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Role of viral vectors in candidate HIV vaccine-induced immune responses

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Role of viral vectors in candidate HIV vaccine-induced immune responses

by

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Role of viral vectors in candidate HIV vaccine-induced immune responses

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Over 30 years since the discovery of HIV, the development of a vaccine for the prevention of HIV/AIDS remains a global research priority**.** Several candidate vaccines have reached clinical efficacy trials in recent years; of these, only the RV144 trial, which utilized the recombinant canarypox vector ALVAC, demonstrated moderate, short-term efficacy, while multiple trials utilizing the recombinant human adenovirus vector Ad5 either demonstrated no efficacy or transiently *increased* the risk of HIV infection in vaccine recipients with pre-existing immunity to the Ad5 vector. The aim of this dissertation is to help fill the gap in our knowledge of host responses to vaccine vectors in HIV vaccination. Using PBMC collected from participants in the RV144 and HVTN204 clinical trials who received an ALVAC- or Ad5-vectored vaccine, respectively, we show that ALVAC-specific CD4 T cells are significantly less susceptible to HIV infection than Ad5-specific CD4 T cells, which could potentially contribute to the efficacy or non-efficacy of the vaccine regimens employing these two vectors. We also show that, compared to Ad5-specific CD4 T cells, ALVAC-specific CD4 T cells have lower surface expression of CCR5 and CXCR4, higher β-chemokine production, and a more Th1-slanted phenotype, all of which are associated with resistance to HIV infection. Unexpectedly, we also found that ALVAC, but not Ad5, induced a robust vector-specific CD8 T cell response which limited the proliferation of autologous vector-specific CD4 T cells and contributed to their reduced HIV susceptibility. We show that ALVAC-primed APCs are sufficient to induce reduced HIV susceptibility and CCR5 expression in autologous CD4 T cells. We also show that ALVAC- but not Ad5-primed APCs significantly upregulate the Th1-promoting cytokine IL-12, which has been previously reported to induce β-chemokine production and reduce surface expression of CCR5 on CD4 T cells. Finally, we show that ALVAC-primed APCs are themselves less susceptible to HIV infection than Ad5-primed APCs, as well as expressing higher levels of HIV restriction genes, particularly TRIM5 and tetherin. Taken together, our findings reveal a previously unappreciated role for vector-induced immune responses in HIV vaccination and provide new insights for rationale design of candidate HIV vaccines.

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Ad5	Human adenovirus serotype 5
ADCC	antibody-dependent cellular cytotoxicity
Ag	antigen
AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen presenting cell
ART	Antiretroviral therapy
AZT	azidothymidine
bnAbs	broadly neutralizing antibodies
CAR	Coxsackie adenovirus receptor
CDC	Centers for Disease Control and Prevention
CFSE	carboxyfluorescein succinimidyl ester
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DC	dendritic cell
dpi	days post-infection
GALT	gut-associated lymphoid tissues
GSBS	Graduate School of Biomedical Science
GZMB	Granzyme B
HAART	Highly active antiretroviral therapy
HIV	human immunodeficiency virus
hpi	Hours post-infection
ICS	intracellular cytokine staining
KO	knockout
MACS	magnetic-activated cell sorting
MDDC	Monocyte-derived dendritic cells
MDM	Monocyte-derived macrophages
MFI	mean fluorescent intensity
nAbs	neutralizing antibodies
NHP	non-human primates
NIH	National Institutes of Health
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptors
qPCR	quantitative PCR
R5	CCR5-tropic
RT	reverse transcriptase
T reg	Regulatory T cell
TCR	T cell receptor
TDC	Thesis and Dissertation Coordinator
TDM	THP-1-derived macrophage
Tfh	Follicular T helper cell

TFV	Transmitted founder virus
Th1	T helper 1
Th17	T helper 17
Th2	T helper 2
UNAIDS	Joint United Nations Programme on HIV and AIDS
UTMB	University of Texas Medical Branch
WHO	World Health Organization
WT	wild type
X4	CXCR4-tropic

Chapter 1: Introduction to HIV/AIDS

HISTORY AND EPIDEMIOLOGY

Evidence from molecular genetics suggests that the human immunodeficiency virus (HIV) jumped from chimpanzees or gorillas into the human population in the early 20th century (1). However, it didn't gain global attention until the summer of 1981, when the Centers for Disease Control and Prevention (CDC) began reporting unusual clusters of rare opportunistic infections in gay men (2, 3). As cases of the strange new syndrome – originally dubbed "gay-related immune deficiency" in the popular press (4) - were diagnosed in IV drug users, blood transfusion recipients, and heterosexual partners of infected patients, the CDC deduced that it was caused by an infectious agent transmitted by blood and other body fluids and named it Acquired Immune Deficiency Syndrome (AIDS) (5). In 1983 the causative agent was discovered by Dr. Luc Montagnier and colleagues at the Pasteur Institute in France (6); the following year Dr. Robert Gallo and colleagues at the National Institutes of Health (NIH) independently isolated the virus (7) and formally established the causal link between the virus and AIDS (8-10).

The discovery of the HIV virus launched an unprecedented global research effort into the pathology of, and potential treatments for, HIV infection. In 1987 the reverse transcriptase (RT) inhibitor azidothymidine (AZT) became the first drug to be FDA approved for the treatment of AIDS (11, 12). Unfortunately, RT inhibitors alone could not suppress HIV replication enough to prevent the emergence of drug-resistant strains (13, 14). The development of protease inhibitors enabled synergistic multi-drug regimens – called highly-active antiretroviral therapy (HAART) - that could suppress HIV replication below the limit of detection indefinitely (14-17). Within fifteen years of the first reported case, HIV infection went from being an inescapable death sentence to a manageable chronic disease – for those who could afford it. In addition to the expense of the drugs

themselves, timely diagnosis and administration of complex drug regimens require a degree of health care infrastructure that did not – and does not – exist in many areas of the world hardest hit by HIV/AIDS (14). By the early 2000's public outcry at the glaring inequality in access to care reached critical mass, and several programs, including the Global Fund and the United States' President's Emergency Plan for AIDS Relief (PEPFAR) were founded to provide ART drugs to the developing world (18).

From 1981 to 2017, the latest data available from Joint United Nations Programme on HIV and AIDS (UNAIDS), an estimated 77.3 million people have been infected with HIV; 35.4 million people have died of AIDS-related causes, leaving 36.9 million people currently living with HIV (19). Of these, 75% know their HIV status, 58.8% of whom are receiving ART, of whom 47% have achieved viral suppression (20).

CLASSIFICATION

HIV is in the genus lentivirus in the family Retroviridae (21). Mature virions are enveloped, roughly spherical, 100 – 120 nm in diameter, and contain a truncated cone-shaped core. There are two strains of HIV: HIV-1, the virus described by Montagnier and Gallo in 1983 (6, 7), and HIV-2, which was discovered in 1986 (22). HIV-2is less pathogenic than HIV-1 and largely confined to West Africa (23). HIV-1 is divided into four groups: the main (M), outlier (O), non-M, non-O (N) and P groups (24). Unless otherwise specified, "HIV" will be used to refer to HIV-1 group M, which accounts for 98% of global isolates. Group M is further subdivided into clades A – K (24); the geographical distribution of these clades is an important factor in HIV vaccine design (Figure 1.2).

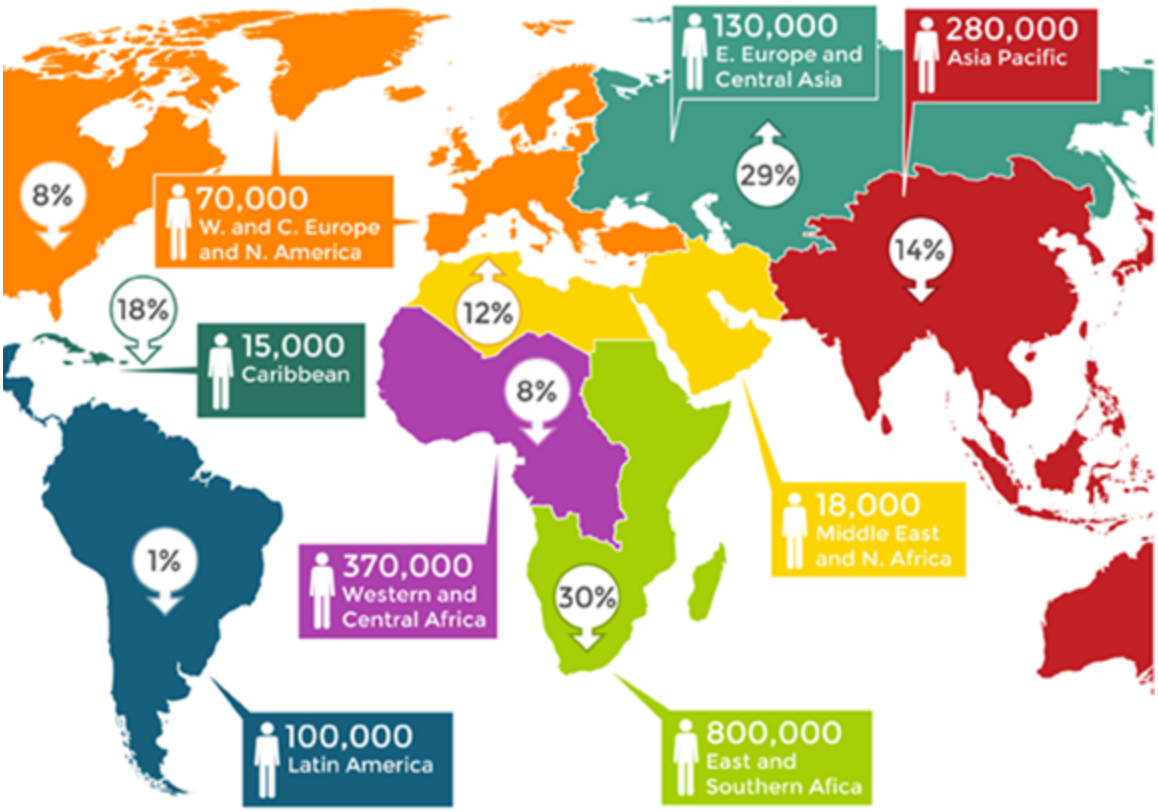

Figure 1.1. New HIV infections in 2017 compared to 2010. Numbers in text boxes give the number of new HIV infections in the named region in 2017. Percentages in white circles give the percent change in new infections compared to the year 2010; upwards- and downwards-pointing arrows indicate whether the infection rate has increased or decreased, respectively. Source: UNAIDS 2018 data. Figure adapted from www.avert.org

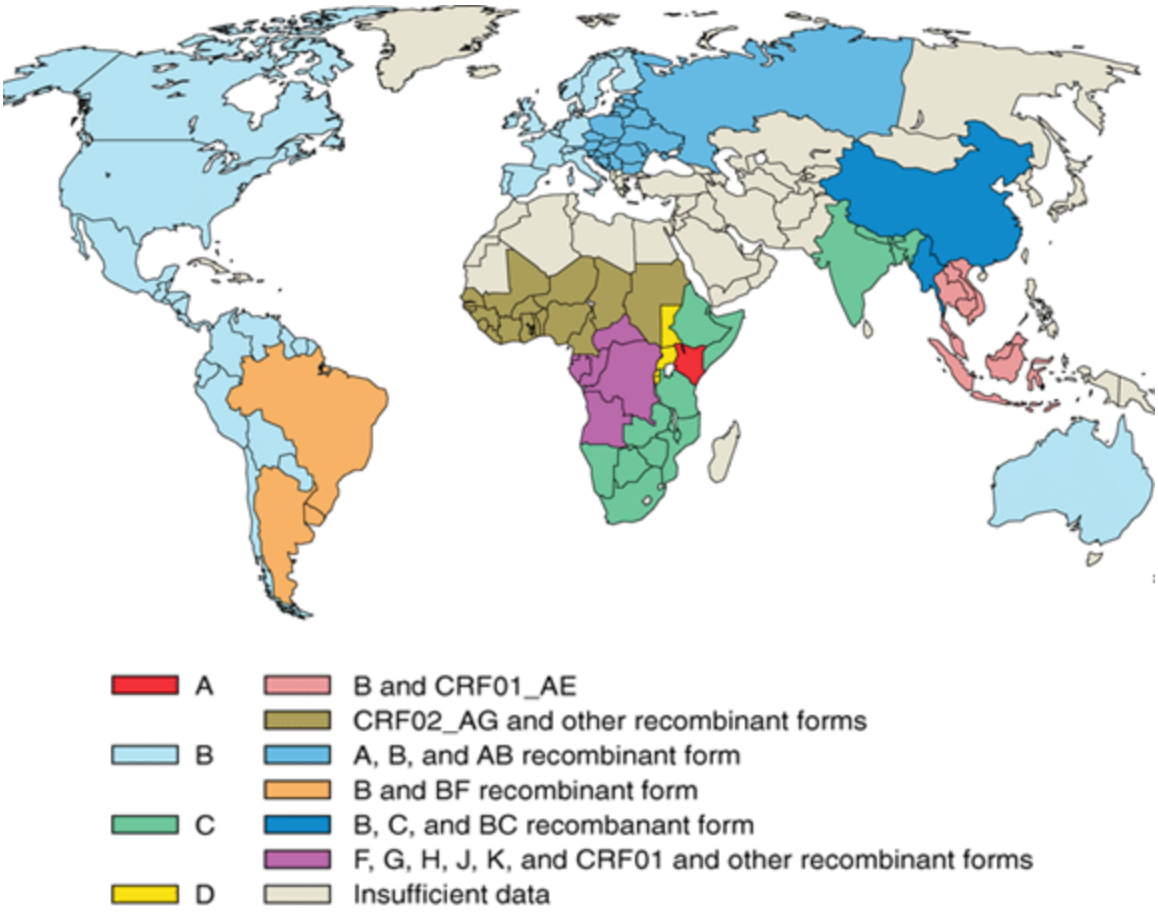

Figure 1.2. Global distribution of HIV-1, M-group clades. HIV antigens used in vaccine clinical trials must reflect the clades prevalent in the area(s) where the trials take place. Adapted from Longo DL, Fauci AS, Kasper DL, Hauser SL, Jameson JL, Loscalzo J. *Harrison's Principles of Internal Medicine,* 18th Ed.

GENOME AND STRUCTURE

Like all retroviruses, HIV has a positive-sense, single-stranded RNA genome that is reverse transcribed into DNA before being integrated into the host genome as a provirus (21). The 10 kb RNA genome consists of nine genes: *gag*, *pol*, *env*, *vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef* (Figure 1.3A) The *gag* gene encodes the gag polyprotein p53, which is further processed to give the structural proteins that make up the matrix and the inner capsid: the matrix protein p17, capsid protein p24, spacer peptides p1 and p2, nucleocapsid protein p7, and p6, which is involved in viral budding. The *pol* gene similarly encodes the pol polyprotein p160, which is processed into the viral enzymes reverse transcriptase, integrase, HIV protease, and RNase H. The *env* gene encodes the envelope protein gp160, which must be cleaved by cellular protease to yield the transmembrane "stalk," gp41, and the surface glycoprotein gp120. The viral envelope is composed of the lipid bilayer acquired from the host cell as the virus buds, studded with protein "spikes" consisting of homotrimers of the non-covalently linked gp41 and gp120 proteins (Figure 1.3B). The remaining genes encode non-structural proteins: *rev* (25) and *tat* (26) encode essential regulatory proteins which are required for HIV replication. *Vif*, *vpr*, *vpu*, and *nef* encode accessory regulatory proteins which enhance HIV replication *in vivo* (27).

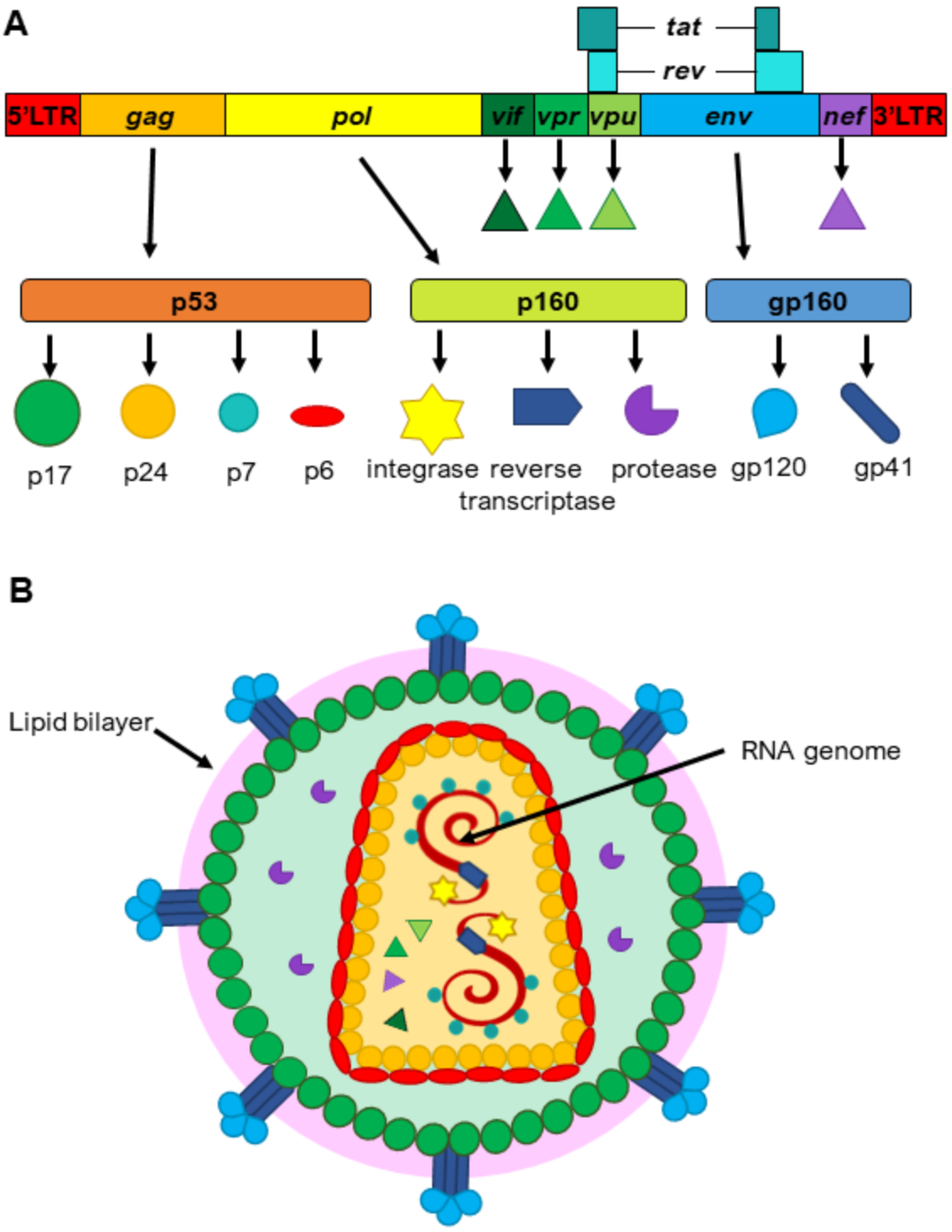

Figure 1.3. HIV genome, gene products, and structure. (A) A simplified representation of the HIV genome and major proteins encoded by each gene. **(B)** The structure of a mature HIV virion showing assembly of the gene products in (A).

REPLICATION CYCLE

HIV infects CD4-expressing cells, which includes T helper cells, monocytes, macrophages, and dendritic cells (DCs) (28, 29). The gp120 envelop protein binds CD4 and then changes conformation to expose a secondary binding site specific for either CCR5 or CXCR4. HIV strains are classified as CCR5-tropic (R5 HIV) or CXCR4-tropic (X4 HIV) according to their coreceptor specificity; some strains (R5X4 HIV) can use both (30). R5 HIV can efficiently infect macrophages and dendritic cells and is the only strain transmitted between individuals, making it an important target for preventative vaccines. X4 HIV is rarely transmitted between individuals but frequently comes to dominate in the late stages of infection because it infects CD4 T cells more efficiently than R5 HIV (30).

After membrane fusion and release of the viral RNA and enzymes into the host cell cytosol, reverse transcriptase transcribes the single-stranded RNA genome into double-stranded DNA. The DNA is then transported into the nucleus, where it is integrated into the host genome by viral integrase. Once integrated, the HIV provirus can remain latent for the life of the cell, or it can be transcribed to produce new virions. Upon activation, the proviral DNA is transcribed into RNA, which is alternatively spliced to give mRNAs for viral proteins as well as complete viral genomes. The viral proteins and RNA genome self-assemble into an immature virion, which then buds out of the host cell, acquiring an Env-studded membrane in the process. After budding, packaged HIV protease cleaves the Gag polyprotein to create the mature structural proteins which form the truncated cone-shaped inner capsid characteristic of the mature virus (28, 29) (Figure 1.4).

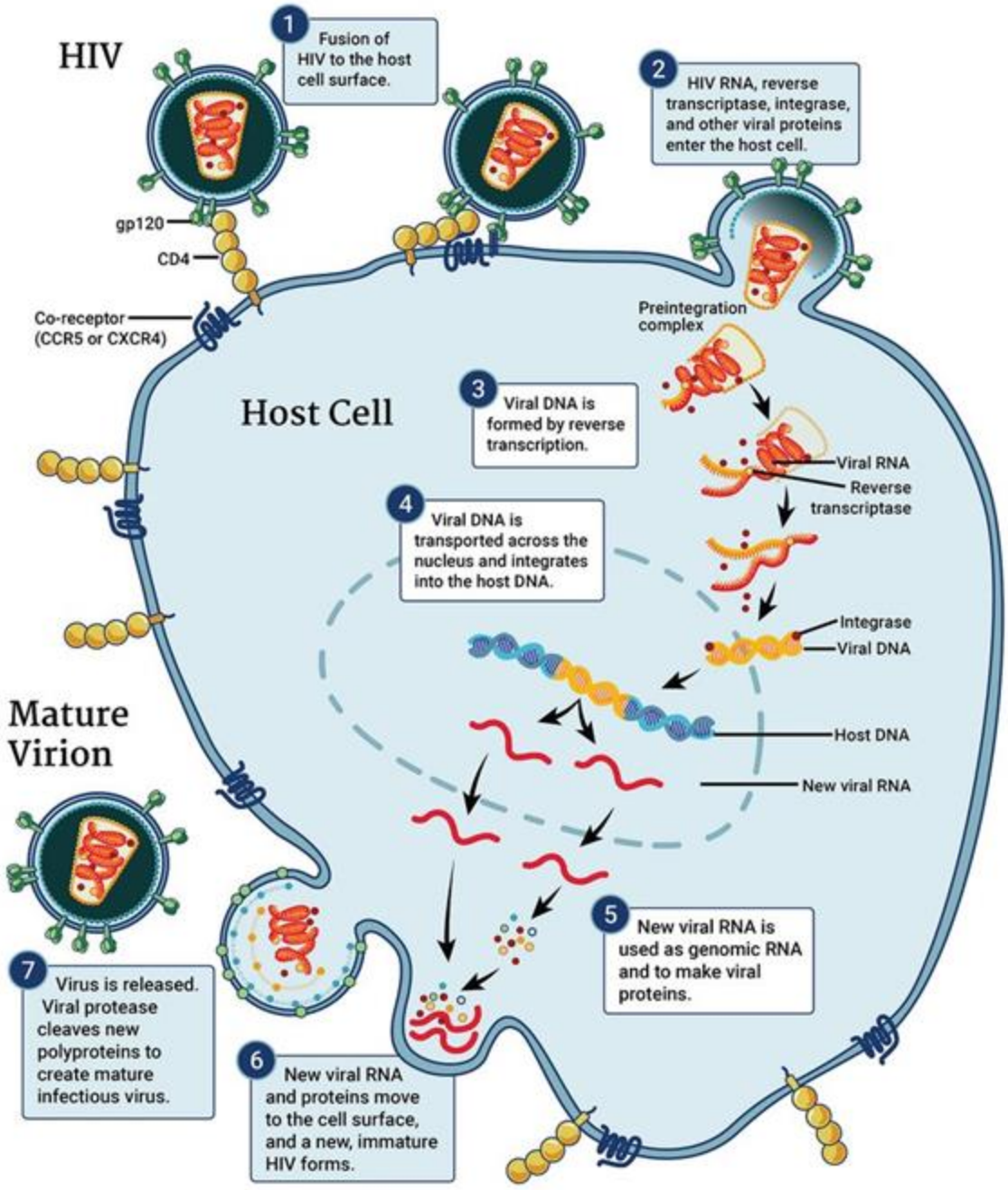

Figure 1.4. HIV replication cycle. Source: National Institute of Allergy and Infectious Disease

PATHOGENESIS AND CLINICAL DISEASE

HIV is transmitted by blood and other body fluids, most commonly via vaginal or rectal intercourse. Infection is initially established in the mucosal tissues, then spreads to the lymphoid tissues (31). The gut-associated lymphoid tissues (GALT) sustain early, intense HIV replication accompanied by severe CD4 T cell depletion; up to 80% of CD4 T cells present in the GALT can be depleted in the first 3 weeks of infection (32). Multiple mechanisms contribute to CD4 T cell depletion, including the direct cytopathic effect of HIV on infected cells (33), apoptosis of uninfected bystander cells (34, 35), and abortive cell-to-cell transfer to non-permissive cells resulting in pyroptosis (36, 37). Plasma viremia peaks at 21 – 28 days post-infection (38) before eliciting a cytotoxic T lymphocyte (CTL) response which is initially effective at controlling viral replication and ending the acute phase of infection (39) (Figure 1.5). The degree to which the CTL response succeeds in reducing plasma viremia establishes a "viral set point" which strongly correlates with the severity of disease (40, 41). Although an antibody response is generated, it takes ≥ 12 weeks to produce neutralizing antibodies (nAbs) to the transmitted strain, by which time escape mutants have already emerged (42, 43). Due to the high genetic diversity of HIV, most nAbs are highly strain specific; only about 20% of patients generate broadly neutralizing antibodies (bnAbs), years after the onset of infection (43-45).

Control of acute viral replication and partial rebound of CD4 T cell counts in the peripheral blood (but not, importantly, the gut mucosa (46)) marks the beginning of clinical latency, also called the chronic phase (Figure 1.5). The ability of HIV to remain latent as a provirus in host cells indefinitely allows the virus to "hide" from the immune system in long-lived memory CD4 T cells (47, 48). The development of extremely sensitive qPCR

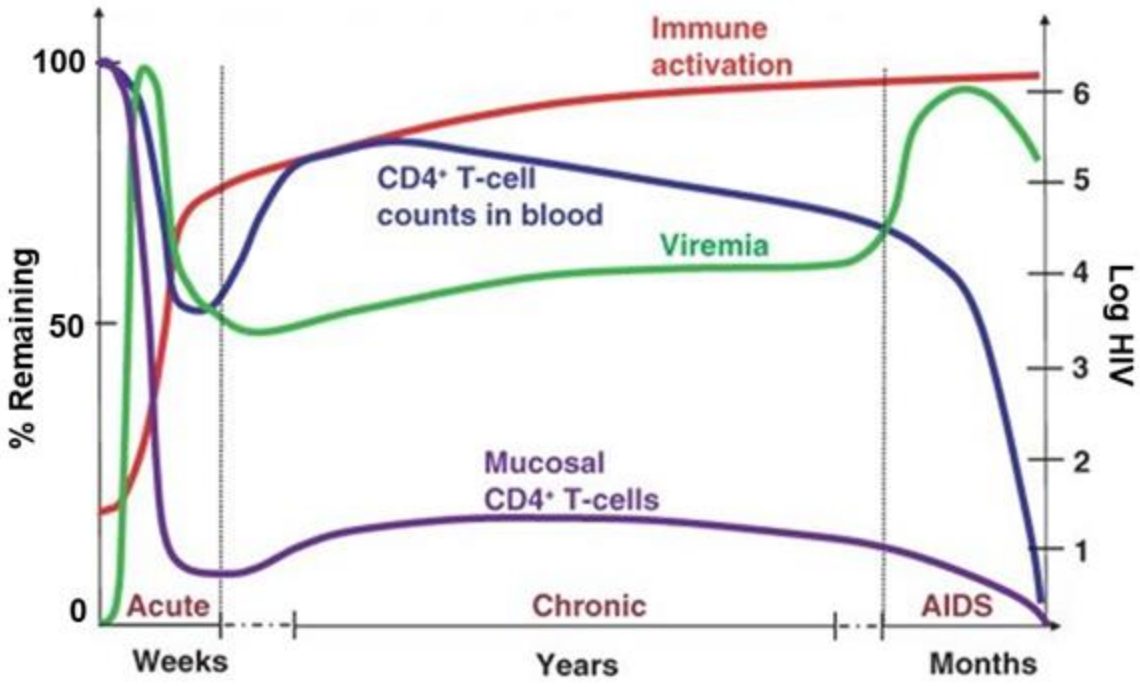

Figure 1.5. HIV disease progression. The acute phase is characterized by rapid viral replication resulting in high plasma viremia (green) and depletion of mucosal (purple) and peripheral (blue) CD4 T cells. Activation of the immune response (red), in particular CTLs, quickly reduces plasma viremia, allowing peripheral CD4 T cells to rebound. In the absence of treatment, chronic immune activation eventually exhausts the immune system, leading to a rebound in viral replication, renewed CD4 T cell depletion, and AIDS. Adapted from Nat Med. 2006 Mar;12(3):289-95

assays revealed that viral replication actually continues at extremely low levels throughout the chronic phase, even in patients undergoing effective HAART (49, 50). In untreated patients, chronic immune activation gradually exhausts the immune system in a manner resembling accelerated immune senescence (52). Several mechanisms are thought to contribute to HIV-induced immune exhaustion: the magnitude of the HIV-specific T cell responses (up to 30% of circulating CD8 T cells can be HIV-specific by the end of the chronic phase (53, 54)), direct activation of lymphocytes and/or macrophages by HIV gene products such as gp120 (55-57) and Nef (58-60), and inflammatory responses to microbial translocation caused by disruption of the gut mucosa during the acute phase (61, 62). Once the immune system is exhausted, viral replication accelerates, peripheral CD4 T cells are rapidly depleted, and the patient progresses to full-blown AIDS (defined by peripheral CD4 T cells levels $\leq 200/\mu L$ and/or the emergence of HIV-associated opportunistic infections) (Figure 1.5). Without HAART nearly all HIV-infected patients (with the rare exception of "elite controllers") progress to AIDS 3 – 20 years post-infection (8 years on average.)

Although extremely low-level HIV replication continues even in patients undergoing HAART (49, 50), effective HAART prevents released virus from infecting new cells, preventing the chronic activation and eventual exhaustion of the immune system that would otherwise lead to AIDS. Despite the remarkable success of HAART, it has important limitations that prevent it from being a "cure" for HIV/AIDS. HAART cannot eliminate the reservoir of latently infected cells, and viral replication, with its attendant pathology, resumes if treatment is discontinued (48, 51, 52). In addition, even successful HAART doesn't completely eliminate HIV-related comorbidities, including elevated rates of metabolic and cardiovascular disease, non-AIDS-related cancers, and neurodegenerative

decline (53, 54). For all of these reasons, as well as the considerable expense of providing life-long treatment for the millions who are currently infected, the development of an HIV vaccine remains a global research priority.

VACCINE DEVELOPMENT

The nature of the HIV virus presents unique challenges for vaccine design. Because of the error-prone nature of reverse transcriptase, HIV exhibits extensive genetic heterogeneity, even within the same individual, making it difficult to find or design a common antigen that will generate a broadly cross-reactive immune response (29). Most currently available vaccines are modeled after natural immunity; however, since HIV doesn't induce natural immunity, the correlates of immunity are unknown. In addition, not only does HIV infect the very CD4 T cells required for adaptive immunity, it preferentially infects HIV-specific CD4 T cells (55).

Nevertheless, several lines of evidence suggest that a vaccine is possible. Non-human primates (NHP) infected with a Nef knockout strain of SIV had attenuated disease which imparted robust resistance to subsequent challenge with the wild-type virus (56). More recently, a cytomegalovirus (CMV)-vectored SIV vaccine successfully protected 50% of vaccinated NHPs from SIV challenge (57). "Elite controllers" who are able to control viral replication without the aid of HAART have been studied extensively in order to determine potential correlates of immunity; one of the best established is high levels of multifunctional HIV-specific CD8 T cells (58-61). Although antibodies don't appear to play a significant role in natural immunity to HIV infection, about 20% of HIV-infected patients eventually generate bnAbs, several of which have been identified in hopes that

they may be more effective in the context of vaccination, where they will ideally be induced quickly upon viral exposure (43-45, 62).

The choice of delivery platform has a profound effect on vaccine design. In general, safety and efficacy are inversely correlated: simple peptide vaccines are extremely safe, but are only effective against a limited number of pathogens, while live attenuated vaccines tend to induce stronger and longer-lasting immune responses, but carry the increased risk associated with deliberately infecting a healthy patient with a live virus (63). This is of particular concern for HIV, since an inadequately inactivated or attenuated HIV virus could integrate permanently into the host cell genome, resulting in the inadvertent, life-long infection of a previously healthy patient. Viral vector vaccines represent a "best of both worlds" approach: a non-pathogenic virus is used to deliver HIV genes into a host cell, where they will be translated into viral proteins and processed for antigen presentation in a manner that mimics natural infection, but without the formation of infectious virions (63). Of the four vaccine strategies that have progressed to late-stage efficacy trials, 3 involve one of two vectors: human adenovirus serotype 5 (Ad5) or a recombinant canarypox called ALVAC.

Ad5 Vector

Adenoviruses are non-enveloped, icosahedral viruses with a linear, non-segmented double-stranded DNA genome; over 50 serotypes are known to infect humans (64, 65). Ad5 is ubiquitous in the human population: prevalence varies from 50 – 80% depending on the geographical region (64, 65). Ad5 is usually harmless in adults but causes mild respiratory illness in children; about 5% of "common colds" are caused by adenoviruses (65). Ad5-based vaccine vectors are attenuated and made replication deficient by deleting

the essential E1A and E1B genes; they are popular vectors for vaccines as well as cancer therapy because they induce strong cellular and humoral responses (64, 65).

ALVAC Vector

One of the most successful vaccines in history, the vaccine used to eradicate smallpox, was based on a poxvirus vector: vaccinia. Although the vaccinia vaccine was extremely effective, it had an unacceptable safety profile by modern standards, causing serious adverse effects in immunocompromised patients and full-blown smallpox in a small but significant percentage of recipients (66). Avian poxvirus vectors were developed to combine the efficacy of poxvirus vectors with an improved safety profile (66). ALVAC is an attenuated canarypox which undergoes abortive replication in human cells (66). Compared to Ad5, the immune response induced by ALVAC is more slanted towards antibodies, with a less robust T cell response (66). The biology of vector infection will be discussed in greater detail in chapter 3.

CLINICAL EFFICACY TRIALS

Vax003 and Vax004

The first candidate HIV vaccine to reach efficacy trials in humans was AIDSVAX: a peptide vaccine consisting of the HIV envelope protein gp120 adsorbed onto alum adjuvant (67, 68). The VAX 003 trial was conducted in Bangkok, Thailand on 2,546 intravenous drug users aged 20 – 60 years (68); the VAX 004 trial was conducted in North America and the Netherlands on 5,403 men and women at elevated risk of HIV infection (67). Both trials consisted of multiple injections at 0, 1, 6, 12, 18, and 24 months; the final injection was at 36 months for the VAX 003 trial and 30 months for the VAX 004 trial.

Neither trial showed any efficacy at preventing HIV infection or reducing the viral set point (67, 68).

HVTN502 and HVTN503

The HVTN502 (STEP) trial was a double-blind, randomized, placebo-controlled phase IIb efficacy trial conducted on 3000 men and women aged 18 – 35 living in the Americas, Caribbean, and Australia who were at high risk for HIV infection (69).

A recombinant, replication-deficient human adenovirus (Ad5) was used as a live vector to deliver the HIV clade C genes *gag, pol,* and *nef* into immune cells (69). Subjects received 3 injections of vaccine or placebo at 0, 4, and 26 weeks (69). The trial was halted for futility after a pre-specified interim analysis found no evidence of efficacy; subsequent analysis showed that circumcised male vaccine recipients who were seropositive for Ad5 prior to vaccination had an increased risk of HIV infection compared to comparable subjects who received a placebo (69).

The HVTN503 (Phambili) trial was a double-blind, randomized, placebo-controlled phase IIb efficacy trial conducted in South Africa on 801 men and women aged 18 – 35 who were at primarily heterosexual risk of HIV infection (70). The vaccine strategy was similar to that of the STEP trial except that clade B *gag, pol,* and *nef* were used. The Phambili trial was halted early and subjects unblinded to their vaccination status after the STEP interim analysis discussed above. At the time the Phambili trial was halted, no significant difference in HIV infection risk was found between vaccine and placebo groups, but vaccine recipients did show a non-significant increase in HIV infection risk that did not appear to be associated with circumcision or preexisting immunity to Ad5 (70). A long-term follow-up analysis conducted at a median of 42 months post-vaccination found a

statistically significant increase in HIV infection risk in vaccine recipients; however, the results must be interpreted with caution since unblinding of subjects may have affected risk behaviors (71).

HVTN505

The HVTN505 trial - the last to use an Ad5-based HIV vaccine strategy - was a randomized, double-blind, placebo-controlled study conducted on 2,504 men and transwomen at elevated risk of HIV infection (72). In an attempt to avoid the elevated HIV infection risk which marred the STEP and Phambili trials, only circumcised subjects without preexisting immunity to Ad5 were enrolled (72). In addition, a slightly different recombinant Ad5 vector developed by the NIH with more deletion to the adenovirus genome was used (72). Lastly, the vaccine strategy utilized a DNA prime/Ad5 boost strategy which differed significantly from the STEP and Phambili Ad5-only regimens. The vaccine group received a DNA prime consisting of 6 separate plasmids encoding HIV clade B *gag, pol, and nef* and clade A, B, and C *env* at weeks 0, 4, and 8, followed by an Ad5 boost bearing the same 6 genes at week 24 (72). The HVTN505 trial was halted for futility after a pre-specified interim analysis showed no significant difference between vaccine and placebo groups for HIV infections or viral set point (72). Although not statistically significant, the vaccine group did have noticeably more HIV infections than the placebo group: 41 HIV infections in the vaccinated group compared to 31 in the placebo group by the 24 month visit (72).

RV144

The RV144 phase III clinical HIV vaccine trial was a double-blind, placebo-controlled efficacy trial conducted in Thailand with 16,402 men and women, aged 18 – 30, at community risk for HIV infection (73). The vaccine group received ALVAC encoding HIV clade B *gag* and *pro* and clade E gp120 at weeks 0, 4, 12, and 24, and AIDSVAX BE (gp120 peptide from HIV clades B and E; see above) at weeks 12 and 24. Trial subjects were tested for HIV infection prior to the first vaccination, 2 weeks after the final vaccination, and every 6 months thereafter for 3 years (73). The results were moderate but promising: vaccine efficacy for the modified intent-to-treat group (which excluded subjects who tested HIV^+ at baseline) was 31.2% at the conclusion of the trial, 42 months after the first vaccination (73). Interestingly, a post-hoc analysis found that the RV144 regimen was 60% effective at preventing HIV infection 6 months after the final vaccination, suggesting that the vaccine elicited an initial potent immune response which waned over time (74). The prespecified immune-correlates analysis conducted after the completion of the trial measured several aspects of the immune response of the 41 vaccine recipients who became infected with HIV over the course of the trial, and 205 controls who did not (75). Of the 6 primary variables that were tested, two significant correlates of immunity were identified: 1) non-neutralizing IgG antibodies directed against the V1/V2 loop of the gp120 protein were positively correlated with protection, and 2) Env-specific serum IgA antibodies were negatively correlated with protection (75). Interestingly, in vaccine recipients who had low levels of serum IgA, nAbs and antibody-dependent cellular cytotoxicity (ADCC) were also correlated with protection, suggesting that the presence of serum IgA interfered with these otherwise protective antibody functions (75). This is consistent with previous studies

showing that serum IgA can interfere with ADCC in both vaccinated (76) and HIV-infected (77) individuals. A detailed analysis of the IgG subclass profile elicited by ALVAC/protein vaccination showed that V1/V2 Env-specific IgG3 antibodies correlated with protection from infection and ADCC (78).

LITERATURE OVERVIEW AND GAPS

The moderate efficacy of the ALVAC prime/gp120 boost strategy employed in the RV144 trials renewed optimism that an HIV vaccine is possible. At the same time, the unexpectedly negative results of the Ad5-vectored vaccine trials – in particular the excess HIV infections observed in some groups of vaccine recipients – were a dramatic demonstration that a deeper understanding of the biology and virus-host interactions of candidate vaccine vectors is required in order to develop safe and effective vaccine strategies. Multiple mechanisms have been proposed to explain the elevated HIV infection risk in Ad5-based vaccine recipients: an increase of $CCR5^+$ CD4 T cells in the gut mucosa following vaccination (79), the design of the insert (80, 81), DC activation by Ad5 immune complexes formed in people with pre-existing immunity to Ad5 (82), and differences between the Ad5 vectors designed by Merck (used in the STEP and Phambili trials) and the NIH (used in the HVTN505 trial.) A more thorough discussion of the relative merits and limitations of these various hypotheses is included in Chapter 4; in summary, no single explanation has been deemed entirely adequate to explain the excess HIV infections seen in the STEP and Phambili trials.

The partial success of the ALVAC/AIDSVAX regimen tested in the RV144 vaccine trial was nearly as surprising as – though obviously more welcome than – the failure of Ad5-based vaccines. Both ALVAC and AIDSVAX had failed individually in previous

human trials (67, 68), and an open letter published in *Science* and signed by 22 established researchers in the field argued that a large-scale efficacy trial combining them was a waste of resources (83). It had been widely believed that the key to inducing HIV immunity lay in a robust CD8 T cell response such as that induced by adenovirus vaccines; however, the efficacy of the ALVAC/AIDSVAX regimen lay in the induction of binding, but non-neutralizing, antibodies against the HIV envelope protein (73, 75), which hadn't even been included in the inserts of the first two Ad5 vaccine trials (69, 70). This, in addition to the identification of bnAbs against HIV, led to a major shift away from T cell responses and towards antibodies as the focus of a renewed optimism towards HIV vaccine studies (84). Unfortunately, in the 10 years since the publication of the results of the RV144 vaccine trial, little progress has been made in extending the protection it induced.

OBJECTIVES OF THIS DISSERTATION

Understandably, the majority of HIV vaccine studies in general, and those attempting to understand the unexpected results of the Ad5- and ALVAC-based vaccine regimens in particular, have focused on the immune responses induced against the HIV insert, with relatively few exploring the role of vector-specific immune responses (82, 85). The objective of this dissertation is to partially fill this knowledge gap by characterizing the innate and adaptive immune responses induced by ALVAC and Ad5 vectors and how they influence HIV susceptibility. Not only will a better understanding of vector-induced immune responses help to explain the results of past clinical trials, it will also be essential to the rational design of new vaccine strategies going forward.

Chapter 2

Distinct susceptibility of HIV vaccine vector-induced CD4 T cells to HIV infection[1]

INTRODUCTION

Over 30 years after the discovery of HIV as the causative agent of acquired immunodeficiency syndrome (AIDS), HIV/AIDS continues to be a significant challenge for global public health. More than 36 million people are currently living with HIV, with over 2 million new infections and 1 million AIDS-related deaths per year (86). Development of a safe and effective HIV vaccine remains a high research priority. Recombinant viral vectors are an important platform for HIV vaccine development. To date, a number of HIV vaccine vectors derived from different viral families have been developed, including adenovirus (87) and poxvirus (88). Several clinical trials (Step and Phambili) testing candidate HIV vaccines based on human Ad5 vector (rAd5) have failed due to lack of efficacy and/or transiently increased HIV infections in some vaccinated individuals (69, 70, 72). These unanticipated results from clinical trials have brought to light the importance of understanding host immune responses induced against viral vectors in HIV vaccination (89, 90).

CD4 T cells are central to host immunity by providing help signals to other components of the immune system (91). The protective role of CD4 T cell responses has been documented for various pathogenic infections, including HIV (92-95). However, CD4 T cells are also major target cells for HIV infection. During an antigen-specific immune response, activation and expansion of responding CD4 T cells is required (38), which is

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usually desired in most vaccine strategies but could become a potential problem in HIV vaccination due to the fact that HIV preferentially infects activated CD4 T cells (96-99). Recent research from our group and others has shown that human CD4 T cells specific for different antigens differ in their susceptibility to HIV infection (55, 100-105). In particular, we have reported that human Ad5-specific CD4 T cells generated in response to both natural Ad5 infection and rAd5 vaccination are highly susceptible to HIV and are preferentially depleted in HIV-infected individuals (101). Although potential mechanisms for Ad5 vector-associated excess HIV infections in the Step and Phambili studies are thought to be complex and could be affected by different factors such as the quantity, quality and in vivo localization of CD4 T cells induced during vaccination, our findings suggest that understanding the HIV susceptibility of vector-specific CD4 T-cell populations induced by different vaccine vectors may provide new insights into our understanding of host immunity in HIV vaccination.

In addition to rAd5, another important HIV vaccine vector that has been tested in late-stage clinical trials is ALVAC, a recombinant canarypox virus vector. The ALVAC prime/gp120 boost HIV vaccine regimen tested in the "Thai" RV144 trial demonstrated modest efficacy (~31%) (73). Building upon the partial success of RV144, multiple ongoing trials further evaluating ALVAC-based HIV vaccine regimens are currently being conducted (106, 107). In this study, we sought to understand anti-vector T cell responses with a focus on the phenotype and in vitro HIV susceptibility of vector-specific CD4 T cells induced by vaccination with ALVAC compared to Ad5. Cryopreserved peripheral blood mononuclear cells (PBMC) from RV144 vaccine recipients were analyzed in comparison with PBMC from HVTN204, a phase II trial evaluating rAd5-HIV vaccine

(DNA prime/Ad5 boost) (77), using the in vitro HIV susceptibility assay reported in our previous studies (100, 101, 103). We also measured vector-induced CD8 T-cell response in these PBMC samples. Our data show that vector-specific CD4 T cells induced by different HIV vaccine vectors manifest marked difference in their susceptibility to HIV infection; compared to Ad5-specific CD4 T cells in HVTN204 PBMC, the ALVAC-specific CD4 T cells in RV144 PBMC are substantially less susceptible to both R5 and X4 HIV infection in vitro. The differential HIV susceptibility between these two groups of vector-specific CD4 T cells is closely associated with their differences in phenotype, cytokine expression, and interestingly, the profiles of vector-specific CD8 vs. CD4 T-cell proliferative response induced by these two vectors.

METHODS

Ethics statement and study participants

The study involves use of PBMC samples from two HIV vaccine clinical trials: RV144 (NCT00223080) (ALVAC-HIV prime/gp120 protein boost) and HVTN204 (NCT00125970) (DNA prime/rAd5 boost). De-identified, cryopreserved PBMC collected from vaccine responders of these two trials were used. All samples were analyzed anonymously and investigators of this study have no access to any subject identification information. The study was determined as non-human subject research and approved by the University of Texas Medical Branch's IRB. Written informed consents were obtained from study participants.

Cells, HIV, and viral vectors

PBMC were maintained at 37°C, 5% CO2 in RPMI medium (Invitrogen) supplemented with 10% human serum, 100 U/mL penicillin G, 100 U/mL streptomycin sulfate, and 1.17mM sodium glutamine. R5 (US1) and X4 (92/UG/029) HIV-1 (original stock from NIH) was used for in vitro infection of PBMC. HIV transmitted founder virus (TFV) strains (including AD17 clone) were a kind gift from Dr. Jason Kimata of Baylor College of Medicine. Empty ALVAC vector was obtained from Sanofi, and empty rAd5 vector was obtained from the Vaccine Research Center (VRC) of NIH.

CFSE staining, vector stimulation, and HIV infection of PBMC

PBMC were CFSE labeled as described previously with slight modifications (100, 101, 103). Thawed and washed PBMC at a concentration of 20 x 10^6 PBMC/mL were stained in 1μM CFSE for 8 minutes at 25°C. Cells were then quenched with 2 mL of warm normal human serum for 5 minutes. Empty ALVAC or rAd5 vector corresponding to the original vaccine was used to re-stimulate CFSE-labeled PBMC (MOI of 3). Unstimulated PBMC were included as a control. Three days after stimulation, cells were exposed to pre-titrated R5 HIV, X4 HIV, or TFV HIV for in vitro infection. Three days after HIV exposure, HIV infection in CD4 T cells was analyzed by flow cytometry based on intracellular HIV p24 expression. For viral kinetics experiments, HIV infection rate was measured at 3 and 9 days post infection. In some experiments, anti-MIP-1α (5μg/mL; clone 93321; R&D Systems), anti-MIP-1β (5μg/mL; clone 24006; R&D Systems), and anti-RANTES (5 μg/mL; clone 21418; R&D Systems) were added to the cultures throughout the experiments to neutralize β-chemokines. In some experiments, anti-human IFNAR antibody (Abcam, final concentration: 5 μg/ml) was added to the cultures throughout the experiments to block type-I IFN signaling.

CD8 T cells depletion, isolation and trans-well co-culture

In some experiments, $CD8^+$ cells were depleted from PBMC using the EasySep Human CD8 Positive Selection Kit (Stem Cell Technologies, cat #17833) for comparison with whole PBMC. In the trans-well co-culture experiment, CD8 T cells were isolated from PBMC of RV144 vaccine recipients using the EasySep™ Human CD8+ T Cell Isolation Kit (StemCell Technologies) according to the manufacturer's protocol after CFSE labeling. After CD8 T cell isolation, CD8 depleted PBMC and the corresponding whole PBMC were infected with ALVAC (MOI = 1), followed by HIV infection as describe above. In addition, isolated CD8 T cells were added back to the trans-well culture to explore mechanisms underlying CD8 T cell-mediated inhibition. Briefly, CD8-depleted PBMC were placed in the bottom chamber of the trans-well co-culture system, and the isolated autologous CD8 T cells were added back to the top chamber. The trans-well culture was also stimulated by ALVAC and infected with HIV as described above. HIV susceptibility and cellular phenotypes for different conditions (whole PBMC, CD8-depleted PBMC, CD8-depleted PBMC with added CD8 T cells in trans-well) were similarly measured by multi-color flow cytometry as described.

Flow cytometric surface, intracellular cytokine and p24 staining and analysis

CFSE staining, vector stimulation and *in vitro* HIV infection of PBMC were conducted as described above. On day 6 after vector stimulation (3 days after HIV infection), cells were subjected to immune staining and flow cytometric analysis to examine the phenotypes and HIV susceptibility of vector-specific CD4 T cells. Cells were

first stained with LIVE/DEAD fixable aqua dead cell stain (ThermoFisher Scientifc, cat #L34957) and antibodies to surface markers including CD3, CD4, CD8, CCR5, α4β7-APC (NIH AIDS Reagent Program), CCR7, PD-1, CD25 and CD45RO. Except α4β7, all surface antibodies were from BD Bioscience. Cells were then fixed, permeabilized (BD Biosciences cat #554722), and stained for HIV p24 (Beckman Coulter) for measuring HIV susceptibility of vector-specific CD4 T cells in PBMC ($p24^+$ rate in $CFSE^{low}$ CD4 T cells). In some experiments that also measured the expression of intracellular cytokines in vector-specific CD4 cells, cells were treated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 5 hours prior to staining in order to stimulate *de novo* cytokine production. After fixation and permeabilization, cells were also stained for intracellular cytokines IFN-γ, IL-2, IL-17, IL-21, (Biolegend), MIP-1β (BD Biosciences). In experiments that measured the antiviral and cytolytic profile of vector-specific CD8 T cells, anti-CD107a antibody (BD Biosciences) was added during cell stimulation. After fixation and permeabilization, cells were also intracellularly stained for perforin and Granzyme B (BD Bioscience). In experiments that measured regulatory T cells, cells were permeabilized using a FoxP3 Staining Buffer Set (eBioscience cat #00-5523-00) and stained for FoxP3 (Biolegend). Antibody capture compensation beads (BD Biosciences) stained with individual antibodies were prepared for compensation. Cell samples and compensation beads were acquired at LSR-II (BD). Flow cytometric data were analyzed using FlowJo Version 10 software (TreeStar).

Cell sorting and real-time PCR for gene expression

Vaccine trial PBMC were CFSE stained and vector stimulated as described above. After 6 days of proliferation, cells were stained for CD3, CD4 and viability (Live/Dead

Fixable Violet). The $CFSE^{low}$, $CD3^{+}CD4^{+}$ T cells were sorted from PBMC using FACSAria IIU (BD Biosciences). Total RNA was isolated from the sorted cells using Quick-RNA MicroPrep Kit (Zymo) according to the manufacturer's protocol. Gene expression was quantified using iTaq Universal SYBR Green Supermix (Bio-Rad) and the CFX Connect Real-Time PCR Detection System (Bio-Rad) after reverse transcription from RNA into cDNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). Primer sequences for quantification of gene expression are shown in Table 1. The relative quantity of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad, Inc.) Two-tailed, unpaired Student's T tests were performed and a p value ≤ 0.05 considered significant. Ratio-paired T tests were performed where appropriate.

Table 2.1: Primer Sequences

Gene	Forward	Reverse
TRIM5	5'- CTGGCATCCTGGGCTCTCAAAGT - 3'	5'- CATACCCCCAGGATCCAAGCAGTT - 3'
APOBEC3G	5' - GGCTCCACA TAAACACGGTTTC - 3'	5' - AAGGGAATCACGTCCAGGAA - 3'
Tetherin	5'- CTGGGGATAGGAATTCTGGTGCTC - 3'	5'- CTCGCTGTTGGCCTTGATGGTGAA - 3'
MxB	5' - GCACAGTGATGAGCAAGCAGTAA - 3'	5' - TCCTATTTTGGCAGATTCTGCTG - 3'
SAMHD1	5′-TTTTGGGATTCCGTTTGTGT-3′	5′-TCACTGAAAGTTGCCAAGAAAA-3′

RESULTS

ALVAC-specific CD4 T cells are less susceptible to HIV infection in vitro than Ad5 vector-specific CD4 T cells

To compare the HIV susceptibility of different HIV vaccine vector-induced CD4 T cells (ALVAC vs. Ad5) in human vaccine recipients, we employed the in vitro HIV infection assay reported in our previous studies (100, 101, 103) (Summarized in Fig 2.1). In brief, PBMC samples of vaccine recipients in RV144 (ALVAC) and HVTN204 (Ad5 vector) were first stained with CFSE, a fluorescent dye used to track T-cell proliferation, and then stimulated with the corresponding empty vector for three days to induce the expansion of vector-reactive CD4 T cells, followed by infection with either CCR5-tropic (R5; US-1 strain) or CXCR4-tropic (X4; 92/UG/029 strain) HIV. Three days post-infection (dpi), flow cytometry was used to measure T-cell proliferation (indicated by decreased CFSE fluorescence intensity; $CFSE^{low}$) and HIV infectivity in vector-specific CD4 T cells (intracellular HIV $p24^{+}$ rate in $CFSE^{low}$ CD4 T cells) (Fig 2.1A). We have previously verified this in vitro system by demonstrating that the CFSE-low, proliferating CD4 T cells are mostly antigen specific (Fig 2.1B) and closely resemble their in vivo phenotypes (Fig 2.1C).

Based on this system, we first observed that both ALVAC and Ad5 vector induced significant levels of CD4 T-cell proliferation in PBMC of vaccine recipients (ALVAC for RV144 and Ad5 for HVTN204) (Fig 2.2). Regarding HIV susceptibility, we found that compared to Ad5 vector-induced CD4 T cells in HVTN204 PBMC, which were highly susceptible to R5 HIV infection (mean $\%p24^{+}$: 26.9%), the ALVAC-induced CD4 T cells in RV144 PBMC were markedly less susceptible to R5 HIV (mean $\%p24^{+}$: 1.27%) ($p<0.01$, day 3 post-infection) (Fig 2.1A). We also monitored HIV infection in

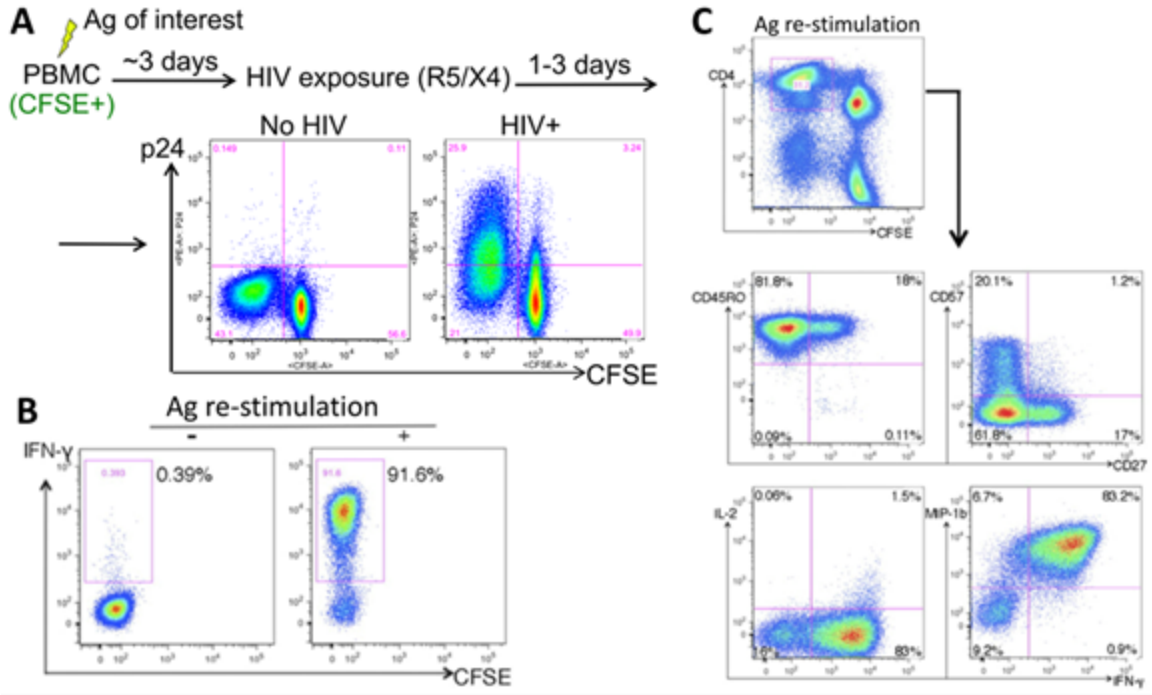

Figure 2.1. Summary and verification of the *in vitro* HIV infection system. (A) PBMCs from human individuals who were positive for CD4 responses to antigen of interest (e.g. to natural infections or vaccination) were CFSE-labeled and then stimulated with recall antigens (antigens from pathogens or vaccines) for ~3 days, followed by exposure to R5 or X4 HIV. Productive HIV infection in antigen (ag)-specific CD4 T cells was determined based on flow cytometric analysis of intracellular p24 in $CFSE^{low}$, proliferating CD4 T cells. **(B)** Assessment of ag specificity of the $CFSE^{low}$, expanded CD4 T cells. We here used CMV antigen as an example, since CMV-specific CD4 T cells manifest a polarized Th1 response with the majority of them producing one same cytokine (IFN-γ), making the assessment of Ag specificity more straightforward. Also, *in vivo* phenotypes of CMV-specific CD4 T cells have been well characterized and can used for comparison with those expanded in vitro in our system. Proliferating T cells were re-stimulated by the same recall antigen (CMV; APC-loaded) on day 6 after initial antigen stimulation. We confirmed that the $CFSE^{low}$ CD4 T cells were mostly antigen specific since >91% of them produced cytokine (IFN-γ) upon Ag re-stimulation. **(C)** *In vitro* expanded antigen-specific CD4 T cells closely resemble their *in vivo* phenotypes. $CFSE^{low}$, CMV-specific CD4 T cells were gated (top) for phenotypic analysis regarding memory differentiation (middle) and cytokine profile (bottom). *In vitro* proliferating CMV-specific cells were largely effector memory cells (CD27−CD45RO+) (81.8%), and a significant fraction of them were terminally differentiated (CD27−CD57+) (20.1%), consistent with their *in vivo* phenotypes. For cytokine expression, a majority of them co-expressed IFN-γ and MIP-1β (83.2%) but very little IL-2 (1.5%). Altogether, the *in vitro* proliferating ag-specific CD4 T cells in our system well mirror their *in vivo* phenotypes.

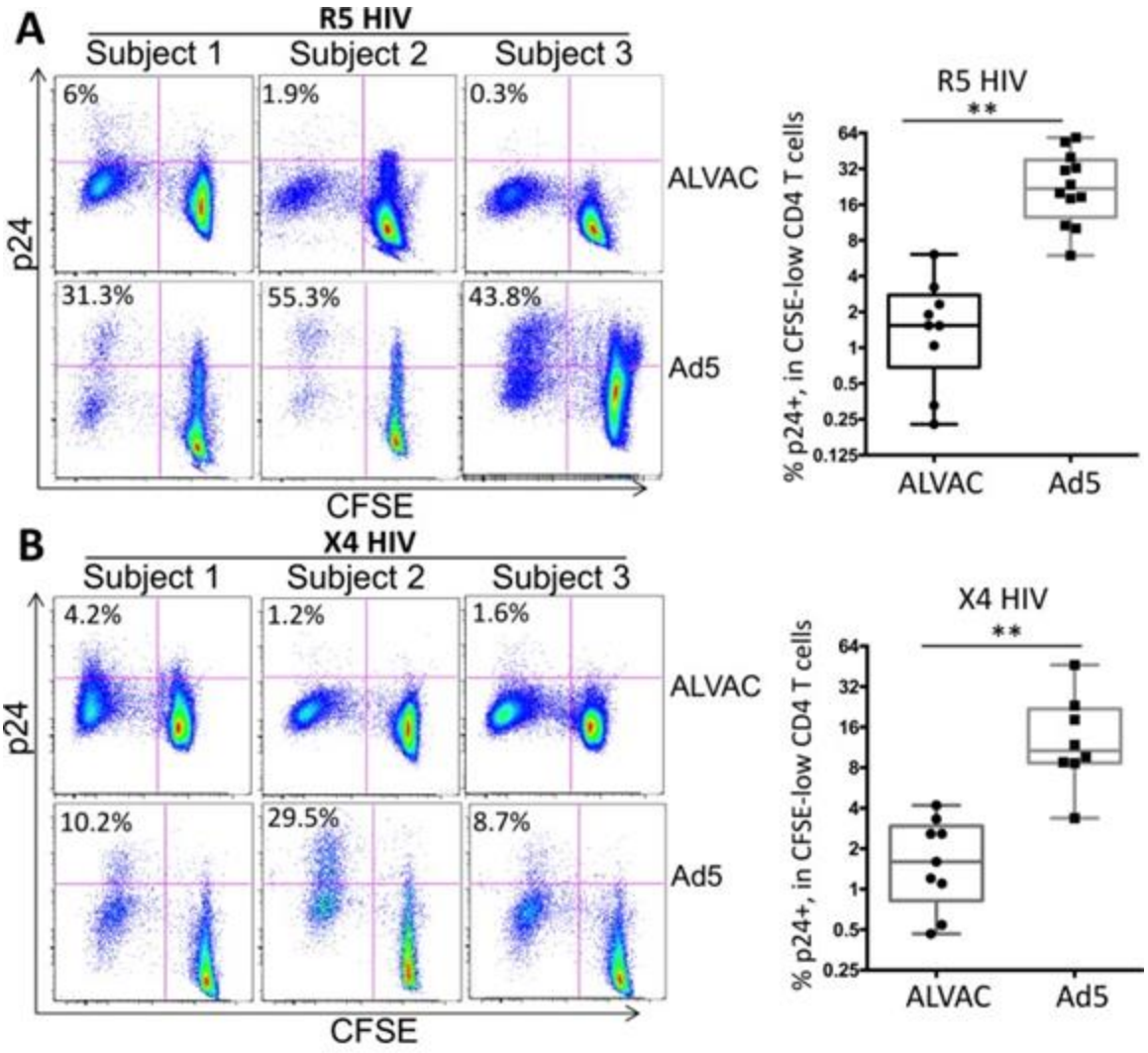

Figure 2.2. ALVAC-specific CD4 T cells are markedly less susceptible to HIV infection in vitro than Ad5 vector-specific CD4 T cells. PBMC collected from ALVAC- (RV144) or Ad5-vectored (HVTN204) HIV vaccine recipients were stained with CFSE and then re-stimulated with the recall vector antigen (ALVAC or Ad5) for three days before being infected with CCR5-tropic (US-1 strain) (A) or CXCR4-tropic (92/UG/029 strain) (B) HIV. HIV infection rate in vector-specific CD4 T cells was determined using flow cytometry to measure p24 expression 3 days post infection and expressed as the percentage of p24+ CFSE-low CD4 T cells. Representative flow cytometry plots shown at left are gated on CD3+CD8- CD4 T cells. Statistical analysis was performed using an unpaired Student's t test. *$p \leq 0.05$, **$p \leq 0.01$.

vector-induced CD4 T cells for up to 9 days post exposure and found that ALVAC-induced CD4 T cells remained resistant to HIV on day 9 post viral exposure ($p24^+$: 0.5%), whereas Ad5 vector-specific CD4 T cells were still readily susceptible ($p24^+$: 11.8%) (Fig 2.3). Consistent with the results of R5 HIV infection, a similar lower susceptibility to X4 HIV (92/UG/029 strain) was also observed for ALVAC-induced CD4 T cells (mean $\%p24^+$: 1.82%) as compared to Ad5 vector-induced CD4 T cells (mean p24+%: 16.2%) ($p<0.01$) (Fig 2.2B). As controls, we showed that the two vectors induced very little T-cell proliferation in pre-vaccine PBMC of the same individuals (Fig 2.4A), suggesting that the T-cell proliferation observed in post-vaccine PBMC in our system were specific to vector with minimal non-specific proliferation. In addition, very little intracellular p24 (<0.1%) was detected in the same proliferating CD4 T cells when HIV was not added, supporting that intracellular p24 staining in our system is specific (Fig 2.4B). As another control, RV144 and HVTN204 PBMC were polyclonally activated by anti-CD3/CD28. We showed that anti-CD3/CD28-activated CD4 T cells in RV144 and HVTN204 PBMC were susceptible to HIV infection at comparable level (Fig 2.5). Furthermore, we noted that in Ad5-stimulated PBMC, the $CFSE^{hi}$ CD4 T cells appeared to be more sensitive to HIV as well compared to those in ALVAC-stimulated PBMC (Fig 2.2A). This might be related to the lower secretion of β-chemokines in the Ad5-stimulated PBMC culture, which will be presented later.

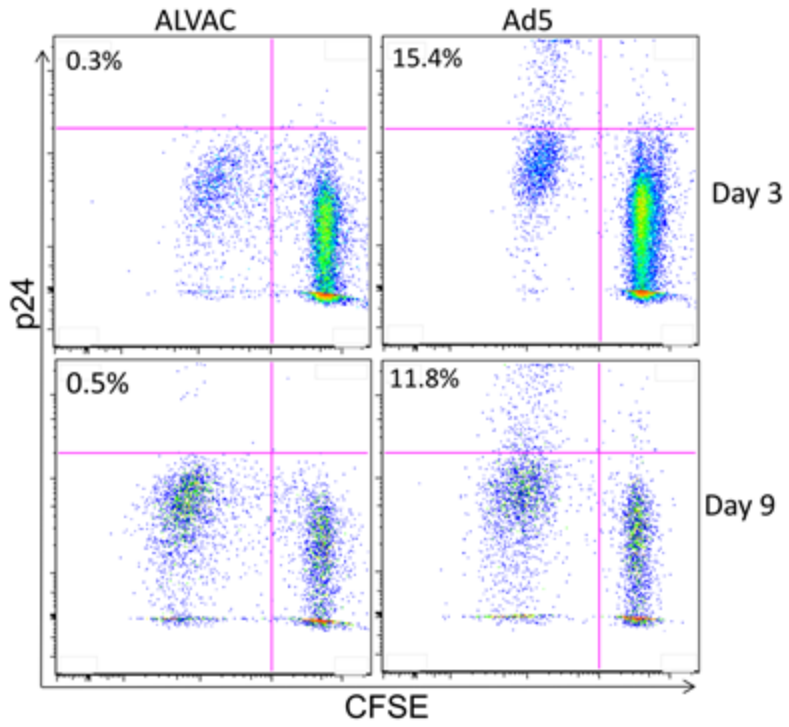

Figure 2.3. HIV infection of CFSE-low vector-induced CD4 T cells at multiple time points after HIV exposure RV144 (left) or HVTN204 (right) PBMC were CFSE-labeled, vector stimulated and HIV-infected as described above. Productive HIV infection in $CFSE^{low}$, vector-induced CD4 T cells was measured by flow cytometry at multiple time points (Day 3 and Day 9) after HIV exposure. Number in each panel shows intracellular $\%p24^{+}$ in $CFSE^{low}$ CD4 T cells.

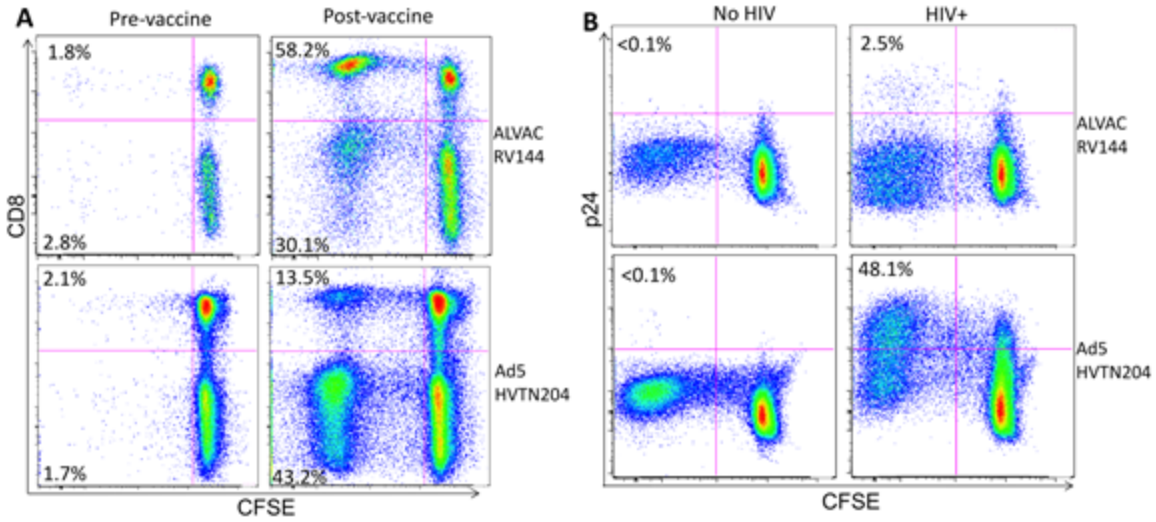

Figure 2.4. Stimulation of T-cell proliferation by vectors in control PBMC and intracellular p24 staining in HIV uninfected CD4 T cells. (A) Pre-vaccine PBMC (left) and post-vaccine PBMC (right) from RV144 (top) and HVTN204 (bottom) vaccine recipients were CFSE-labeled, and respectively stimulated with ALVAC or Ad5 vector. $CD3^+$ total T cells were gated and T-cell proliferation (CD8 and CD4) was analyzed on day 6 after stimulation by flow cytometry. **(B)** Post-vaccine PBMC from RV144 (top) and HVTN204 (bottom) were CFSE-labeled and respectively stimulated with ALVAC or Ad5 vector for 3 days, followed by HIV infection (R5; US-1) or not. 3 days after infection, $CD3^+CD8^-$ T cells were gated and HIV infection in $CFSE^{low}$ $CD3^+CD8^-$ T cells was analyzed by flow cytometry based on intracellular p24 expression. Cells with no HIV infection were used to set up the gate for intracellular p24 staining (left panels).

Transmitted founder virus (TFV) is important in HIV transmission. In addition to R5 US-1 and X4 92/UG/029 strains used, we also tested the susceptibility of vector-induced CD4 T cells to AD17 HIV molecular clone, a TFV (108, 109). Consistently, we observed that ALVAC-induced CD4 T cells were also less susceptible to AD17 TFV infection (%$p24^+$: 0.6%) as compared to Ad5 vector-induced CD4 T cells (%$p24^+$: 3.8%) (Fig 2.6), although the overall infectivity of AD17 TFV in these CD4 T cells was lower than that of the US-1 and 92/UG/029 strains (Fig 2.6).

In vector HIV vaccination, insert-specific CD4 T cells are also induced in addition to vector-specific CD4 T cells. Therefore, we measured HIV susceptibility of vaccine Env-specific CD4 T cells using the same assay and found that unlike vector-specific CD4 T cells, Env-specific CD4 T cells in both RV144 and HVTN204 PBMC were readily susceptible to R5 and X4 HIV infection with no significant difference detected (Fig 2.7). Taken together, these data suggest that the vector-specific CD4 T cells induced by different HIV vaccine vectors manifest marked differences in their susceptibility to both R5 and X4 HIV infection *in vitro*, with ALVAC-specific CD4 T cells being less susceptible than Ad5 vector-specific CD4 T cells.

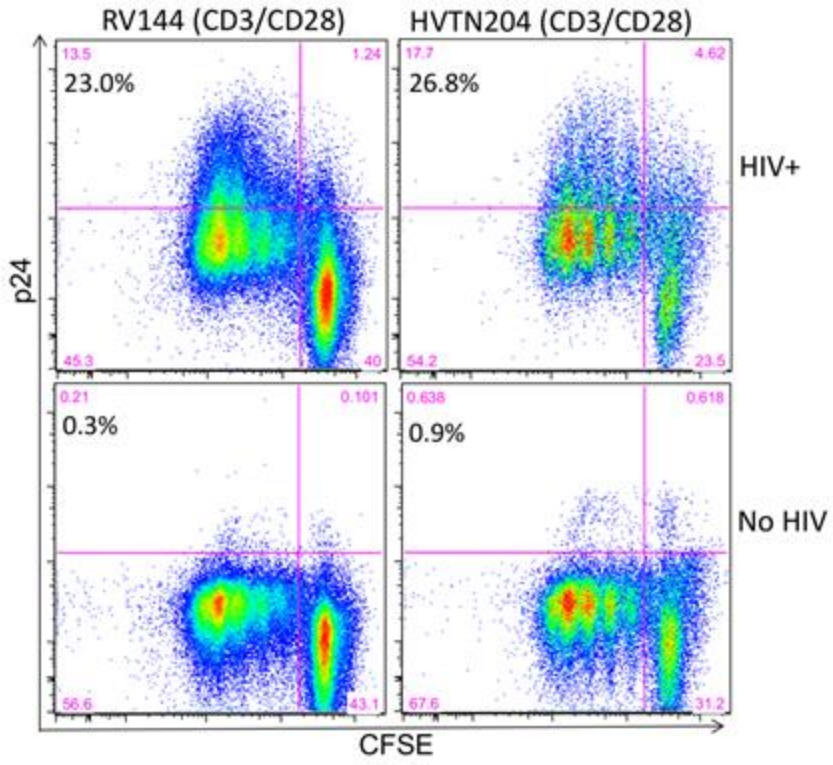

Figure 2.5. HIV susceptibility of polyclonally stimulated CD4 T cells in PBMC. RV144 (left) and HVTN204 (right) PBMC were CFSE-labeled and then polyclonally stimulated with anti-CD3/CD28, followed by HIV infection (US-1) or not. HIV infection in proliferating CFSE-low CD4 T cells was measured by flow cytometry on day 6 as described above.

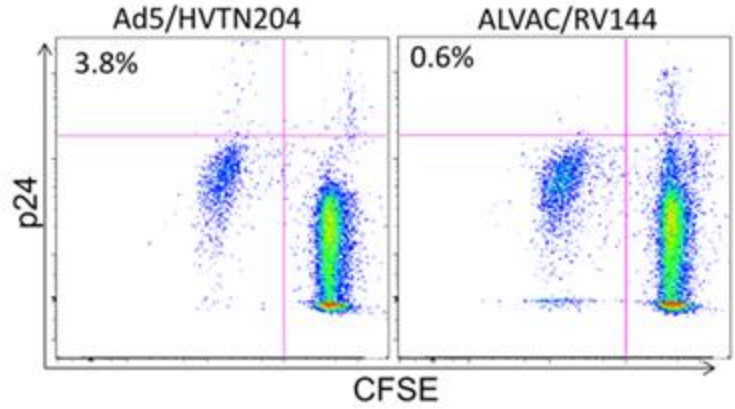

Figure 2.6. *In vitro* HIV susceptibility of vector-induced CD4 T cells to transmitted/founder virus HIV infection (TFV). HIV infection was conducted as described above, except that the transmitted/founder virus (TFV) (AD17 clone; virus prepared by Jason T. Kimata) was used for infection. Productive HIV infection in $CFSE^{low}$, vector-induced CD4 T cells in HVTN204 (left) or RV144 (right) PBMC was determined as described above.

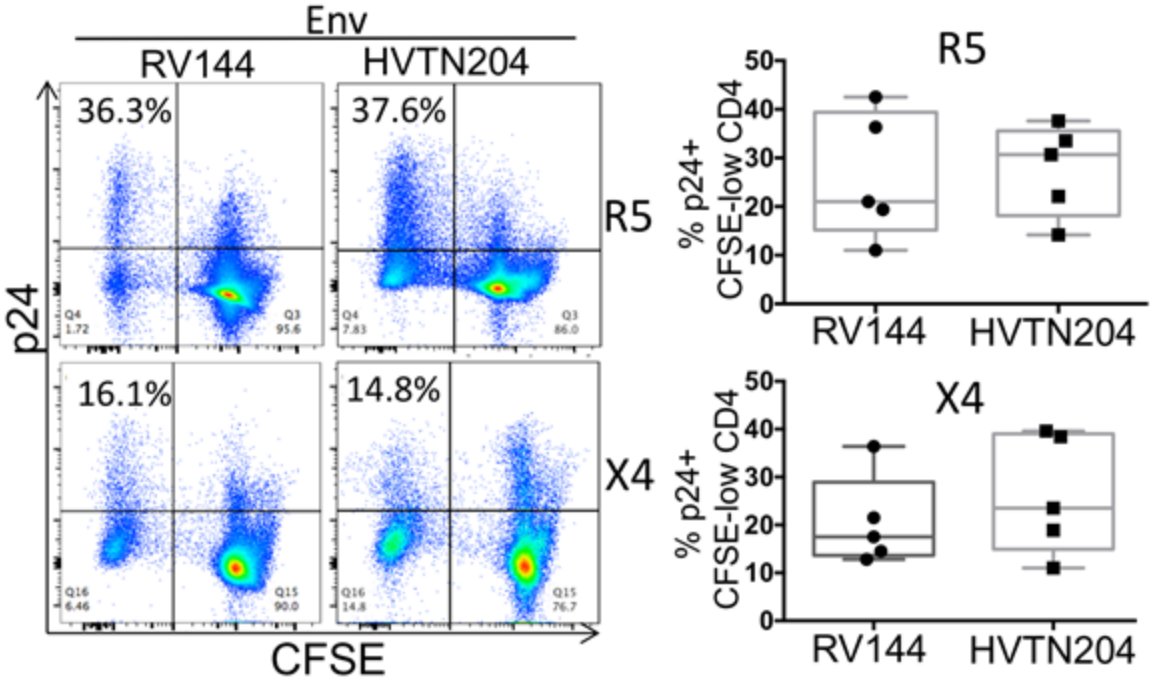

Figure 2.7. In vitro HIV susceptibility of vaccine Env-specific CD4 T cells in PBMC of RV144 and HVTN204. PBMC of RV144 or HVTN204 HIV vaccine recipients were stained with CFSE and then re-stimulated with Env peptides for three days before being infected with R5 (top) or X4 (bottom) HIV. HIV infection rate in Env-specific CD4 T cells was determined using flow cytometry to measure p24 expression 3 days post infection and expressed as the %$p24^+$ $CFSE^{low}$ CD4 T cells. Representative flow cytometry plots shown at left were gated on $CD3^+CD8^-$ T cells.

ALVAC-specific CD4 T cells express lower levels of HIV co-receptor CCR5 and CXCR4 than Ad5 vector-specific CD4 T cells.

We and others have shown that differential HIV susceptibility of human antigen-specific CD4 T cells can occur at both HIV entry and post-entry levels (100, 110). An important factor that influences HIV infection of target cells at the entry level is the surface expression of the HIV co-receptors CCR5 and CXCR4. To understand potential mechanisms underlying the differential HIV susceptibility of ALVAC and Ad5 vector-specific CD4 T cells described above, we examined CCR5 and CXCR4 expression on these two groups of vector-specific CD4 T cells. We found that ALVAC-specific CD4 T cells expressed significantly lower frequencies of CCR5+ CD4 T cells (%$CCR5^+$: 8.4 ± 1.8) than Ad5 vector-specific CD4 T cells (%$CCR5^+$: 31.9 ± 5.1) ($p<0.005$) (Fig 2.8A). A similar difference was also observed for CXCR4 expression on ALVAC- and Ad5 vector-specific CD4 T cells (%$CXCR4^+$ for ALVAC vs. Ad5: 8.3 ± 1.6 vs. 38.6 ± 7.4) ($p<0.001$) (Fig 2.8B). These data suggest that limited expression of CCR5 and CXCR4 represents an important mechanism for the lower susceptibility of ALVAC-specific CD4 T cells to R5 and X4 HIV, respectively, compared to Ad5 vector-specific CD4 T cells.

To better understand the relative contribution of co-receptor expression to the overall HIV susceptibility of vector-induced CD4 T cells in our system, we further analyzed HIV infection in co-receptor$^+$ and co-receptor$^-$ ($CCR5^{+/-}$ and $CXCR4^{+/-}$) subsets of Ad5-specific CD4 T cells as compared to that in ALVAC-specific CD4 T cells. Not surprisingly, we found that majority of HIV infection was observed in $CCR5^+$ or $CXCR4^+$ subsets of Ad5-specific CD4 T cells (Fig 2.8C). We also noted that the HIV infection rate in the $CCR5^-$ subset ($p24^+$: 23%) or CXCR4- subset ($p24^+$: 5.3%) of Ad5-specific CD4 T

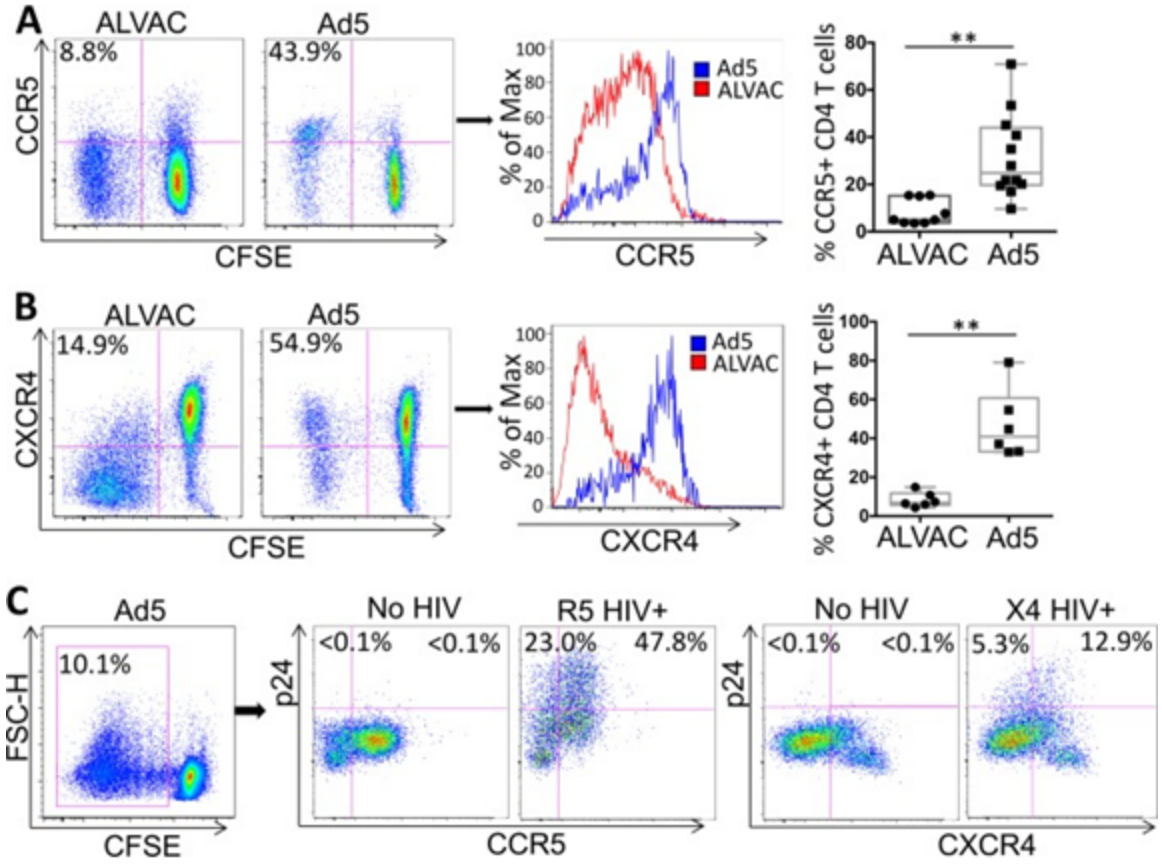

Figure 2.8. ALVAC vector-specific CD4 T cells express lower levels of the HIV co-receptors CCR5 and CXCR4 than Ad5 vector-specific CD4 T cells. PBMC of RV144 and HVTN204 vaccine recipients were stained with CFSE and stimulated with vector (ALVAC or Ad5) for 6 days. Surface expression of CCR5 **(A)** and CXCR4 **(B)** was measured by flow cytometry. Representative flow cytometry dot plots (left; gated on $CD3^+CD8^-$ CD4 T cells) and histogram for co-receptor expression on ALVAC and Ad5 vector-specific CD4 T cells are shown. Comparison of $\%CCR5^+$ or $CXCR4^+$ vector-specific CD4 T cells from multiple subjects is shown (right). **(C)** HIV infection in co-$receptor^+$ vs. co-$receptor^-$ Ad5-specific CD4 T cells. $CFSE^{low}$, Ad5-specific CD4 T cells were gated for analysis (left). HIV infection rate ($\%p24^+$) in $CCR5^+$ vs. $CCR5^-$ Ad5-specific CD4 T cells infected with R5 HIV (middle) or in $CXCR4^+$ vs $CXCR4^-$ Ad5-specific CD4 T cells infected with X4 HIV (right) were shown. For both R5 and X4, no HIV infection was included as control to set p24 staining gate. Statistical analysis was performed using an unpaired Student's t test; $*p \leq 0.05$, $**p \leq 0.01$.

cells (Fig 2.8C) remained higher than the overall HIV infection rate in ALVAC-specific CD4 T cells (Fig 2.2). This data suggests that other factors may also contribute to the differential HIV susceptibility between Ad5- and ALVAC-specific CD4 T cells besides co-receptor expression.

Innate antiviral state and immune activation status of ALVAC- and Ad5-specific CD4 T cells

At the post-entry level of viral infection, HIV infectivity is associated with innate antiviral status and the activation state of target cells. Our recent study has demonstrated that ALVAC and Ad5 vector manifest distinct innate stimulatory properties with ALVAC being able to activate strong innate responses in antigen-presenting cells (APCs) (111). This could potentially affect the antiviral status of CD4 T cells in vector-stimulated PBMC. We therefore compared the antiviral status of vector-specific CD4 T cells in our system. $CFSE^{low}$ CD4 T cells were sorted from vector-stimulated PBMC and subjected to gene-expression analysis for antiviral genes and common HIV restriction factors, including A3G, MxB, SAMHD1, Tetherin and TRIM5. We found that expression of the genes was comparable between Ad5- and ALVAC-specific CD4 T cells (Fig 2.9A). Consistent with this result, blockade of type-I IFN signaling in ALVAC-stimulated PBMC (111) did not significantly alter the HIV infection in ALVAC-specific CD4 T cells (Fig 2.9B). These data suggest that the differential HIV susceptibility of vector-specific CD4 T cells may not be related to their innate antiviral status. Next, we assessed immune activation status of vector-specific CD4 T cells by examining the expression of T-cell activation markers (CD25 and CD69). While no significant difference CD69 expression was observed between ALVAC- and Ad5-specific CD4 T cells, Ad5-specific CD4 T cells appeared to

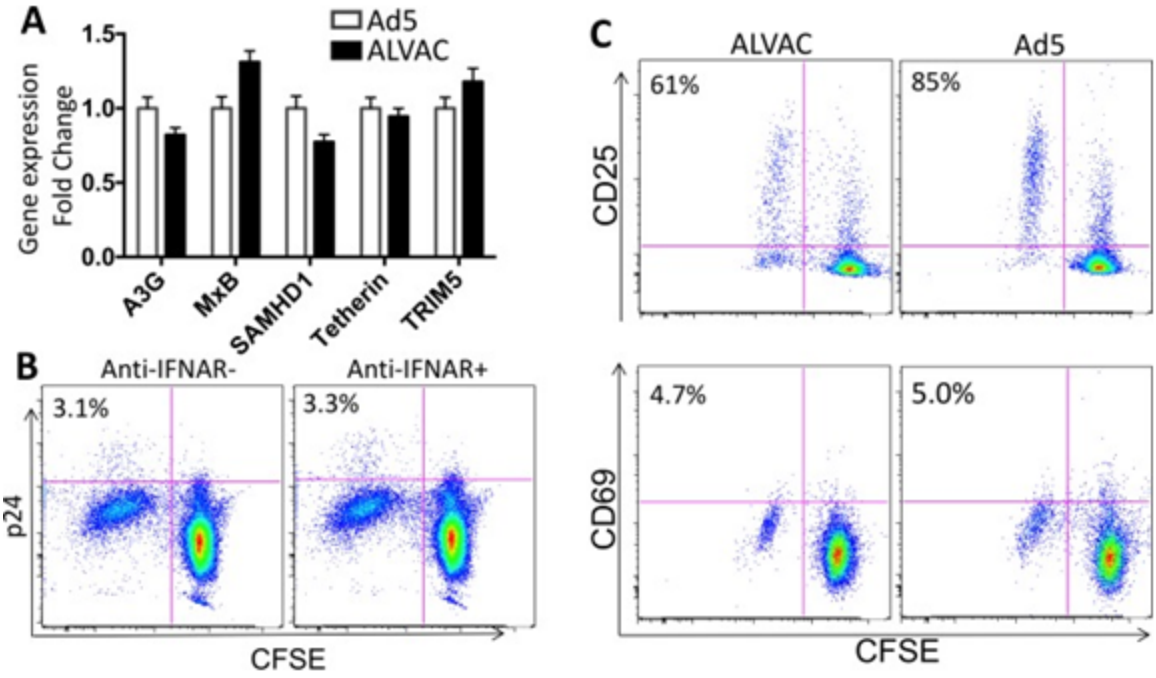

Figure 2.9. ALVAC- and Ad5-specific CD4 T cells show similar levels of innate antiviral gene expression and immune activation. **(A)** Relative expression of innate antiviral genes in ALVAC- and Ad5-specific CD4 T cells. RV144 and HVTN204 PBMC were CFSE-labeled and vector stimulated as described above. On day 6 ALVAC- and Ad5-specific CD4 T cells were sorted from PBMC based on $CFSE^{low}$ and subjected to qPCR for analysis of gene expression. The results were shown as fold change of ALVAC relative to Ad5. **(B)** HIV infection of ALVAC-specific CD4 T cells in RV144 PBMC in the presence or absence of anti-human IFNAR antibody blockade (gated on $CD3^{+}CD8^{-}$ CD4 T cells). Number in each plot shows $\%p24^{+}$ in $CFSE^{low}$ CD4 T cells. **(C)** Surface expression of T-cell activation markers CD25 (top) and CD69 (bottom) on ALVAC- vs. Ad5-specific CD4 T cells 6 days after stimulation with the corresponding vector (gated on $CD3^{+}CD8^{-}$ CD4 T cells). Number in each plot shows $\%CD25^{+}$ or $\%CD69^{+}$ in $CFSE^{low}$ CD4 T cells.

express slightly higher level of CD25 than ALVAC-specific cells (Ad5 vs. ALVAC: 81% vs. 65%) (Fig 2.9C). This activation status of vector-specific CD4 T cells is generally consistent with their susceptibility to HIV infection.

ALVAC-specific CD4 T cells display distinct phenotypic characteristics from Ad5 vector-specific CD4 T cells

Human antigen-specific CD4 T cell populations manifest different phenotypes in memory differentiation, T helper (Th) lineages, and cytokine profiles which are associated with their susceptibility to HIV infection (100-103, 105, 112, 113). We next characterized major phenotypes of ALVAC- and Ad5 vector-specific CD4 T cells. Based on expression of CCR7 and CD45RO, human CD4 T cells can be categorized into central memory (CM: $CCR7^{+}CD45RO^{+}$) and effector memory subsets (EM: $CCR7^{-}CD45RO^{+}$). By focusing on the $CFSE^{low}$ CD4 T cells, we found that both ALVAC- and Ad5 vector-specific CD4 T cells predominantly manifested an EM-like phenotype 2 weeks after the final vaccination, and no significant difference in memory phenotypes was observed between ALVAC- and Ad5 vector-specific CD4 T cells (Fig 2.10A). Mucosal homing is another important characteristic of CD4 T cells that influences HIV pathogenesis. Mucosal compartments represent a major site for HIV infection and CD4 depletion in HIV disease (46). Integrin α4β7 is an important mucosal homing receptor, directing migration of CD4 T cells to gut mucosa (114). We found that compared to Ad5 vector-specific CD4 T cells, which expressed high levels of α4β7 as reported in previous studies (96, 101), ALVAC-specific CD4 T cells expressed significantly lower levels of α4β7 (Fig 2.10B).

Next, we examined T-helper lineage and cytokine profile of ALVAC- and Ad5 vector -specific CD4 T cells. As described, CFSE-stained PBMC from vaccine recipients

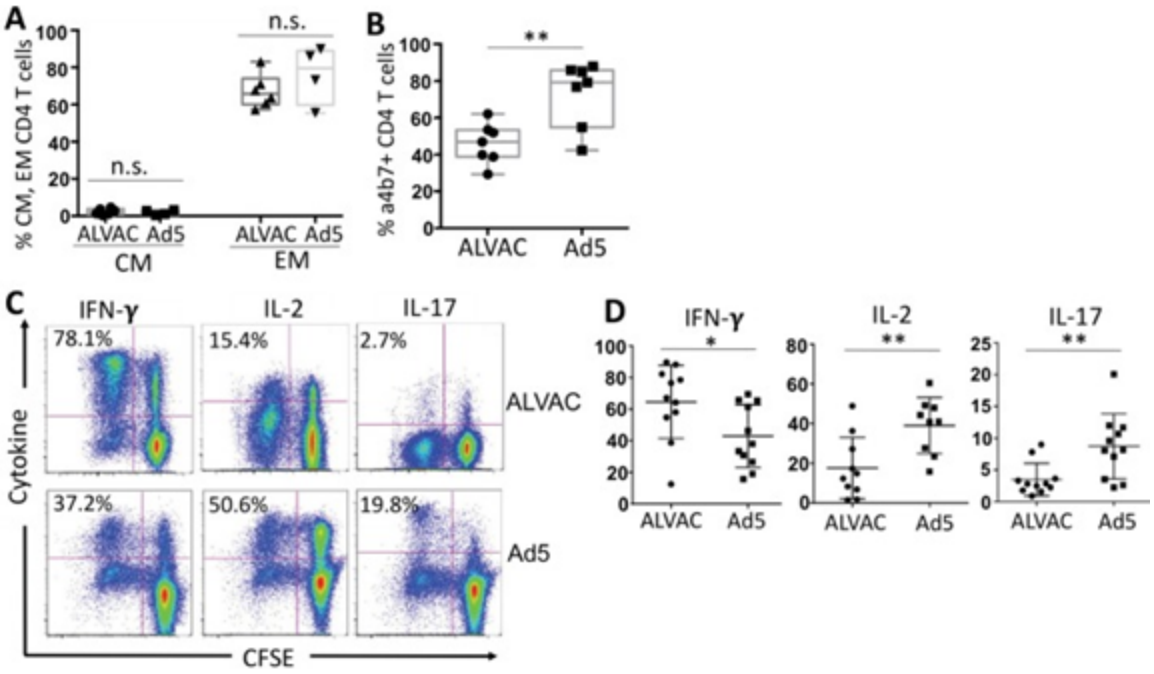

Figure 2.10. Phenotypic characterization of ALVAC- and Ad5 vector-specific CD4 T cells. PBMC from RV144 or HVTN204 vaccine recipients were stained with CFSE and re-stimulated with vector for 6 days. Phenotypes and cytokine profile of $CFSE^{low}$, vector-specific CD4 T cells were measured by flow cytometry. **(A)** Comparison for percent of central memory (CM: $CCR7^{+}CD45RO^{+}$) and effector memory (EM: $CCR7^{-}CD45RO^{+}$) subsets in $CFSE^{low}$, ALVAC- and Ad5 vector-specific CD4 T cells; **(B)** Comparison for $\%\alpha4\beta7^{+}$ in $CFSE^{low}$, ALVAC- and Ad5 vector-specific CD4 T cells; **(C)** Representative flow cytometric plots for cytokine expression (IFN-γ, IL-2, and IL-17) in $CFSE^{low}$, ALVAC-specific (top) or Ad5 vector-specific (bottom) CD4 T cells; **(D)** Comparison for cytokine expression in $CFSE^{low}$, vector-specific CD4 T cells ($\%cytokine^{+}$ $CFSE^{low}$) between ALVAC and Ad5 vector from multiple vaccine recipients (n = 11). n.s.: not significant, $*p \leq 0.05$, $**p \leq 0.01$.

were stimulated with ALVAC or Ad5 vector to induce vector-specific CD4 T cell expansion. Since cytokine expression in activated T cells is usually transient and the $CFSE^{low}$, vector-specific CD4 T cells in our system undergo days of proliferation, in order to measure cytokine production in $CFSE^{low}$ CD4 T cells the culture was re-stimulated with the global PMA/ionomycin stimulus on day 6 for cytokine de novo re-synthesis in T cells as we reported previously (101, 103). Since Th17 CD4 T-cell subset has been shown to be highly susceptible to HIV as compared to Th1 subset (101, 103), we first measured expression of IFN-γ, IL-17 and IL-2 in vector-specific CD4 T cells (Fig 2.10C), and found that a significantly higher fraction of ALVAC-specific CD4 T cells expressed IFN-γ than Ad5 vector-specific CD4 T cells (64.6% ± 6.98 vs. 43.0% ± 5.96; $p<0.05$), typical of a strong Th1-like response. In contrast, a higher fraction of Ad5-specific CD4 T cells expressed IL-2 (39.0% ± 4.68 vs. 17.6% ± 4.90; $p<0.01$) and IL-17 (8.71% ± 1.55 vs. 3.50% ± 0.77; $p<0.01$), suggesting a mixed Th1/Th17 response (Fig 2.10C and D). This result is in agreement with our previous report that examined Ad5 vector-specific CD4 T cells in PBMC from the RV156A trial (101). Therefore, since IL-17- and IL-2-producing CD4 T cells are known to be more susceptible to HIV infection than IFN-γ-producing CD4 T cells, this differential Th1 vs. Th1/Th17 phenotype for ALVAC- and Ad5-specific CD4 T cells is consistent with their susceptibility to HIV infection.

Besides Th1 and Th17 markers, we also examined other major T-cell associated phenotypes for vector-specific CD4 T cells, including T-follicular helper (Tfh), regulatory T cells (Treg) and PD-1 (T-cell exhaustion marker). First, we observed that a significant fraction of both ALVAC- and Ad5-specific CD4 T cells expressed IL-21, a lineage-specific cytokine for Tfh cells. However, unlike IFN-γ and IL-17, no significant difference in IL-

21 expression was found between ALVAC- and Ad5-specific cells (Fig 2.11A). Further analysis identified that HIV infection in the IL-21^{+} Tfh-like subset (p24^{+}: 7.6%) was not higher than the IL-21^{-} subset (p24^{+}: 10.3%) (Fig 2.11B), suggesting that in our system HIV does not preferentially infect Tfh-like CD4 subset (115). Furthermore, we measured expression of Treg markers (CD25 and FoxP3) and the exhaustion marker PD-1 in vector-specific CD4 T cells and found that, similar to Tfh, no significant difference in expression of Treg markers (Fig 2.11C) and PD-1 (Fig 2.11D) was observed between ALVAC- and Ad5-specific CD4 T cells. Altogether, these data suggest that Tfh, Treg and PD-1 phenotypes may not account for the differential HIV susceptibility of ALVAC- and Ad5-specific CD4 T cells in our system.

Higher levels of MIP-1β in ALVAC-specific CD4 T cells contributes to their enhanced HIV resistance

β-chemokines (MIP-1α, MIP-1β, and RANTES) are CCR5 ligands and can block CCR5-tropic (R5) HIV infection at entry level by competitively binding to CCR5 (110, 116, 117). Therefore, we examined MIP-1β (CCL4) expression in the CFSE^{low}, vector-specific CD4 T cells. Not surprisingly, we found that a significantly higher fraction of ALVAC-specific CD4 T cells expressed MIP-1β than Ad5 vector-specific CD4 T cells (57.10% ± 5.67 vs. 36.84% ± 4.16; $p<0.01$) (Fig 2.12A). To evaluate the potential impact of β-chemokine production on HIV susceptibility of vector-specific CD4 T cells in our system, *in vitro* HIV infection (CCR5-tropic; US-1) was conducted in the presence of neutralizing antibodies against these β-chemokines (CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES). We found that blocking β-chemokines could modestly, but significantly, increase the susceptibility of ALVAC-specific CD4 T cells to R5 HIV ($p <0.01$) (Fig

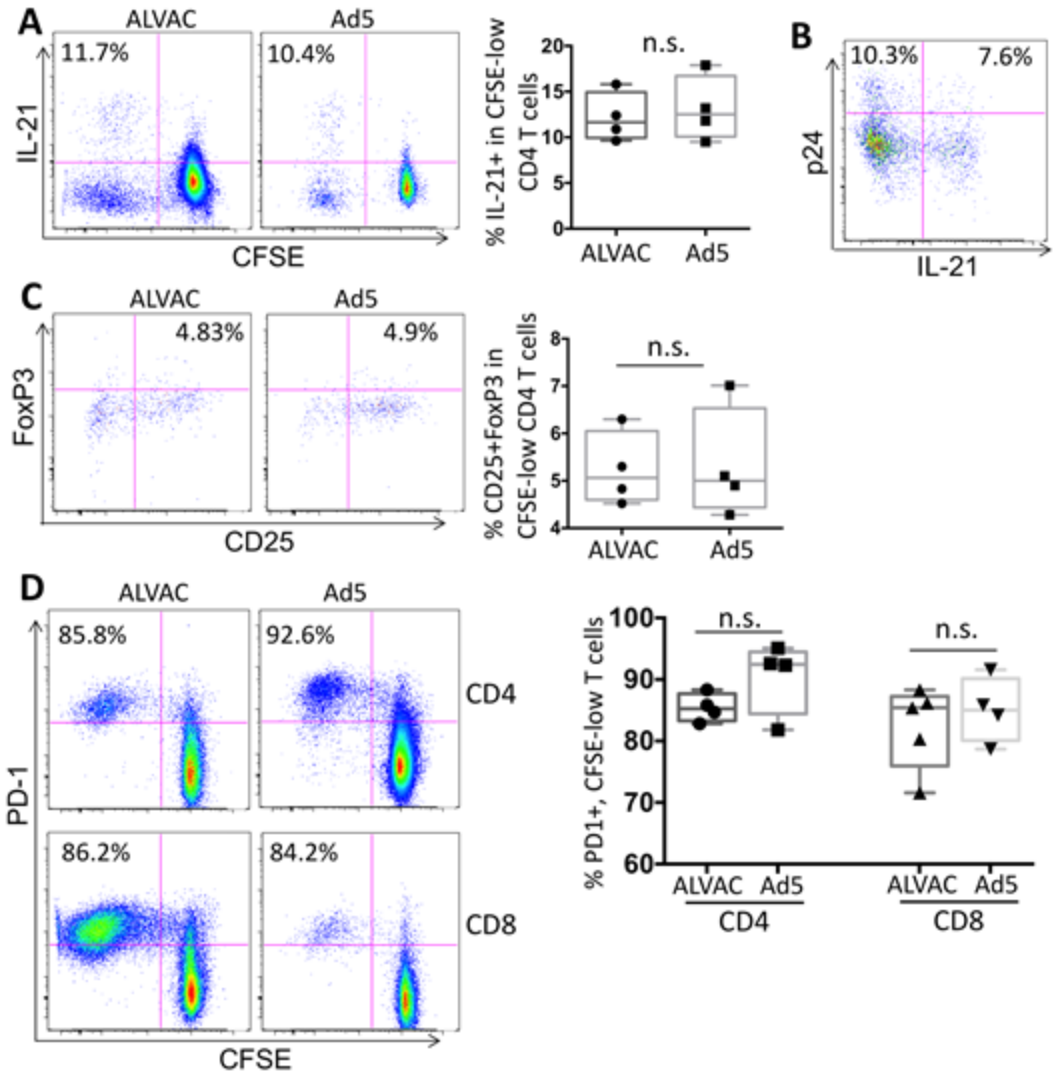

Figure 2.11. Tfh, Treg and PD-1 analysis of vector-specific CD4 T cells. CFSE-labeled RV144 and HVTN204 PBMC were respectively stimulated with ALVAC or Ad5 as described for 6 days. Cells were analyzed for expression of different markers as indicated by flow cytometry. **(A)** Expression of Tfh cytokine IL-21 in $CFSE^{low}$ CD4 T cells. Representative flow cytometry plots and cumulative results comparing the $\%IL\text{-}21^{+}$, $CFSE^{low}$ CD4 T cells between ALVAC- and Ad5-specific CD4 T cells are shown. **(B)** Flow cytometric analysis of HIV infection (intracellular p24) in $IL\text{-}21^{+}$ and $IL\text{-}21^{-}$ subsets of $CFSE^{low}$, Ad5-specific CD4 T cells. Numbers in the plots show $\%p24^{+}$, in $IL\text{-}21^{+}$ (upper right quadrant) and $IL\text{-}21^{-}$ (upper left quadrant) subset of Ad5-specific CD4 T cells. **(C)** Expression of Treg markers (CD25 and FoxP3) in $CFSE^{low}$ CD4 T cells. Representative flow cytometry plots and cumulative results comparing the $\%CD25^{+}FoxP3^{+}$ CD4 T cells between ALVAC- and Ad5-specific CD4 T cells were shown. **(D)** PD-1 expression on vector-specific CD4 and CD8 T cells. Representative flow cytometry plots and cumulative results comparing the $\%PD\text{-}1^{+}$ between ALVAC- and Ad5-specific CD4 and CD8 T cells were shown. n.s.: non-significant.

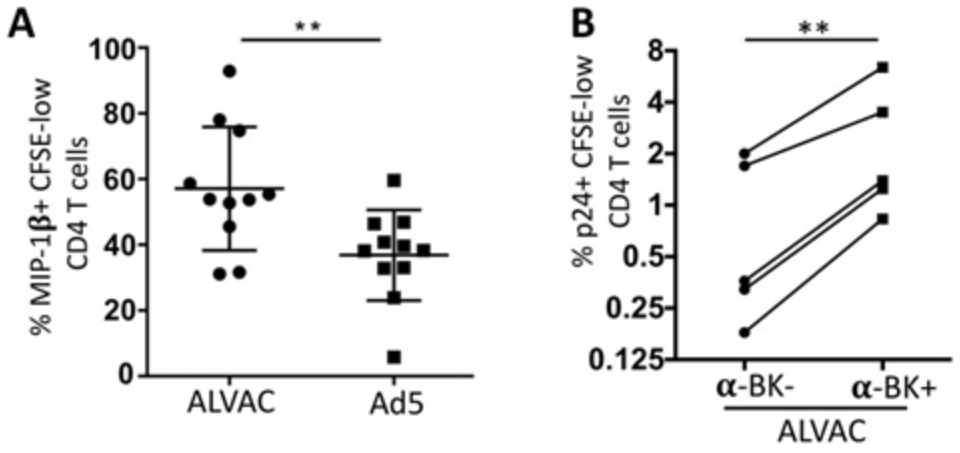

Figure 2.12. ALVAC-specific CD4 T cells produce higher levels of MIP-1β than Ad5-specific CD4 T cells, which contributes partly to their lower susceptibility to in vitro HIV infection. (A) MIP-1β expression in $CFSE^{low}$ vector-specific CD4 T cells was determined by intracellular cytokine staining and flow cytometric analysis as described above; results are expressed as $\%MIP\text{-}1\beta^{+}$ $CFSE^{low}$ CD4 T cells (n = 11). **(B)** Impact of MIP-1β neutralization on HIV infection of ALVAC-specific CD4 T cells. PBMC were stained with CFSE and re-stimulated *in vitro* with ALVAC vector in the absence or presence of β-chemokine neutralizing antibodies (CCL3/4/5). 3 days after vector stimulation, PBMC were infected with R5 HIV, followed by measurement of HIV infection in vector-specific CD4 T cells ($CFSE^{low}$ CD4 T cells) on day 6 after initial vector stimulation. HIV infection was expressed as the percentage of $p24^{+}$ in $CFSE^{low}$ CD4 T cells (n = 4). $*p \leq 0.05$, $**p \leq 0.01$.

2.12B), suggesting a role for β-chemokines in protecting ALVAC-specific CD4 T cells from R5 HIV. However, we also found that even in the presence of β-chemokine neutralization, ALVAC-specific CD4 T cells were still significantly less susceptible to R5 HIV than Ad5 vector-specific CD4 T cells (Fig 2.12B; Fig 2.2A), suggesting that the higher production of β-chemokines contributes only partly to the lower susceptibility of ALVAC-specific CD4 T cells to HIV as compared to Ad5 vector-specific CD4 T cells in our system.

ALVAC and Ad5 vectors elicit distinct profiles of vector-specific CD8 vs. CD4 T-cell proliferative response in PBMC of vaccine recipients

By simultaneous analyses of both CD8 and CD4 T cells, we found that ALVAC and Ad5 vector elicited distinct profiles of vector-specific CD8 vs. CD4 T-cell proliferative response in PBMC. ALVAC stimulated robust vector-specific CD8, but relatively weak vector-specific CD4, T-cell proliferation in RV144 PBMC, whereas Ad5 vector predominantly induced vector-specific CD4, but not CD8, T-cell proliferation in HVTN204 PBMC (Fig 2.13A). When we analyzed the cumulative results from multiple vaccine recipients (n = 14), although no significant difference in the magnitudes of vector-specific CD4 T-cell proliferation was observed between ALVAC and Ad5 (13.43 ± 3.118 vs 19.62 ± 4.633, respectively; p = 0.2776), ALVAC induced significantly higher levels of vector-specific CD8 T-cell proliferation in RV144 PBMC (31.94 ± 5.085 vs 8.908 ± 2.172; p = 0.0004) than Ad5 vector did in HVTN204 PBMC (Fig 2.13B). We further analyzed the ratio of vector-induced CD8 vs. CD4 T-cell proliferation within the same individuals and compared between ALVAC and Ad5 (Fig 2.13C), and found that ALVAC induced a much higher ratio of CD8/CD4 T-cell proliferation than Ad5 vector did (3.137 ± 0.5696 vs 0.5615 ± 0.1364; p = 0.0003) (Fig 2.13C). In contrast, the vaccine insert antigen envelope

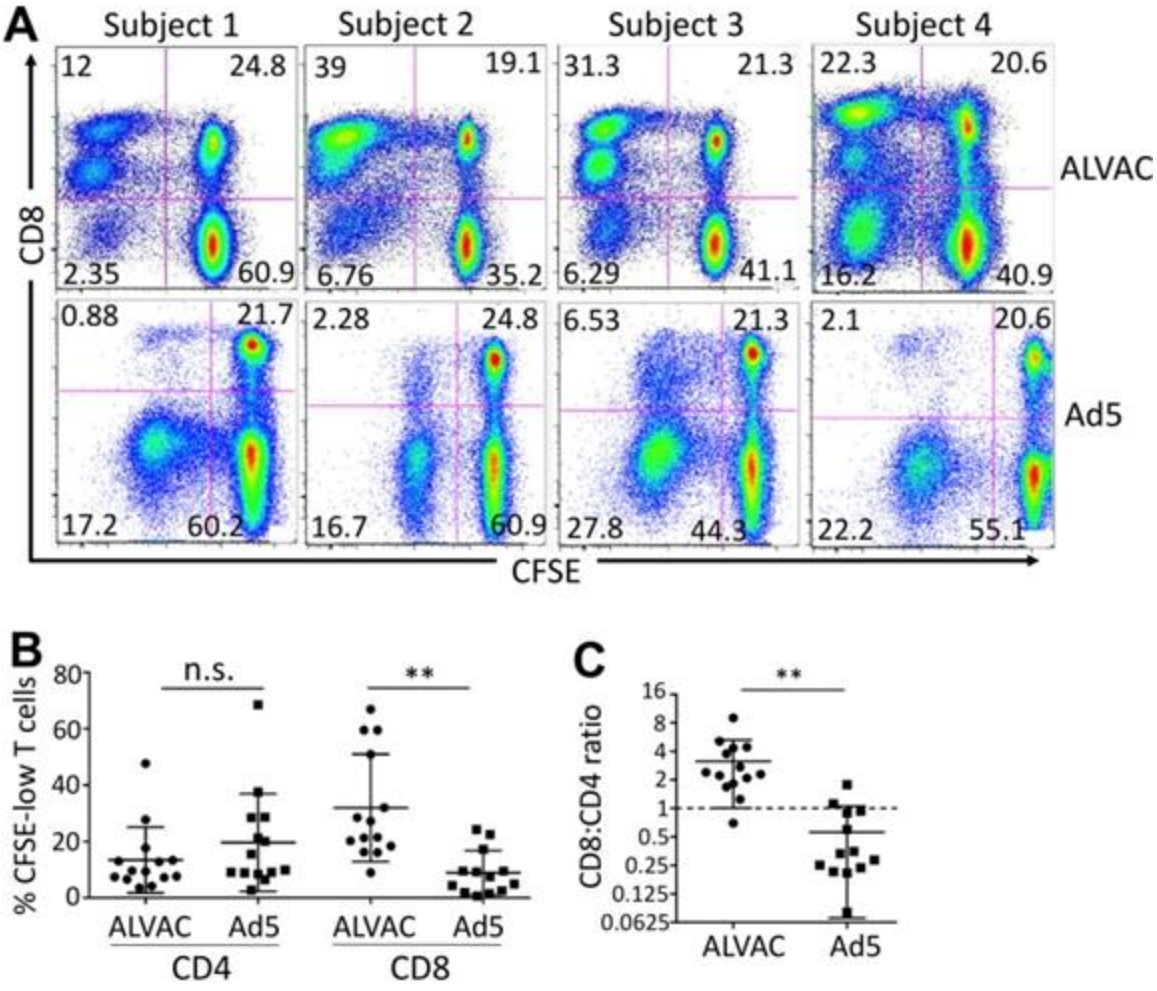

Figure 2.13. ALVAC elicits distinct profile of vector-specific CD8 vs. CD4 T-cell proliferative response compared to Ad5 vector. PBMC were stained with CFSE and re-stimulated with the corresponding vector for 6 days. **(A)** Representative flow cytometry plots for PBMC of multiple subjects showing vector-induced CD8 vs. CD4 T-cell proliferative responses in PBMC of RV144 (top) and HVTN204 (bottom) vaccine recipients. **(B)** Comparison for vector-specific CD8 and CD4 T-cell proliferative responses (%$CFSE^{low}$) in PBMC of RV144 and HVTN204 after corresponding vector stimulation. **(C)** Ratio of vector-specific CD8/CD4 T-cell proliferation in RV144 (ALVAC) and HVTN204 (Ad5) PBMC. Statistical analysis was performed using an unpaired Student's t test; n = 14. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

(Env) induced strong CD4 and weak CD8 T-cell proliferation in RV144 PBMC, but comparable levels of CD4 and CD8 T-cell proliferation in HVTN204 PBMC (Fig 2.14), consistent with the results of Env-specific CD4/CD8 T-cell response measured by *ex vivo* ICS in previous studies (73, 77). Taken together, these data suggest that ALVAC induces a distinct profile of vector-specific CD8 to CD4 T-cell proliferative response from that induced by Ad5 vector *in vitro.*

ALVAC-, but not Ad5-, induced CD8 T cells inhibit the expansion of autologous vector-specific CD4 T cells

The importance of CD8 T cells in anti-HIV immunity, including control of viral replication and limiting HIV-infected CD4 T cells, has been well established (58, 59, 118). In our system, we have observed low levels of CD4 T-cell proliferation in RV144 PBMC after ALVAC stimulation as compared to that in HVTN204 PBMC after Ad5 vector stimulation, which could reflect the inhibition of CD4 T cell proliferation by ALVAC-induced CD8 T cells. Therefore, we next explored the potential impact of vector-induced CD8 T cells on vector-specific CD4 T cell proliferation. CD8 T cells were depleted from PBMC using magnetic cell sorting (MACS) prior to CFSE staining and vector re-stimulation. Efficient depletion of CD8 T cells from PBMC was confirmed (Fig 2.15A). Subsequently, proliferation of CD4 and CD8 T cells in the whole or CD8-depleted PBMC was measured on day 6 by flow cytometry. We showed that depletion of CD8 T cells from ALVAC-stimulated PBMC led to a significant increase in the proliferation of ALVAC-specific CD4 T cells in RV144 PBMC ($p = 0.0068$), whereas no such effect was seen in CD8-depleted HVTN204 PBMC when stimulated by Ad5 vector

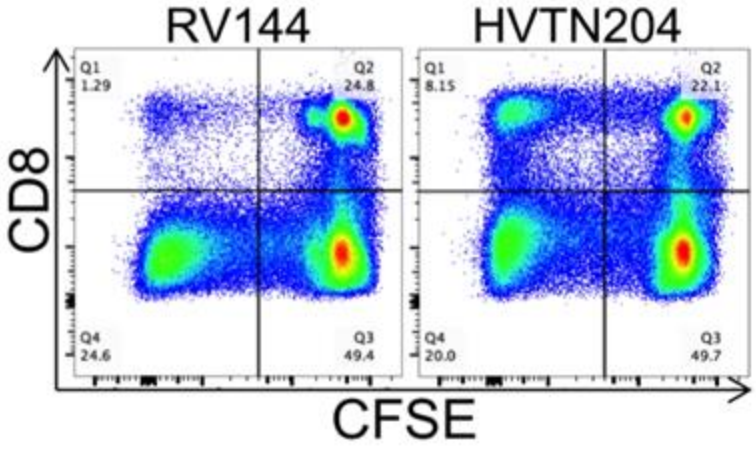

Figure 2.14. Profile of vaccine Env-specific CD8 vs. CD4 T-cell proliferative response in RV144 and HVTN204 PBMC. PBMC were stained with CFSE and re-stimulated with Env peptides for 6 days. CD8 and CD4 T cell proliferation in stimulated PBMC was measured by flow cytometry. Live $CD3^+$ T cells were gated for analysis.

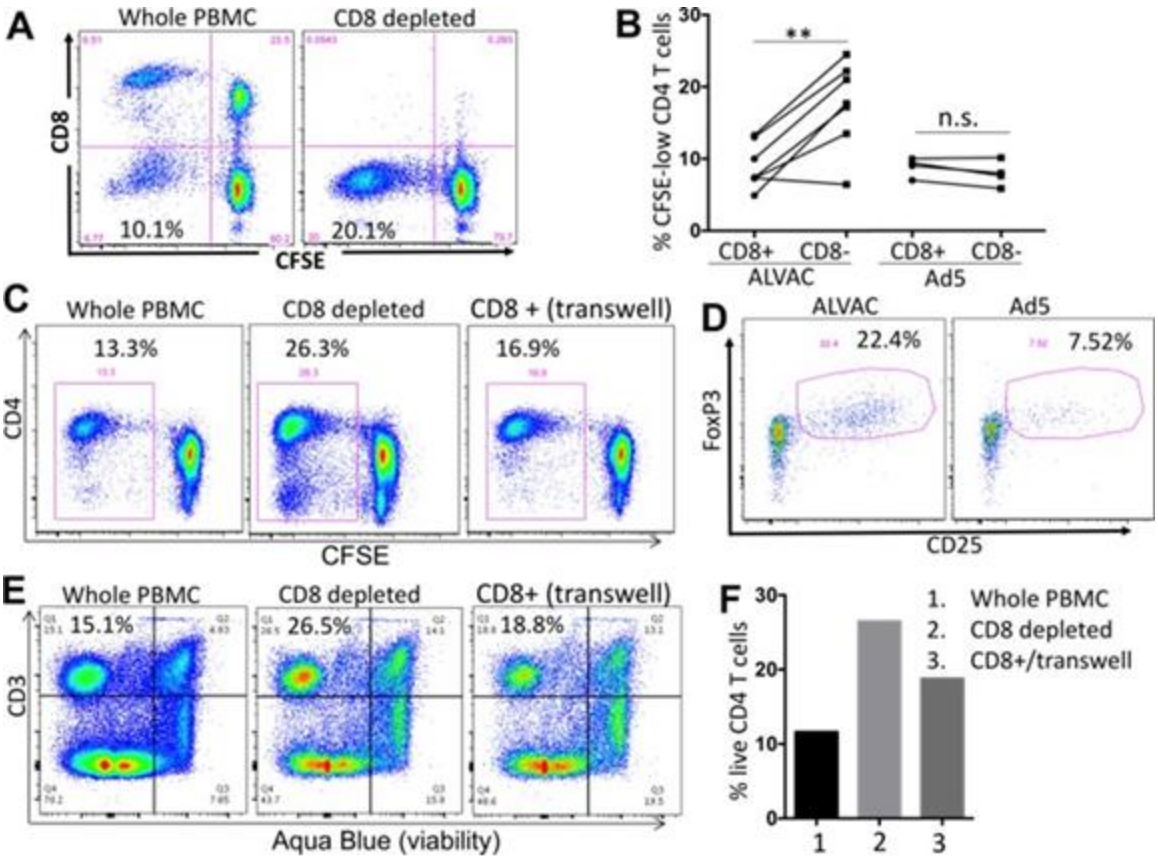

Figure 2.15. ALVAC-induced CD8 T cells inhibit the expansion of autologous vector-specific CD4 T cells. (A) CD8+ cells were depleted from PBMC of vaccine recipients using magnetic beads. CD8-depleted or whole PBMC were CFSE stained and re-stimulated with the appropriate vector for 6 days. Efficient CD8 depletion was verified by flow cytometry. Number in the bottom-left quadrant shows %CFSE^{low}, proliferating CD4 T cells in total CD4 T cells. **(B)** Comparison for vector-specific CD4 T cell proliferation (%CFSE^{low}) in PBMC with or without CD8 depletion (n = 7 for ALVAC; n = 4 for Ad5). **(C)** CD4 T-cell proliferation in RV144 PBMC 6 days after stimulation with ALVAC. Comparison of whole PBMC, CD8-depleted PBMC, and PBMC from which CD8 T cells were depleted and then added back to culture in trans-well (gated on CD3$^+$ T cells). **(D)** CD25 and FoxP3 expression in ALVAC- versus Ad5-specific CD8 T cells 6 days after stimulation with the corresponding vector (gated on CD3$^+$CD8$^+$ CFSE^{low} T cells). **(E)** Flow cytometry plot and **(F)** bar graph showing CD4 T cell viability (%viable cells) in RV144 PBMC 3 days after stimulation with ALVAC (before significant T-cell proliferation occurs), as determined by Aqua Blue dye exclusion. Comparison of cell viability in whole PBMC, CD8-depleted PBMC, and PBMC from which CD8 T cells were depleted then added back to culture in trans-well. Statistical analysis was performed using an unpaired Student's t test; n = 2 (Ad5) or 7 (ALVAC). n.s.: non-significant; *$p \leq 0.05$, **$p \leq 0.01$.

($p = 0.1747$) (Fig 2.15B). These results suggest that ALVAC-induced CD8 T cells can inhibit the expansion of autologous vector- specific CD4 T cells in PBMC.

To explore potential mechanisms by which ALVAC-stimulated CD8 T cells inhibit autologous ALVAC-specific CD4 T-cell proliferation, we conducted trans-well experiments where CD8 T cells were first depleted from PBMC and then added back to the culture in trans-well. We found that addition of CD8 T cells in trans-well could largely, though not completely, restore the inhibitory effect of CD8 T cells on ALVAC-specific CD4 T-cell proliferation (from 26.3% to 16.9%, compared to 13.3% for whole PBMC) (Fig 2.15C), suggesting that CD8 T cells inhibit ALVAC-specific CD4 proliferation via a cell-contact-independent mechanism. $CD25^{+}FoxP3^{+}$ regulatory CD8 T cells are an emerging CD8 subset with strong suppressive activities (119). We measured CD25 and FoxP3 expression in vector-activated CD8 T cells on day 6 after initial vector stimulation and found that a much higher fraction of ALVAC-activated CD8 T cells were $CD25^{+}FoxP3^{+}$ (22.4%) as compared to Ad5-activated CD8 T cells (7.52%) (Fig 2.15D), suggesting that ALVAC-induced $CD25^{+}FoxP3^{+}$ CD8 T cells could play a role in inhibition of autologous vector-specific CD4 T-cell proliferation.

In addition to CD4 T cell inhibition, we also explored potential cytolytic effects of CD8 T cells from RV144 vaccine recipients on autologous CD4 T cells in response to ALVAC stimulation. Three conditions of RV144 PBMC were prepared as described above, including whole PBMC, CD8-depleted PBMC, and CD8 T cell addition back to trans-well culture (Fig 2.15E). On day 3 after ALVAC stimulation, before significant cell proliferation occurred in the culture (Fig 2.16), the viability of total cells ($CD3^{+}$ T cells and $CD3^{-}$ non-T cells) was measured by flow cytometry based on aqua blue staining (Fig

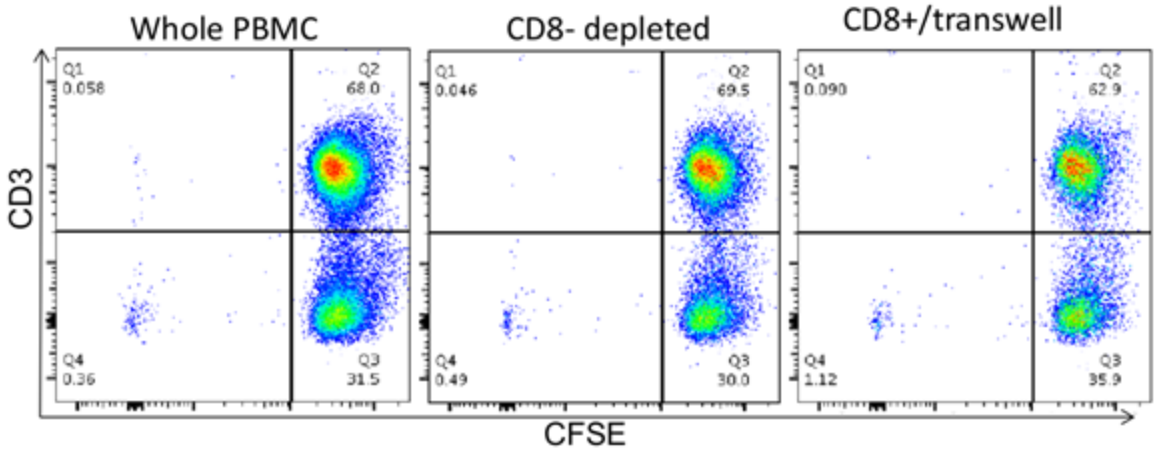

Figure 2.16. No significant cell proliferation was detected in RV144 PBMC early at day 3 post ALVAC stimulation. RV144 PBMC were CFSE-labeled and stimulated with ALVAC for 3 days as described. Cell proliferation ($CD3^+$ T cells and $CD3^-$ non-T cells) was measured by flow cytometry based on CFSE intensity.

2.15E). We observed that compared to the whole PBMC that had only 15.1% live T cells, CD8-depleted PBMC had higher levels of live T cells (26.5%) (Fig 2.15E and F). Addition of the depleted CD8 T cells back to the trans-well culture decreased the level of live T cells (18.8%) (Fig 2.15 E and F). The percent of live CD4 T cells (after subtracting CD8 T cells from the total live CD3+ T cells) in each condition was summarized and shown in Fig 2.15F. This data suggests that in ALVAC-stimulated PBMC, CD8 T cells can manifest a cytotoxic effect on the autologous CD4 T cells, which involves a cell-contact-independent mechanism. This cytotoxic effect of CD8 T cells may also contribute to the overall inhibition of ALVAC-specific CD4 T-cell expansion in our system.

ALVAC-, but not Ad5-, induced CD8 T cells limit HIV infection of autologous vector-specific CD4 T cells

In the context of vector HIV vaccination, it has been speculated that vector-induced CD4 T cells can be potential targets for HIV, which may affect the risk of HIV acquisition in vaccine recipients and overall outcome of vaccination (89). Therefore, limiting the numbers and/or HIV susceptibility of vector-induced CD4 T cells in HIV vaccination is thought to be critical. We next explored the impact of vector-induced CD8 T cells on HIV susceptibility of autologous vector-specific CD4 T cells in PBMC, by using the above CD8-depletion assay. Whole or CD8-depleted PBMC were CFSE-labeled, and stimulated with vector antigen for 3 days, followed by infection with R5 or X4 HIV. Three days after infection, HIV infectivity in vector-specific CD4 T cells was measured by flow cytometry based on intracellular HIV p24 expression in CFSE-low CD4 T cells. We found that compared to whole PBMC, depletion of CD8 T cells from ALVAC-stimulated PBMC led to considerable increase in both R5 and X4 HIV infection of ALVAC-specific CD4 T cells

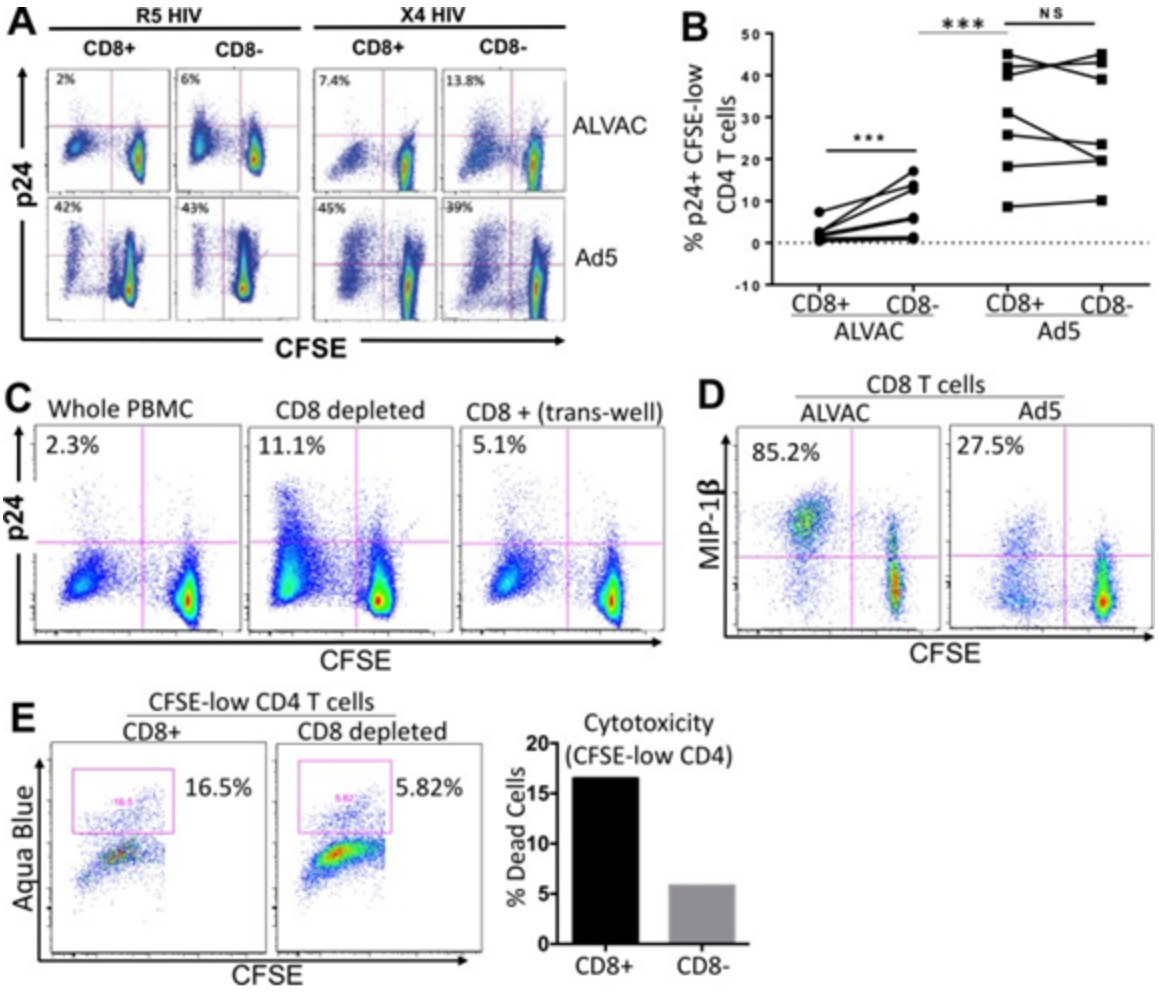

Figure 2.17. CD8 depletion increases HIV susceptibility of ALVAC-specific CD4 T cells.(A) Representative flow cytometry plots showing HIV infection in $CFSE^{low}$, vector-specific CD4 T cells in whole ($CD8^+$) or CD8-depleted ($CD8^-$) PBMC. Whole and CD8-depleted PBMC were CFSE stained and stimulated with vector antigen for 3 days before being infected with R5 or X4 HIV. HIV infection rate was determined using flow cytometry to measure intracellular p24 and expressed as $\%p24^+$ in $CFSE^{low}$ CD4 T cells. $CD3^+CD8^-$ T cells were gated for analysis. **(B)** Comparison for HIV infection rates in $CFSE^{low}$ vector-specific CD4 T cells ($\%\ p24^+$) in whole or CD8-depleted PBMC from multiple vaccine recipients. **(C)** HIV infection ($\%\ p24^+$) in ALVAC-specific CD4 T cells in whole PBMC, CD8-depleted PBMC or PBMC from which CD8 T cells have been depleted and then added back to culture in trans-well (gated on $CD3^+CD8^-$ CD4 T cells). $\%p24^+$ in $CFSE^{low}$ cells was shown. **(D)** MIP-1β expression in ALVAC- versus Ad5-specific CD8 T cells 6 days after stimulation with the corresponding vector (gated on $CD3^+CD8^+$ T cells). **(E)** Flow cytometry plot (left) and bar graph (right) showing the viability of ALVAC-specific CD4 T cells (based on Aqua Blue staining) 6 days after vector stimulation with or without CD8 T-cell depletion (gated on $CD3^+CD8^-$ CD4 T cells). Statistical analysis was performed using an unpaired Student's t test; $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

(R5 HIV for $CD8^+$ and $CD8^-$: 2% vs. 6%; X4 HIV for $CD8^+$ and $CD8^-$: 7.4% vs. 13.8%) (Fig 2.17A). Analyses of PBMC from multiple subjects showed strong statistical significance between whole and CD8-depleted PBMC ($p = 0.0006$) (Fig 2.17B); in contrast, depletion of CD8 T cells in Ad5 vector-stimulated PBMC (HVTN204) had no significant impact on HIV infection rate of Ad5 vector-specific CD4 T cells (Fig 2.17A and B). Of interest, it should be noted that even in the absence of CD8 T cells (CD8 depletion), ALVAC-specific CD4 T cells were still significantly less susceptible to HIV infection than Ad5 vector-specific CD4 T cells (5.63 ± 1.84 vs 28.56 ± 5.16; $p = 0.0002$) (Fig 2.17B), suggesting that CD8 T cells contributed only partly to the low HIV susceptibility of ALVAC-specific CD4 T cells as compared to Ad5 vector-specific CD4 T cells. These data indicate that unlike Ad5 vector, ALVAC may induce vector-specific CD8 T cells that can not only inhibit the expansion of autologous vector-specific CD4 T cells, but also limit their susceptibility to HIV infection.

CD8 T cells can control viral infections through various mechanisms, including cytolytic activity and the secretion of soluble HIV-suppressive factors (120). We next characterized potential mechanisms underlying CD8-mediated HIV inhibition in autologous ALVAC-specific CD4 T cells. First, we observed that CD8 depletion did not significantly affect the expression of CCR5 and T-cell activation markers (CD25 and CD69) on ALVAC-specific CD4 T cells (Fig 2.18). We then performed a similar CD8 trans-well experiment to explore if the HIV inhibition by CD8 T cells is dependent of cell contact or soluble factors. We found that CD8 T cells could still inhibit R5 HIV infection in ALVAC-specific CD4 T cells even in the absence of direct cell contact (p24% for CD8- vs. trans-well CD8+: 11.1% vs. 5.1%) (Fig 2.17C), indicating that soluble HIV suppressive factors

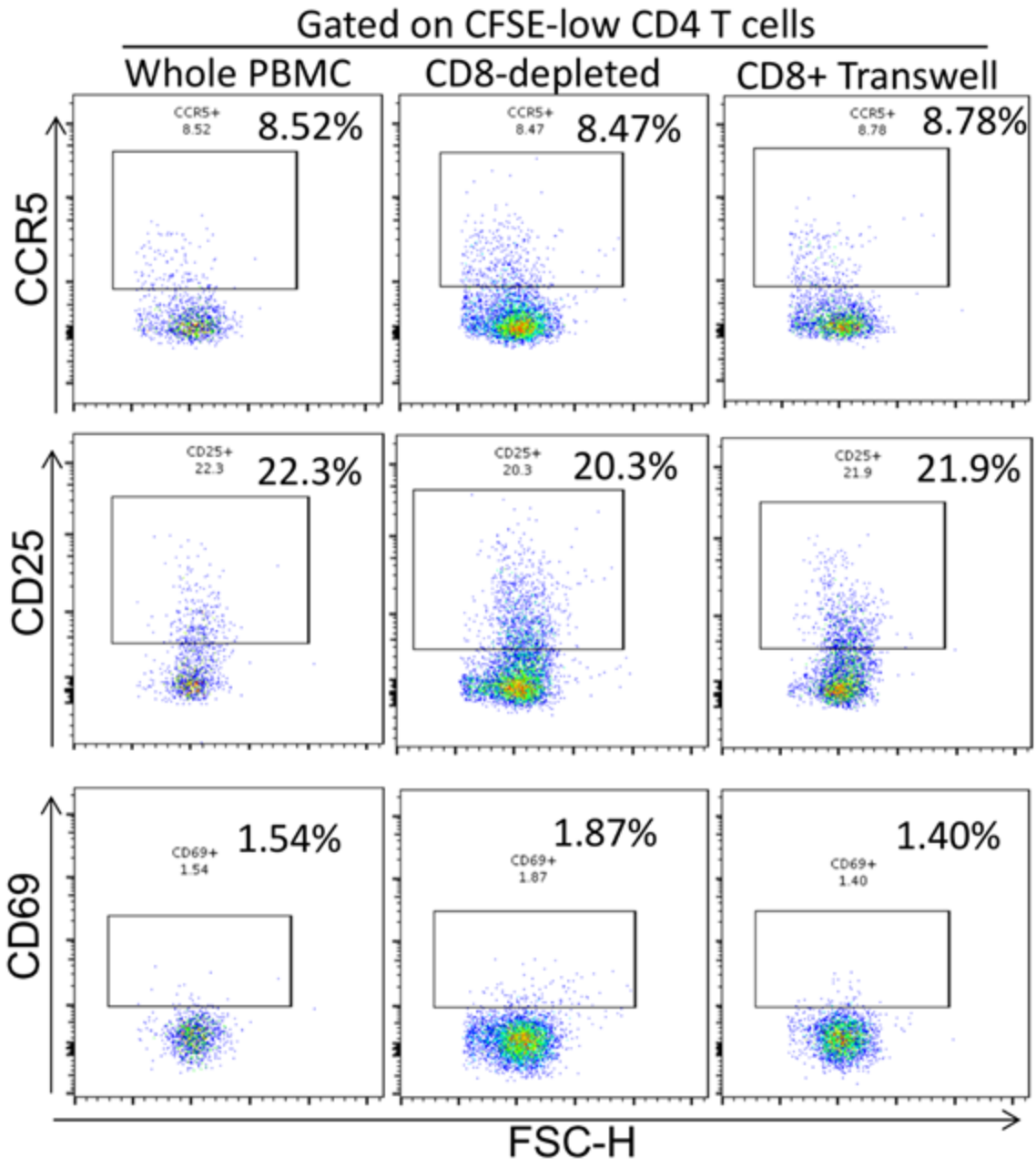

Figure 2.18. Impact of CD8 depletion on expression of CCR5 and activation markers (CD25 and CD69) on CFSE-low, ALVAC-specific CD4 T cells. Three conditions of one RV144 PBMC (Whole PBMC, CD8-depleted PBMC, and CD8 addition back in trans-well) were CFSE-labeled and stimulated ALVAC as described. On day 6, CCR5 (top), CD25 (middle) and CD69 (bottom) expression on CFSE-low CD4 T cells was measured by flow cytometry.

may play a role in this process. Consistent with this observation, we found that compared to Ad5 vector, ALVAC-induced CD8 T cells produced markedly higher levels of MIP-1β (MIP-1β+ % in Ad5 vs. ALVAC-induced CD8 T cells: 27.5% vs. 85.2%) (Fig 2.17D). Since in ALVAC-stimulated PBMC, high levels of CD8 T cells were induced (Fig 2.13), and β-chemokines were shown to mediate R5 HIV inhibition in ALVAC-specific CD4 T cells in our system (Fig 2.12), secretion of more β-chemokines might represent a mechanism for HIV inhibition in ALVAC-specific CD4 T cells by CD8 T cells. Lastly, we observed that compared to CD8-depleted PBMC, the presence of CD8 T cells in PBMC led to higher level of cell death (based on aqua blue staining) in CFSE-low, ALVAC-specific CD4 T cells (aqua blue staining in $CD8^+$ vs. $CD8^-$: 16.5% vs. 5.82%) (Fig 2.17E). These data suggests that the cytotoxic effects of CD8 T cells may also contribute to overall HIV inhibition in ALVAC-specific CD4 T cells.

ALVAC-induced CD8 T cells manifest a stronger antiviral and cytotoxic phenotype than Ad5 vector-induced CD8 T cells

Lastly, we characterized the poly-functional profile of ALVAC- and Ad5 vector-induced CD8 T cells by examining expression of antiviral and cytolytic effectors. CFSE-stained PBMC from RV144 or HVTN204 were re-stimulated with ALVAC or Ad5 vector, respectively, as described above. Six days after stimulation, cells were briefly treated with PMA and ionomycin for 6 hours to induce de novo re-synthesis of cytokines or effector molecules. Expression of IFN-γ, MIP-1β, CD107a, granzyme B (GZMB), and perforin in CFSE-low, vector-induced CD8 T cells was measured by flow cytometry. We found that compared to Ad5 vector, significantly higher percentages of ALVAC-induced CD8 T cells expressed IFN-γ (78.74 ± 12.50 vs 36.86 ± 7.57; $p = 0.0210$), MIP-1β (88.38 ± 4.753 vs

33.00 ± 4.51; $p < 0.0001$) and perforin (75.86 ± 9.139 vs 27.91 ± 8.369; $p = 0.0047$) (Fig 2.19). No significant difference in expression of GZMB (ALVAC vs. Ad5: 31.60 ± 9.720 vs 19.94 ± 5.913; $p = 0.4261$) and CD107a (ALVAC vs. Ad5: 15.32 ± 6.853 vs 6.893 ± 1.199; $p = 0.2713$) was observed between ALVAC- and Ad5-induced CD8 T cells (Fig 2.19). Altogether, these data suggest that ALVAC-induced CD8 T cells manifest a stronger antiviral and cytolytic phenotype than Ad5 vector-induced CD8 T cells.

DISCUSSION

In the present study, by using PBMC samples from two important HIV vaccine trials, we investigated host anti-vector T-cell responses induced by ALVAC and Ad5 vector in human vaccine recipients with a focus on the HIV susceptibility of vector-specific CD4 T cells. Our major finding is that different HIV vaccine vector-induced CD4 T cells manifest distinct susceptibility to HIV infection; while Ad5 vector-specific CD4 T cells are readily susceptible to HIV (101), ALVAC-specific CD4 T cells in RV144 PBMC are more resistant to both R5 and X4 HIV infection. Associated with this are the differences in phenotypes and cytokine profiles of these two groups of vector-specific CD4 T cells. Another major finding of our study is that in contrast to the lack of vaccine insert-specific CD8 T-cell response reported from the RV144 trial (73, 75), we demonstrate that ALVAC vector induces strong proliferative response of vector-specific CD8 T cells, which can limit the proliferation and HIV susceptibility of the autologous ALVAC-specific CD4 T cells.

The unexpected outcomes of human trials testing HIV vaccine regimens involving different viral vectors have suggested that assessment of both protective and potentially detrimental immune responses induced by vaccination is important (80, 89). Development of a safe and efficacious HIV vaccine poses a unique challenge in that HIV infects the very

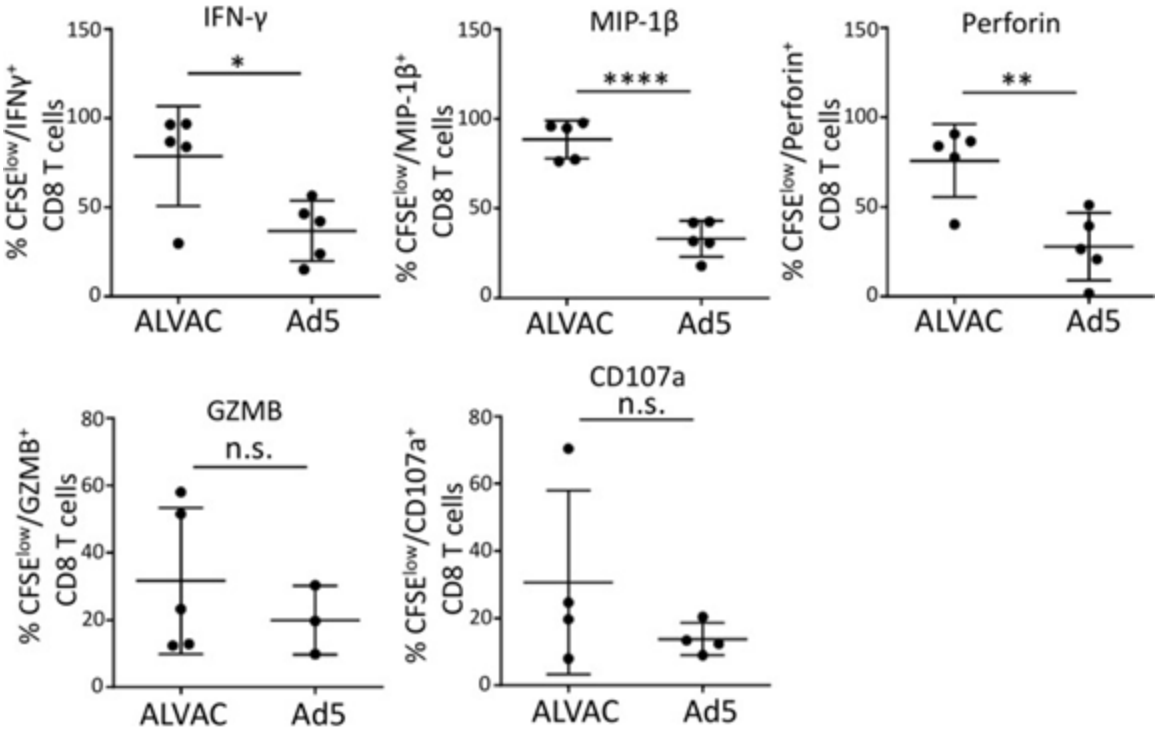

Figure 2.19. ALVAC-induced CD8 T cells manifest stronger antiviral and cytotoxic phenotype than Ad5 vector-induced CD8 T cells. PBMC of vaccine recipients were stained with CFSE and then stimulated with vector antigen for 6 days, followed by brief PMA/Ionomycin re-stimulation (6 hours) for cytokine/effector molecule re-synthesis. Intracellular staining and flow cytometry were used to measure the production of IFN-γ, MIP-1β, perforin, granzyme B (GZMB), and CD107a; results are expressed as %cytokine$^+$ in CFSE^{low} CD8 T cells. Statistical analysis was performed using an unpaired Student's t test; n = 3–5. n.s.: not significant; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

CD4 T cells which are usually required to mount an effective adaptive response; this is of especial concern for viral vector vaccines because the expansion of vector-specific CD4 T cells following immunization can provide potential HIV target cells (89, 101), while presumably not contributing to anti-HIV immunity. From this point of view, it would be advantageous to employ vectors which generate fewer and/or less HIV-susceptible vector-specific CD4 T cells. Human CD4 T cells specific for different antigens or pathogens manifest differential susceptibility to HIV (55, 100-105). Our previous study has reported that human Ad5-specific CD4 T cells induced by natural infection or rAd5 vaccination are more susceptible to HIV infection (101). This finding suggests that although Ad5 vectors have been commonly employed for vaccine development due to their potent immunogenicity (121), the advantages of Ad5 as a vector may be dampened by the high HIV susceptibility of CD4 T cells it induces. Our current study shows that unlike Ad5 vector, the vector-specific CD4 T cells induced by ALVAC in RV144 are markedly less susceptible to HIV infection (Fig 2.2). This finding is relevant to HIV vaccine development, considering that in the context of HIV vaccination, if vaccine-induced protective immunity is comparable between different vaccine regimens, the relative HIV susceptibility of vector-specific CD4 T cells may be an important factor that can affect the overall outcome of HIV vaccination. Future studies are being planned to examine the HIV susceptibility of CD4 T cells induced by other important HIV vaccine vectors, especially the adenovirus rare serotypes Ad26 and Ad35.

Parameters that influence HIV acquisition risk in HIV vaccination are thought to be complex, among which the level, quality (e.g. phenotypes, cytokine profile, and HIV susceptibility) and *in vivo* localization of induced CD4 T cells play important roles. Our

data suggest that the high HIV susceptibility of Ad5 vector-specific CD4 T cells may be a contributing factor for the observed excess HIV infections in some Ad5-HIV vaccine recipients (69, 70, 72). In addition, our ongoing studies examining *in vivo* localization and phenotypes of CD4 T cells following ALVAC and Ad5 immunization show that ALVAC immunization induces substantial lower levels of $CCR5^{+}CD4^{+}$ and $CCR5^{+}$ $\alpha4\beta7^{+}CD4^{+}$ T cells in various immune compartments, especially in the gut mucosa, of the immunized mice as compared to Ad5 immunization. Based on these findings, we propose that to better understand immune parameters associated with HIV acquisition risk in vector HIV vaccination, future studies are warranted to more thoroughly assess the frequency, quality and *in vivo* localization of vaccine-induced CD4 T cells in animal models and/or human trials.

HIV infection of antigen-specific CD4 T cells can be regulated at both entry and post-entry levels, and is closely associated with the phenotypic and functional characteristics of these CD4 T cells (100, 105, 110, 112). CCR5 and CXCR4 as HIV entry co-receptors play major roles in regulating the susceptibility of target cells to HIV at entry level (30). Our data show that ALVAC-specific CD4 T cells express markedly lower levels of CCR5 and CXCR4 than Ad5 vector-specific CD4 T cells (Fig 2.8), providing an explanation for the lower HIV susceptibility of ALVAC-specific CD4 T cells. We further identified that HIV infection rate in $CCR5^{-}/CXCR4^{-}$ subset of Ad5-specific CD4 T cells remained higher than that in ALVAC-specific CD4 T cells (Fig 2.8C), suggesting that factors other than co-receptor expression are also involved in regulating the differential HIV susceptibility of vector-specific CD4 T cells in our system. Regulation of HIV co-receptor expression on target cells has been investigated previously in HIV pathogenesis

(110, 122). However, currently little is known about co-receptor regulation in HIV vaccination. Evidence from our ongoing studies suggests that innate signals derived from vector-infected APCs play a role in regulating CCR5 on CD4 T cells. Further understanding mechanisms that regulate HIV co-receptor expression on vaccine-induced cells is an interesting topic and should be pursued in future studies.

Another important factor that regulates HIV infection of CD4 T cells at entry level is β-chemokines, including CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) (110, 116, 117). Our data show that compared to Ad5 vector, ALVAC-induced T cells (CD4 and CD8) produce much higher levels of β-chemokines (MIP-1β) (Figs 2.12A and 2.19, respectively); however, interestingly, neutralization of β-chemokines in ALVAC-stimulated PBMC only slightly increased HIV infection in ALVAC-specific CD4 T cells (Fig 2.12B), suggesting a modest role of β-chemokines in this process. In addition to co-receptors and β-chemokines, cytokine profiles of CD4 T cells are closely associated with HIV infection. It has been shown that IL-17-producing CD4 T cells are more susceptible to HIV than IFN-γ-producing CD4 T cells (100, 101, 103, 123, 124). In our study, we demonstrate that while Ad5 vector-specific CD4 T cells manifest a mixed Th1/Th17 phenotype, producing high levels of IL-17 and IFN-γ (101), ALVAC-specific CD4 T cells display a polarized Th1-like phenotype, producing high level of IFN-γ but very little IL-17 (Fig 2.10C and D). This different cytokine profile of ALVAC- and Ad5-specific CD4 T cells is consistent with their susceptibility to HIV infection in our system.

CD8 T cells play important roles in anti-HIV immunity, including control of HIV replication and limiting HIV-infected cells (58, 59). An interesting observation in the current study is that ALVAC and Ad5 vector stimulate distinct CD8 vs. CD4 T-cell

proliferative responses; Ad5 vector stimulates predominantly CD4 T-cell proliferation, whereas ALVAC stimulates strong CD8 T-cell proliferation (Fig 2.13). This finding is somewhat unexpected since the ALVAC/gp120 vaccine regimen in the RV144 trial was reported to elicit a weak insert-specific CD8 response (73), whereas Ad5-HIV vaccines have been shown to induce a strong insert-specific CD8 response (69, 72, 77). These findings suggest that the induction of anti-vector and anti-insert T-cell responses in vector HIV vaccination may be differentially regulated. In this study, mechanisms for differential stimulation of vector-specific CD8 vs. CD4 T-cell proliferation by ALVAC and Ad5 remain unknown. However, a prominent difference between ALVAC and Ad5 vector is related to their intracellular locations for replication. After entry into target APCs, poxvirus replicates in cytoplasm (125), whereas adenovirus replicates in nucleus (126). This may lead to engagement of different antigen presentation pathways (e.g. MHC class I vs. II) and therefore differential induction of CD8 vs. CD4 T-cell responses to these two vectors. Nevertheless, elicitation of vector-specific CD8 vs. CD4 responses by different vaccine vectors in vivo and the immune pathways involved remain less clear and should be further investigated.

Another interesting finding of this study is that unlike Ad5 vector, ALVAC-activated CD8 T cells can inhibit the proliferation and HIV infection of autologous vector-specific CD4 T cells (Figs 2.15 and 2.17, respectively). Evidence presented in our study supports that the process may involve both lytic and non-lytic effects of CD8 T cells (120). First, our trans-well experiments showed that CD8 T cells could still inhibit ALVAC-specific CD4 T cell proliferation (Fig 2.15C) and HIV susceptibility (Fig 2.17C) even in the absence of cell contact, indicating that soluble factors play a role in mediating the

inhibitory effects of CD8 T cells. Indeed, we demonstrate that compared to Ad5 vector, ALVAC-activated CD8 T cells manifest a stronger Treg potential ($CD25^{+}FoxP3^{+}$) (Fig 2.15D) and produce higher levels of β-chemokines (Fig 2.17D), which may inhibit ALVAC-specific CD4 T-cell proliferation and HIV susceptibility, respectively. Other than the non-lytic mechanisms, our data suggest that the cytotoxic effects of CD8 T cells may also play a role. We found that the presence of CD8 T cells in either whole PBMC or in trans-well culture (depleted CD8 T cells were added back) caused significant cytotoxic effect on total CD4 T cells (Fig 2.15E and F) as well as on ALVAC-specific CD4 T cells (Fig 2.17E). In support, we further demonstrate that compared to Ad5 vector, ALVAC-activated CD8 T cells manifest a stronger cytolytic and antiviral phenotype, expressing elevated levels of perforin, IFN-γ, and MIP-1β (Fig 2.19). Collectively, our observation that preferential induction of strong vector-specific CD8, but not CD4, T-cell proliferation by ALVAC as compared to Ad5 vector provides some new insights into our understanding of vaccine-induced immunity in HIV vaccination.

In summary, we here present strong evidence that CD4 T cells activated via different HIV vaccine vectors manifest distinct susceptibility to HIV infection, which is closely associated with their phenotypic and functional characteristics. Our findings suggest that future efforts should focus on candidate vaccine vectors that can maximize immunogenicity while minimizing potential HIV susceptibility, for example, by inducing low levels of vector-specific CD4 T cells with high HIV resistance. Future studies will seek to extend this analysis to other important HIV vaccine vectors and to further explicate the mechanism underlying differential HIV susceptibility of vector-specific CD4 T cells. Research that aims to understand how vector-specific CD8 T cells may exert anti-HIV

activity and the immune pathways by which ALVAC stimulates strong vector-specific CD8 T-cell proliferation should also be of interest.

Chapter 3

Distinct innate immune responses elicited by ALVAC versus Ad5 vaccine vectors

INTRODUCTION

Role of APCs in HIV and infection and immunity

Antigen presenting cells (APCs) play several roles in HIV pathogenesis and in the generation of immune response to HIV in vaccination: by directing the activation and differentiation of T cells, as direct targets of HIV infection, and by mediating cell-to-cell HIV transmission.

All nucleated cells have the potential to present antigen: infected cells display antigens on class I major histocompatibility complex (MHC I) on their surface, marking themselves for destruction by CTLs. However, only "professional" antigen presenting cells, including macrophages, dendritic cells (DCs), and B cells, have the capacity to present captured antigen to CD4 T cells via MHC class II. Of these, only DCs have the ability to activate naïve T cells and to cross-present captured antigen to CD8 T cells, making them a crucial gateway between the innate and adaptive immune responses (127, 128). Many factors influence T cell fate, including the nature of the DC maturation stimulus (128), the strength and duration of the immunological synapse (127, 128), and cytokines produced by the DCs (the "third signal") (128).

Mucosal APCs are among the first cells to encounter HIV virus after exposure, and macrophages are particularly important target cells in the early stages of HIV infection (129). Macrophages are more resistant to the cytopathic effects of HIV than CD4 T cells, allowing them to produce virus for longer (129, 130). Macrophages also

have access to otherwise immune-privileged tissues such as the brain, which makes them important mediators of the neurological complication of HIV infection (129, 130). Dendritic cells are typically more resistant to HIV infection than macrophages due to their higher production of HIV restriction factors such as SAMHD1.

Cell-to-cell transmission of HIV refers to the transfer of virions directly from one cell to another, as opposed to cell-free transfer where virions are released into the extracellular fluid (blood or lymph) and encounter another permissible cell. Cell-to-cell transfer is thought to be more efficient than cell-free transfer, as well as protecting the virus from nAbs and ART (131). APCs can transfer HIV to CD4 T cells via two mechanisms: *de novo*, in which productively infected APCs transfer newly assembled virions, or *in trans*, in which APCs transfer captured virions in the course of antigen presentation (130, 132).

Differential innate immune activation by ALVAC vs Ad5 vectors

Studies reported by our lab as well as others show that ALVAC and Ad5 vectors induce significantly different innate responses in infected APCs. ALVAC infects cells of the myeloid lineage and shows a marked tropism for immature monocytes (133). Although the pattern recognition receptors (PRRs) activated by ALVAC are not well characterized, it is known that genes carried by ALVAC are expressed in the cytoplasm of host cells, making it likely that cytosolic sensing occurs (134). ALVAC has been shown to upregulate IFN-related genes (135) and induce higher levels of proinflammatory cytokines - including TNFα, IL-6, and IL-1β - than other poxvirus vectors (136, 137).

Ad5 infects a wide range of host cells (64), including monocytes and dendritic cells (138). In contrast to ALVAC, genes carried by Ad5 are expressed in the nucleus rather than the cytosol. Ad5 has been reported to induce DC maturation and production of proinflammatory cytokines such as IL-6 and IL-12 (64, 139); however, our lab has reported that Ad5 is relatively inefficient at inducing DC maturation compared to ALVAC (111). Some of this discrepancy may be explained by the fact that Ad5-ab immune complexes have been reported to induce more robust DC maturation compared to Ad5 alone (82), predicting different results *in vivo* (where immune complexes may be formed) versus *in vitro*.

HYPOTHESIS AND OBJECTIVES

We have previously reported that ALVAC-specific CD4 T cells are less susceptible to HIV infection than Ad5-specific CD4 T cells (140). We also reported several characteristics of ALVAC- versus Ad5-specific CD4 T cells that appeared to contribute to this reduced susceptibility: lower surface expression of the HIV coreceptors CCR5 and CXCR4, higher levels of the β-chemokine MIP-1β, and a predominantly Th1 phenotype (as opposed to the mixed Th1/Th17 phenotype shown by Ad5-specific CD4 T cells) (140). APCs, especially DCs, play a crucial role in the activation and differentiation of naïve T cells, and the conditions under which they mature can influence T cell fate. Our lab has previously reported that ALVAC, but not Ad5, induces robust maturation, inflammasome activation, and pyroptosis in infected MDDCs (111). This led us to hypothesize that the differential HIV susceptibility and related phenotypes we observed in ALVAC- vs Ad5-specific CD4 T cells collected from vaccine recipients could be caused by the differential maturation of the ALVAC- vs Ad5- primed APCs which activated them. Since APCs are

also HIV target cells as well as important mediators of cell-to-cell transfer, we further speculated that the short-term efficacy of the RV144 vaccine regimen (60% efficacy at 6 months post-vaccination compared to 31.2% efficacy at 42 months) could be due in part to the vector-induced inflammatory state of the APCs themselves making them less susceptible to HIV infection and/or less likely to transfer HIV to CD4 T cells. In this study, we use *in vitro* vector prime and HIV infection of MDDCs, MDMs, and TDMs to investigate the impact of different vectors on phenotype and HIV susceptibility of APCs.

METHODS

Cells, HIV, and viral vectors.

PBMC and THP-1 cells were maintained at 37°C, 5% CO_2 in RPMI-1640 medium (Invitrogen) supplemented with 10% human serum, 100 U/mL penicillin G, 100 U/mL streptomycin sulfate, and 1.17mM sodium glutamine (complete medium.) R5 (US1) HIV-1 (original stock from NIH) was used for *in vitro* infection of PBMC. Empty ALVAC vector was obtained from Sanofi, and empty rAd5 vector was obtained from the Vaccine Research Center (VRC) of NIH.

Generation of MDDCs and MDMs.

Cryopreserved human PBMCs were enriched for monocytes using the adhesion method: briefly, PBMCs were thawed, washed twice with serum-free RPMI-1640 medium, then resuspended in serum free medium and plated at a density of 10 $x10^6$ cells/well in a 6 well tissue culture plate. The plates were then incubated for 1 hour at 37°C, 5% CO_2 to allow the monocytes to adhere. The nonadherent cells were then washed away and complete RPMI-1640 containing 50 ng/mL human rGM-CSF and 100 ng/mL human rIL-

4 for MDDCs or 100 ng/mL human rGM-CSF for MDMs. Monocytes were incubated at 37°C, 5% CO_2 for 6 days, adding fresh cytokine-containing medium on day 3. Suspended MDDCs were collected by aspiration. Adherent MDMs were washed 2x with PBS and incubated for 10 minutes in non-enzymatic cell dissociation solution (Sigma-Aldrich #C5789-100ML) before being removed from the plastic by washing and gentle scraping.

Generation of THP-1-derived macrophages (THP-MΦs)

THP-1 cells (ATCC #TIB-202) were plated at a density of 0.5 x 10^6 cells/well in a 24 well plate and stimulated with PMA for 2 days to generate adherent macrophage-like cells. Cells were then rested for 1 day before performing vector prime and HIV infection directly in the plate. Cells were lysed directly in the plate for RNA/DNA isolation or detached with non-enzymatic cell dissociation solution as described above for antibody staining.

Vector prime and HIV infection

ALVAC was added to plates at a multiplicity of infection (MOI) of 5, Ad5 at MOI = 10. Cells were incubated with vector at 37°C, 5% CO_2 overnight, then washed 3x with medium to remove free vector. For HIV infection, HIV stocks were diluted 1:4 in medium and added to cell cultures. Infected cells were incubated at 37°C, 5% CO_2 overnight, then washed 3x with medium to remove free virus. Days post-infection were counted from cell wash.

Flow cytometric surface and intracellular p24 staining and analysis.

Cells were first stained with LIVE/DEAD fixable aqua dead cell stain (Thermo Fisher Scientific, cat #L34957) and antibodies to surface markers including CD3, CD14,

CD16, and CCR5. Cells were then fixed, permeabilized (BD Biosciences cat #554722), and stained for HIV p24 (Beckman Coulter) to measure HIV infection. Antibody capture compensation beads (BD Biosciences) stained with individual antibodies were used for compensation. Cell samples and compensation beads were acquired on a Fortessa LSR-II (BD). Flow cytometric data were analyzed using FlowJo Version 10 software (TreeStar).

Real-time PCR for gene expression.

Total RNA was extracted from APCs collected 24 hours post-infection using Quick-RNA MicroPrep kit (Zymo # R1050) according to the manufacturer's protocol. Gene expression was quantified using iTaq Universal SYBR Green Supermix (Bio-rad) and the CFX Connect Real-Time PCR Detection System (Bio-rad) after reverse transcription from RNA into cDNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-rad). Primer sequences for quantification of gene expression are shown in Table x. The relative quantity of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Measurement of relative HIV integration by *Alu-Gag* PCR

Total DNA was extracted from APCs collected 24 hours post-infection with HIV using Quick-DNA Miniprep Kit (Zymo # D3024) according to manufacturer's directions and *Alu-Gag* PCR performed as previously described (141), with some modifications. In order to correct for cell death caused by ALVAC prime leading to unequal numbers of target cells at the HIV infection step, *Gag* Ct values were normalized to GAPDH Ct values from the same sample as a proxy for cell number. Instead of using a standard curve to obtain absolute quantification of HIV provirus, we used the $2^{-\Delta\Delta Ct}$ method to measure

integrated Gag in ALVAC- vs. Ad5-primed, HIV-infected APCs relative to unprimed HIV infected APCs. Uninfected APCs were included as a negative control.

RESULTS

ALVAC-primed MDDCs reduce HIV susceptibility of CD4 T cells

Our previously published paper showed that ALVAC-specific CD4 T cells are less susceptible to HIV infection than Ad5-specific CD4 T cells using PBMCs collected from vaccine recipients (140). In order to investigate possible mechanisms for this observation, we co-cultured vector-primed MDDCs generated from normal human PBMCs with autologous CD4 T cells for 24 hours, then infected with R5 HIV *in vitro*. Cells were stained for lineage surface markers and intracellular p24 expression 3 days post-infection (dpi) with HIV (Fig 3.1A). Our results show that CD4 T cells cultured with ALVAC-primed MDDCs were significantly less susceptible to R5 HIV infection than those cultured with Ad5-primed MDDCs and non-primed control MDDCs (Fig 3.1B).

ALVAC-primed MDDCs reduce CCR5 expression on CD4 T cells

A major contributor to HIV susceptibility is surface expression of HIV coreceptors CCR5 and CXCR4, and our previous paper showed that ALVAC-specific CD4 T cells had lower surface expression of CCR5 compared to Ad5-specific CD4 T cells (140). To determine if coculture with vector-primed MDDCs was sufficient to induce differential expression of CCR5 expression on CD4 T cells, we co-cultured vector-primed MDDCs with autologous CD4 T cells as described above, then used flow cytometry to measure CCR5 expression on CD4 T cells. Our results show that CD4 T cells cultured with

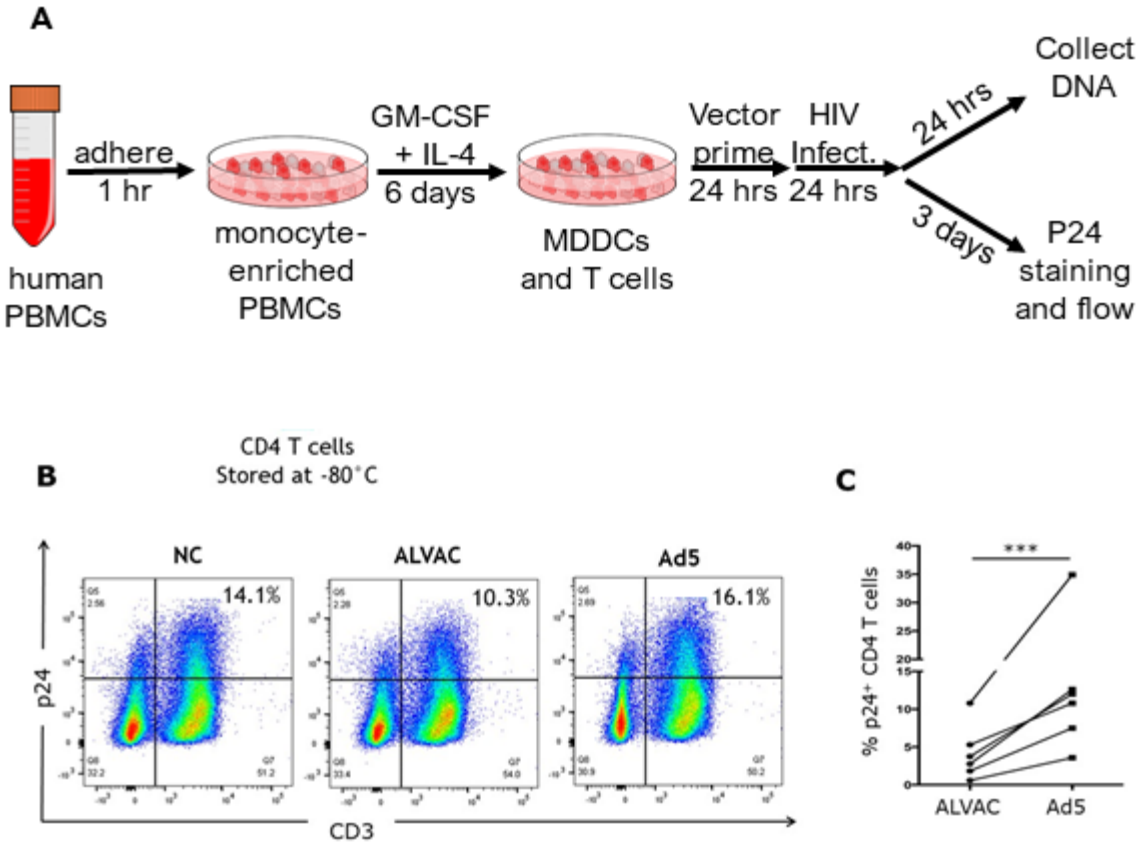

Figure 3.1. ALVAC-infected MDDCs reduce HIV susceptibility of CD4 T cells. **(A)** Diagram showing experimental design. Briefly, normal human PBMCs were enriched for monocytes by allowing them to adhere to plastic tissue-culture plates for 1 hour and washing away non-adherent cells. Monocytes were then differentiated into MDDCs by culturing with GM-CSF and IL-4 for 5 - 6 days. The PBMCs were then infected with ALVAC or Ad5 vector for 24hrs, washed to remove free virus, and infected with R5 HIV for 3 days. HIV infection was measured by using flow cytometry to detect intracellular p24 expression **(B)** A flow cytometry plot representative of 3 independent repeats. An HIV^- control (not shown) was used to set the gate. **(C)** Comparison of % $p24^+$ CD4 T cells from multiple subjects (including experiments performed by Dr. Fengliang Liu.) Statistical analysis was performed using a paired Student's t test ($p < 0.001$).

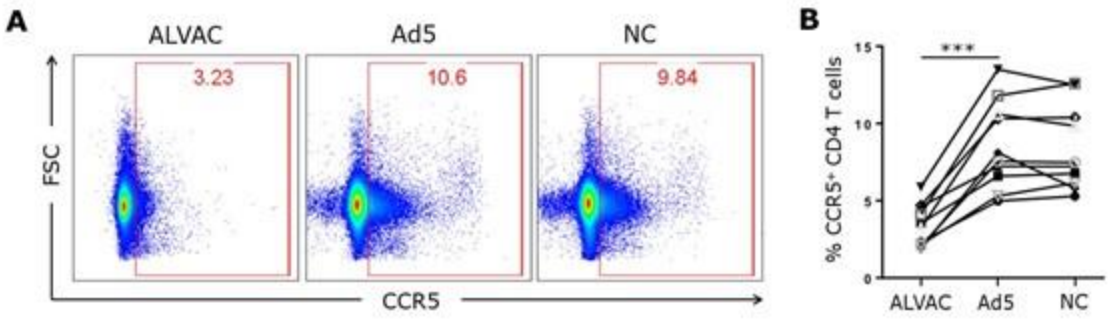

Figure 3.2. ALVAC-infected MDDCs reduce CCR5 expression on CD4 T cells. (A) Representative flow plot showing CCR5 expression. MDDC-T cell coculture was performed as described in Fig. 1, *except* cells were antibody-stained and analyzed via flow cytometry 24 hrs post-vector infection; no HIV infection was performed. **(B)** Comparison of % $CCR5^+$ CD4 T cells from multiple subjects (including experiments performed by Dr. Fengliang Liu.) Statistical analysis was performed using a paired Student's t test ($p < 0.001$).

ALVAC-primed MDDCs had significantly lower CCR5 expression than those cultured with unprimed or Ad5-primed MDDCs (Fig 3.2).

ALVAC-primed MDDCs show decreased HIV susceptibility compared to Ad5 vector-primed MDDCs

In addition to their role in inducing T cell activation and differentiation, APCs can also be directly infected by HIV. DCs are usually resistant to HIV infection, however, susceptibility can vary significantly depending on donor, maturation stage, and activation state. In addition, DCs can capture and internalize HIV which they can then transfer to CD4 T cells in the absence of active infection (*trans* infection.) To determine whether vector priming can influence MDDCs' susceptibility to HIV infection or ability to capture HIV, we primed MDDCs with vector for 24 hours before exposing them to R5 HIV. Cells were stained 3 dpi for intracellular p24 and analyzed via flow cytometry. Our results thus far show that ALVAC-primed MDDCs have decreased susceptibility to R5 HIV infection compared to ALVAC-primed MDDCs (Fig 3.3 A-B).

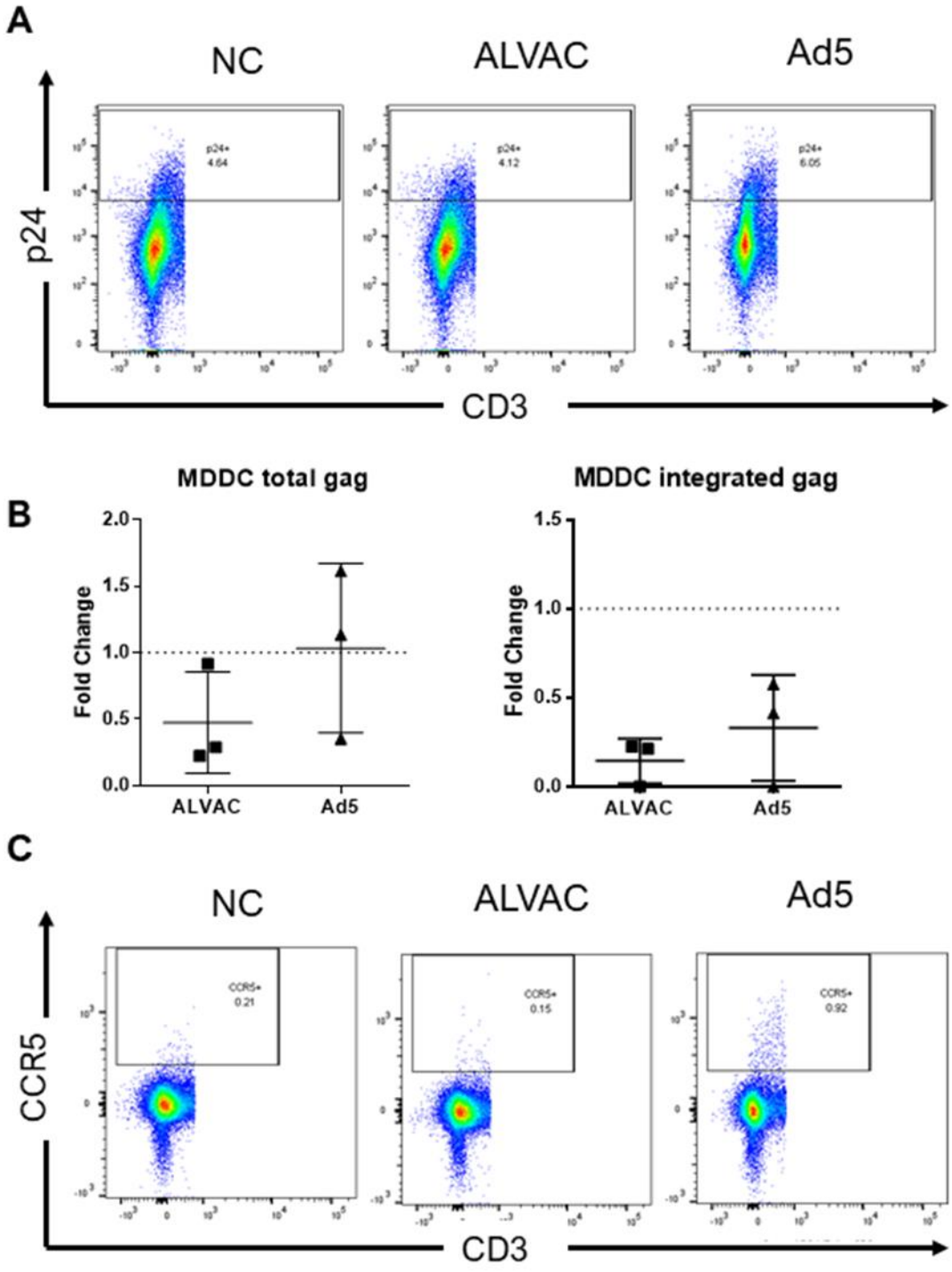

Figure 3.3. Ad5 increases the HIV susceptibility of MDDC (A) Representative flow plots showing intracellular p24 **(B)** Integrated and total gag. **(C)** Representative flow plots showing surface expression of CCR5 (1 experiment)

Surface expression of HIV coreceptors is a significant factor influencing susceptibility to HIV infection. In order to determine whether increased CCR5 expression could contribute to the increased HIV infection observed in Ad5-primed MDDCs, we vector-primed MDDCs for 24 hours as described above, then used flow cytometry to measure surface CCR5 expression (no HIV infection.) Our results show that Ad-primed MDDCs display higher levels of CCR5 than unprimed or ALVAC-primed MDDCs, consistent with their increased susceptibility to HIV infection (Fig 3.3C).

ALVAC induces higher antiviral gene expression in MDDCs than Ad5 does

As discussed above, DCs are naturally resistant to HIV infection due to their expression of innate HIV restriction factors, especially SAMHD1. Interestingly, Ad5 appears to increase both the HIV susceptibility and CCR5 expression of MDDCs compared to baseline, suggesting that Ad5 infection abrogates existing defense mechanisms. As a preliminary test of this hypothesis, we analyzed data from a microarray experiment previously performed by Dr. Fengliang Liu in our laboratory. Briefly, MDDCs were generated from PBMCs from three normal donors and infected with ALVAC or Ad5 for 24 hours before collecting RNA for the microarray. We found that 4 out of 5 of the HIV restriction factors analyzed were more highly expressed in ALVAC-primed MDDCs than Ad5 MDDCs, however, due to the lack of an unprimed control these data cannot tell us whether these restriction factors were upregulated in ALVAC-primed MDDCs or downregulated in Ad5-primed MDDCs (Fig 3.4A). Accordingly, we analyzed the gene expression of ALVAC- and Ad5-primed MDDCs in comparison to unprimed MDDCs. While more donors are required for statistical significance, there appears to be a strong

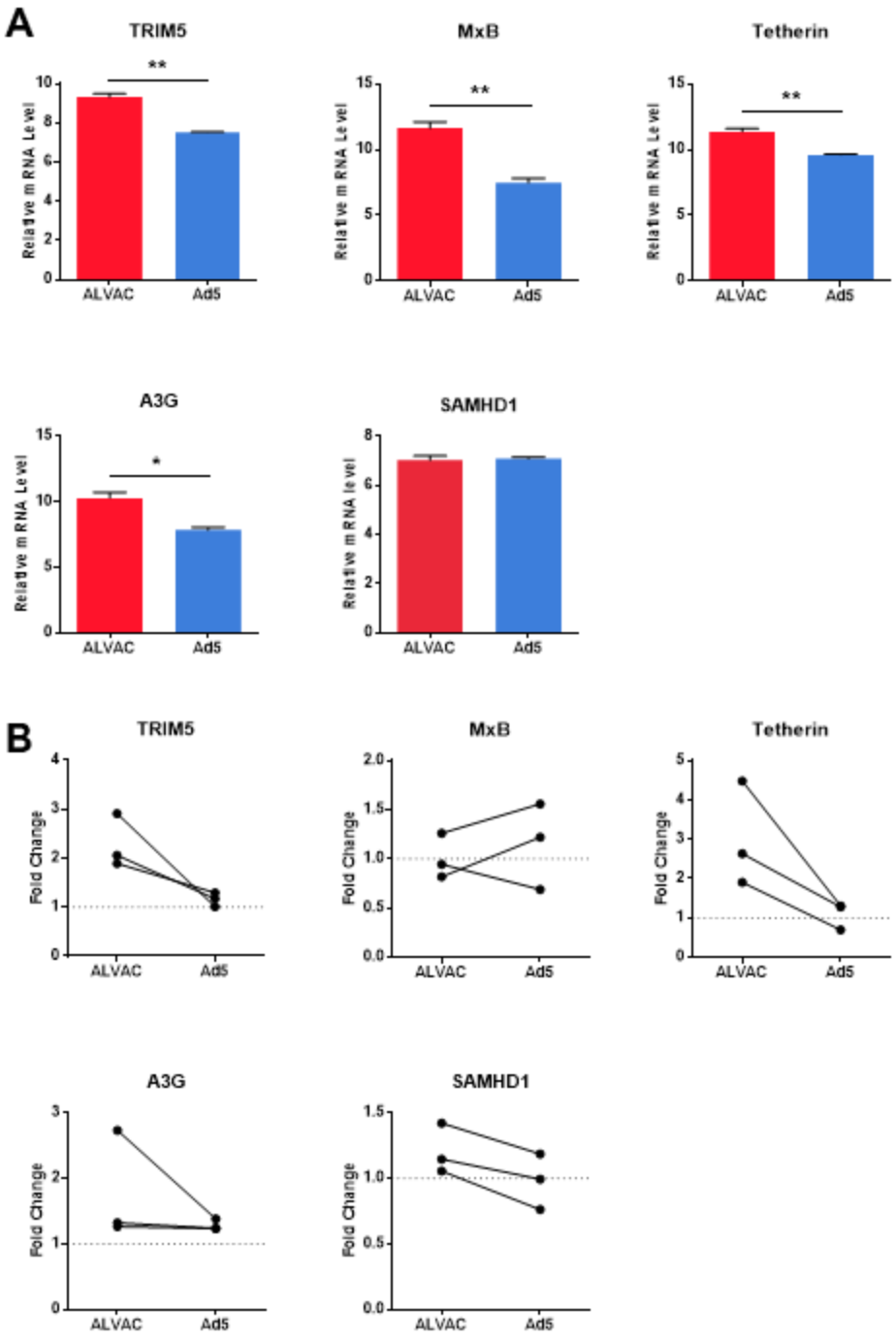

Figure 3.4. ALVAC infection increases HIV restriction factor gene expression in MDDCs. (A) Relative mRNA levels for HIV restriction factors expressed by ALVAC- compared to Ad5-infected MDDCs, measured by microarray (n = 3). **(B)** Fold change in HIV restriction factor expression in ALVAC- and Ad5-primed MDDCs compared to unprimed MDDCs. Statistics were calculated using a paired Student's t test. *$p < 0.05$, **$p < 0.01$

trend for ALVAC to significantly upregulate TRIM5 and tetherin expression, while Ad5-primed MDDCs have gene expression similar to unprimed controls (Fig. 3.4B).

ALVAC decreases the HIV susceptibility of THP-1-derived macrophages

Macrophages are an important HIV target cell in the early stages of infection. To test the HIV susceptibility of vector-primed macrophages we used PMA stimulation to induce the THP-1 monocytic cell line to differentiate into THP-1-derived macrophages (TDMs). TDMs were primed with vector for 24 hours, then infected with R5 HIV. DNA was collected at 24 hpi for PCR quantification of HIV infection, and remaining cells were stained for intracellular p24 3dpi. Our results show that ALVAC reduces the HIV susceptibility of TDMs (Fig 3.5A). Interestingly, a more dramatic decrease is seen in integrated *gag* measured by the alu-gag method compared to total *gag* and intracellular p24, suggesting that HIV restriction may occur at both entry and post-entry levels (Fig 3.5B-C).

ALVAC decreases the HIV susceptibility of monocyte-derived macrophages

Although THP-1 cells are convenient and easy to work with, they may not accurately reflect primary macrophages *in vivo.* In order to develop a more physiologically relevant model of HIV infection of vector-primed macrophages, we generated monocyte-derived macrophages (MDMs) from monocytes isolated from normal human PBMCs. MDMs were then primed with vector for 24 hours before infected with R5 HIV. DNA was collected 24 hpi for and qPCR and alu-gag PCR used to measure total and integrated gag, respectively. Remaining cells were stained for intracellular 24 3 dpi. Our results show that

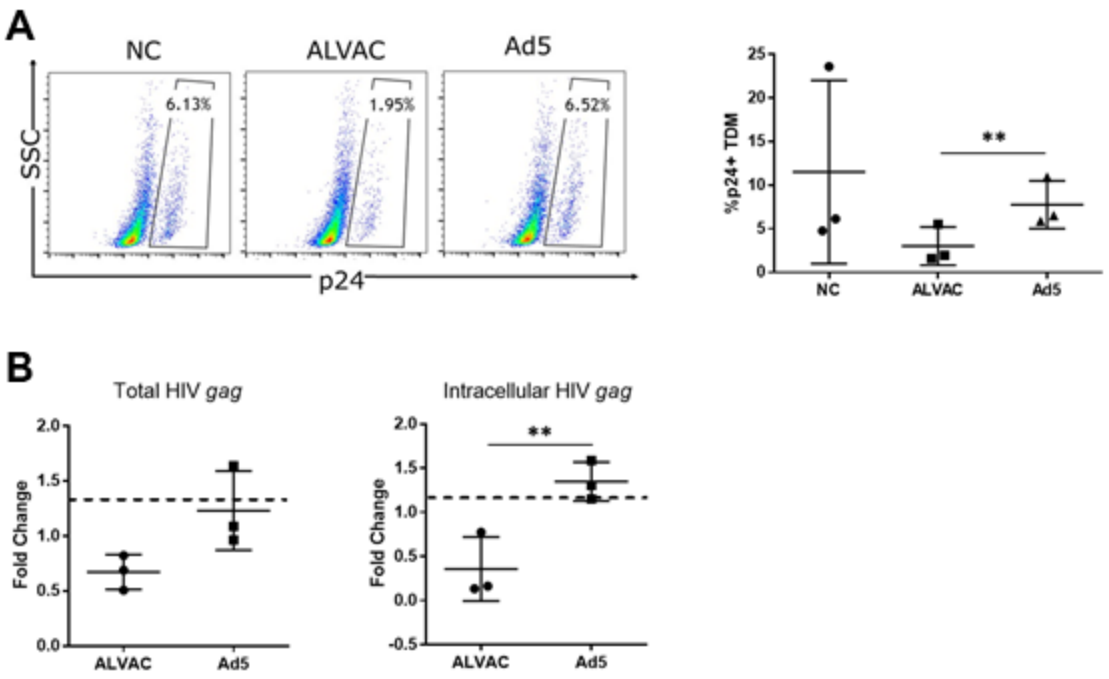

Figure 3.5 ALVAC infection decreases the HIV susceptibility of THP-1-derived macrophages (TDMs) (A) Flow plot showing %p24^{+} THP-MΦs. THP-MΦs were generated by stimulating THP-1 cells with PMA for 2 days, then resting 1 day prior to vector infection. THP-MΦ were then infected with vector for 1 day, washed, and then infected with R5 HIV. Total genomic DNA was collected 24hrs post-infection. Cells were antibody-stained and analyzed via flow cytometry for intracellular p24 3 days post-infection. Plot is representative of 3 independent experiments. **(B)** Diagram illustrating the principle behind the *Alu-Gag* PCR method for measuring integrated HIV *gag*. **(C)** Fold change in total (left) and integrated (right) *gag* DNA compared to a non-vector-infected control.

ALVAC reduces HIV susceptibility (Fig 3.6A) and CCR5 expression (Fig 3.6B) compared to unprimed or Ad5-primed MDMs.

Discussion

In the present study, we used human PBMCs to generate MDDCs and test their ability to influence the HIV susceptibility and CCR5 expression of autologous CD4 T cells as well as their own HIV susceptibility. We also generated macrophages from the monocytic cell line THP-1 and human PBMCs and tested their susceptibility to HIV infection. Our previous study found differences between ALVAC- and Ad5-specific CD4 T cells in susceptibility to both R5 and X4 HIV (140); however, in this study we chose to focus on R5 HIV because it is the strain responsible for HIV transmission and thus of especial relevance to vaccine design.

Consistent with our previous observations, we found that co-culture with ALVAC-primed MDDCs induced decreased HIV susceptibility (Fig 3.1B) as well as decreased CCR5 expression in CD4 T cells (Fig 3.2). Consistent with previously published literature, we found that HIV infection rates in MDDCs were very low (Fig 3.4.) Interestingly, however, we found that Ad5-primed MDDCs had higher HIV susceptibility and CCR5 expression than either ALVAC-primed or unprimed control MDDCs (Fig 3.4). This led us to speculate that Ad5 might downregulate the innate restriction factors that typically make DCs resistant to HIV infection. There is some indirect support for this hypothesis in the literature: adenovirus E4 ORF3 protein has been shown to inhibit the interferon-mediated antiviral response in interferon-treated Vero cells (142). We also found tentative support for this hypothesis by analyzing the results of a microarray previously performed in our lab which compared the gene expression of ALVAC- vs Ad5-primed MDDCs: of the five HIV

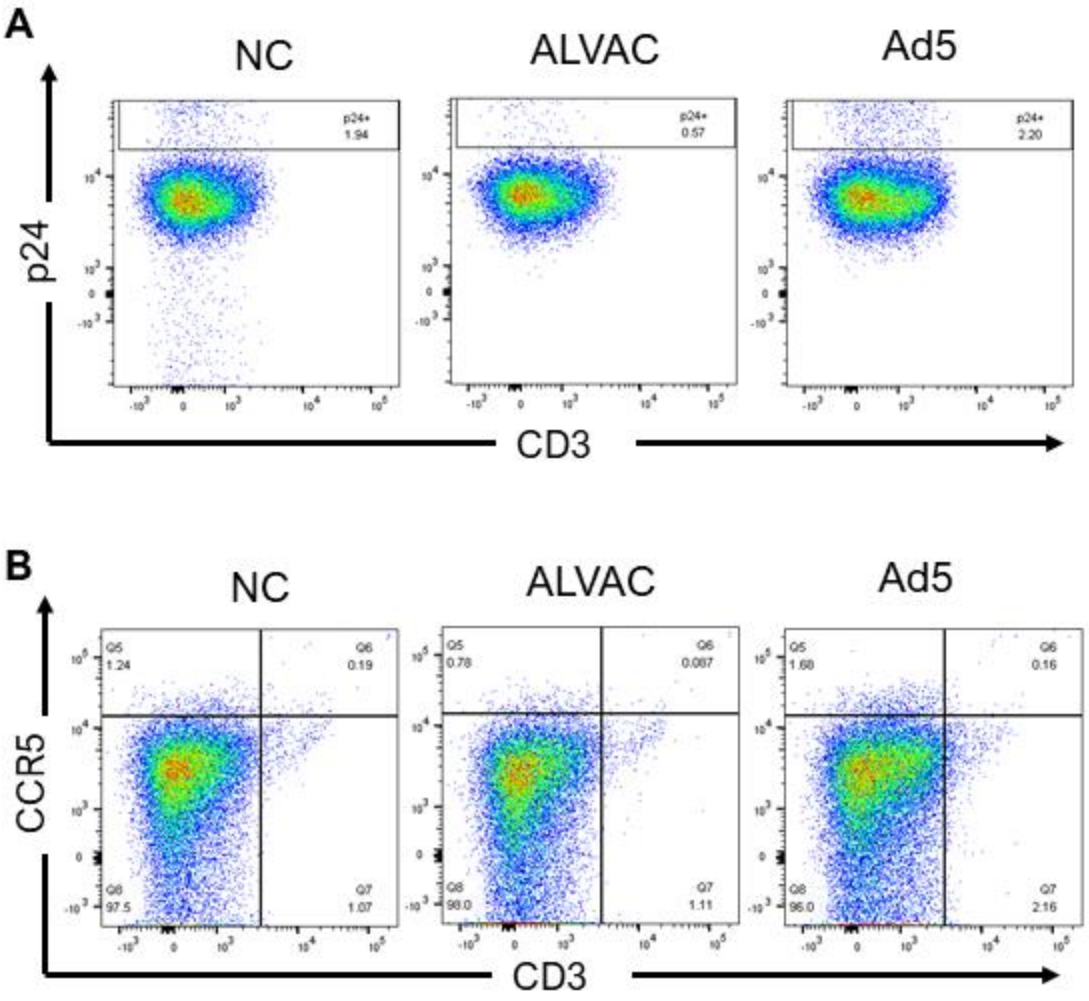

Figure 3.6. ALVAC reduces the HIV susceptibility of MDMs. (A) Representative flow plots showing intracellular p24. **(B)** Representative flow plots showing surface expression of CCR5 (1 experiment)

restriction factors which we analyzed, four had higher expression in ALVAC-primed MDDCs compared to Ad5-primed MDDCs: TRIM5, MxB, tetherin, and APOBEC3G (A3G); the fifth, SAMHD1, showed no difference in expression (Fig 3.5A). However, subsequent gene expression analysis using qPCR indicates that these restriction factors are increased by ALVAC infection rather than decreased by Ad5 expression (Fig 3.5B).

Macrophages can act as APCs as well as DCs; however, unlike DCs, macrophages cannot activate naïve T cells. On the other hand, macrophages are more susceptible to R5 HIV infection than DCs, and are thought to be important HIV target cells in the early stages of infection. In this study, we generated THP-1-derived macrophages (TDMs) by using PMA stimulation to induce THP-1 differentiation and tested their susceptibility to HIV infection. We found that ALVAC-primed TDM were less susceptible to HIV infection than Ad5-primed or unprimed control TDMs (Fig 3.5). Because APCs can capture HIV without being productively infected, we used alu-gag PCR to measure integrated HIV. Interestingly, we found that ALVAC reduced integrated gag significantly more than total gag compared to unprimed controls, suggesting that ALVAC reduced HIV infection at the post-entry level (Fig 3.5C). In addition, while total gag was similar between Ad5-primed and unprimed control TDMs, Ad5 elevated integrated gag compared to unprimed TDMs (Fig 3.5C). CCR5 was undetectable in TDMs (data not shown,) suggesting that CCR5 expression is likely not a significant factor in the differential HIV susceptibility of ALVAC- vs Ad5-primed TDMs.

THP-1 cells have the advantage of being readily available and easy to work with, but are arguably less physiologically relevant than primary cells. Therefore, we also generated monocyte-derived macrophages (MDMs) from human PBMCs and tested their

susceptibility to HIV infection *in vitro*. Although further replication is still necessary, our results thus far are consistent with those found in TDMs: ALVAC-primed MDMs were less susceptible to HIV infection than Ad5-primed or unprimed control MDMs (Fig 3.6A). Unlike THP-1 cells, CCR5 expression in MDMs was low but detectable, with lower CCR5 expression in ALVAC-primed MDMs compared to Ad5-primed or unprimed control MDMs (Fig 3.6B). Interestingly, the ALVAC-induced reduction in HIV infection is greater than the reduction in CCR5 expression (70.6% vs 54.2%, reduction compared to unprimed MDMs, respectively), suggesting that ALVAC inhibits HIV infection at post-entry as well as entry stages of infection (Fig 3.6).

In summary, our results thus far suggest that ALVAC decreases the HIV susceptibility of APCs themselves, while also priming them to induce HIV-resistant or -susceptible phenotypes, respectively, in CD4 T cells. Future work will focus on replicating our results thus far as well as exploring potential mechanisms by which ALVAC-primed APCs promote an HIV-resistant phenotype in CD4 T cells (more details and preliminary data are included in Chapter 4.) Although DCs and macrophages can both present antigen and be infected by HIV, DCs are relatively more important as APC and mediators of *in trans* infection of CD4 T cells, while macrophages are relatively more important as HIV target cells. For this reason, we prioritized MDDC-T cell coculture and TDM/MDM HIV susceptibility; future work will also include MDM-T cell coculture to determine their ability to mediate cell-to-cell transfer to CD4 T cells.

Chapter 4: Results Summary, Limitations, and Future Directions

In my dissertation, we investigated the innate and adaptive immune responses directed against two non-replicating viral vectors employed in multiple late-stage HIV vaccine trials: ALVAC and Ad5. In this chapter, I summarize our major findings and discuss their significance, study limitations, and future directions, including preliminary data which will inform future work.

MAJOR FINDINGS AND SIGNIFICANCE

ALVAC-specific CD4 T cells are less susceptible to HIV infection than Ad5-specific CD4 T cells

Prior to this study, most research investigating the immunological mechanisms contributing to the results of the recent HIV efficacy trials focused on the role of insert-specific responses, with some limited work done on Ad5-specific innate and adaptive responses (82, 85, 97, 98, 101). To the best of our knowledge, ours was the first study to describe ALVAC-specific CD4 T cells, with the major finding being that ALVAC-specific CD4 T cells are less susceptible to HIV infection than either Ad5-specific or polyclonally stimulated CD4 T cells (140). Since Ad5-specific CD4 T cells had HIV susceptibility comparable to that of polyclonally stimulated cells (Fig 2.5) and lower than that of Env-specific CD4 T cells (which were comparable between RV144 and HVTN204 vaccine recipients (Fig 2.7)), this result suggests a possible contributing factor to the efficacy of the ALVAC/gp120 regimen tested in the RV144 trial, but not necessarily for the elevated HIV infection risk observed in Ad5-based vaccine recipients.

It should be noted that the HIV susceptibility of polyclonally stimulated CD4 T cells was only measured for 1 donor per trial. Ideally, this result would be confirmed with

multiple replicates; however, the limited number of vaccine trial samples available makes this impractical. In addition, the trials in which a statistically significant increase in HIV infections was observed (the STEP and Phambili trials) used a recombinant Ad5 vector developed by Merck (MRK-rAd5,) whereas the HVTN204 trial from which we acquired our samples used a different Ad5 vector developed by the NIH Vaccine Research Center (VRC-rAd5) in a DNA prime/Ad5 boost regimen. The sole late-stage efficacy trial to use this vaccine regimen, HVTN505, showed no statistically significant increase in HIV infection risk; however, noticeably more HIV infections were observed in the vaccine group compared to the placebo group. This is consistent with our findings; if Ad5-specific CD4 T cells are innately susceptible to HIV infection compared to other antigen-specific CD4 T cells, we would expect to see greater *in vivo* susceptibility in vaccinated individuals with pre-existing immunity to Ad5, as vaccination would induce a faster and more powerful secondary response – and consequently more Ad5-specific CD4 T cells - in these patients than in vaccine recipients with no pre-existing immunity. However, even a primary response to Ad5 would be expected to generate some Ad5-specific CD4 T cells, thus increasing the vaccine recipient's susceptibility to HIV infection. Thus, our findings suggest that vaccine strategies based on the assumption that increased HIV susceptibility is confined to Ad5-seropositive individuals are likely to fail and should be avoided. This includes the recent trend favoring viral vectors based on rare adenovirus serotypes such as Ad35 and Ad26; our research suggests that the HIV susceptibility of the vector-specific CD4 T cells generated by these vectors (and any other viral vectors being considered for use in HIV vaccines) should be evaluated before performing large-scale trials in humans.

Vaccination with ALVAC/gp120, but not DNA/Ad5, induces a strong vector-specific CD8 T cell response

Multiple analyses of HIV vaccine trials using the ALVAC and Ad5 vectors reported that Ad5, but not ALVAC, generated strong CD8 T cell responses against the vaccine insert. This being the case, we were surprised to find that vector-specific T cell responses showed precisely the opposite pattern: the ALVAC vaccine induced significantly more vector-specific CD8 T cell proliferation than the Ad5, while vector-specific CD4 T cell proliferation was comparable between the two groups (Fig 2.13.) In addition, ALVAC-specific CD8 T cells produced significantly higher levels of IFN-γ, MIP-1β, and perforin than Ad5-specific CD8 T cells, indicating a qualitatively as well as quantitatively stronger CD8 T cell response (Fig 2.19).

The ability of the Ad5 vector to generate balanced cellular and humoral responses (in contrast to ALVAC, which generated primarily antibody responses) was one of the primary reasons for its use in multiple HIV trials; the unexpected failure of multiple Ad5-based trials and partial success of the ALVAC-based RV144 trial led to a significant realignment of the field towards antibody-mediated immunity and away from cellular immunity. Our results suggest that the full picture is more complex, with ALVAC inducing a vector-specific CD8 T cell response that may contribute to the enhanced short-term efficacy seen in the RV144 trial (73).

ALVAC-primed MDDCs reduce the HIV susceptibility of autologous CD4 T cells

This study aimed to answer the question generated by our previously published study (discussed above): why does ALVAC vaccination generate HIV-resistant vector-specific CD4 T cells, while Ad5 vaccination generates HIV-susceptible vector-specific

CD4 T cells? Another study published by our lab showed that ALVAC, but not Ad5, induces robust MDDC maturation, pyroptosis, and inflammasome activation, leading us to hypothesize that the distinct phenotypes of ALVAC- vs Ad5-specific CD4 T cells had their roots in the distinct phenotypes of the vector-primed APCs that originally activated them. Our results thus far support this hypothesis, but much remains to be determined.

An important caveat to these results lies in the fact that we used MDDCs and CD4 T cells isolated from normal human PBMCs. Since Ad5 is ubiquitous in the human population, some of these donors likely have Ad5-, but not ALVAC-specific memory T cells, which could influence the results. If practicable, future studies should be performed with PBMC from donors who are known to be seronegative for anti-Ad5 antibodies.

ALVAC-primed APCs are less susceptible to HIV infection than Ad5-primed APCs

APCs are typically the first cells to encounter transmitted HIV virus at the mucosal surfaces and are thought to play an important role in transporting HIV from the mucosal entry point to the lymphoid tissues where the virus can encounter CD4 T cells. Macrophages, in particular, are thought to be an important early, long-lived HIV reservoir due to their susceptibility to infection coupled with their resistance to HIV-mediated cell death. Based on the fact that both the initially high (≈ 60%) efficacy of the ALVAC/gp120 vaccine and the increased infection risk induced by the Ad5-based vaccines quickly waned after 6 months post-vaccination, we hypothesized that differential HIV susceptibility of vector-primed APCs might be a contributing factor.

Although DCs are usually resistant to HIV infection, we found that Ad5 increased both the HIV susceptibility and CCR5 expression of MDDCs compared to both ALVAC-primed and unprimed MDDCs. This is particularly significant as it is the only finding in

this study to show a potential mechanism by which vaccination with Ad5 could increase HIV infection over baseline. This result should be interpreted with caution, however, since it represents a single experiment using PBMCs from 1 donor. Future work will focus on replicating this result.

Synthesis

While much work remains to be done, based on the data presented thus far we may begin developing a tentative model of how ALVAC- and Ad5-based vaccine regimens induce differential vector-specific immune responses and how these responses contribute to vaccine efficacy (Fig. 4.1): First, ALVAC, but not Ad5, induces inflammasome activation in infected APCs, leading to pyroptosis and more robust maturation of surviving APCs than Ad5 (113) (Fig. 4.1A). Next, mature APCs migrate to the lymphoid organs and present vector antigens to naïve, vector-specific T cells. ALVAC-infected APCs promote Th1 differentiation, while Ad5-infected APCs promote a mixture of Th1 and Th17 differentiation (Figure 4.1B). In addition, ALVAC-, but not Ad5-primed APCs induce a robust antigen-specific CD8 T cell response which limits the proliferation of ALVAC-specific CD4 T cells (Figure 4.1B). The final result is that ALVAC vaccination produces a relatively small population of HIV-resistant vector-specific CD4 T cells, while Ad5 vaccination produces a relatively large population of HIV-susceptible vector-specific CD4 T cells (Figure 4.1C). In addition, ALVAC-, but not Ad5-specific CD8 T cells also contribute IFNγ and MIP-1β to the local cytokine milieu, further promoting anti-viral responses and blocking HIV entry, respectively (Fig. 4.1C).

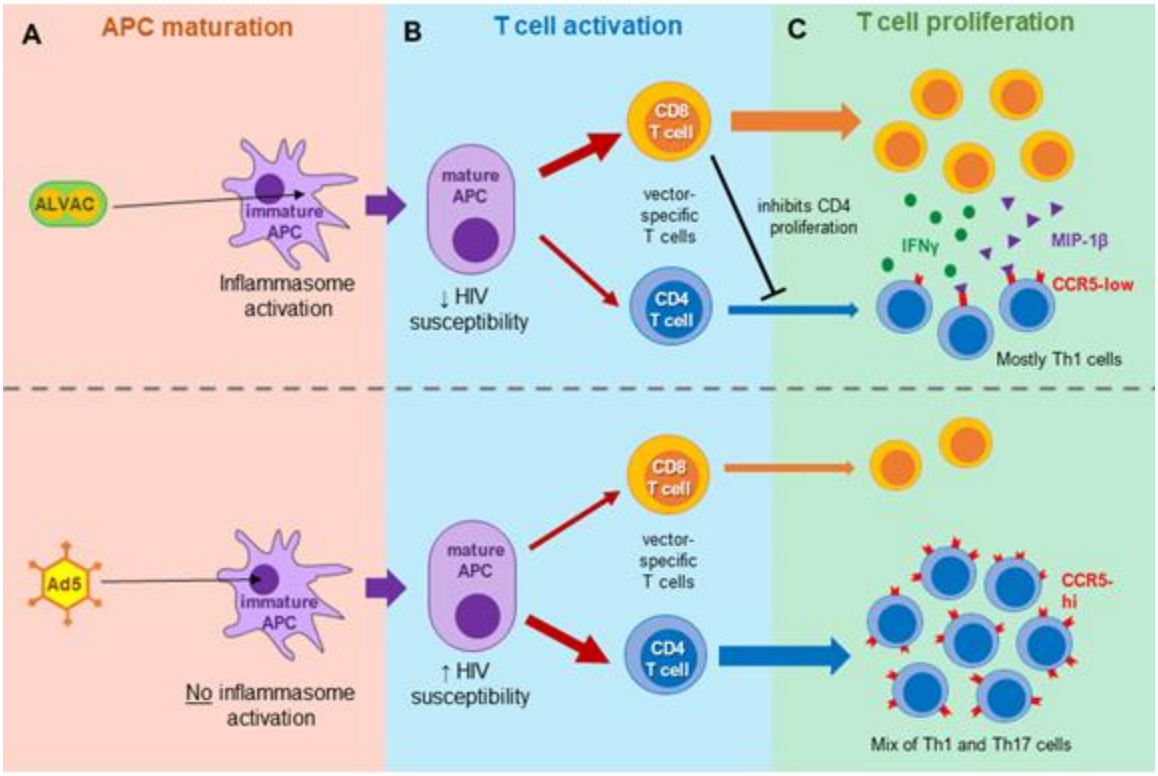

Figure 4.1. Proposed model for differential vector-specific responses induced by ALVAC- vs Ad5-based vaccine regimens.

STUDY LIMITATIONS AND DIRECTIONS FOR FUTURE WORK

Extrapolating from *in vitro* results to *in vivo* outcomes

One major limitation of this study is the fact that all experiments were performed *in vitro*, meaning that care must be taken in attempting to use our results to explain the *in vivo* results obtained in the course of the vaccine trials. Therefore, an important direction for future research will be confirming and expanding on our results in a humanized mouse model. For example, transferring vector-specific CD4 T cells from vaccinated to unvaccinated mice prior to HIV infection would allow us to determine whether vector-specific CD4 T cells are necessary and/or sufficient to replicate the differential susceptibility to HIV observed in the vaccine trials.

In vivo studies would also allow us to put our *in vitro* results in their physiological temporal and spatial contexts. Most HIV transmission events take place at the vaginal or rectal mucosa; thus, an important direction for *in vivo* studies would be to determine the number vector-specific T cells at these sites, how soon after vaccination they appear, and how long after vaccination they persist. It would also be informative to track the migration of APCs after vaccination to determine whether vector-infected APCs migrate to the mucosa where they would encounter HIV and, if so, whether there is a difference between ALVAC- and Ad5-infected APCs in the magnitude or timing of migration.

Differential antigen presentation of insert vs vector proteins

Perhaps the most surprising result to come out of this study was the finding that ALVAC, but not Ad5, induces a robust CD8 T cell response, given that studies of the insert-specific response gave the exact opposite result. An interesting and potentially important direction for future study would be to investigate the antigen presentation

mechanisms which lead to the induction of such different T cell responses against the viral vector and the insert that it carries.

One important difference between ALVAC and Ad5 is that the former replicates in the cytoplasm while the latter replicates in the nucleus, which could result in the insert antigens being sensed by different PRRs, which in turn would lead to these antigens being processed via different pathways. The vector antigens, on the other hand, would be sensed via both intracellular pathways and phagocytotic pathways activated by the engulfment of the viral particle itself. It is tempting to speculate that a similar mechanism could account for the efficacy of the ALVAC prime/gp120 protein boost vaccine regimen, in spite of the fact that neither ALVAC nor gp120 alone showed any efficacy: combining the two resulted in the gp120 antigen being presented via intracellular and phagocytotic pathways at the same time.

Another possibility is that the inclusion of a CMV promoter to ensure robust expression of insert proteins resulted in much higher levels of insert antigens compared to vector antigens, which may have influenced the antigen presentation pathways by which they were processed. It should also be noted that non-physiological levels of antigen have been reported to generate less sensitive antigen-specific CD8 T cells, which subsequently fail to respond when later exposed to physiological antigen levels. This could offer an alternative explanation for why Ad5-based vaccines proved ineffective in spite of generating a strong CD8 T cell response, which has long been considered an important correlate of immunity: Env-specific CD8 T cells generated by Ad5 vaccination respond when restimulated with relatively high levels of antigen *in vitro,* but not when exposed to physiological levels *in vivo.* ALVAC-specific CD8 T cells, on the other hand,

showed a robust anti-viral and cytolytic phenotype, but are only be present for a short time after vaccination. If the mechanism responsible for this response could be elucidated, and a vaccine designed to introduce HIV-specific antigens via the appropriate pathway, it might be possible to design a vaccine capable of eliciting a robust and effective CD8 T cell response against HIV antigens.

Mechanisms for the induction of HIV-resistant vs -susceptible CD4 T cells by vector-primed APCs

Another major limitation of this study is the lack of mechanistic studies to explain the observed results. In particular, we know that ALVAC-infected MDDCs promote an HIV-resistant phenotype in the vector-specific T cells they activate, but we don't yet know the mechanism(s) responsible for this observation. A better understanding of this process would advance vaccine design in several ways: it would allow us to design vaccine delivery systems with a specific APC phenotype in mind, which would allow more efficient screening of vaccine candidates at an earlier stage which is more amenable to *in vitro* analysis. Below I present preliminary data which will guide our future studies.

It has been reported that APCs can downregulate CCR5 expression on CD4 T cells by producing IL-12, which induces CD4 T cells to produce β-chemokines that bind to CCR5 receptors, causing them to be internalized (142). We collected RNA from vector-primed MDDC 24 hours post-prime and used qPCR to measure expression of the p35 subunit of IL-12 (active IL-12 is a heterodimer; the p40 subunit is shared with IL-23 (143)). Our results show that ALVAC, but not Ad5, significantly upregulates IL-12p35 expression relative to unprimed MDDCs (Fig 4.2A). This qPCR result suggests a plausible mechanism for the downregulation of CCR5 in ALVAC-specific CD4 T cells, which in turn contributes to their decreased HIV susceptibility relative to Ad5-specific CD4 T cells. It should be noted, however, that our previous report showed that CCR5- Ad5-specific CD4 T cells

were still more susceptible to HIV infection than CCR5+/- ALVAC-specific CD4 T cells, suggesting that coreceptor expression is not the only factor responsible for their differential HIV susceptibility (140). Interestingly, IL-12 also induces Th1 differentiation and CD8 T cell activation, which also characterized the vector-specific T cell response induced by ALVAC vs Ad5 (140). Priorities for ongoing studies will include measuring active IL-12p70 (the active heterodimer) in the cell culture supernatants of vector-infected MDDCs and using neutralizing antibodies to determine the effect of blocking IL-12p70 on the CCR5 expression and HIV susceptibility of CD4 T cells cultured with ALVAC-infected MDDCs.

Our lab has previously reported that ALVAC infection of MDDCs induces inflammasome activation, including the production and release of IL-1β and IL-18 into the cell culture supernatants (111). Hypothesizing that these cytokines might contribute to the decreased CCR5 expression observed in CD4 T cells cocultured with ALVAC-infected MDDCs, the author of that paper (Dr. FengLiang Liu) used neutralizing antibodies to block IL-1β/IL-18, the β-chemokines CCL3, 4, and 5, or both. His results show that blocking β-chemokines alone only slightly increased CCR5 expression on CD4 T cells cultured with ALVAC-primed MDDCs, but blocking both β-chemokines and IL-1β/IL-18 increased CCR5 expression to a level comparable to that of CD4 T cells cultured with Ad5-primed MDDCs (Fig 4.2B-C). Future work will focus on replicating this result, as well as determining the effect of blocking IL-1β/IL-18 alone. Unlike IL-12, to the best of my knowledge there is no report in the current literature of IL-1β and/or IL-18 downregulating CCR5 expression on CD4 T cells, making this an important direction for novel research.

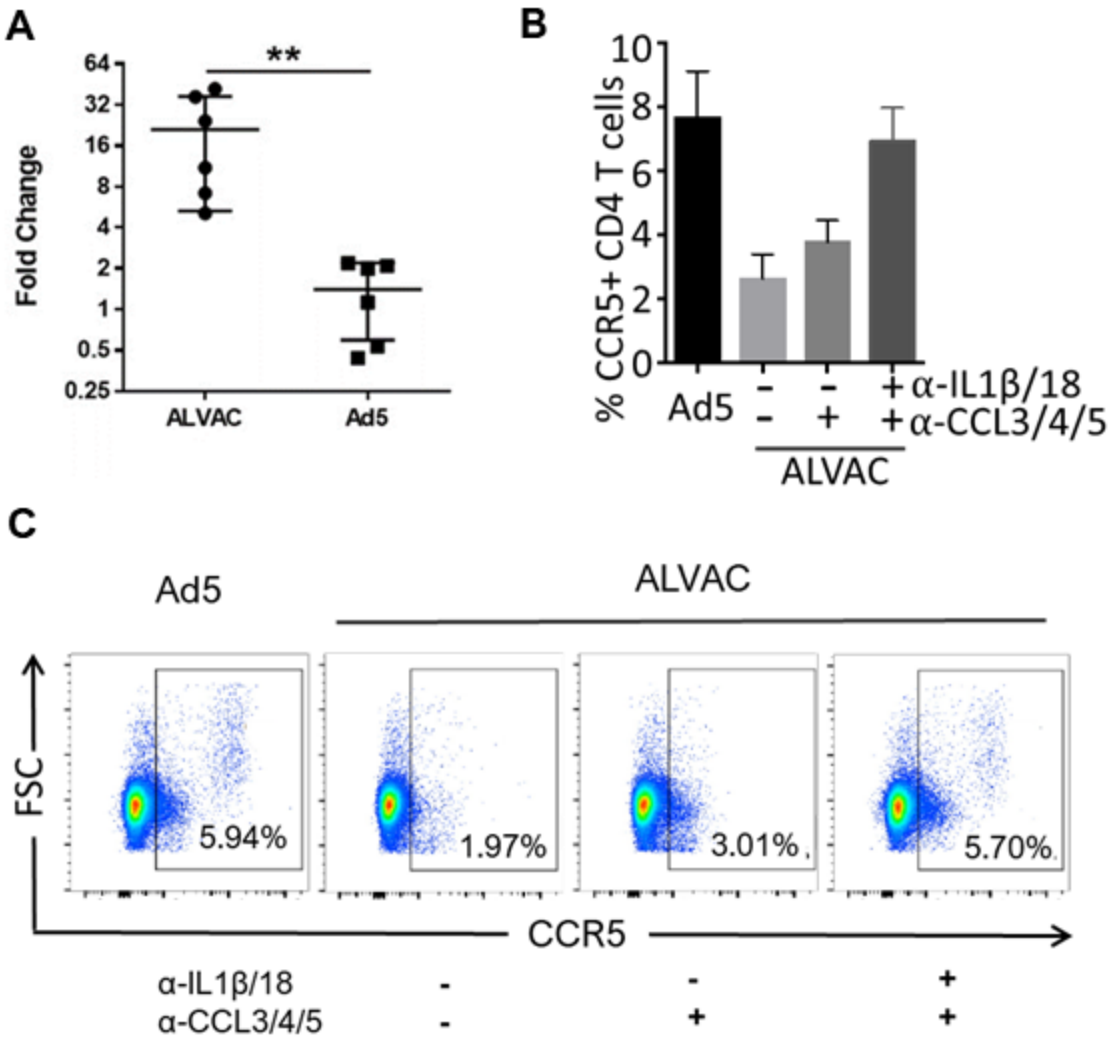

Figure 4.2. Preliminary data suggesting potential mechanisms for CCR5 downregulation/decreased HIV susceptibility of ALVAC-specific CD4 T cells. (A) Fold change in IL-12 mRNA expression in MDDCs infected with ALVAC vs Ad5, compared to an uninfected control. **(B)** CCR5 expression of CD4 T cells cultured with ALVAC-primed MDDCs in the presence or absence of IL-1β/IL-18 and/or β-chemokine nAbs compared to that of CD4 T cells cultured with Ad5-primed MDDCs. **(C)** Representative flow plot for the data shown in (B). Statistics were performed using an unpaired Student's t test. $P < 0.01$.

CONCLUDING REMARKS

The development of a safe and effective HIV vaccine has been a major global research priority for over 30 years. After decades of failure, the partial success of the RV144 "Thai" trial renewed optimism in the field, and several large-scale efficacy trials attempting to prolong the short-lived protection generated by the ALVAC prime/gp120 boost vaccine regimen with additional booster vaccinations are ongoing. However, the fact that the primary correlate of immunity was short-lived IgG3 antibodies poses a significant obstacle to this approach, since prolonged antigen exposure tends to decrease IgG3 production in favor of IgG2 and IgG4, which were not associated with protection from HIV. Our study takes a different approach, investigating the protective and detrimental immune responses to the vectors themselves in hopes of a) minimizing detrimental responses generated by future candidate vaccines, and b) redirect protective responses towards HIV antigens. Overall, the results presented in this dissertation help to partially fill the gap in our understanding of how viral vectors influence vaccine outcomes as well as suggesting potentially fruitful areas for future research in a previously neglected field.

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Vita

Sarah Rebekah Auclair was born in Woodbridge, Virginia on January 13, 1983, to David and Anita Auclair. She completed a Bachelor's degree in Philosophy at the University of Virginia in 2005 with the intent to go on to medical school. After working as a patient care aid at UVA hospital, however, she decided to pursue a career in research rather than clinical medicine. She completed a second Bachelor's degree in Biomedical Sciences via James Madison University's Adult Degree Program, during which she became involved in undergraduate research in the laboratory of Dr. Chris Lantz, using interleukin-3 (IL-3) knockout mice to investigate the role of IL-3 in the immune response to blood-stage infection with *Plasmodium*, the single-cell, eukaryotic parasite that causes malaria. After completing her Bachelor's degree, Sarah continued her research in Dr. Lantz's lab as a Master's student, receiving her Master's Degree in Biology from James Madison University in the summer of 2014. In the fall of 2014 she entered the Graduate School of Biomedical Sciences (GSBS) at the University of Texas Medical Branch in Galveston, Texas as a McLaughlin Scholar. After completing the first year GSBS program for Microbiology and Immunology, she joined the laboratory of Dr. Haitao Hu in January of 2016. Sarah's dissertation research was supported by a McLaughlin Fellowship (2016 – 2018) followed by the American Association of Immunologists (AAI) Careers in Immunology Fellowship (2018 – 2019). While completing her dissertation research, Sarah also received a scholarship from the Del Papa Distributing College Endowment (2017), an AAI Trainee Abstract Award for her abstract which was presented as a poster at the 2018 AAI National Meeting in Austin, Texas, and McLaughlin Travel Awards for her poster presentations at the 2017 and 2018 McLaughlin Colloquium's in Galveston, TX.

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PUBLICATIONS

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ABSTRACTS

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ABSTRACTS

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