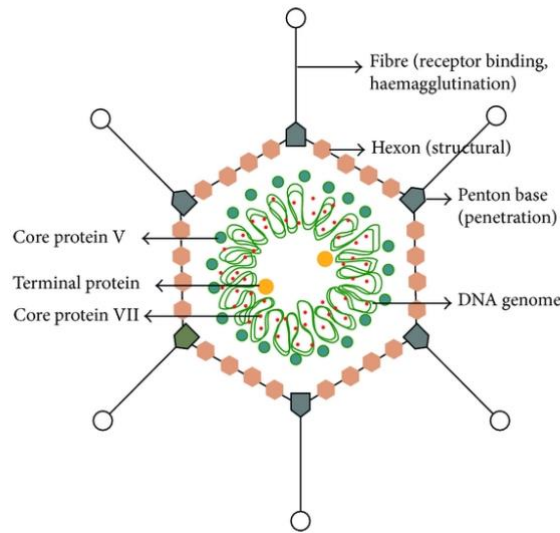
Figure 1.2 Structure of Adenovirus [128] (Copyright © 2013 W.WYIp and W. Qasim. This Figure is an open access under the Creative Commons Attribution Liscense, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Original figure is attached at the end of this thesis). Adenoviruses were isolated from human adenoid tissue firstly in 1953. AdVs are non-enveloped, double-stranded DNA viruses, the size of AdVs range from 65~ 80 nm in diameter [128]. For more detailed explanations see text.

AdVs infect a broad range of mammalian cells, including dividing and non-dividing cells [129]. The replication of AdV is epi-chromosomal, which is not like retroviruses that need to integrate into genome for replication. The infection of AdV is initiated by the contact between its fiber proteins in the capsid and coxsackie AdV receptor (CAR), which exists on an epithelial cell surface, DCs, macrophages, and integrin [129, 130]. After attachment, adenovirus is endocytosed and then transported into the nucleus for gene transcription. AdVs are highly antigenic, and they can induce strong cell-mediated immune responses.

Also, the capsid of AdVs can be easily modified and used to target the particular type of cells. We use "AdEasy-like" system to produce AdVs, combine the gene of interest and shuttle vector (4-8kb) firstly. Shuttle vector transfers the gene of interest to AdV backbone cosmid by homologous recombination, and then the recombined plasmid is ready for replication in HEK293 cell line and purification (overviewed in Figure 1). We use "AdEasy-like" strategy to produce AdV_{IL-21}, AdV_{OVA}, and AdV_{41BBL} (explained later in text) in our laboratory.

1.3.3.3 Exosome

Exosomes (EXOs) are small lipid bilayers vesicles (30-100nm in diameter) released by red blood cell, dendritic cell, tumor cell and other cell types [131]. Exosomes are formed through the fusion between the endosomal external membrane and plasma membrane [132]. EXOs are eliminated as waste from cells but, they are crucial in transferring membrane-associated molecules between cells as multiple receptors and ligands present on the surface [133]. Due to immune stimulatory properties, EXOs have attracted lots of attention nowadays especially in the immunological context [134]. Lysosomal or endosomal proteins are observed in DC- and B-cell released EXOs. Enzymes involved in metabolism and tetraspanin family proteins such as CD81, CD63, and CD82, are also found in EXOs [135].

DC-released EXOs have attracted greater attention of immunologists because of the expression of MHC I, MHC II molecules, costimulatory molecules (CD80 and CD86), and adhesion molecules (CD11c and CD54), which have been proven to induce antigen-specific T cell responses *in vivo*

[136]. According to Zitvgel et al., EXOs derived from DCs or tumors cells are involved in transferring Ag-specific MHC complexes to mature DCs, thus stimulating Ag-specific T cell responses [125]. Some studies also show that EXOs derived from the tumor can cause regression of tumors in skin and lymph nodes [137]. DC-derived EXOs are being used in a phase-I clinical trial to treat metastatic malignancies in human at Institute Gustave Roussy and Institute Curie, France. It is also demonstrated that CD4^{+} T cells vaccine, which has acquired DC-derived EXOs, stimulates stronger Ag-specific immune responses than mature DC-based vaccine [138].

1.3.3 EXO-targeted T cell-based vaccine

Membrane molecules are exchanged between different types of immune cells including DCs [139], T cells [140], B cells, NK cells [141], basophils [139] and macrophages [142]. An immunological synapse form between APCs and Ag-specific CD4^{+} T cell is a critical event for activation of T cells [143, 144]. It has been reported that, during formation of the immunological synapse, APC surface molecules get transferred to T helper cells through the process of internalization and recycling pathways [145, 146]. Based on this principle, our lab has proposed a new dynamic model of Ag presentation [147]. According to this model, CD4^{+} T cells stimulated with ovalbumin (OVA)- specific DCs (DC$_{OVA}$) acquire Ag-presenting machinery, such as pMHC-I and –II complexes, and co-stimulatory molecules (CD54 and CD80) from APCs (DC$_{OVA}$), and function like APCs (called CD4^{+} T helper Ag-presenting cells (Th-APCs)) [148]. These Th-APCs can stimulate naïve Ag-specific CD8^{+} T cells to become effector and memory CD8^{+} T cells, thus able to provide protection against tumors.

Our lab [138] previously developed a novel EXO-targeted T cell vaccine using Concanavalin A (ConA-stimulated) polyclonal CD4^{+} T cells co-cultured with EXOs derived from Ag-stimulated DC. The novel DC-released EXO-targeted T cell-based vaccine with the uptake of antigen-specific EXOs stimulated antigen-specific CTL responses and antitumor immunity [149]. T cells have the ability to take up DC-derived EXOs, but with lower efficiency compared to direct cell-to-cell interactions [138, 150]. T cells acquire the ability of Ag-presenting activity through uptake of EXOs. Activated T cells taking up Ag-specific mature DC-derived EXOs are equipped with all exosomal molecules and become T-APCs. Later, we established an adenovirus-induced chronic

infection model by i.v. infection of C57BL/6 mice with a recombinant adenovirus (AdV$_{OVA}$) expressing ovalbumin (OVA). Similar to the situation in the LCMV clone 13-induced chronic infection, our mice with the AdV$_{OVA}$-induced chronic infection demonstrated that OVA-specific CD44$^+$PD-1$^+$LAG-3$^+$ memory CTL (mCTL) inflation. These mCTLs were also functionally defective and exhausted [151]. We also found that the PD-1 blockade efficiently converts CTL exhaustion in the OVA-specific chronic infection model [152].

We recently developed a novel ovalbumin (OVA)-specific exosome (EXO)-targeted T cell-based (OVA-T$_{EXO}$) vaccine by using non-specific polyclonal T cells with the uptake of OVA-specific dendritic cell (DC)-released EXO *via* the CD54/LFA-1 interaction [138]. We demonstrated that the OVA-T$_{EXO}$ vaccine was able to directly stimulate potent OVA-specific CTL responses in the absence of CD4$^+$ T cell help by counteracting CD4$^+$25$^+$FoxP3$^+$ regulatory T (Treg) cell suppression [138, 149]. We also developed an HIV-1 Gag-specific T cell-based vaccine, Gag-T$_{EXO}$, by using non-specific polyclonal T cells with the uptake of Gag-specific DC-released EXO and demonstrated that the Gag-T$_{EXO}$ vaccine triggered potent Gag-specific immunity against Gag-expressing tumors in transgenic human leukocyte antigen (HLA)-A2 mice [153]. To enhance its immunogenicity, we generated 4-1BBL-expressing OVA-T$_{EXO}$ and Gag-T$_{EXO}$ vaccines, demonstrated that the former one triggered potent therapeutic immunity [154]. It also induced an efficient conversion of CTL exhaustion via its CD40L-dependent signaling activation of the mammalian target of rapamycin complex 1 (mTORC1) pathway in chronic infection models [151].

1.4 HYPOTHESIS

We hypothesize that novel transgene IL-21-engineered OVA- and Gag-specific T cell-based OVA-T$_{EXO/IL-21}$ and Gag-T$_{EXO/IL-21}$ vaccines efficiently stimulate therapeutic immunity and convert CTL exhaustion significantly in chronic infection.

1.5 OBJECTIVES

1. Construction of recombinant adenovirus AdV_{IL-21} expressing IL-21;

2. Generation of novel transgene IL-21-engineered OVA- and Gag-specific T cell-based OVA-$T_{EXO/IL-21}$ and Gag-$T_{EXO/IL-21}$ vaccines;

3. Assessment of OVA-$T_{EXO/IL-21}$-induced OVA-specific CTL responses and anti-tumor immunity in wild-type C57BL/6 mice;

4. Development of a chronic infection model with OVA-specific CTL exhaustion by i.v. infection of C57BL/6 mice with recombinant AdV_{OVA};

5. Assessment of OVA-$T_{EXO/IL-21}$-converted CTL exhaustion in chronic infection;

6. Assessment of Gag-$T_{EXO/IL-21}$-stimulated therapeutic immunity in chronic infection.

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CHAPTER 2 A TRANSGENE IL-21-ENGINEERED T CELL-BASED VACCINE POTENTLY CONVERTS CTL EXHAUSTION VIA THE ACTIVATION OF THE mTORC1 PATHWAY IN CHRONIC INFECTION

Xueying Zhang[1,2], Aizhang Xu[1,3], and Jim Xiang[1,2,3]

[1]Cancer Research Unit, Saskatchewan Cancer Agency, Saskatoon, Saskatchewan, Canada;

[2]School of Public Health, University of Saskatchewan, Saskatoon, Saskatchewan, Canada;

[3]Department of Oncology, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada;

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Correspondence: Dr. Jim Xiang, 4D30.1, Health Science Building, 107 Wiggins Road, Saskatoon S7N 5E5, Saskatchewan, Canada. E-mail: jim.xiang@usask.ca

All experiments were performed by Xueying Zhang under supervision of Dr. Jim Xiang and Dr. Aizhang Xu

2.1 Abstract

$CD8^+$ cytotoxic T lymphocyte (CTL) exhaustion is one of the major obstacles for the effectiveness of virus control in chronic infectious diseases. We previously generated novel ovalbumin (OVA)-specific 41BBL-expressing OVA-T_{EXO} and human immunodeficiency virus (HIV-1) Gag-specific Gag-T_{EXO} vaccines, inducing therapeutic immunity in wild-type C57BL/6 (B6) mice, and converting CTL exhaustion in recombinant OVA-specific adenovirus AdV_{OVA}-infected B6 (AdV_{OVA}-B6) mice with chronic infection. IL-21 cytokine plays an important role in controlling chronic infections. Therefore, in this study, we constructed recombinant transgene IL-21-expressing AdV_{IL-21}, and generated IL-21-expressing OVA-$T_{EXO/IL-21}$ and Gag-$T_{EXO/IL21}$ vaccines, or control vaccines (OVA-$T_{EXO/Null}$ and Gag-$T_{EXO/Null}$) by infecting OVA-T_{EXO} and Gag-T_{EXO} cells with AdV_{IL-21} or the control AdV_{Null}, lacking transgene, and assessed their effects in B6 or AdV_{OVA}-B6 mice. We demonstrate that both OVA-$T_{EXO/IL-21}$ and control OVA-$T_{EXO/Null}$ vaccines are capable of converting CTL exhaustion in chronic infection. However, the OVA-$T_{EXO/IL-21}$ vaccine rescues exhausted CTLs more efficiently by increasing stronger CTL proliferation and effector cytokine IFN-γ expression than the control OVA-$T_{EXO/Null}$ vaccine in AdV_{OVA}-B6 mice with chronic infection, though both vaccines stimulated comparable OVA-specific CTL responses and protective immunity against OVA-expressing BL6-10$_{OVA}$ melanoma lung metastasis in wild-type B6 mice. In vivo, the OVA-$T_{EXO/IL-21}$-stimulated CTLs up-regulate phosphorylation of mTORC1-controlled EIFE and expression of mTORC1-regulated T-bet molecule more efficiently than the control OVA-$T_{EXO/Null}$-stimulated ones. Importantly, the Gag-$T_{EXO/IL21}$ vaccine induces stronger Gag-specific therapeutic immunity against established Gag-expressing BL6-10$_{Gag}$ melanoma lung metastases than the control Gag-$T_{EXO/Null}$ vaccine in chronic infection. Therefore, this study should have a strong impact on developing new therapeutic vaccines for patients with chronic infections.

2.2 Introduction

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

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

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

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

We previously developed a novel ovalbumin (OVA)-specific exosome (EXO)-targeted T cell-based (OVA-T$_{EXO}$) vaccine by using non-specific polyclonal T cells with the uptake of OVA-specific dendritic cell (DC)-released EXO *via* the CD54/LFA-1 interaction [29]. We demonstrated that the OVA-Texo vaccine was able to directly stimulate potent OVA-specific CTL responses in the absence of CD4$^+$ T helper cell by counteracting CD4$^+$25$^+$FoxP3$^+$ regulatory

T (Treg) cell suppression [29, 30]. We also developed an HIV-1 Gag-specific T cell-based vaccine, Gag-T$_{EXO}$, by using non-specific polyclonal T cells with the uptake of Gag-specific DC-released EXO and demonstrated that the Gag-T$_{EXO}$ vaccine triggered potent Gag-specific immunity against Gag-expressing tumors in transgenic HLA-A2 mice [31]. To enhance its immunogenicity, we generated 4-1BBL-expressing OVA-T$_{EXO}$ and Gag-T$_{EXO}$ vaccines, and we demonstrated that the former one triggered potent therapeutic immunity [32]. It also induced an efficient conversion of CTL exhaustion via its CD40L-dependent signaling activation of the mTORC1 pathway in chronic infection models [18].

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

2.3 Materials and methods

2.3.1 Reagents, cell lines and animals

Chicken ovalbumin (OVA) and carboxylfluorescein succinimidyl ester (CFSE) were obtained from SIGMA-Aldrich Canada Ltd, Oakville, Ontario, Canada. Phycoerythrin [17]-labeled anti-T-bet and PE-Cy5-labeled anti-IFN-γ antibodies (Abs) were obtained from BD Biosciences (Mississauga, ON, Canada). Biotin-labeled anti-T-bet, anti-phosphor-EIF4E (S209), anti-CD44, anti-PD-1, anti-PD-L1 and anti-LAG3 Abs were obtained from eBioscience (San Diego, CA). PE-labeled H-2K^b/OVA$_{257-264}$ tetramer (PE-tetramer) and Fluorescein isothiocyanate (FITC)-labeled anti-CD8 Ab were obtained from Beckman Coulter (Miami, FL). Rabbit anti-CD9 and rabbit anti-LAMP-1 Abs were obtained from BD Bioscience. The H-2K^b-restricted OVAI (OVA$_{257-264}$, SIINFEKL) peptide and the control Lewis lung carcinoma H-2K^b-restricted MutI (FEQNTAQP) peptide were obtained from Multiple Peptide Systems (San Diego, CA). All cytokines were obtained from Peprotech (Peprotech, Rocky Hill, NJ). Recombinant adenoviral vectors, including transgene OVA-expressing AdV$_{OVA}$, transgene 41BBL-expressing AdV$_{41BBL}$ and the no transgene-expressing AdV$_{Null}$ were available in our laboratory [18, 32]. Adenoviral vector AdV$_{IL-21}$ expressing mouse IL-21 was constructed by the insertion of IL-21 open reading frame of pUNO1-mIL-21 vector (InVivoGen, San Diego, CA) into the pShuttle-CMV vector (Stratagene, La Jolla, CA) by recombinant technology (**Figure 2.1A**). The Pmel-digested pShuttle-CMV-mIL-21 was transformed into BJ5183 *Escherichia coli* cells containing pAdEasy-1 backbone vector for the homologous recombination. The recombinant AdV vector was linealized by PacI digestion, it was transfected into HEK-293 cells using Lipofectamine 2000 (Invitrogene, Carlsbad, CA) to generate AdVIL-21 expressing transgene IL-21. AdV$_{IL-21}$ was amplified in HEK-293 cells, it was purified by a series of cesium chloride ultracentrifugation (**Figure 2.1A**). The highly lung metastatic, OVA- and Gag-expressing BL6-10$_{OVA}$ and BL6-10Gag mouse B16 melanoma cell lines were available in our laboratory [31-33]. IL-21 enzyme-linked immunosorbent assay (ELISA) kit was obtained from R&D Systems Inc, Minneapolis, MN. Female C57BL/6 (B6) and OVA-specific T cell receptor (TCR) transgenic OTI mice were obtained from the Jackson Laboratory (Bar Harbor, MA). All experiments were performed

according to protocols and guidelines approved by the Animal Research Ethics Board, University of Saskatchewan.
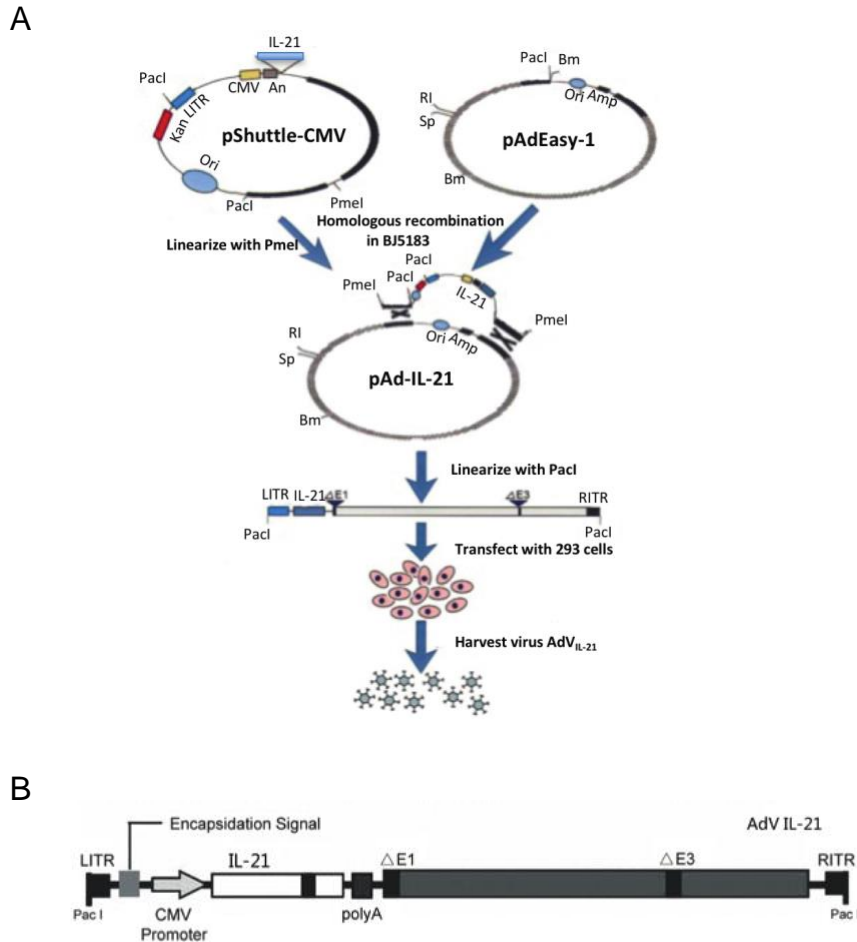

Figure 2.1 Diagram of the production of AdV$_{IL-21}$. **(A)** Mouse IL-21 open reading frame was inserted pShuttle-CMV vector by recombinant technology to get pShuttle-CMV-mIL-21. The Pmel-digested pShuttle-CMV-mIL-21 was then transformed into BJ5183 *Escherichia coli* cells for homologous recombination. The recombinant AdV vector was linealized by PacI digestion, and then transfected into HEK-293 cells to generate AdV$_{IL-21}$. AdV$_{IL-21}$ was purified by a series of cesium chloride ultracentrifugation. **(B)** Schematic representation of adenoviral (AdV) vector AdV$_{IL-21}$. The E1/E3 depleted replication-deficient AdV is under the regulation of the cytomegalovirus (CMV) early promoter. ITR, inverted terminal repeat.

2.3.2 Preparation of dendritic cells and dendritic cell-released exosomes

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

2.3.3 Assessment of the uptake of EXO by polyclonal T cells

Polyclonal naïve $CD8^+$ T cells were isolated from WT B6 mouse spleens, enriched by passage through nylon wool columns, and then purified by negative selection using anti-mouse CD4 paramagnetic beads (Life Technologies, Waltham, MA), as previously described [34, 35]. Purified polyclonal $CD8^+$ T cells were cultured for 3 days in RPMI 1640 medium, containing IL-2 (20 U/ml) and ConA (1 µg/ml). $CD8^+$ T cells were then purified from ConA-activated T cells using MACS anti-CD8 microbeads (Miltenyi Biotech, Auburn, CA) to yield $CD8^+$ T cell populations with >95% purity [29], and termed ConA-T cells. To assess the uptake of EXO by ConA-T cells, DCs were initially labeled with CFSE [29] to form DC_{CFSE}. EXOs were purified from DC_{CFSE} culture supernatants by ultracentrifugation and termed EXO_{CFSE}. $CD8^+$ ConA-T cells were then incubated with EXO_{CFSE} at $1X10^6$ cells/µg EXO_{CFSE} for 3 hrs, it was analyzed for the uptake of EXO_{CFSE} by flow cytometry, as described [29].

2.3.4 Preparation of the OVA-$T_{EXO/IL-21}$ and Gag-$T_{EXO/IL-21}$ vaccines and the control OVA-$T_{EXO/Null}$ and Gag-$T_{EXO/Null}$ vaccines

The OVA-T$_{EXO}$ and Gag-T$_{EXO}$ vaccines were generated by the incubation of CD8$^+$ ConA-T cells with EXO$_{OVA}$ or EXO$_{Gag}$ at 3 x 10^6 cells/10 µg for 1 hour, followed by the transfection of T cells with the uptake of EXO$_{OVA}$ or EXO$_{Gag}$ with AdV$_{41BBL}$ at 100 pfu/cell for another 2 hours to form the vaccines, as previously described [32]. To prepare the OVA-T$_{EXO/IL-21}$ or Gag-T$_{EXO/IL-21}$ and the control OVA-T$_{EXO/Null}$ or Gag-T$_{EXO/Null}$ vaccines, the above OVA-T$_{EXO}$ or Gag-T$_{EXO}$ cells were further transfected with AdV$_{IL-21}$ and AdV$_{Null}$ [100 plaque forming unit (pfu)/cell] for 2 hours to form OVA-T$_{EXO/IL-21}$ or Gag-T$_{EXO/IL-21}$ and the control OVA-T$_{EXO/Null}$ or Gag-T$_{EXO/Null}$ vaccine respectively, as we previously described [32].

2.3.5 Establishment of AdV$_{OVA}$-induced chronic infection animal model

To develop a chronic infection model, B6 mice (4/group) were intravenously (i.v.) injected with anti-CD4 Ab (300 µg/mouse) to deplete CD4$^+$ T cells, followed by i.v. infection of mice with AdV$_{OVA}$ (1×10^6 pfu/mouse) one day after the anti-CD4 Ab treatment performed for CD4$^+$ T cell depletion. This experimental procedure was aimed to develop a more stringent mouse model of chronic infection, since AdV$_{OVA}$ infection of mice was performed under the condition of CD4$^+$ T cell depletion such that the 'helpless' CD8$^+$ T cells activated by AdV$_{OVA}$ infection became functionally exhausted with severe defects in IFN-γ production and cytotoxicity [18]. These mice were chronically AdV$_{OVA}$-infected B6 (AdV$_{OVA}$-B6) mice and termed AdV$_{OVA}$-B6 mice with chronic infection. To develop an acute infection model, B6 mice (4/group) were i.v. infected with recombinant OVA-expressing rLmOVA bacteria [2,000 colony forming unit (cfu)/mouse]. These mice were termed rLmOVA-B6 mice. After infections, OVA-specific CD8$^+$ CTL responses in AdV$_{OVA}$-B6 and rLmOVA-B6 mice were kinetically analyzed by flow cytometry.

2.3.6 Flow cytometric analysis

Peripheral blood samples derived from AdV$_{OVA}$-B6 and rLmOVA-B6 mice were double stained with FITC-CD8 Ab and PE-tetramer and analyzed by flow cytometry to assess CD8$^+$ T cell responses at different days after infection. To increase the amount of OVA-specific CD8$^+$ memory T (Tm) cells formed 30 days or over 30 days post infection, such that phenotypes of OVA-specific CD8$^+$ Tm cells can be more accurately analyzed, B6 mice were infected with an

increased amount of AdV$_{OVA}$ (1.2-1.4×10^6 pfu/mouse) for the development of chronic infection AdV$_{OVA}$-B6 mice, while B6 mice with prior i.v. injection of naïve CD8$^+$ T cells (1X10^3/mouse) purified from OTI mouse spelnocytes were infected with rLmOVA bacteria for the development of acute infection rLmOVA-B6 mice [18]. To assess the phenotype of OVA-specific CD8$^+$ Tm cells, peripheral blood samples derived from AdV$_{OVA}$-B6 and rLmOVA-B6 mice 60 days post the primary infection were triply stained with FITC-CD8 Ab, PE-tetramer and PE-Cy5-Abs for various immune molecules, and then analyzed by flow cytometry. To assess the conversion of CTL exhaustion, AdV$_{OVA}$-B6 mice with chronic infection were i.v. immunized with OVA-T$_{EXO/IL-21}$ or the control OVA-T$_{EXO/Null}$ vaccine (1X10^6 cells/mouse), followed by the analysis of OVA-specific CD8$^+$ T cell proliferation and phenotypes 4 days post immunization. To stain the intracellular molecules, mouse splenocytes derived from AdV$_{OVA}$-B6 and rLmOVA-B6 mice were first incubated with FcR blocking anti-16/32 Ab (eBioscience) for 30 min on ice for eliminating any nonspecific staining. The cells were then stained with FITC-CD8 Ab and PE-tetramer, followed by the fixation and permeabilization with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instruction. The cells were further stained with PE-Cy5-antibodies specific for various molecules such as EIF4E and T-bet, and the expression of these molecules was assessed by flow cytometry. For intracellular staining of IFN-γ, mouse splenocytes were first incubated in culture medium containing OVAI peptide (2 µg/ml) and Golgi-stop (0.7 µg/ml) (BD Biosciences) at 37°C for 5 hrs, followed by the incubation with FcR blocking anti-16/32 Ab for 30 min on ice. The cells were then stained with FITC-CD8 Ab and PE-tetramer, followed by fixation and permeabilization with Cytofix/Cytoperm (BD Biosciences). Intracellular staining of IFN-γ was conducted using PE-Cy5-anti-IFN-γ Ab, and the expression of IFN-γ was assessed by flow cytometry. Data were acquired by CytoFlex (Beckman Coulter) and analyzed with FlowJo software (TreeStar, San Diego, CA).

2.3.7 Western blot analysis

EXO (10 µg/well) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred onto the nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked with 5% bovine serum albumin (BSA) in PBS, immunoblotted with rabbit anti-CD9 or anti-LAMP-1 Ab, followed by the incubation

with anti-rabbit IRDyeR800CW Ab, and then, scanning with the ODYSSEY imager according to manufacturer's instruction (Li-COR Bioscience, Lincoln, NE).

2.3.8 Electron microscopy

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

2.3.9 Animal studies

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

2.3.10 Statistical analyses

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

2.4 Results

2.4.1. Preparation of the transgene IL-21-engineered T cell-based OVA-T$_{EXO/IL-21}$ vaccine

B6 mouse dendritic cells were generated by culturing B6 mouse bone marrow cells in the culture medium containing GM-CSF and IL-4 for six days, pulsed with OVA for overnight and termed DC$_{OVA}$ [18, 32]. To assess their phenotype, we analyzed these DCs by flow cytometry. We showed that these DCs displayed cell surface DC maturation markers such as CD40, CD80 and Ia^b (**Figure 2.2A**), indicating that they are mature DCs. OVA-specific exosomes (EXO$_{OVA}$) were purified from DC$_{OVA}$ culture supernatants by ultracentrifugation, and analyzed by electron microscopy and Western blotting analyses, respectively. We demonstrated that EXO$_{OVA}$ had exosomal "saucer" or round shape with 50-90 nm in diameter (**Figure 2.2B**) and contained EXO-associated proteins such as CD9 and LAMP-1 (**Figure 2.2C**) [36]. To assess the uptake of EXOs by T cells, we incubated polyclonal ConA-T cells with CFSE-labeled DC (DC$_{CFSE}$)-released EXO (EXO$_{CFSE}$) for 3 hours, analyzed T cells for EXO$_{CFSE}$ uptake by flow cytometry. We found that ConA-T cells acquired CFSE after incubation with EXO$_{CFSE}$ (**Figure 2.2D**), indicating that ConA-T cells can uptake EXO$_{CFSE}$ through *in vitro* incubation process. We constructed a replication-deficient transgene IL-21-expressing recombinant adenovirus AdV$_{IL-21}$ by recombinant DNA technology (**Figure 2.1B**). We infected the OVA-T$_{EXO}$ cells [32] with AdV$_{IL-21}$ to generate a transgene IL-21-engineered T cell-based vaccine, OVA-T$_{EXO/IL-21}$. IL-21 secretion in OVA-T$_{EXO/IL-21}$ culture supernatants was estimated to be ~200 pg/ml using IL-21 ELISA kit.
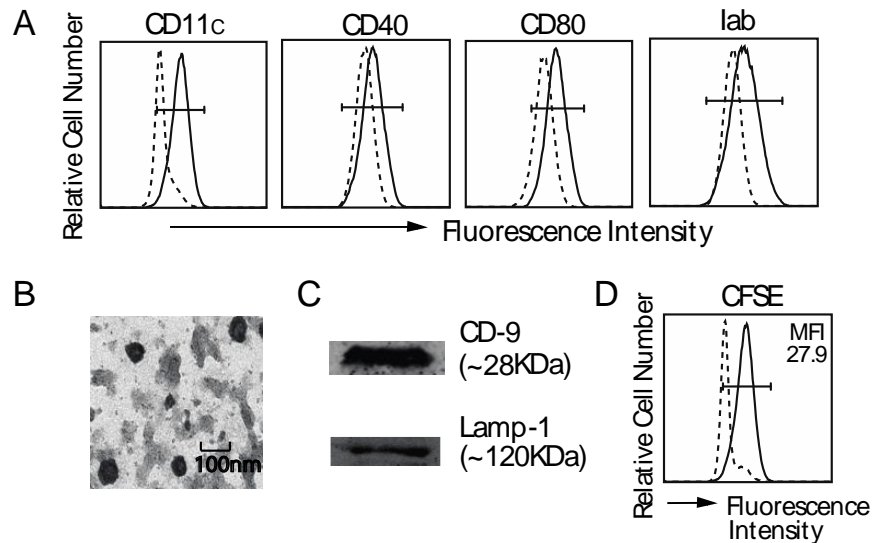
Figure 2.2 Characterization of EXO$_{OVA}$. (**A**) EXO$_{OVA}$ were stained with a panel of Abs (solid lines) or isotype Abs (dotted lines) and analyzed by flow cytometry. (**B**) Electron micrograph of EXO$_{OVA}$. Scale bar, 100 nm. (**C**) Western blot analysis of EXO$_{OVA}$ for the expression of EXO markers CD9 and LAMP-1. (**D**) ConA-T cells incubated with EXO$_{CFSE}$ (solid line) or EXO (dotted line) were analyzed by flow cytometry. Mean fluorescence intensity (MFI) values are indicated in each panel. Dotted lines (on the left) represent isotype controls. One representative experiment of two is shown.

2.4.2 OVA-T$_{EXO/IL-21}$ vaccine stimulates OVA-specific CTL responses in wild-type C57BL/6 mice

To examine the immunogenicity, we i.v. immunized B6 mice with OVA-T$_{EXO/IL-21}$ or the control OVA-T$_{EXO/Null}$ vaccine. We demonstrated that both OVA-T$_{EXO/IL-21}$ and control OVA-T$_{EXO/Null}$ vaccines stimulated comparable OVA-specific CTL responses (**Figure 2.3A**). To assess, whether these CTLs are of functional effect, we challenged immunized mice with OVA-expressing BL6-10$_{OVA}$ tumor cells and examined its protective immunity against lung BL6-10$_{OVA}$ tumor metastases. We showed that OVA-T$_{EXO/IL-21}$ or the control OVA-T$_{EXO/Null}$ vaccine induced complete protection from lung tumor metastases in all 6/6 mice (**Figure 2.3B** & **2.3C**), indicating

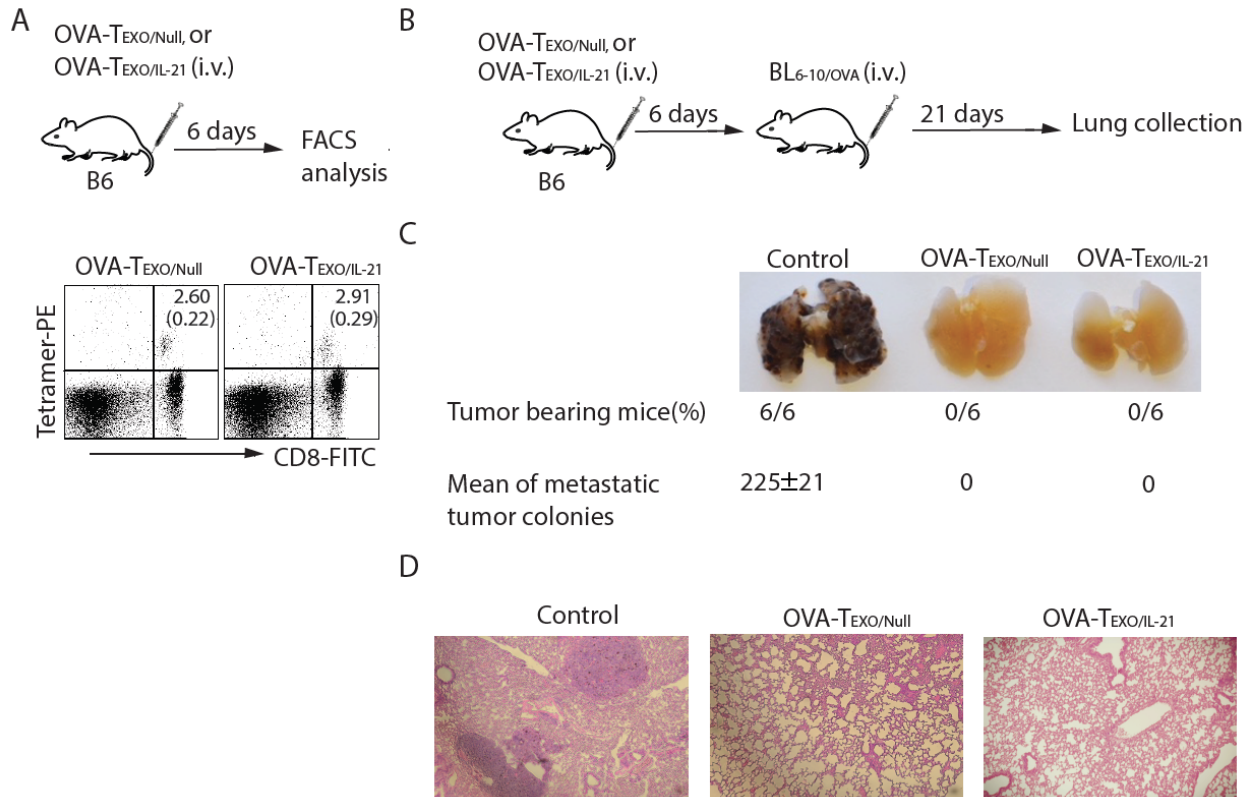that OVA-T$_{EXO/IL-21}$ vaccine stimulates functionally effective CTL responses in wild-type B6 mice.

Figure 2.3 AdV$_{IL-21}$ does not enhance OVA-T$_{EXO}$ vaccine immunity in wild-type (WT) B6 mice. (**A**) WT B6 mice (n = 5) were injected with OVA-T$_{EXO}$ or OVA-T$_{EXO/IL-21}$. Six days after the injection, blood samples were collected, stained with FITC-CD8 Ab and PE-tetramer, and then analyzed by flow cytometry. The percentages of tetramer$^+$CD8$^+$ T cells in the total CD8$^+$ T cell population are indicated. (**B**) Experimental set-up to test the protective anti-tumor immunity of T$_{EXO}$ vaccine. WT B6 mice were immunized with OVA-T$_{EXO}$ or OVA-T$_{EXO/IL-21}$, followed by injection with BL$_{6-10/OVA}$ tumor cells 6 day later. (**C**) Murine lungs were collected at day 21 following tumor cell injection. Metastatic tumor colonies were counted. (**D**) The lung tissues of immunized mice were fixed in 10% neutral-buffered formalin and then embedded in paraffin. Tissue sections were stained with H&E and examined by microscopy. Magnification, ×100. One representative experiment of two is shown.

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2.4.3. OVA-expressing adenovirus induces an OVA-specific chronic infection in mice with CTL exhaustion

We previously found that infection of B6 mice with AdV$_{OVA}$, led to establishment of an OVA-specific chronic infection mouse model with OVA-specific CTL inflation and exhaustion [18]. To develop acute and chronic infection models, B6 mice were i.v. infected with OVA-expressing recombinant rLmOVA bacteria and AdV$_{OVA}$, respectively, followed by the kinetic analysis of OVA-specific CTL responses [18]. We demonstrated that AdV$_{OVA}$ infection resulted in OVA-specific memory CD8$^+$ T cell inflation, when compared to CTLs developed in rLmOVA-immunized mice (**Figure 2.4A**), suggesting that rLmOVA induces an acute infection, while AdV$_{OVA}$ induces a chronic infection with CD8$^+$ CTL inflation [18, 19]. To confirm it, the phenotypes of OVA-specific memory CTLs were analyzed by flow cytometry. We demonstrated that memory CTLs expressed cell surface T-cell memory markers IL-7R and CD44 as well as IL-21R sixty days after infection in both AdV$_{OVA}$-B6 and rLmOVA-B6 mice (**Figure 2.4B**). Importantly, we also found that these CTLs up-regulated inhibitory molecules, such as PD-1, PD-L1 and LAG-3 AdV$_{OVA}$, when compared to CTLs developed in rLmOVA-immunized mice (**Figure 2.4B**), indicating that these CTLs may be exhausted. To further confirm it, we assessed the functional effect (expression of the effector cytokine IFN-γ) of these CTLs by flow cytometry. We showed that ~72% OVA-specific CTLs were IFN-γ positive in rLmOVA-B6 mice, compared to only ~10% OVA-specific CTLs were IFN-γ positive in rLmOVA-B6 mice (**Figure 2.4C**), thus confirming that those OVA-specific CTLs in AdV$_{OVA}$-B6 mice are exhausted CTLs [18, 19].
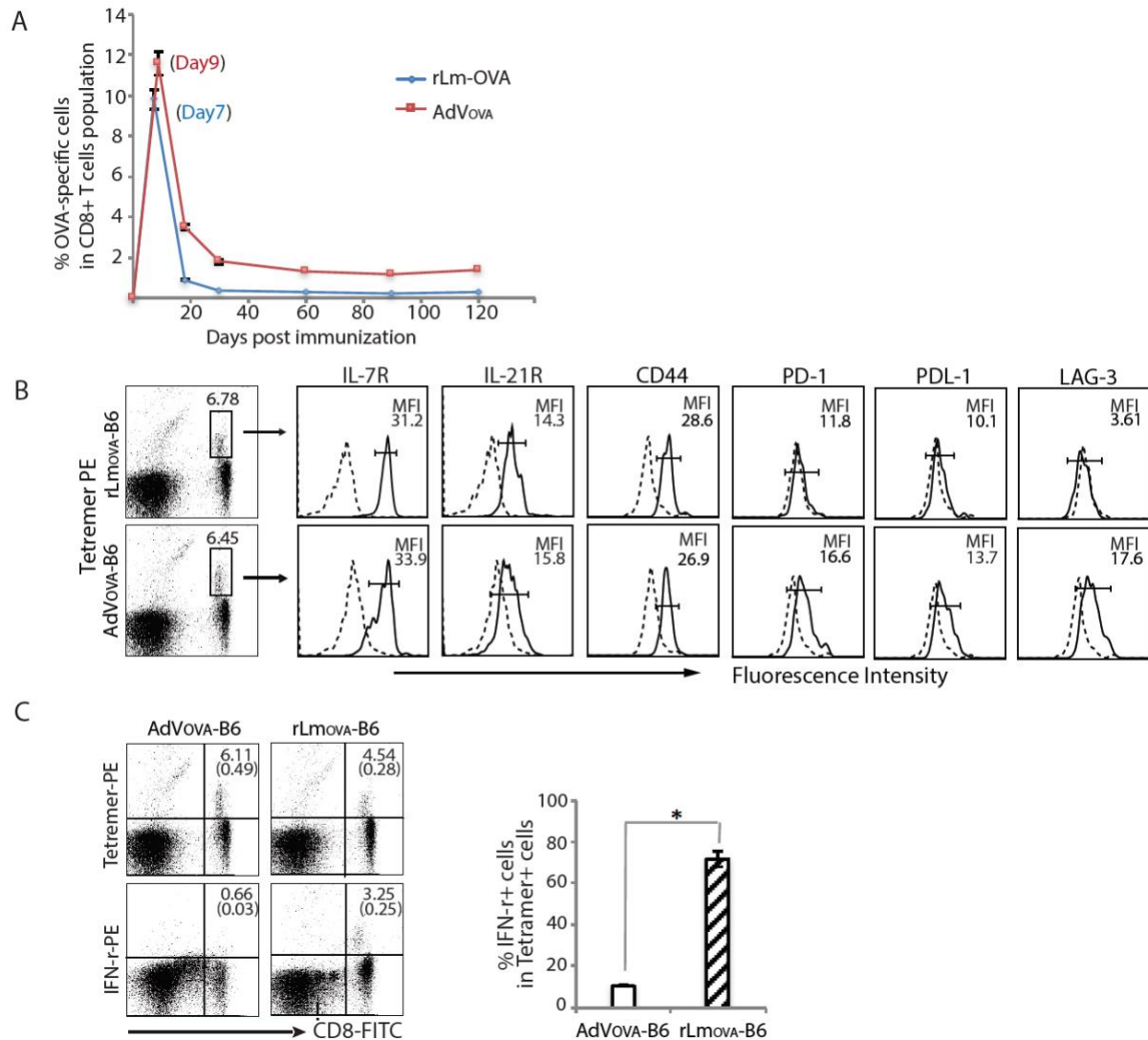
Figure 2.4 AdV$_{OVA}$ induces chronic infection in B6 mice. **(A)** OVA-specific CTL responses were analyzed by flow cytometry (n = 5) at the indicated days following initial AdV$_{OVA}$ or rLmOVA infections. **(B)** Sixty days after the AdV$_{OVA}$ or the rLmOVA infection, flow cytometric analyses were performed. PE-tetramer and FITC-CD8 double positive cells were gated (rectangle) for further assessment of the expression of the indicated molecules (solid lines on the right). Mean fluorescence intensity (MFI) values are indicated in each panel. Dotted lines (on the left) represent isotype controls. **(C)** Splenocytes were stained with PE-tetramer and FITC-CD8 or permeabilized for the assessment of intracellular IFN-γ by flow cytometry. The value in each panel represents the percentage of CD8$^+$ T cells producing IFN-γ in the total CD8$^+$ T-cell population. One representative experiment of two is shown.

2.4.4. The OVA-T$_{EXO/IL-21}$ vaccine converts CTL exhaustion in chronic infection

We examined in our next set of experiments, whether the OVA-T$_{EXO/IL-21}$ vaccine converts CTL exhaustion in chronic infection. The AdV$_{OVA}$-B6 mice were boosted with OVA-T$_{EXO/IL-21}$ and the control OVA-T$_{EXO}$ vaccine. Four days after the boost, cell proliferation and intracellular IFN-γ expression of OVA-specific CTLs were assessed by flow cytometry (**Figure 2.5A**). We demonstrated that there were ~6-fold (5.20% vs 0.81%) of CTL increase in OVA-T$_{EXO/IL-21}$-boosted mouse peripheral blood, which is more than ~3-fold of CTL increase in the control OVA-T$_{EXO}$-boosted mouse peripheral blood at day 4 after the boost (**Figure 2.5B**), indicating that OVA-T$_{EXO/IL-21}$ vaccine can more potently convert CTL exhaustion by significantly stimulating the proliferation of previously exhausted CTLs. We next assessed expression of an effector T cell cytokine, IFN-γ, in exhausted CTLs on a 'per-cell' basis by intracellular staining of T cell IFN-γ. We demonstrated that only ~20% of OVA-specific CTLs were IFN-γ positive in AdV$_{OVA}$-B6 mice (**Figure 2.5C**). In OVA-T$_{EXO}$- and OVA-T$_{EXO/IL-21}$-boosted AdV$_{OVA}$-B6 mice, however, we found ~60% and ~75% of IFN-γ-producing CTLs in the total OVA-specific CTLs, respectively (**Figure 2.5C**), confirming that OVA-T$_{EXO/IL-21}$ vaccine more potently converts CTL exhaustion in chronic infection by not only increasing the number of CTLs, but also restoring CTL functional effect.
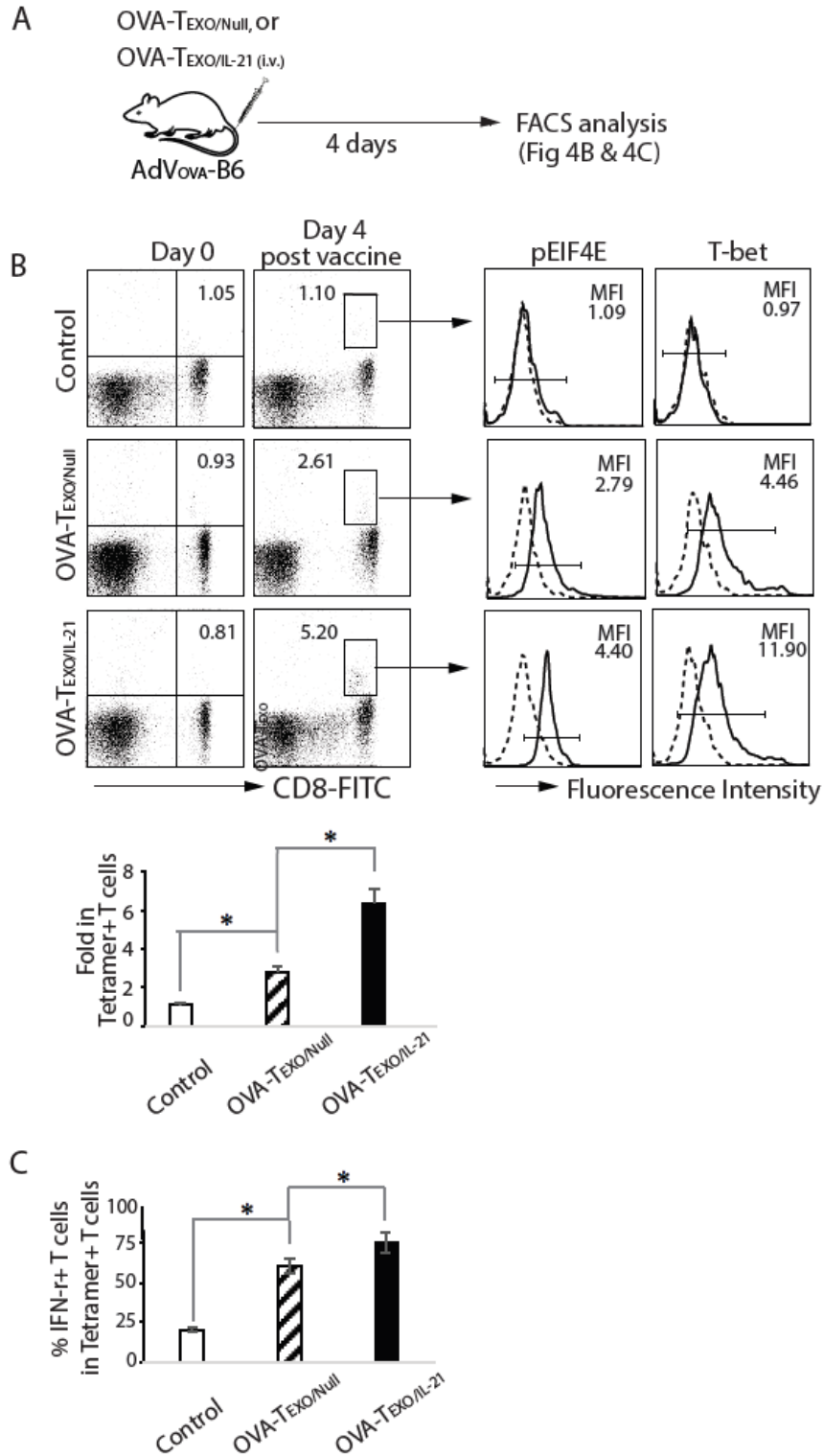
Figure 2.5 AdV$_{IL-21}$ enhance the capability of OVA- T$_{EXO}$ vaccine in converting CTL exhaustion conversion. (**A**) Experimental set-up. AdVova-infected B6 mice (n = 5) were immunized with

OVA-T$_{EXO}$ or OVA-T$_{EXO/IL-21}$ at day 60 after the infection. (**B**) One day prior to (day 0) and 4 days post the immunization, periphery blood samples were analyzed for OVA-specific CTL responses by flow cytometry. The value in each panel represents a percentage of PE-tetramer-positive CD8$^+$ T cells in the total peripheral CD8$^+$ T cell population. Four days after immunization, mouse splenocytes were stained with PE-Tetramer, FITC-CD8, and PE-Cy5-labeled Abs. The tetramer$^+$CD8$^+$ T cells were gated and assessed for expression of peIF4E and T-bet (solid lines). MFI values are indicated in each panel. (**C**) The fold increases (%Tetramer$^+$ T cells of day 4 post vaccine / %Tetramer$^+$ T cells of day 0) of tetramer$^+$CD8$^+$ T cells are indicated in the graph. (**D**) Splenocytes were permeabilized for the assessment of intracellular IFN-γ by flow cytometry at day 4 after immunization. *$p < 0.05$. One representative experiment of two is shown.

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

We previously showed that the OVA-T$_{EXO}$ vaccine rescued exhausted CTLs in chronic infection via its CD40L signaling, inducing the activation of the mTORC1 pathway [18]. Four days after the boost, cell phenotypes of the OVA-specific CTLs were assessed by flow cytometry to examine, whether the OVA-T$_{EXO/IL-21}$ vaccine activates the mTORC1 pathway (**Figure 2.5A**). We analyzed CTLs for the phosphorylation status of mTORC1-regulated EIF4E (pEIF4E) and for the intracellular expression of the transcription factor T-bet by flow cytometry. We determined that OVA-specific CTLs up-regulated levels of phospho-EIF4E and T-bet in OVA-T$_{EXO}$-boosted AdV$_{OVA}$-B6 mice (**Figure 2.5B**), which was consistent with our previous report [18]. Interestingly, the intracellular expression of pEIF4E and T-bet in OVA-specific CTLs was significantly higher in the OVA-T$_{EXO/IL-21}$-boosted AdV$_{OVA}$-B6 mice than that in the OVA-T$_{EXO/Null}$-boosted AdV$_{OVA}$-B6 mice (**Figure 2.5B**), confirming that OVA-T$_{EXO/IL-21}$ vaccine converts CTL exhaustion mostly through the activation of the mTORC1 pathway by both CD40L and IL-21 signaling.

2.4.6. The Gag-T$_{EXO/IL-21}$ vaccine induces Gag-specific therapeutic immunity in chronic infection model

To assess a potential therapeutic immunity of the Gag-T$_{EXO/IL-21}$ vaccine in chronic infection, AdV$_{OVA}$-B6 mice with chronic infection were first i.v. injected with Gag-expressing BL6-10$_{Gag}$ melanoma cells. Four days post B16 melanoma cell challenge, mice were i.v. immunized with the Gag-T$_{EXO/IL-21}$ or the control Gag-T$_{EXO/Null}$ vaccine. Importantly, the Gag-T$_{EXO/IL-21}$ vaccine demonstrated a complete eradication of established BL6-10$_{Gag}$ lung metastases in 5/6 AdV$_{OVA}$-B6 mice, thus stimulating more efficient therapeutic immunity against Gag-expressing BL6-10$_{Gag}$ melanoma than the control Gag-T$_{EXO/Null}$ vaccination (**Figure 2.6**). Our data indicate that Gag-T$_{EXO/IL-21}$ vaccine is capable of inducing potent therapeutic immunity against established Gag-expressing tumors in the presence of chronic infection.

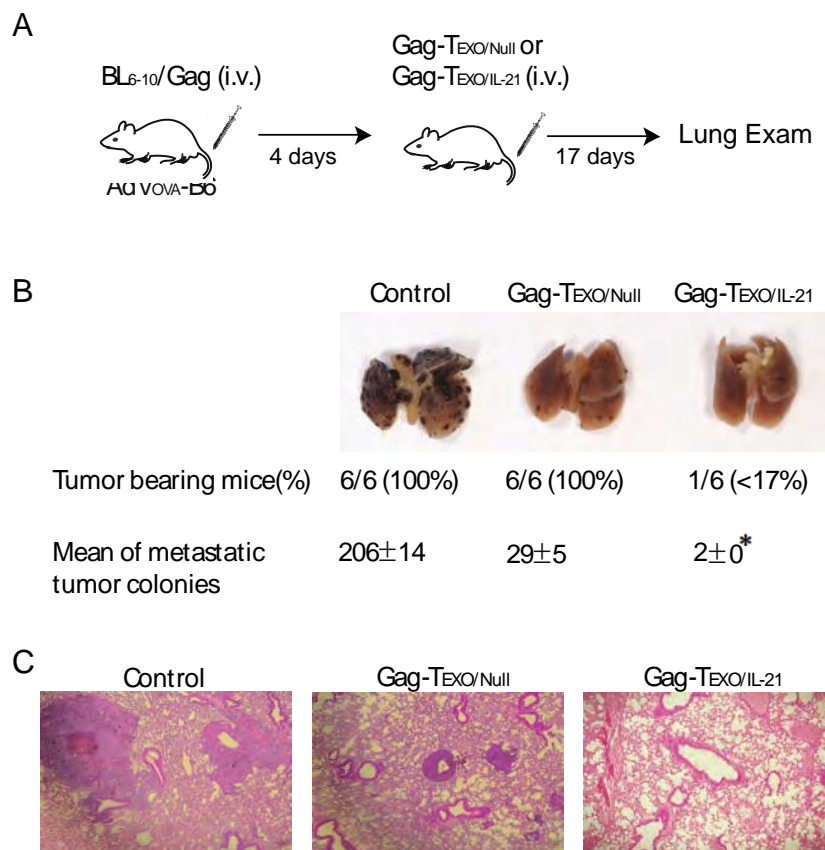**Figure 2.6** Gag-T$_{EXO/IL-21}$ vaccine stimulates enhanced antitumor immunity in chronic infection mice. **(A)** Experimental set-up to examine the therapeutic antitumor immunity of T$_{EXO}$ vaccines. Chronic AdV$_{OVA}$-B6 (n = 6) were i.v. injected with BL6-10$_{OVA}$ cells. Four days after tumor challenge, mice were vaccinated with OVA-T$_{EXO}$, OVA-T$_{EXO/IL-21}$ or control ConA-T. The mice

were sacrificed 3 weeks after tumor cell challenge. **(B)** The average number of lung metastatic tumor colonies were counted. *$p < 0.05$ versus cohorts of ConA-T cells. **(C)** H&E staining of the 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. Magnification, ×100. One representative experiment of two is shown.

2.5 Discussion

The inhibitory PD-1 molecule was originally found on active T cells following TCR engagement, it became a T cell-intrinsic mechanism for negatively regulating CTL responses [37, 38]. In chronic infection, PD-1 expression is sustained on CTLs due to persistent antigen stimulation. Expression of PD-1 suppresses T cell functions through inhibiting TCR signaling by recruiting phosphatases [39, 40] and modulating the mTORC1 pathway responsible for T cell proliferation and effector function [37, 38]. Later, expression of other inhibitory molecules such as LAG-3 and TIM-3 has also been found on exhausted CTLs [41]. It has been demonstrated that PD-1 blockade rescued exhausted CTLs in chronic viral infection [17]. An enhanced effect on conversion of CTL exhaustion was observed upon combination treatments with antagonists of various inhibitory molecules, such as PD-1, LAG-3 and Tim-3 [41, 42]. In addition to blockade therapies, costimulatory signaling, such as 41BB-, OX40- and CD27- mediated costimulation also synergized with PD-L1 blockade by forcing exhausted CTLs to exit quiescence [4, 43, 44]. Moreover, costimulating CD40L signaling has been shown to assure T cell activation by recruiting tumor necrosis factor (TNF) receptor-associated factor (TRAF), which leads to the activation of the mTORC1 pathway [45]. Consistent with this, it has recently been demonstrated that CD40 agonist enhances PD-1 blockade's effect in rescuing exhausted CTLs in chronic infection [19].

IL-21 belongs to the common γ-chain cytokine family and is closely related to another family member, IL-2, which is encoded upstream of IL-21 on chromosome 3 [46]. IL-21 binds to a heterodimeric receptor CD123 encoded on chromosome 7 [47], which is widely expressed by B cells, natural killer cells, DCs, macrophages and T cells [46, 47]. The IL-21 cytokine was originally found to be produced by CD4$^+$ T cells [20] and to serve as a "third" signal, functioning in cooperation with TCR ligation and costimulation, to trigger CD8$^+$ T cell responses [21]. IL-21 promotes CTL activation and survival by inducing signaling through the phosphatidylinositol-3 kinase (PI3K) and the mTORC1-regulated T-bet pathway [48, 49]. In addition, IL-21 also upregulates granzyme-B expression in CTLs [50] and triggers proliferation of B cells and NK cells [51]. Recently, it has been shown that IL-21 plays a significant role in controlling chronic LCMV infection by rescuing exhausted CTL via diminishing their exhaustion phenotype and

maintaining their ability to proliferate [22-24]. IL-21 also restricts virus-driven regulatory T cell expansion in chronic LCMV infection [52], enhances cytolytic and virus-controlling abilities of HIV-specific CTLs *in vitro* [25, 26], and enhances virus control in immunodeficiency virus - infected rhesus macaques or in HCV- and HIV-1-infected patients [4, 7, 28, 53, 54].

We previously developed a novel OVA-specific EXO-targeted T cell-based OVA-T$_{EXO}$ vaccine by using non-specific polyclonal T cells with the uptake of OVA-specific DC-released EXO [29]. We found that the OVA-Texo vaccine was able to directly stimulate potent OVA-specific CTL responses by counteracting CD4$^+$25$^+$FoxP3$^+$ Treg-induced CTL suppression [29, 30]. We also demonstrated that the 4-1BBL-expressing OVA-T$_{EXO}$ vaccine triggered an enhanced therapeutic immunity in WT B6 mice [32] and induced an efficient conversion of CTL exhaustion in chronic infection model via the CD40L-initiated signaling through the mTORC1 pathway [18]. In this study, we generated new OVA-T$_{EXO/IL-21}$ and Gag-T$_{EXO/IL-21}$ vaccines, expressing IL-21, by infecting the above 41BBL-expressing OVA-T$_{EXO}$ and Gag-T$_{EXO}$ cells with AdV$_{IL-21}$, as previously described [32]. We assessed the effectiveness of the OVA-T$_{EXO/IL-21}$ vaccine in the conversion of CTL exhaustion and examined the effectiveness of the Gag-T$_{EXO/IL-21}$ vaccine in therapeutic immunity against Gag-expressing tumor in chronic infection. We discovered that in chronic infection, the OVA-T$_{EXO/IL-21}$ vaccination more strongly rescued CTL exhaustion than the OVA-T$_{EXO}$ vaccine. In addition, we also found that OVA-T$_{EXO/IL-21}$-boosted CTLs strongly up-regulated phosphorylation of mTORC1-controlled EIF4E. OVA-T$_{EXO/IL-21}$ also very effectively enhanced mTORC1-controlled expression of T-bet that regulates T cell activation. These OVA-T$_{EXO/IL-21}$-induced responses were significantly stronger than OVA-T$_{EXO}$-boosted ones. It has been shown that IL-21 promotes CTL survival via activation of the PI3K signaling cascade [48]. Our data showing that OVA-T$_{EXO/IL-21}$ vaccination rescued CTL exhaustion more strongly than the OVA-T$_{EXO}$ one indicates that the IL-21 signaling of OVA-T$_{EXO/IL-21}$ vaccine plays an important role in conversion of CTL exhaustion *via* the activation of the mTORC1 pathway in chronic infection. Our data thus provide the first evidence that our novel T cell-based vaccine is capable of converting CTL exhaustion in chronic infection *via* its CD40L and IL-21 signaling through the mTORC1 pathway.

CD8$^+$ CTLs are important effector T cells capable of directly destroying HIV-1-infected cells, and their activity correlates with acute viral control and long-term non-progression [55-57]. CD8$^+$ CTLs play a critical role in controlling HIV-1 proliferation and disease progression even in the absence of neutralizing antibodies [58, 59]. Stimulation of HIV-1-specific CTLs has been also reported to facilitate elimination of latent viral reservoirs [60, 61]. We originally generated HIV-1 Gag-specific Gag-T$_{EXO}$ vaccine by using polyclonal ConA-T cells with the uptake of Gag-specific DC-released EXO, and demonstrated that Gag-T$_{EXO}$ stimulated Gag-specific CTL responses in transgenic HLA-A2 mice [31]. We also generated a 4-1BBL-expressing Gag-T$_{EXO}$ vaccine capable of triggering more efficient CTL responses and therapeutic immunity against Gag-expressing tumor challenges than the original Gag-T$_{EXO}$ vaccine [32]. In this study, we have generated a new Gag-T$_{EXO/IL-21}$ vaccine engineered to express IL-21 by infection of the 41BBL-expressing Gag-T$_{EXO}$ cells with AdV$_{IL-21}$ as [32], and demonstrated that the Gag-T$_{EXO/IL-21}$ vaccine triggered more effective therapeutic immunity against established Gag-specific BL6-10$_{Gag}$ melanoma lung metastases in chronic infection. We expect that combinations of similarly designed vaccines with blockades against various inhibitory molecules, such as PD-1, TIM-3 and LAG-3, may become new startegies for combined immunotherapies to covert CTL exhaustion in chronic infections such as HIV-1.

Taken together, our data demonstrate that our novel transgene IL-21-engineered T cell-based vaccine OVA-T$_{EXO/IL-21}$ is capable of strongly converting the exhaustion of CD40-expressing CTLs in chronic infection *via* the activation of the mTORC1 pathway caused by endogenous CD40L- and transgene IL-21-triggered signals. Therefore, this study is likely to have a strong impact on developing new therapeutic vaccines that might be used as monotherapies or in combination with other HIV-1 blockades for treating immune deficiency syndrome (AIDS) patients.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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CHAPTER 3 CONCLUSIONS AND FUTURE DIRECTIONS

Our data demonstrates that OVA-T$_{EXO/IL-21}$ vaccination rescued CTL exhaustion more strongly than the OVA-T$_{EXO}$ one, indicating that the IL-21 signaling of OVA-T$_{EXO/IL-21}$ vaccine plays an important role in conversion of CTL exhaustion *via* the activation of the mTORC1 pathway in chronic infection. Our data thus provide the first evidence that our novel T cell-based vaccine is capable of converting CTL exhaustion in chronic infection *via* its CD40L and IL-21 signaling through the mTORC1 pathway.

The inhibitory PD-1 molecule was originally found on active T cells following TCR engagement, and it became a T cell-intrinsic mechanism for negatively regulating CTL responses [1, 2]. In chronic infection, PD-1 expression is sustained on CTLs due to persistent antigen stimulation. Expression of PD-1 suppresses T cell functions through inhibiting TCR signaling by recruiting phosphatases [3, 4] and modulating the mTORC1 pathway responsible for T cell proliferation and effector function [1, 2]. Later, expression of other inhibitory molecules such as LAG-3 and Tim-3 has also been found on exhausted CTLs [5]. In chronic infection, CD8$^+$CD122$^+$ regulatory T (Treg) cells secreting inhibitory cytokine IL-10 also play important role in induction of CTL exhaustion [6-9]. It has been demonstrated that PD-1 blockade rescued exhausted CTLs in chronic viral infection [10]. An enhanced effect on conversion of CTL exhaustion by a combinational treatment with antagonists (blockades for blocking inhibition) for different inhibitory molecules such as PD-1, LAG-3 and Tim-3 [5, 11]. It has also been shown that depletion of CD8$^+$CD122$^+$ regulatory T (Treg) cells [8] or IL-10 blockade improved reactivation of exhausted CTLs and efficiently reduced chronic retroviral loads [6, 7, 9].

In addition to above inhibitory blockade therapies, which put on the "brake" for blocking inhibition of CTL responses, costimulatory signaling such as 41BB-, OX40-, CD27- and glucocorticoid-induced tumor necrosis factor receptor (TNFR)-relation protein (GITR)-mediated co-stimulations also converted CTL exhaustion and synergized with PD-1 blockade by forcing exhausted CTLs to exit quiescence [12-15]. Another costimulatory CD40L signaling (CD40

agonist) has been shown to assure T cell activation through recruiting TNFR-associated factor (TRAF), leading to activation of the mTORC1 pathway [16]. It has recently been shown that CD40 agonist plays a critical CD8-intrinsic and CD8-extrinsic role during rescue of exhausted CTLs [13] and enhanced PD-1 blockade's effect on conversion of CTL exhaustion in chronic infection [17]. Dr. Xiang's laboratory has recently demonstrated that the novel OVA-specific exosome-targeted T cell vaccine converts $CD8^+CD40^+$ CTL exhaustion in chronic infection via its CD40L signaling the activation of the PI3K-Akt-mTORC1 pathway [18]. This was further supported by their recent finding that CD40 agonist alone converts CTL exhaustion and also synergizes the PD-1 blockade in rescue of exhausted CTLs in chronic infection [19]. These costimulatory agonists can be used as stimulatory therapy against CTL exhaustion in chronic infection, which put on the "engine" for stimulation of CTL responses.

In addition to the stimulatory co-stimulation therapy, stimulatory cytokine signaling especially those derived from common γ-chain cytokine family members such as IL-2, IL-7, IL-15 and IL-21 have been found to exert their conversional effects on rescuing exhausted CTLs in chronic infection [20]. For example, cytokine IL-2 with stimulatory effect on T cell activation and proliferation has been found to synergize with PD-1 blockade in reinvigorating exhausted CTLs in chronic LCMV infection [21, 22]. Clinical trials using IL-2 therapy in acquired immune deficiency syndrome (AIDS) patients, receiving antiretroviral therapy (ART), demonstrated significant increase in $CD4^+$ T cell counts [23, 24]. Cytokine IL-7 with functional effect on promoting homeostatic proliferation and survival of naïve and memory $CD8^+$ T cells [25]. IL-7 treatment induced expansion of virus-specific $CD8^+$ T cells in LCMV chronic infection and in HIV-1 patients [26, 27]. In clinical trials, IL-7 treatment reduced viremia and PD-1 expression on CTLs in HIV-1 patients receiving ART [26, 28, 29]. IL-15 promotes effector $CD8^+$ T cell survival and memory $CD8^+$ T cell formation [30, 31]. Higher serum levels of IL-15 have been found to be correlated with better controlling HBV and HIV in patients with chronic infection [32]. IL-15 treatment in SIV-infected Macaques receiving ART increased both effector memory $CD4^+$ and $CD8^+$ T cells [33]. Cytokine IL-21 with stimulatory effect on T cell activation and memory T cell differentiation plays a significant role in controlling chronic LCMV infection through rescuing CTL exhaustion via diminishing their exhaustion phenotype and maintaining their ability to proliferate [34-36]. IL-21 also restricts virus-driven regulatory T cell expansion in chronic LCMV infection [37] and enhances cytolytic and viral control abilities of HIV-specific

CTLs *in vitro* [38, 39] and enhances the viral control in SIV-infected rhesus macaques and HCV- and HIV-1-infected patients [40-43]. Dr. Xiang's laboratory has recently demonstrated that the novel transgene IL-21-engineered OVA-specific exosome-targeted T cell vaccine enhances the conversion of $CD8^+CD40^+IL\text{-}21R^+$ CTL exhaustion in chronic infection via its IL-21 signaling the activation of the PI3K-Akt-mTORC1 pathway [18]. These stimulatory cytokines can also be used as stimulatory therapy against CTL exhaustion in chronic infection, which also put on the "engine" for triggering CTL responses.

Taken together, we speculate that a combinational therapy by using the above the inhibitory blockade reagents as well as the stimulatory co-stimulation and cytokine reagents may represent a future direction in developing potent immunotherapeutic strategies in overcome of CTL exhaustion in chronically infectious diseases such as HIV-1 infection.

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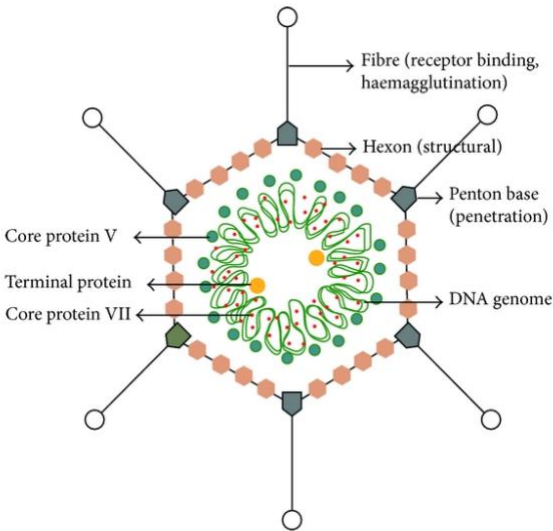
Original figure of Structure of adenovirus

FIGURE 1: Structure of adenovirus

Management of Adenovirus in Children after Allogeneic Hematopoietic Stem Cell Transplantation. Available from:
https://www.researchgate.net/publication/259003696_Management_of_Adenovirus_in_Children_after_Allogeneic_Hematopoietic_Stem_Cell_Transplantation?_sg=CMBfmheP97hW7_JVq1suYwccmy-T0NHg13lhnEFbSijAw6UqCAHGvgwfzVWlRhYcwUIJ1tEh4g