CONSEQUENCES OF ADENOVIRUS VECTOR VACCINATION ON T CELL ACTIVATION AND SIV SUSCEPTIBILITY IN RHESUS MACAQUES

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DEDICATION

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ABSTRACT

CONSEQUENCES OF ADENOVIRUS VECTOR VACCINATION ON T CELL ACTIVATION AND SIV SUSCEPTIBILITY IN RHESUS MACAQUES

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Michael R. Betts

The quest for an efficacious HIV vaccine has resulted in several clinical trial failures, including the Step Trial, which used a replication-incompetent adenovirus (AdV) vector called human adenovirus type 5 (HAdV-5). Despite eliciting strong cellular immune responses, these trials were prematurely halted due to statistical futility resulting from increased HIV acquisition in vaccinated individuals. The Step Study showed increased HIV susceptibility in HAdV-5 baseline seropositive subjects, which complicates the use of HAdV-5 vectors since pre-existing neutralizing antibodies (nAb) to HAdV-5 are prevalent worldwide. Sampling the unique immunological phenotypes of the rectal mucosa – the site of HIV infection in the Step Study, and of AdV persistence and trafficking – could help explain this trial, since only peripheral blood (PBMC) was collected from subjects. We obtained rectal lamina propria T lymphocytes (rLPL) from a rhesus macaque (RM) model vaccinated with a species-specific simian Ad type 7 (SAdV-7) or HAdV-5 vector. We hypothesized that AdV-based vector vaccination in macaques previously exposed to AdV would activate AdV-specific CD4+ memory T cells that are either already harbored in the gastrointestinal mucosa or traffic to this original site of infection. The increased percentage of activated target cells upon SIV exposure would create a favorable environment for increased SIV susceptibility. We show an increase in both AdV-specific and activated CD4+ memory T cells in the rLPL after AdV vector vaccination relative to baseline, without significant changes in PBMCs. This led us to a proof-of-concept study where we collected PBMCs and rLPL from 10 SAdV-7 vector

vaccinated and 10 placebo vaccinated RMs at baseline and week 2 post-vaccination. We performed low-dose escalating intra-rectal challenges weekly with SIVmac251 until SIV acquisition or 16 weeks post-vaccination. If we demonstrated increased SIV infection in the vaccine group, this would show evidence of activation-induced SIV susceptibility. Although we did not see an alteration in CD4+ memory T cell activation, we saw a trend towards SIV acquisition in the SAdV-7 vector vaccinated group versus placebo. Future studies should be cautious with the use of AdV vectors for mucosally transmitted pathogens, even for rare AdV serotypes due to T cell cross-reactivity and CD4+ memory T cell activation-induced susceptibility within mucosal sites.

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CHAPTER 1

General Introduction

Origins of HIV and Epidemiology

Two laboratories identified Human Immunodeficiency Virus (HIV), the etiologic agent that causes Acquired Immunodeficiency Syndrome (AIDS), in 1983 [1,2]. HIV remains a significant public health concern, despite research seeking a cure for over 30 years. It is estimated that 34 million people are living with HIV globally, which includes approximately 2.5 million new infections and 1.7 million AIDS-related deaths yearly. Although there appears to be a slight decrease in the total number of new infections and deaths, and an increase in the life expectancy of HIV-infected individuals due to better therapeutics and access to anti-retroviral therapy (ART), there is still no cure. In particular, sub-Saharan Africa has been dramatically affected by this epidemic, where it is estimated that a fifth of the population may be HIV positive [34]. The limited access to treatment or care in impoverished regions across the world is a major issue that must be tackled in conjunction with continued research towards HIV eradication.

HIV is acquired via the transfer of bodily fluids, including blood, vaginal fluid, semen, and breast milk. HIV-positive individuals are most infectious during the acute phase, when the viral load is highest in the transmitting individual, through unprotected sex, intravenous drugs using contaminated needles, perinatally, and during breast-feeding [5-8]. While the predominant route of transmission varies in different parts of the world, over half of the infections that occur in the U.S. are a result of men who have sex with men (MSM), and ~25% are due to heterosexual contact (2).

There are two strains of HIV in humans – HIV-1 and HIV-2 – both of which

originated from zoonotic transfers from Simian Immunodeficiency Viruses (SIV) (SIVcpz and SIVsmm, respectively) in African non-human primates (NHP) [9,10]. Molecular clock estimates indicate that HIV-1 group M transfer to humans occurred in the early 20th century, but the first evidence of HIV infection was determined to be from 1959 in the Democratic Republic of Congo [11-14]. Comparative sequence analysis has revealed that HIV-1 group M, which represents the large majority of human HIV infections, is the consequence of cross-species transmission of an SIV found in chimpanzees (*Pan troglodyte troglodyte*), SIVcpz (which also includes HIV-1 group N) [15]. Further, SIVcpz contains a recombinant genome made up of two similar strains of SIV – SIVrcm from red-capped mangabeys (*Cercocebus torquatus*) and SIVgsn from greater spot-nosed monkeys (*Cercopithecus nictitans*) [16,17]. Thus, while it was originally believed that chimpanzees were a natural host of SIV, we now know they are intermediate hosts with increased mortality and morbidity compared to uninfected counterparts [18]. Additionally, HIV-1 groups O and P likely originated from gorilla SIV transmission, which also appears to trace back to chimpanzee SIV [19-21]. On the other hand, HIV-2 infection, which is much less prevalent relative to HIV-1, originated from a cross-species transmission of SIV found in sooty mangabeys (*Cercocebus atys*), occurring probably around the 1930s [10,12,22]. This strain, also known as SIVsmm, crossed over into the rhesus macaque (RM) population during a separate transmission event [23,24]. HIV-2 infection displays relatively milder symptoms compared to HIV-1, with slower disease progression and less severe immunodeficiency [25-27].

These aforementioned SIV strains are just a handful of more than 40 different species-specific SIVs that currently exist in African NHPs [reviewed in [28]]. This species-specificity means that while HIV originated in NHPs, in the majority of cases it is unable to effectively replicate in the host it was transmitted from, as a consequence of rapid viral

evolution and various host-restriction factors [29-32]. For the hosts in which SIV is endemic, namely African non-human primates, these animals generally exhibit a normal lifespan with high viral loads, but a lack of symptoms [30,33-36]. This largely contrasts with non-natural SIV hosts such as chimpanzees, rhesus macaques and other Asian macaques, as well as humans, which tend to progress to the opportunistic infections characteristic of AIDS if left untreated [18,37,38]. Some of the mechanisms governing the asymptomatic nature of SIV in natural NHP hosts have been described [36,39], although additional research aims to understand this further for use as a stepping-stone towards a therapy and/or cure for HIV.

The SIV transmission event from sooty mangabeys (SM) to rhesus macaques, briefly mentioned already, occurred during cohousing of these two species in US primate centers. The SMs had been infected with SIVsmm prior, and the SM/RM contact initiated an outbreak that was first recognized due to pathogenic lymphomas that developed in the Asian macaques [23,24]. It was this transmission which led to the development of the well-known SIVmac251 and SIVmac239 strains now used as a model for human HIV infection, since SIV-infected RMs follow a disease course which resembles AIDS resulting from HIV [38,40,41]. I will describe the SIV viruses used in research in a later section, as this is the animal model upon which the entirety of this dissertation is based upon.

HIV Biology and Disease Pathogenesis

HIV and SIV belong to the Retroviridae family, and are more specifically in the lentivirus genus [1,2]. These lentiviruses have two identical copies of a single-stranded RNA genome with nine open reading frames and encode two general classes of proteins. These include viral structural proteins (gag, pol, env) and accessory proteins (tat, rev, nef, vpr, vif, vpu) [reviewed in [42-44]]. It is the envelope (Env) glycoprotein, made up of gp41 and gp120 to form gp160, which assists with viral attachment and fusion to the cells targeted for HIV infection. More specifically, HIV infects CD4+ T lymphocytes and macrophages, and infection and subsequent depletion of the former is the main driver towards pathology associated with AIDS. Interaction of Env gp120 and the CD4 cellular receptor initiates infection, subsequently altering the conformation of gp120, and exposing co-receptor binding sites such as the predominant CCR5, and CXCR4, which is sometimes seen in chronic infections [45-52]. Further, other co-receptors have been discovered in addition to CCR5 and CXCR4 [53-56], which, in SIV, include CCR2b, CXCR6, GPR15, GPR1 and APJ [57-59].

HIV infection generally progresses through three phases **(Figure 1.1)**, starting with the acute phase, which lasts several weeks. During this time there is a peak in viral load and an increase in immune activation, both of which can lead to flu-like symptoms [60]. Mucosal CD4+ T cells undergo a significant decrease and a similar, but less pronounced, decrease in seen in peripheral blood (PBMC) CD4+ T cells. The phenotype of the mucosal CD4+ T cells, those found in the gut associated lymphoid tissue (GALT), tends to lend itself to HIV infection since they are activated and express sufficient CCR5 to become infected. Mucosal activated memory CD4+ T cells become depleted very rapidly and do not experience the slight rebound that is seen in the peripheral blood population [61,62]. During the chronic phase, which can span many years, there is typically

a slow climb in viremia and an opposing drop in CD4+ T cells [63]. Most CD4+ T cells are lost due to direct infection and subsequent killing, but there is also bystander cell killing, as well as immune exhaustion due to chronic activation [64]. The onset of AIDS is a consequence of a severe drop in CD4+ T cell counts, which was one of the earliest observations seen in patients before HIV was identified as the causative agent of AIDS. This drop sets the stage for an immune compromised state where opportunistic infections can take over [65,66].

These aforementioned pathological consequences of HIV were difficult to eliminate partly as a result of HIV's high rate of mutation. This feature is a serendipitous attribute for the virus due to the lack of polymerase proofreading, and has led to the necessity for highly active antiretroviral therapy (HAART), which is the use of three or more antiretroviral (ARV) drugs together. Single ARVs produced drug-resistant strains, but consistent use of HAART is able to reduce viral load below detectable levels [67-69]. There exists a small percentage of HIV-infected individuals that can control infection on their own while exhibiting detectable viral loads, called long-term non-progressors, and further, a smaller subset of those called elite controllers that have undetectable viral loads [70]. Trying to understand the mechanisms of control in these individuals, as well as certain NHPs including sooty mangabeys (SM) and African green monkeys (AGM) that are natural hosts of SIV and do not progress to AIDS (I will touch on the natural NHP model more in the next section), is an active field of research [33,35].

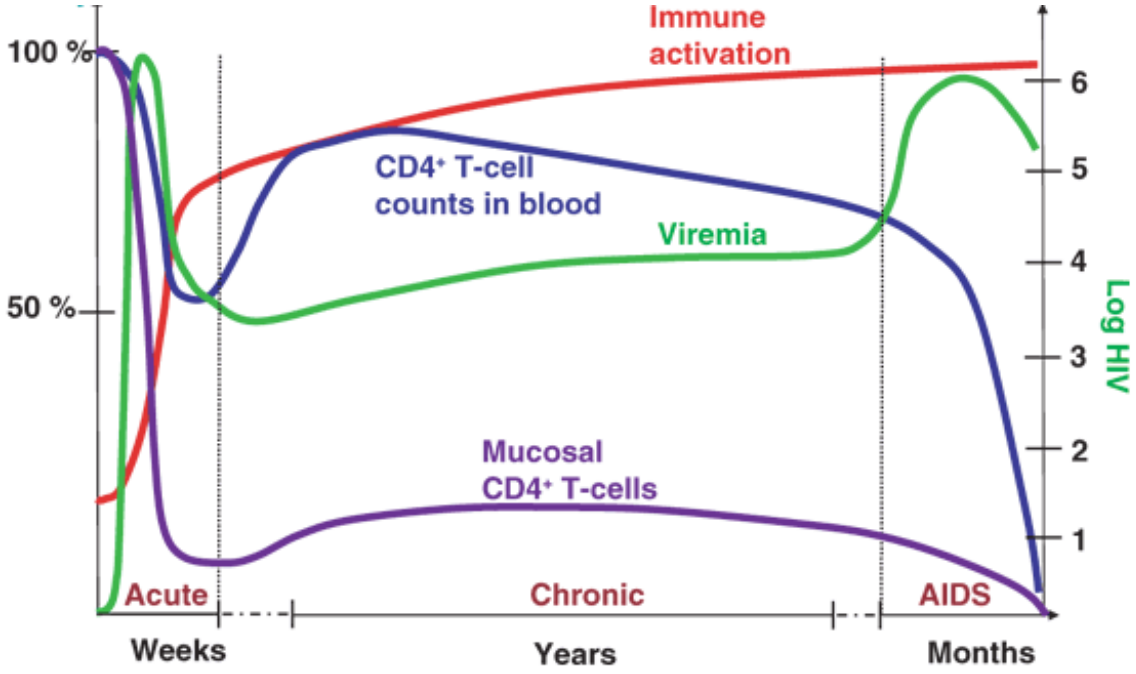

Figure 1.1 – Quantitative and qualitative measures of HIV disease progression. Mucosal and blood CD4+ T cell numbers, viremia, and immune activation are shown over the course of progression from acute to chronic HIV infection and ultimately AIDS. Adapted from Grossman et al., 2006 [71].

Innate and Cell-Mediated Immune Responses to HIV

Most HIV infections are mucosally transmitted, and in approximately ¾ of these cases, a single virus or virus-infected cell leads to productive clinical infection [72]. The genital and rectal mucosa is home to various immunological barriers to HIV. If the virus can bypass the thick layer of mucus, which is first line of defense in the female genital tract [73], then the next barrier is the mucosal epithelial barrier, followed by the innate immune system. The main players in innate immunity to HIV include Langerhans cells, dendritic cells (DCs), type I interferons (IFN), cellular restriction factors, and natural killer (NK) cells. Langerhans cells express Langerin, a receptor that can capture HIV virions, internalize, and then degrade it, thus preventing infection [74,75]. DCs express DC-SIGN, a receptor that helps DCs capture and internalize, but not degrade, HIV [76,77]. DCs then carry HIV to the lymph nodes where they can present it to CD4+ T cells, which are the main target cell of HIV. Although this promotes infection on one hand, DCs also help induce additional immune responses to attempt to eliminate HIV [78]. Type I IFNs regulate immune activation, apoptosis, and can upregulate cellular restriction factors (APOBEC3G, TRIM5a, tetherin), all of which contribute to antiviral activity [79-87]. NK cells are activated quickly after HIV infection and can mediate effector functions, limit viral replication through various methods, two of which include antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent viral inhibition (ADCVI) [88], although these functions are likely to only be seen in previously exposed individuals.

Compared to innate responses, the cell-mediated responses of the adaptive immune system take more time to initiate. Cell-mediated immunity consists of CD4+ and CD8+ T lymphocytes that interact with antigens presented to them by antigen-presenting cells (APCs) within the context of major histocompatibility (MHC) alleles. After this interaction, T cells proliferate and differentiate, eventually maturing from naïve into

effector T cells [89]. Effector T cells produce molecules that function to kill infected cells, either directly or indirectly. Another subset, known as memory T cells, are formed slowly after initial antigenic encounter to provide protection after re-infection, at which point they multiply rapidly to differentiate into secondary effector and memory populations [90]. Within the CD8+ T cell population exist cytotoxic T lymphocytes (CTLs), which have been associated with an initial drop in HIV viral load after infection [91-93]. CTLs recognize viral peptides presented by MHC Class I molecules found on the surface infected cells and destroy them. HIV can escape from CTL-induced pressure, and if that escape mutation doesn't lead to a cost in viral fitness, there is a faster progression to AIDS[94,95]. CD4+ T cells have an even wider range of activities compared to CD8+ T cells. They are able to differentiate into several subsets, including Th1, Th2, T follicular helper (Tfh), and T regulatory (Treg) cells. These subsets carry with them different functions and are induced by different conditions [96]. CD4+ T cells are heavily depleted in HIV infection, particularly in the gastrointestinal mucosa, which can ultimately lead to AIDS [48,97].

Primate Models of HIV and Limitations

In general, SIV infection in NHPs can be grouped into those that are pathogenic in non-natural hosts (such as RMs) and those that are non-pathogenic in natural hosts (such as SMs and AGMs) [reviewed in [28]]. SIVmac239 and SIVmac251 are the predominant isolates used to infect macaques, as mentioned previously, to model HIV in humans. These two isolates are closely related, as SIVmac239 is a clonal virus that has a single genotype, which was passaged *in vivo* from SIVmac251. SIVmac251 is an uncloned heterogenous swarm, and was used in the challenge studies we performed in Chapter 3 [23,98,99]. Depending on whom the SIVmac251 was obtained from, it has the potential to create slight phenotypic variability as it is actually a collection of unique,

albeit very similar, viral stocks. In total, there is up to 2% diversity within the SIVmac251 pool seen in the US, which may affect the neutralization sensitivity and receptor tropism [100-102]. Within the SIVmac251 swarm, the viruses are still more similar to each other than they are to SIVmac239. Even so, the results of SIV challenge studies leading to infection, investigating pathogenesis, and vaccine utility are still thought to be an incredibly powerful tool when using either of these isolates [41,100].

Why did challenging macaques with SIV in order to model human HIV infection become so predominant? First, the HIV and SIV viral structures are similar, with differences displayed in **Figure 1.2.** The structural proteins are nearly identical, with some variability in accessory genes – namely *vpu* in HIV and *vpx* in SIVmac. As mentioned above, there is ~2% sequence diversity in SIVmac, although >10% diversity is seen in HIV [reviewed in [40,103,104]]. As far as disease course and pathogenesis, experimental transmission can be modeled after human infection to mimic sexual (mucosal) or intravenous (blood) infection, which leads to a dramatic decrease in mucosal CD4+ T cells and a less pronounced drop in the blood in both RMs and humans. One important distinction is progression to AIDS, which in humans it takes 8-10 years, while RMs experience a more rapid timeline of 1-2 years. Overall, though, the progressive loss of CD4+ T cells systemically, increased lymphocyte turnover, similar peak viral loads, chronic immune activation followed by immune exhaustion, amongst other shared features, indicate that SIV infection in RMs is the most appropriate model currently known for investigating HIV infection in humans [38,105-109].

Briefly, non-pathogenic natural SIV infection seen in sooty mangabeys and African green monkeys is interesting because it exhibits the same features of high levels of viral replication, with viral loads equal to or even greater than what is seen in pathogenic RM/human infection [33,35]. Unlike pathogenic infection, though, SMs and

AGMs do not display chronic immune activation and display more moderate lymphocyte turnover [64,110-114]. Further, there is no evidence for microbial translocation, which is when a breach occurs in the mucosal barrier, which likely leads to chronic immune activation [115,116]. How CD4+ T cells are maintained and progression to AIDS is countered in these species is still an active field of research.

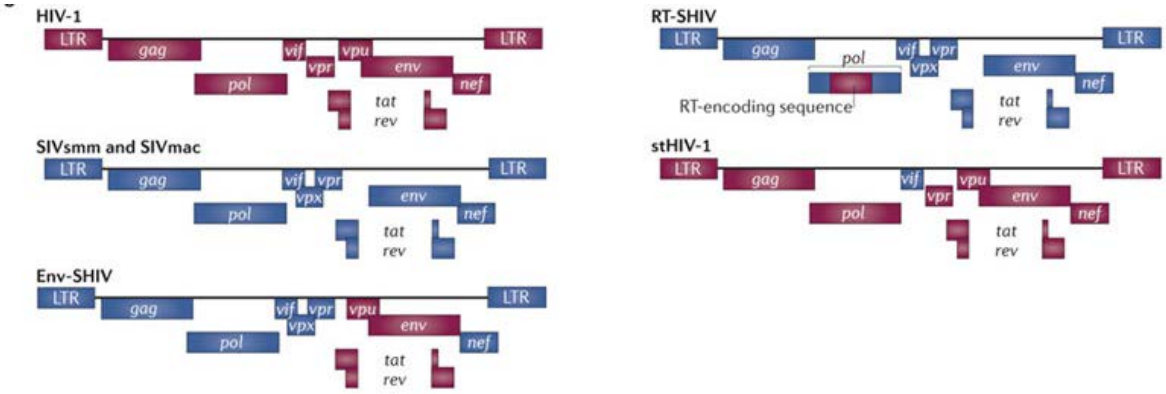

Figure 1.2 Structure of commonly used HIV and SIV strains Schematic representation of HIV-1, SIV and chimeric viral genomes. Maroon and blue boxes indicate HIV-1- and SIV-derived sequences, respectively. Env, envelope glycoprotein; LTR, long terminal repeat; Mm, *Macaca mulatta* (rhesus macaque); Mn, *Macaca nemestrina* (pig-tailed macaque); SHIV, simian–human immunodeficiency virus; SIVmac, macaque SIV; SIVmne, pig-tailed macaque SIV; SIVsmm, sooty mangabey SIV; stHIV-1, simian-tropic HIV-1; RT, reverse transcriptase. Figure was adapted from Hatziioannou and Evans, 2012 [104].

Progress in HIV Vaccines

Edward Jenner invented the first vaccine over 200 years ago for smallpox by inoculating an 8-year old with material from a cowpox lesion taken from another person. When challenged later with smallpox, there was no indication of disease [117]. Nearly a century later in 1885, Pasteur developed the rabies vaccine to be used in humans. He was the first scientist to artificially attenuate viruses for use in vaccines [118,119]. Since then, over 20 vaccines have been developed and regularly used for diseases including smallpox, polio, measles, mumps, rubella, and others [119]. These generally use a killed or attenuated virus, protein, or toxin that induces neutralizing antibodies (nAb) and protects from subsequent pathogen exposure by antibody-mediated immunity. Although great strides have been made in the decrease or even eradication of certain diseases, the worldwide impact of tuberculosis, malaria, and HIV are significant and necessitate further research towards a cure.

In general, there are currently two main approaches towards a vaccine for HIV – elicitation of the humoral immune response with broadly neutralizing antibodies (bnAbs), and induction of cellular immunity with CD8+ cytotoxic T cells largely directed towards conserved epitopes of the virus [120]. The focus is on inducing protection through T cells because multiple lines of evidence point to CD8+ T cells as vital in the control of HIV infection. The first reason is that depletion of CD8+ T cells via antibodies in SIV-infected macaques resulted in a dramatic rise in plasma viremia, which was controlled after reconstitution of CD8+ T cells [121]. Also, the drop in viremia during acute HIV infection occurs concomitantly with the generation of HIV-specific CD8+ T cells [122]. Further, certain human MHC class I alleles found in HIV non-progressors are associated with control of HIV and slower progression to AIDS due to highly functional HIV-specific CD8+ T cells, relative to non-progressors [123,124]. Finally, escape mutations in HIV are

known to be a response to immunological pressure on dominantly targeted cytotoxic T lymphocyte epitopes [95,125]. The ultimate long-term solution to the HIV pandemic would be to develop a vaccine that prevents HIV acquisition in the first place (sterilizing immunity). A more plausible, but still valuable, alternative may be to lower viral load and thus decrease transmission since the risk is greatest when viremia is highest. Beyond those options, Picker et al. has recently found that half of the macaques vaccinated with a cytomegalovirus (CMV) derived vector encoding SIV genes were able to suppress viral load to undetectable levels after repeated rectal SIV challenge. This suppression is likely due to the induction of unusual and broad effector memory T cell responses [126,127]. The same outcome has also been reported after vaginal and intravenous challenge, implying that control through the immune response spreads beyond mucosal tissues to the blood [128].

The earliest HIV vaccine candidates were subunit vaccines, which include a specific antigen shown to be immunogenic and safe, meant to induce nAb responses to HIV Env. The first two vaccine candidates were a recombinant gp120 protein and a recombinant soluble gp160 with aluminum hydroxide as an adjuvant. To test these vaccines, chimpanzees were immunized 3x (0, 1, 8 months) with one of these vaccines, then challenged intravenously 35 weeks after immunization. Two animals were vaccinated with each candidate, and only the recombinant gp120 vaccine showed protection. Therefore, it moved on to phase I clinical trials where 50 human volunteers were immunized and sera from almost all of the vaccine recipients neutralized at least one lab-adapted HIV-1 strain (but not primary HIV-1 isolates) [129]. With this outcome, this AIDSVAX vaccine moved on to phase II and phase III testing by VaxGen. These were double-blinded, placebo-controlled clinical trials in two sites. First, Vax003 in Thailand recruited 2,546 intravenous drug users and combined a clade B and clade E adjuvanted

recombinant gp120 together (AIDSVAX B/E) [130]. Subjects were vaccinated seven times, but showed approximately the same amount of infections in placebo (105 infections) and vaccine (106 infections) groups and thus demonstrated no protection or effect on viral load [131]. The second study, Vax004 used two different isolates of clade B viruses and was tested among 5,400 people at risk of sexual HIV transmission in the US, Canada, Puerto Rico and the Netherlands (of which ~90% were men who have sex with men). Participants were again vaccinated seven times, but the trial showed no evidence of protection (241 vaccine group and 127 placebo group infections) or reduction in viral load, although non-neutralizing HIV antibodies were elicited [132].

Despite the lack of success in these AIDSVAX trials, a later study known as the RV144 'Thai' trial used the ALVAC-based (canary-pox) vaccine, in combination with the AIDSVAX gp120 vaccine as the protein boost. It showed 31.2% efficacy against HIV infection in a low-risk heterosexual population in Thailand. This was a large phase III HIV-1 vaccine trial that used a canary-pox vector expressing gag, protease, and Env proteins and immunized volunteers at weeks 0, 4, 12 and 24. The 12 and 24 week time points also included an AIDSVAX B/E gp120 (used in the previous Vax003 trial) as a boost. While >16,000 people participated in the study, approximately 4,000 of those were excluded since they did not receive all four immunizations. Once these volunteers were removed, the vaccine efficacy was found to be statistically insignificant. But a "modified intent to treat (mITT)" analysis was statistically significant, with the 31.2% efficacy previously mentioned. Interestingly, it appeared that only CD4+ T cell and gp120 non-neutralizing antibody responses were generated after giving the viral vector-based vaccine, with very low levels of both CD8+ T cell and neutralizing antibody induction [133].

Numerous vaccine platforms have been studied, including adjuvanted protein vaccines [134], virus-like particles (VLPs) [135], plasmid vectors [136-141], viral [142-146] and

bacterial recombinant vectors [147,148]. As for the viral recombinants, there are an expansive amount of them, including those that are based on poxviruses (such as modified vaccinia Ankara (MVA) [142], vaccinia virus [144], AAV [146], VSV [149], measles-virus based vaccines [143], and different Ad vectors [145] (which I will expand on in upcoming sections of this thesis). In addition to RV144, vaccine regimens including MVA and NYVAC, which all encode SIV proteins, have only exerted modest levels of control over SIVmac251 replication [150]. In many HIV vaccine trials, prime-boost strategies are often employed to increase the HIV antigen-specific immune responses, and allow for reduced vaccine dosage to minimize toxicity. These often combine DNA or protein vaccines, or use heterologous viral vectors [147,148]. DNA vaccines, such as those being pursued by Weiner et al., have potential since they induce both cellular and humoral immunity potently [151], though they are typically used in combination with another modality [152,153].

Adenoviruses and Their Use As HIV Vaccine Vectors

Adenoviruses (Ad) have been used for three decades in gene therapy, and now are used in the development of vectors for HIV, malaria, and hepatitis C vaccines, as well as tumor therapies. Ad-based vectors have shown superior immunogenicity, particularly in CD8+ T cell induction. They can be derived from bacterial molecular clones, and are thus easily modified [154]. Adenovirus is icosahedral, non-enveloped, and the capsid contains a double-stranded, linear DNA genome of ~34-43 kb [155] **(Figure 1.3)**. The capsid faces are composed of 240 hexons, each with three identical proteins, and 12 pentons sit on the vertices, each with five protein chains. A long fiber extends from each vertex, made up of three identical chains that form a knob at the end [156-158]. The hexon protein contains seven hypervariable regions, though the remaining ~80% of

the hexon sequence is conserved between Ad serotypes [159-161]. The genome contains two inverted terminal repeats, with early (E1A, E1B, E2-E5) and late transcription (L1-L5) genes. When Ad infection occurs, E1a is expressed and encodes polypeptides that initiate transcription of other early genes [162,163]. Thus, the most common way to make Ad replication-incompetent is to delete E1a – which is considered a safer alternative to replication-competent vectors [164,165]. Another common deletion performed in order to make more space for transgenes is the E3 domain, a region that encodes polypeptides that subvert the immune response, although this deletion on its own does not render it replication-incompetent [166]. Studies have shown that whether E1-deleted alone, or E1-plus E3-deleted, there does not appear to be a variation in vector infectivity, or an effect on the vector's ability to induce transgene product-specific CD8+ T cell responses [167].

Adenoviruses are classified in the genus *Mastadenovirus*, which currently contains 7 known human adenoviral species classified into 7 subgroups, A-G [168]. Although human Ads were historically further broken down into 51 serotypes based on hemagglutination and serum neutralization reactions, there now exist 65 serotypes based on genomic data [169-176]. In the past few years, a New World monkey Ad was able to cross into humans to cause infection [177]. Adenovirus can cause respiratory disease, conjunctivitis, and gastroenteritis, and as such, subgroups tend to be associated with tissue tropism since unique cellular receptors are expressed in different parts of the body [178]. The main receptors used for viral binding and entry are CD46 for group B Ads, and the coxsackie adenovirus receptor (CAR) for nearly all the other serotypes including human adenovirus type 5 (HAdV-5) [179]. The Ad fiber protein "knob" engages cells through interaction with CAR, which is found in numerous tissues including the heart, brain, pancreas, intestine, lung, liver, testis and prostate [180-182].

When adenoviral infection occurs, Ad-infected macrophages and dendritic cells

transport Ad to lymph nodes present antigen, which leads to adaptive immune responses. These include CD8+ cytotoxic T cell (CTL) responses to viral proteins and vector-encoded transgene products expressed by transduced cells, and production of antibodies against capsid proteins by B cells [183]. If the individual has previously encountered Ad, primed memory CD8+ T cells will recognize antigen-expressing tissue cells and rapidly eliminate them [184]. This is one difficulty, which I will discuss in more detail later, with the use of the same vector to boost or re-challenge, since circulating neutralizing antibodies prevent virus transduction and memory T cells decrease transgene expression.

Despite this, there are numerous features of Ad that make it an attractive candidate for a vaccine vector. These include ease of manufacture (Ad does well in large-scale cell culture), good safety profile, and the ability to elicit broad immune responses. Ad is able to target the mucosa, and infect and persist in a wide variety of both dividing and non-dividing cells [185,186]. The transgene can be expressed at a high level, induce strong chemokine and cytokine responses, all without integration of the viral genome into the host genome which would carry the risk of insertional mutagenesis [187]. Immunogenicity testing with Ad vectors in mice, NHPs, and humans have shown induction of potent and sustained transgene product-specific CD8+ T cells, which is likely a consequence of Ad persistence in general [185,188-190]. Persistence occurs largely in activated T cells, where Ad vectors continue to be transcriptionally active, leading to more activation and preventing contraction of vector-induced T cells. On one hand, this is advantageous, as it creates high frequencies of effector/effector memory T cells, but it's also possible that the extended availability of activated CD4+ T cells can increase susceptibility to HIV by increasing target cell infection. Furthermore, it's possible that the greater presence of effector/effector memory CD8+ T cells may hinder the development

of central memory cells, which proliferate faster upon antigen re-exposure [191].

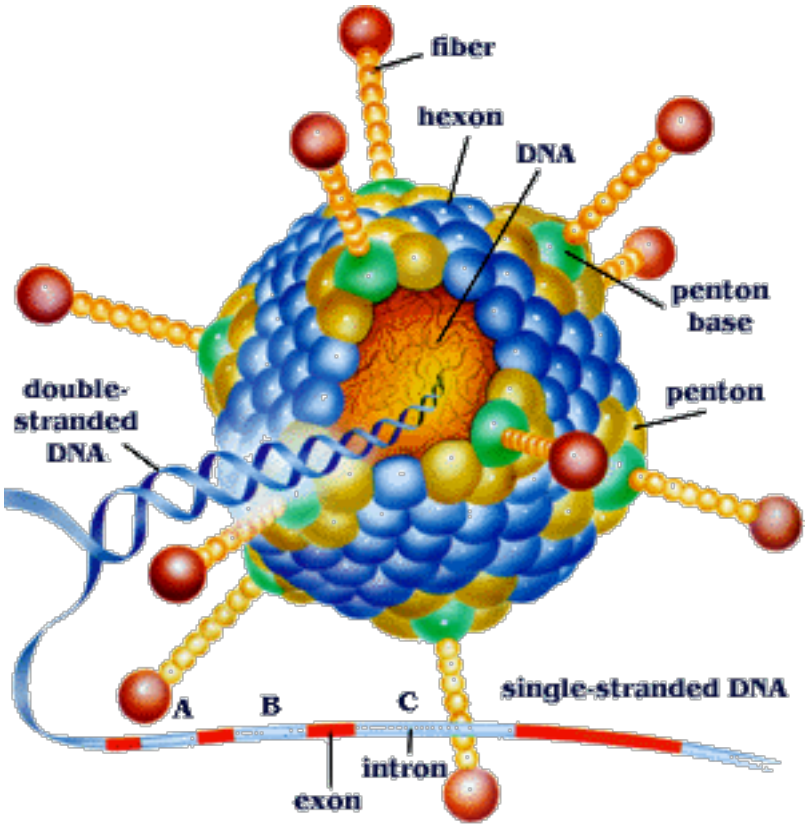

Figure 1.3 Adenovirus structure [192]

Adenoviral Pre-Existing Immunity

Levels of pre-existing immunity to adenovirus varies among the serotypes, and between different populations worldwide. Humans are infected frequently with Ad viruses throughout their life, and as expected, greater exposure occurs with age. In the United States, between 40-60% of people carry detectable neutralizing antibodies (nAbs) to HAdV-5, and this figure is much higher in developing countries [193]. Ad-specific nAbs are directed against the hexon loops and are serotype-specific. High levels of nAbs can significantly influence the immunogenicity of the vector as it can restrict the number of HAdV-5 particles that can infect target cells and produce transgene-derived protein products. This leads to lower levels of antigen presentation, thus decreased adaptive immune responses [194]. The high levels of human anti-HAdV-5 antibodies, especially in sub-Saharan Africa, are likely part of the difficulty encountered with HAdV-5-vectored HIV vaccines. Further, even if nAbs are not present initially, most vaccine strategies involve at least one booster to amplify memory T cells, which may be hindered by nAbs that developed during the prime [195].

In addition to concerns about nAbs, T cell responses also pose a difficulty with AdV vector immunization due to broad cross-reactivity. Human AdV epitopes have been found in both CD4+ and CD8+ T cells. In individuals previously exposed to AdV, anamnestic AdV-specific CD8+ T cells initially take over the response to AdV vectored vaccines. These pre-existing HAdV-5-specific CD8+ T cell responses are capable of reducing the potency and breadth of vaccine-induced HIV-specific CD8+ T cell responses [196-201] and this reduction in transgene-specific responses by AdV-specific T cells has been shown to occur in cross-reactive serotypes [196]. Previous work from our lab has found that AdV-reactive T cells possess an effector and effector memory-like

phenotype. Ad-specific CTLs largely produce effector functions such as MIP1α and perforin, which may indicate efficiency in targeting and eliminating vector-infected cells to decrease vaccine efficacy [202]. There are 65 known strains of human adenovirus, and at least 25 non-human primate strains [203-206], so it is likely that AdV-specific T cell memory and cross-reactivity may affect more rare serotypes, in addition to common AdV serotypes [207]. Therefore, trepidation towards the use of rare AdV vectored vaccines may still be warranted. Furthermore, human intestinal tissue, particularly intestinal lymphocytes, can frequently be found to harbor adenoviral DNA [206] and captive macaques are chronically infected by adenoviruses [208]. Adenovirus has been shown to persist for years in humans, and replication-defective HAdV-5 vectors can persist over a year in mice [185,209], potentially resulting in prolonged antigen presentation and stimulation of T cells at a common site of entry for HIV.

Step Trial and other AdV Vectored Clinical Trials for HIV

Adenoviruses have contributed to a significant proportion of viral vector-based HIV vaccine trials partially due to the high-frequency CD4+ and CD8+ T cell responses they stimulate. Most other viruses used as vaccine vectors have proven to be less immunogenic than AdV-based platforms, including adeno-associated virus (AAV), vesicular stomatitis virus (VSV), lymphocytic choriomeningitis virus (LCMV), herpes simplex virus (HSV), semliki forest virus (SFV), Venezuelan equine encephalitis virus (VEE), and others [191]. Despite the initial positive anticipation for AdV vectored vaccines for HIV, the outcome of several studies, including the Step Trial, have been unsuccessful. This has raised many questions as to why the vaccine regimens failed, the usefulness of cellular immune responses as HIV vaccine targets, and the predictive

efficacy of NHP models.

Prior to pursuing human trials for AdV vectored vaccination, AdVs have had a three-decade history of development in gene therapy. Before the Step Study, several primate challenge studies were conducted to investigate the efficacy of a recombinant HAdV-5-based HIV vaccine. AdV-vectored vaccines have consistently induced strong cell-mediated immune responses and have thus been prime candidates for further development. An early pre-clinical study by Merck compared several vaccine vectors that encoded SIV gag. These vectors included DNA, a modified vaccinia Ankara virus vector, and a replication-incompetent HAdV-5 alone or in combination with the DNA vector. Results demonstrated the largest percentage of gag-specific CD8+ T cells were induced by either HAdV-5 alone or with the DNA prime. [188]. The HAdV-5 encoding only gag led to substantial and durable control of SHIV-89.6P, but it appears that SHIV-89.6P might not be the most appropriate virus to use for modeling human HIV infection [210,211]. This strain has a different target cell tropism and pathogenesis than most SIV/HIV strains as it causes rapid CD4+ T cell depletion. Additionally, all macaques were Mamu-A*01 positive, an MHC class I allele which is known to be naturally protective in SIV infection, so make-up of the NHPs was likely not representative of a human population [212-214]. Another study vaccinating with HAdV-5 encoding gag and subsequently challenging with a single high-dose of the CCR5-tropic SIVmac239 clone led to transient viral control, but only in Mamu-A*01 positive macaques [215]. An analogous outcome occurred when a trivalent HAdV-5 vaccine was tested in another group of Mamu-A*01 positive macaques and challenged with SIVmac239 [216]. Further challenge studies prior to Step believed that including more viral proteins and/or a DNA prime could induce protection [217-219].

Despite what seems to be, in retrospect, less than conclusive evidence for

moving towards a human Phase 1 study, a trial using an IM-administered trivalent HAdV-5-based HIV vector expressing gag, pol and nef was initiated in May 2003 [220]. These proteins are relatively well conserved across different HIV clades and recognized by many infected individuals. Phase 1 studies are designed to test dose safety and immunogenicity, so study participants received between $3x10^6$ – $1x10^{11}$ viral particles (vp) of vector at week 0, 4, and 26, with no serious adverse effects following vaccination. Nearly ¾ of subjects had HIV responses at week 30 until week 78. Based on these results indicating safe and robust immune responses, a phase IIb proof-of-efficacy trial called Step began to enroll subjects at high risk for HIV-1 infection in 2004.

The Step Study was a multi-center, randomized, double-blind and placebo-controlled study with enrollment sites including North America, South America, the Caribbean, and Australia, where clade B HIV-1 is predominant. The study population was HIV-negative when enrolled and included mostly men who have sex with men (MSM) and heterosexual women at high risk for HIV acquisition. It used the same replication-defective recombinant HAdV-5 vector as in the Phase 1 study to target the induction of T cell responses against HIV proteins. This was the first large-scale study to assess the efficacy of a T cell-based HIV vaccine, with 3,000 participants split in half between vaccine and placebo recipients. Since it appeared that pre-existing AdV seropositivity did not limit vaccine efficacy in the Phase 1 study, both HAdV-5 seropositive and seronegative subjects were equally included in the Step Study. As before, participants were immunized at weeks 0, 4, and 26, using an optimized dose of $3x10^{10}$ vp. The goal was to either prevent infection, or control viral replication after infection in order to limit pathogenesis and reduce secondary transmission [221].

Unfortunately, despite early optimism, the study was prematurely halted during a planned interim analysis in September 2007. It was revealed that the vaccine regimen

was unable to prevent infection or lower viral replication in vaccinated individuals. Even worse, by spring of 2008, the placebo group had 33/922 infected men versus 49/914 men in the vaccine group, a statistically significant increase in HIV acquisition in the vaccinated individuals. Viral set points initially revealed no significant difference between placebo and vaccine recipients. Further analyses indicated a trend towards HIV acquisition in those with pre-existing HAdV-5 neutralizing antibodies – for titers <18; 20/382 vaccinated men versus 20/394 placebo controls became infected, and in titers >18; 21/392 vaccinated men versus 9/386 placebo controls became infected. Beyond that, circumcision was shown to lower the risk of HIV infection. It is important to note, though, that nAbs to HAdV-5 prior to vaccination were lower in circumcised men [221,222].

Another Phase IIb clinical efficacy trial for HIV commenced in South Africa around the same time called Phambili where clade C is predominant. This study tested individuals with both low and high pre-existing HAdV-5 titers at high risk for HIV-1, although the predominant mode of transmission was via unprotected heterosexual sex. In light of the Step Trial interim reports, this study was halted and unblinded in September 2007, with many subjects not having received the full complement of vaccine doses. In total, 801 participants had been enrolled, 11 HIV-infections were confirmed (10/11 were women), with 4 in the placebo group and 7 in the vaccine group. Participants with HAdV-5 nAb titers >18 revealed 6 vaccinated-group HIV infections versus 3 in the placebo-group. These aforementioned differences in vaccine and placebo group infections were only trends, and not significant differences [223,224].

Despite the lack of efficacy seen in both Step and Phambili, another HAdV-5-based vaccine advanced to a clinical trial in 2009 called HVTN 505. This study initially aimed to examine the vaccine's ability to moderate the course of HIV infection after vaccination. Once enrollment began, researchers decided to also detect the efficacy of

preventing HIV acquisition during the first 18 months after a completed vaccination course. HVTN 505 used the Vaccine Research Center's multi-clade HIV-1 DNA plasmid (EnvA, EnvB, EnvC, gagB, polB, nefB) and boosted with recombinant HAdV-5 (EnvA, EnvB, EnvC, gag/polB). They enrolled 2,504 HAdV-5 seronegative, circumcised MSM in order to attempt to prevent the risks seen in Step and Phambili. In April 2013, this study followed the fate of previous HAdV-5-vectored HIV vaccines and was stopped. The Data Safety Monitoring Board revealed statistical futility, meaning there was no chance that the vaccine would prevent HIV acquisition or lower viral load set-point in those who became HIV infected after vaccination. At this time, there were 71 cases of HIV (41 vaccine-group, 30 placebo group) and 48/71 of those occurred after 28 weeks when the vaccine regimen was already completed. It is important to keep in mind that of those 48 infections where participants actually received the intended immunization course (27 vaccine-group and 21 placebo-group), there was no statistical significance between groups [225]. Follow-up of the HVTN 505 study participants is currently underway.

Theories for the Step Study Failure

Why did these trials fail and were the outcomes potentially predictable? Although the results of these trials are disappointing, prior analogous SIV vaccine studies in rhesus macaques had not been able to show long-lasting protection. One proposed hypothesis for the lack of efficacy seen in Step was the inability for the series of vaccines to elicit sufficiently broad immune responses for the variety of HIV strains in circulation. Although the vaccine was shown to be immunogenic in 75% of participants, vaccinated individuals made a median of only one CD8+ T cell response per protein when using pools of vaccine-matched 9-mer peptides [221,222,226]. This narrow breadth seems too modest to handle the extensive variability of circulating HIV strains [227,228]. As already

mentioned, vaccination with a viral vector induces vector-specific neutralizing antibodies. Any pre-existing immunity to AdV can restrict the number of HAdV-5 particles that can infect target cells, and also limit both magnitude and breadth of cellular responses to the HIV gag, pol, and nef antigens being delivered [220,222]. Further, homologous prime-boost regimens with viral vectors are suboptimal compared to heterologous prime-boost regimens, since individuals who may have been seronegative at the start of the trial develop vector-specific nAb after the first immunization leading to anti-vector immunity [195,229]. Therefore, during the Step Trial, all participants would have been exposed to HAdV-5 after the first vaccination, hypothetically producing an equal likelihood of targeted nAb and T cell responses. Another proposed hypothesis to explain the increased HIV acquisition is the activation of dendritic cells through immune complexes composed of HAdV-5 particles and HAdV-5-NAbs, which may facilitate HIV infection of DC and spreading to CD4+ T cells at the site of virus entry, such as mucosal surfaces [230]. However, again, Step Study vaccine recipients that were baseline HAdV-5 seronegative would develop HAdV-5 nAb after the first vaccination. Most enrolled individuals received the complete course of three immunizations, thus all vaccinees should show evidence of HAdV-5 seropositivity and thus increased risk of HIV infection.

Only peripheral blood mononuclear cells (PBMCs) were collected during the Step Trial, and no baseline samples were obtained [221]. Data from two studies among Step participants (both of which also did not have access to mucosal samples) concluded that HAdV-5 specific CD4+ T cells were unlikely to be responsible for increased HIV susceptibility [231,232]. In one, the presence of pre-existing HAdV-5 nAbs was shown to not predict the presence of HAdV-5-specific CD4+ T cells both before and after vaccination. Also, HAdV-5-specific CD4+ T cells in HAdV-5 seropositive and seronegative individuals expanded after HAdV-5 vector immunization, and HAdV-5 seronegative patients all

converted to HAdV-5-seropositive after the first vaccination [231]. The second study used the Step Study HAdV-5 vaccine and found no evidence of CD4+ T cell activation after vaccination. It showed that HAdV-5-specific nAbs before vaccination did not correlate with HAdV-5-specific cellular immune responses. It also said that HAdV-5-specific T cell responses after vaccination were lower in HAdV-5 seropositive individuals compared to HAdV-5 seronegative ones [232].

Thus, although multiple theories have been considered and tested in attempts to understand the failure of the Step Study, the main one I will argue for in this dissertation is the influence of activated mucosal CD4+ T cells on HIV acquisition. In those subjects with previous exposure to adenovirus, whether it be HAdV-5 or another strain to which T cells are cross-reactive, immunization with a HAdV-5 vector will cause memory AdV-specific CD4+ T cells to become activated. Since AdV is a fecal-oral pathogen, activation of these memory cells will cause trafficking to the gastrointestinal mucosa, which is the primary site of initial HIV encounter and subsequent infection [233]. A greater frequency of CD4+ T cell targets that are activated and largely express the co-receptor CCR5 would hypothetically increase the risk of HIV acquisition, as shown in the model below **(Figure 1.4)**. Benlahrech et al. had previously proposed that increased rates of HIV infection in the Step Study are due to activation and expansion of HAdV-5-specific mucosal-homing memory CD4+ T cells. They found that memory CD4+ T cells from HAdV-5 seropositive subjects cultured for 5 days with HAdV-5 pulsed DCs preferentially expanded AdV-specific memory CD4+ T cells expressing $\alpha 4\beta 7$ integrins, CCR9 and CCR5, which indicated a mucosal homing phenotype [234]. Investigation of T cells within the mucosa, though, still provides the most reliable method of determining T cell phenotypes within this compartment, since previous studies have shown no increase in $\alpha 4\beta 7$ in peripheral

blood CD4+ T cells from vaccinees in the Step Study [231].

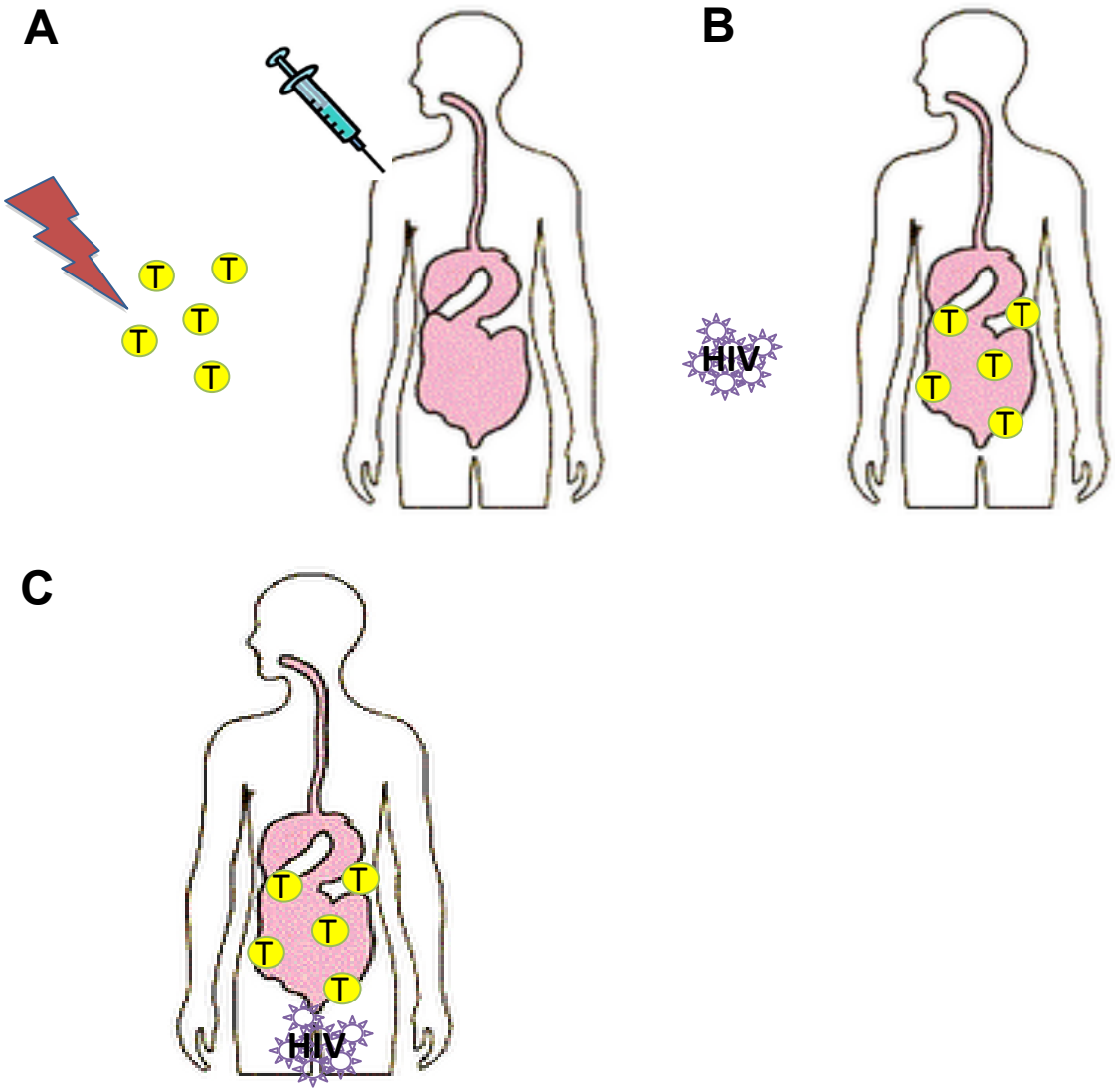

Figure 1.4

A. Natural exposure to Ad will create cross-reactive AdV-specific CD4+ T cells that are activated upon AdV-based vector vaccination.
B. These activated CD4+ T cells will traffic to the GI mucosa, since Ad is naturally found there due to fecal-oral transmission.
C. Those at high risk for HIV infection due to sexual practices may have come in contact with HIV and the larger concentration of preferential CD4+ activated T cell targets in the GI mucosa can increase HIV susceptibility.

Figure 1.4 Model for AdV-based vector vaccination induced HIV acquisition

Use of Alternative Adenoviral Serotype Vaccines

In light of the evidence against using HAdV-5 for an HIV vaccine, researchers have started leaning towards other adenovirus serotypes with lower seroprevalence. These are viewed as promising since the rare frequency would hypothetically lead to lower rates of pre-existing immunity, although cross-reactive T cell responses may still hinder use of rare serotypes. There are biological differences, too, between Ad serotypes [229], including *in vivo* tropism [235], use of different cellular receptors [236], interaction with dendritic cells [237,238], innate immune profiles [239], and adaptive immune phenotypes [240]. Some options for alternative serotypes are HAdV-11, HAdV-24, HAdV-26, HAdV-28 HAdV-35, which are all less common worldwide with lower titers of pre-existing nAbs compared to HAdV-5 (subgroup C). For HAdV-26 (subgroup D) and HAdV-35 (subgroup B), they are rare in the States and SE Asia, and are lower than HAdV-5 in Africa. Also, relative to HAdV-5, HAdV-26 and HAdV-35 induce significantly higher innate cytokine responses *in vivo*, but some studies indicate that they are less potent as vaccine vectors in mice and NHPs, with a lower protective efficacy in NHPs. Nonetheless, these rare Ad vectors have moved on to human trials [236,241-244].

A recent Phase 1 dose-escalation study using a HAdV-26-based vector HIV vaccine containing EnvA was demonstrated to elicit transgene- and vector-specific

responses that persisted for at least 1 year post-vaccination [245]. They saw a dose-dependent expansion of the magnitude, breadth, and epitopic diversity of Env-specific binding antibody responses. Further, they observed antibody-dependent cell-mediated phagocytosis, virus inhibition, and degranulation. The vaccine induced Env-specific cellular immune responses in both CD4+ and CD8+ memory T cells, albeit with a limited breadth [246]. Interestingly, baseline vector-specific T cell responses did not impair Env-specific immune responses in this study and other HAdV-26-based HIV vaccines [245].

Similarly to HAdV-26, HAdV-35 has a low seroprevalence, with only 2-16% of individuals exposed worldwide [247]. Preclinical studies using heterologous vectors and gene inserts with HAdV-35 and recombinant MVA have shown reasonable cellular immune responses [248]. A Phase 1 dose-escalation study using a 1) HAdV-35 with HIV-1 subtype A gag, RT, integrase, and nef and 2) HAdV-35 with EnvA were both well-tolerated and immunogenic in >90% of participants [249]. Despite these results, HAdV-5 and HAdV-35 share ~90% hexon sequence homology, which would predict that cross-reactive T cells may hinder protection from HIV [250]. Nonetheless, additional studies, including a HAdV-26/HAdV-35 prime-boost phase 1 study and others are ongoing.

Non-human primate Ad vectors are also being pursued for possible use in HIV vaccines, in particular, chimpanzee Ad (AdC) vectors. AdC vectors are attractive in that they have low or no seroprevalence in humans (0-4% in US, up to 20% in developing countries) [193,251,252], have low nAb titers in humans (<10% of those with AdC nAb have titers >200), can grow in human cell lines including HEK 293 and PerC6 cell lines, and exhibit similar immunological potency to HAdV-5 and HAdV-6 in mice and NHPs [253,254]. In NHP, vaccination with AdC vectors has displayed long-lived responses persisting over 5 years [253]. In pre-clinical studies, AdC-based vector vaccines were able to protect from Ebola virus [255], influenza [256], malaria [257], and so it appears that their development as HIV

vectors will remain to be seen. Recent studies of prime-boost immunizations with AdC serotypes 6 and 7 as vectors expressing SIV Gag/Tat have induced robust T cell responses, although without significant protection from SIV transmission. Notably, breakthrough infections in macaques that were AdC7 followed by AdC6 SIV vector-immunized has a significant, but transient, reduction in set-point viral load relative to unvaccinated controls [258]. These two AdC vectors appear to be moving forward in clinical trials.

Interplay of Mucosal Immunity and T Cell Phenotype During HIV Infection

The mucosal surface of the gastrointestinal tract (GI) is often the initial site of HIV exposure in men who have sex with men (MSM) and a major site of HIV replication. The anatomical and physiological characteristics of the GI tract, including its function as a barrier to the outside world and the source of most of the body's T cells [259], has led to a boom of research interest which is just starting to illuminate the unique immunology of this site. HIV infection leads to loss of CD4+ T cells systemically, but the most striking depletion is of the lamina propria CD4+ T cells during acute infection, which does not rebound effectively like the peripheral blood and over time can lead to AIDS-related pathologies [260-262].

It was the initial observation of enteropathy seen very quickly after HIV infection that led to an interest in what was occurring at the mucosal surface [263]. One possible cause for HIV enteropathy appears to be activation in the gut [264-266]. Proinflammatory mediators including beta-chemokines [267], IL-6, IL-10 and IFNg [268] are found at high levels in the lamina propria of HIV-positive individuals. Also, studies have shown that the degree of GI inflammation correlates with viral replication [260,262,268]. The principal hypothesis for the systemic immune activation seen with HIV stems from initial damage to the tight epithelial barrier of the GI mucosa, leading to bacterial translocation and their

microbial products stimulating the immune system. In turn, the increased levels of inflammation lead to increased CD4+ T cell activation and create more targets for HIV [115,269]. Immune activation is actually a better predictor of disease progression than either peripheral blood CD4+ T cell count or plasma viral load [270]. Notably, the majority of T cells in the GI mucosal tissues of healthy individuals express the HIV co-receptor CCR5, furthering their permissiveness to infection [271,272]. In fact, HIV infects CD4+ T cells in the gut mucosa 10-fold more frequently than CD4+ T cells in the peripheral blood [273,274]. To develop a successful HIV vaccine, it appears that we would need to prevent early CD4+ T cell loss within the gastrointestinal tract, preserve barrier integrity, and develop a better understanding of the complex mucosal microenvironment in general.

Polyfunctional HIV-specific CD8+ T cells, in both the peripheral blood and GI mucosa, are probably one aspect of a successful HIV vaccine [121,125,275]. When measuring the functional capacity of HIV-specific CD8+ T cells in the peripheral blood of HIV-positive individuals, it was found that those CD8+ T cells that were able to control viral replication were producing several effector cytokines (IFNg, MIP-1b, TNFa, IL-2, and/or CD107a). Since polyfunctionality was seen to be lost in the peripheral blood with progressive chronic infection, this led to studies investigating SIV/HIV-specific T cells in the mucosa [276]. Similarly to the blood, these cells in the mucosa were limited in functionality during chronic HIV infection. Several rhesus macaque studies have further illustrated vaccine-induced functional SIV-specific CD8+ T cells in the GI mucosa, the frequency of which negatively correlated with peak plasma viral load after SHIV challenge [277-279]. This leads one to believe in the benefit of an HIV vaccine that induces a polyfunctional CD8+ T cell response.

Although it is clear that some similarities exist between T cells in the peripheral blood and those in the intestinal mucosa, this thesis will emphasize that drawing

conclusions from the latter based on the former is not always warranted. One variable may be the magnitude of a T cell response, where several functions are commonly measured. Most often, IFNg expression is the gold standard assay, but IFNg alone will typically underestimate the magnitude of the response [280-283]. In this thesis, IFNg, TNFa, and IL-2 were combined to assess Ad-specific T cell responses. IFNg and TNFa are both pro-inflammatory cytokines with immunomodulatory and anti-viral effects [284]. IL-2 is a cytokine-signaling molecule required for the growth, proliferation, and differentiation of T cells to become effector cells and generate immunologic memory [285].

Another potential difference between mucosal and peripheral blood T cells may be the expression of activation markers. As mentioned, HIV preferentially infects activated CD4+ T cells, and we used the well-established activation markers HLA-DR, CD25, CD69, and Ki67 to understand compartmental differences. HLA-DR is an MHC Class II cell surface receptor with the primary purpose of presenting peptide antigens to T cells, which leads to T cell responses. In addition to this, the frequency of MHC-II+ CD4+ T cells increases after T cell activation, resulting in HLA-DR being regarded as a marker of activation [286]. CD25 is the alpha-chain of the IL-2 receptor, a trans-membrane protein present on activated T and B cells [287,288]. CD69 is the earliest inducible cell surface glycoprotein acquired during lymphoid activation, and is involved in lymphocyte proliferation [289]. Finally, Ki67 is a nuclear antigen that is a cellular marker of proliferation and is absent in resting cells [290].

Developing a vaccine-induced SIV acquisition model using a low-dose challenge

In order to determine whether vaccination with a species-specific AdV vector increases susceptibility in RMs to SIV infection through induction of CD4+ T cell activation in the rectal mucosa, we designed a model using a low-dose intra-rectal SIV

challenge. In this thesis, I show evidence for increased AdV-specific CD4+ memory T cells and activated CD4+ memory T cells in the rLPL after AdV vector vaccination. These changes in CD4+ memory T cells were evident as early as 2 weeks and gradually contracted by 16 weeks after AdV-vector vaccination. This led to the development of a RM model that aimed to determine if AdV-induced activation levels are sufficient to increase SIV susceptibility. To clarify this question, it was necessary to titrate SIV to a very low dose that would generally be insufficient to infect animals when administered intra-rectally. The titration is described in Chapter 3, which used SIVmac251 at a beginning dose of 1:5000 or 4$TCID_{50}$ and was tested in 15 macaques. Prior to this, titration data on this stock of SIVmac251 only used a few macaques, which produced fairly inconclusive results. Using our titration results, the AdV vector vaccine study set-up would demonstrate activation-induced susceptibility to SIV if more vaccinated animals became infected at the same dose as placebo controls. Our biggest hurdle in the design of this study was to synchronize the window of increased CD4+ T cell activation we expected to see in the AdV vector vaccine group with the intra-rectal challenges. This feat was complicated, as the rectal mucosa required a few weeks to heal properly after biopsies, prior to initiating intra-rectal SIV challenges. Therefore, we were only able to obtain a single week 2 post-vaccination rectal biopsy time point, in order to begin SIV challenges by week 5. We recognize that immunological dynamics might be slightly different in macaques obtained from different sources and housed in different settings, thus we risked missing the ability to collect rLPL samples during the CD4+ memory T cell activation in order to begin SIV challenges within the desired timeframe. Unfortunately, at n=10/group, our statistical power was very limited, and a similarly designed study with larger macaque numbers may be able to produce a statistically significant outcome, rather than just the trend we were able to show.

Thesis goals

The failure of the Step Study, and the consequent halting of Phambili and HVTN-505, demonstrated that HAdV-5 vectors were not attractive candidates for HIV vaccines. Further, the presence of pre-existing AdV-specific immunity resulted in an increased risk of HIV acquisition, and a myriad of studies have sought to investigate the mechanism for this risk. In this thesis, I advocate for the use of the rhesus macaque as a model that will optimally become increasingly refined in future studies. Unlike previous studies that employed a human AdV vector in macaques to simulate vaccination with AdV vectors in humans, we argue that using a macaque-specific AdV vectored vaccine (based on SAdV-7) is a more appropriate model. Also, obtaining samples from the peripheral blood alongside the rectal lamina propria offers insight into the unique immunology of the gastrointestinal mucosa. This method can clarify previously disputed assumptions based on PBMC data alone. The rhesus macaque model allows us to characterize the phenotype and functionality of CD4+ T cells in various compartments with frequent time points, which leads to a greater probability of detecting rapidly changing immunological dynamics. We hypothesized that AdV-based vector vaccination would result in increased AdV-specific CD4+ memory T cells and activated CD4+ memory T cells in the rectal mucosa, which we successfully demonstrated together with a lack of significant changes

in the peripheral blood. Further, we used these results to design a SAdV-7 vectored vaccination followed by a low-dose intra-rectal SIV challenge study to determine whether AdV vectored vaccination leads to activation-induced susceptibility to SIV. We were able to show a trend towards SIV acquisition in the vaccine group, while also initiating the first step towards developing a vaccine-induced SIV acquisition model using a low-dose challenge. These studies have established the groundwork for use of macaques as a valuable tool to further enhance pre-clinical understanding of HIV vaccine dynamics.

CHAPTER 2

Increased Mucosal CD4+ T cell Activation Following Vaccination With an Adenoviral Vector in Rhesus Macaques

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Abstract

The possibility that vaccination with adenoviral (AdV) vectors increased mucosal T cell activation remains a central hypothesis to explain the potential enhancement of HIV acquisition within the Step trial. Modeling this within rhesus macaques is complicated because human adenoviruses, including adenovirus type 5 (HAdV-5), are not endogenous to macaques. Here, we tested whether vaccination with a rhesus macaque-derived adenoviral vector (simian adenovirus 7, SAdV-7) enhances mucosal T cell activation within rhesus macaques. Following intramuscular SAdV-7 vaccination, we observed a pronounced increase in SAdV-7-specific CD4+ T cell responses in the peripheral blood, and more dramatically, in the rectal mucosa tissue. Vaccination also induced a significant increase in the frequency of activated memory CD4+ T cells in SAdV-7 and HAdV-5-vaccinated animals in the rectal mucosa, but not in the peripheral

blood. These fluctuations within the rectal mucosa were also associated with a pronounced decrease in the relative frequency of naïve resting CD4+ T cells. Together these results indicate that peripheral vaccination with an AdV vector can increase the activation of mucosal CD4+ T cells, potentially providing an experimental model to further evaluate the role of host-vector interactions on increased HIV acquisition after AdV vector vaccination.

Introduction

The pursuit of an efficacious HIV-1 vaccine is an ongoing effort to minimize or halt the burden of this disease worldwide. Numerous T cell directed HIV vaccines have aimed to target immune responses that would either decrease viral load if infected or restrict HIV replication entirely [291,292]. Notably, Merck's Step Study was a large, phase IIb placebo-controlled clinical trial testing a replication-defective recombinant human adenovirus serotype 5 (HAdV-5) vector to deliver HIV-1 antigens *gag*, *pol*, and *nef*, with the intention of preventing HIV-1 infection by generating T cell immunity. This candidate HIV vaccine trial was halted in September 2007 due to statistical futility and the finding of a trend towards increased rates of HIV acquisition among vaccinated men with anti-HAdV-5 seropositivity [221]. Another phase IIb clinical trial using the same adenovirus vector is referred to as the Phambili trial, which yielded the same results of increased acquisition of HIV infection in some vaccinees [293]. The underlying mechanism for these apparent increases in acquisition remains to be defined.

During the Step trial, only peripheral blood mononuclear cells (PBMC) were collected, and many follow-up studies seeking to elucidate the Step outcome have investigated this compartment exclusively without the use of mucosal biopsy specimens [221,222,231,234]**.** Importantly, the gut mucosa is both the potential site of HIV transmission in

infected Step participants [231,234] as well as a site of AdV persistence [209]. Expansion of the enteric virome, including mucosal adenovirus infection, has even been associated with pathogenic SIV infection [294]. Compartmental differences in the immunological milieu between the blood and mucosa are well documented, and alterations in phenotype, functionality, activation state, or distribution of CD4+ T cells within these compartments may offer clues to the Step outcome. In the peripheral blood of HAdV-5 vaccine recipients, we found no difference in the expression of Ki67, a proliferation marker, or $\alpha_4\beta_7$, a gut homing marker [231], in agreement with others who saw no increase in cellular activation [222,232]. Similarly, there was no increase in $\alpha_4\beta_7$ or CCR5 expression on peripheral blood CD4+ T cells in vaccinees during the Step Study [222,231]; however *in vitro* studies suggest that HAdV-5 can induce $\alpha_4\beta_7$ and CCR5 expression on expanded AdV-specific memory CD4+ T cells [234,295], raising the possibility that HAdV-5 could have a specific effect in the mucosa. In these studies immune responses to adenovirus were evaluated using HAdV-5. It is important to note that there are currently 65 serologically distinct human adenoviruses and responses to adenovirus may be influenced by the type of adenovirus used in the study. Moreover similar studies in monkeys used human adenovirus to reproduce the Step trial in monkeys [188,215,219]. It is important to note that also adenovirus can be found in macaques and that these adenoviruses are different from those isolated from human [208] which suggest that using a vector based on an endogenous rhesus macaque AdV may more accurately model host-vector interactions compared to an endogenous human adenoviral vector, such as HAdV-5. Vaccination with human AdV vector in the macaque model may produce an incomplete understanding of potential outcomes, as was seen with studies leading up to Step [188,215,219].

To address this, we examined whether vaccination of rhesus macaques with a simian adenovirus SAdV-7-based vaccine vector differentially influences peripheral and mucosal CD4+ T cell responses and cellular activation compared to a HAdV-5 based vector. We find that both SAdV-7 and HAdV-5 vector vaccination can increase AdV-specific CD4+ T cell cytokine responses in blood and, more dramatically, rectal mucosa. Importantly, however, both vectors heightened the cellular activation profile of total and AdV-specific CD4+ T cells specifically within the rectal mucosa. This increase in rectal mucosa CD4+ T cell activation was most pronounced after the vaccine prime, and in SAdV-7 vaccinated animals was accompanied by a prolonged decrease in resident naïve CD4+ T cells within the rectal mucosa. These data highlight the importance of assessing tissue-specific effects induced by vaccine platforms, and provide a potential mechanism by which adenovirus vaccine vectors might influence susceptibility to HIV as was observed in the Step study [221].

Materials and Methods

Adenovirus vectors

Wild-type SAdV-7 was purchased from the ATCC (VR-201, originally isolated from rhesus monkey kidney cells). HAdV-5 was generated from a molecular clone (Clontech) by Vector Core (University of Pennsylvania). SAdV-7-based vectors were constructed as previously described [296]. Briefly, the viral genome was molecularly cloned and expression cassettes (SARS coronavirus spike protein driven by a chicken beta-actin promoter, or beta-galactosidase driven by a CMV promoter) were inserted in place of an E1-deletion to generate replication incompetent vectors SAdV-7 SARS spike and SAdV-7LacZ respectively. These, as well as the HAdV-5 vectors used in this study were propagated on HEK 293 cells and purified on CsCl gradients.

Animals

17 rhesus macaques of Indian origin and captive bred were purchased from Covance Research Products (Alice, TX) and enrolled in this study. All animals were treated and cared for at the Nonhuman Primate Research Program (NPRP) facility of the Gene Therapy Program of the University of Pennsylvania (Philadelphia, PA) during the study. The study was performed according to a protocol approved by the Environmental Health and Radiation Safety Office, the Institutional Biosafety Committee, and the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania. All macaques were intramuscularly (IM) immunized 3 times with SAdV-7 or HAdV-5 at a dose of $1x10^{11}$ particles/injection (see **Figure 2.2** for the immunization schedule).

Endoscopic sampling of macaque rectum and isolation of Lamina Propria Lymphocytes (LPLs) All nonhuman primates (NHP) were fasted the evening before the procedure, with free access to water at all times. NHPs were sedated and biopsies were obtained utilizing an alligator-jaw style endoscopic biopsy pinch held free hand. 20 biopsies were taken spaced far enough apart so as not to weaken the rectal wall. Biopsies were placed in RPMI medium and LPLs isolated using collagenase type II as previously described [297].

Blood collection and isolation of peripheral blood mononuclear cells

PBMCs were isolated from whole blood collected in heparin-containing Vacutainer tubes after Ficoll (Amersham Bioscience) density-gradient centrifugation at 1000g for 25 minutes. Cells were collected from the interphase and washed with PBS. PBMCs were incubated with ACK lysing buffer to lyse red blood cells, washed and resuspended in complete RPMI medium (Mediatech) containing 10% FBS, 2 mM glutamine, 10 mM

HEPES, 50 ug/ml Gentamycin sulfate and Pen/strep.

AdV neutralizing antibody assay

Anti-SAdV-7 or anti-HAdV-5 neutralizing antibody titers in serum samples were measured by assessing the ability of serum to inhibit transduction of the corresponding reporter vector, SAdV-7LacZ or HAdV-5LacZ, respectively, into HEK 293 cells. The reporter vector was incubated with two-fold serial dilutions (initial dilution, 1/5) of heat-inactivated sera for 1h at 37 °C. Serum samples were diluted with naïve mouse serum (Sigma Aldrich) so that the final serum concentration at all dilutions was 5%. Subsequently, the serum–vector mixture was added onto HEK 293 cells in 96-well flat-bottomed plates (at an m.o.i. of 10 virus particles per cell) and incubated for 18–22 h. Cells were then washed twice in PBS and lysed, and the lysate was developed with the mammalian β-galactosidase assay kit for bioluminescence, in accordance with the manufacturers' protocol (Applied Biosystems), and measured in a microplate luminometer (Clarity [BioTek]). The NAb titer was reported as the highest serum dilution that inhibited AdV.CMV.LacZ transduction (β-gal expression) by 50%, compared with the mouse serum control (Sigma S3509).

Antibody reagents

Antibodies used for surface staining included: anti-CD14 Qdot 655, anti-CD20 Qdot 655, anti-CD8 PE Texas Red (Invitrogen; Carlsbad, California), anti-CD28 PE-Cy7 (eBioscience; San Diego, California), anti-CD4+ Pac Blue (Biolegend; San Diego, California), anti-CD25 APC-Cy7, anti-CD95 PE-Cy5, anti-HLA-DR PerCP-Cy5.5 (BD Pharmingen; San Diego, California). Antibodies used for intracellular staining included: anti-interleukin-2 (IL-2) Alexa700 (Biolegend), anti-interferon-gamma (IFNg) Alexa 700

(Invitrogen), anti-tumor necrosis factor-alpha (TNFa) Alexa 700, anti-CD69 APC, anti-Ki67 FITC (BD Pharmingen).

Cell processing and stimulation

Rectal biopsies were processed within 6 hours of being collected. Rhesus macaque PBMC were cryopreserved in fetal bovine serum (FBS; ICS Hyclone, Logan, Utah) containing 10% dimethyl sulfoxide (DMSO; Fisher Scientific, Pittsburgh, Pennsylvania) and stored in liquid nitrogen until use. After washing fresh LPL cells or thawed PBMCs once in RPMI (Mediatech Inc; Manassas, Virginia), both PBMC and LPL cells were resuspended in complete medium [(RPMI supplemented with 10% FBS, 1% L-glutamine (Mediatech Inc) and 1% penicillin-streptomycin (Lonza; Walkersville, Maryland), sterile filtered] at a concentration of 1-2x10^6 cells/mL medium in FACS tubes. Cells were split into three stimulation conditions, at a volume of 1 mL each, with either: no stimulation, 1 ml Staphylococcus Enterotoxin B (SEB) at a concentration of 1 mg/mL (Sigma-Aldrich; St. Louis, Missouri) as a positive control, or 1x10^{10} particles/mL of the SAdV-7 vector. Cells were stimulated overnight at 37°C, 5% CO_2.

FACS Staining Assay

Stimulation tubes were removed from the incubator in the early morning to add monensin (0.7 µg/ml final concentration; BD Biosciences) and brefeldin A (1 µg/ml final concentration; Sigma-Aldrich; St. Louis, Missouri) and incubated for an additional 6 hours. Cells were then washed once with PBS and stained for viability with Aqua amine-reactive dye **(**Invitrogen**)** for 10 minutes in the dark at room temperature. A mixture of antibodies used for staining surface markers were added to the cells and kept at room

temperature for 20 minutes. Cells were washed with PBS containing 1% bovine serum albumin (BSA, Fisher Scientific) and 0.1% sodium azide (Fisher Scientific) and permeabilized for an additional 20 minutes at room temperature using the Cytofix/Cytoperm kit (BD Pharmingen). Next, cells were washed in Perm/Wash buffer (BD Pharmingen). A mixture of antibodies used for staining intracellular markers was added to the cells and incubated in the dark for one hour at room temperature. Cells were again washed with Perm/Wash buffer and fixed with PBS containing 1% paraformaldehyde (Sigma-Aldrich). Fixed cells were stored in the dark at 4°C until being collected for flow cytometric analysis. Biopsy samples from four animals at the baseline time point were stained with a different fluorophore panel and were not included in the analysis. These samples were identified in results section as “lost”.

Flow Cytometric Analysis

For each sample, between $3x10^5$-$1x10^6$ total events were acquired on a modified flow cytometer (LSRII; BD Immunocytometry Systems; San Jose, CA) equipped to detect up to 18 fluorescent parameters. Antibody capture beads (BD Biosciences) were used to prepare individual compensation tubes for each antibody used in the experiment. Data analysis was performed using FlowJo version 9.0.1 (TreeStar, Ashland, Oregon). Percent expression is shown after background subtraction, where values are calculated as the difference between cells that were stimulated with SAdV-7 vector overnight minus those that were left unstimulated. AdV-specific percentages are reported as the population of CD4+ memory T cells which express IL-2, IFNγ, and/or TNFα within each compartment. While we did not include a placebo-only group, AdV-specific T cell responses prior to immunization at baseline should reflect this appropriately. To

separate naïve cells from memory, effector memory, and effector T cells, we stained with fluorochrome-conjugated antibodies for CD28 and CD95, where CD28+ CD95- CD4+ T cells indicated the naïve subset, whereas all other cells were grouped as memory. Naïve CD4+ T cells were gated using FlowJo for each macaque at all time points in both compartments. Although a CD3 antibody was not included in the panel, a CD14/CD20 exclusion gate as well as careful gating including CD4+ bright cells, while excluding CD8- cells, provided assurance in the gating strategy.

Figures

Prism software, version 5.0 (Graphpad; La Jolla, California) was used to create the figures.

Statistical Analysis

Activation levels were summarized overall and at each time point. Graphical methods were employed to assess distributional assumptions of the data. SAdV-7 and HAdV-5 group levels were modeled over time using GEEs (generalized estimating equations), which are a standard statistical approach for testing hypotheses involving repeated measures data [298]. They have the advantage over traditional repeated measures ANOVA of being able to handle randomly missing observations from animals. GEEs adjust for the fact one has data from an animal over time by linking that data. They are similar to repeated measures ANOVA models, in that they model the inherent correlation from measurements made on the same animal at different times, however they can handle data that is missing completely at random. GEE models are flexible allowing overall group comparisons, comparisons at each time point, as well as within group comparisons to their respective baseline levels. The GEE models used were of the form:

f(outcome) = constant + group + time + group * time. Time was included as a categorical variable (rather than continuous) to allow for potentially non-linear changes from one time point to another. The transformation (f) used for the outcome was based on whether the outcome of interest was normally distributed, Poisson, or binomial (standard transformation for each type were used). Once the model was fitted (and assessed for goodness-of-fit), the average differences between groups at each time point, and also the differences within a group from baseline, were calculated. The p-values for these pair-wise comparisons were calculated using the Holm-Bonferroni method in order to preserve the overall Type I error rate of 0.05. This was to make sure we did not incorrectly find a comparison significant by chance just because of the number of comparisons being performed. To investigate naïve CD4+ T cells over the time course, four analyses were completed. Namely, the LPL outcome was evaluated separately for the two groups of macaques determined by the type of vaccine administered (SAdV-7 and HAdV-5) and a similar series of analyses were conducted for PBMC in the two vaccine groups. For each analysis, GEE models were fit to accommodate the repeated measures of macaques over time. Specifically, linear GEE models were fit for each continuous outcome (LPL and PBMC) adjusting for a categorical 'time' effect and assuming an exchangeable correlation structure. Model-based differences of predicted outcomes based on each respective fitted model are presented, by vaccine type. Finally, raw p-values from the pair-wise differences comparing outcomes to each subsequent follow-up timepoint as well as comparing week 16 and week 33 have been adjusted using the Holm-Bonferroni method to control for multiple comparisons.

Results

Simian adenovirus type 7 immunity and vaccination

Adenoviral vectors are known to elicit potent T cell responses to the encoded transgenes and have the potential to be an important component a defense strategy against infectious agents such as HIV where T cell-based immunity can have an important part to play in either preventing infection or in containing its replication. However prior exposure to adenoviruses in vaccinees can result in a memory response to the payload adenoviral capsid antigens [207]. This response is likely to be strongly influenced by the epitopes that are shared between capsid antigens present in the adenoviral vaccine and the epitopes in adenoviruses that have caused prior infections in the host. Furthermore, it is likely that both humans and macaques are chronically infected by adenoviruses [203,206], resulting in ongoing restimulation of memory responses. The use of an adenovirus homologous to the species used in the study may be important to restimulate this response and properly model the immune parameters consequent on adenoviral vaccination, including the magnitude and the homing of the response. A phylogenetic analysis of monkey adenovirus hexon protein sequences (**Figure 2.1**) indicates that they are divergent from all human adenoviral species. Other macaque adenovirus proteins also form clusters distinct from human adenoviruses (data not shown). Thus it is plausible that vaccination of monkeys by a macaque-derived adenovirus such as SAdV-7 would result in anamnestic responses that may similarly replicate what might be expected in human subjects vaccinated with a HAdV-5 vector.

We vaccinated 12 rhesus macaques three times intramuscularly with $1x10^{11}$ viral particles (VP) of E1-deleted replication defective SAdV-7-derived vector expressing SARS spike (see Materials and Methods for description of construct). As a control, we vaccinated 5 additional rhesus macaques with an E1-delected replication defective HAdV-5-derived vector containing the same transgene and promoter. Ten sample collection time points were interspersed around the vaccinations (**Figure 2.2**). Prior to

vaccination, baseline levels of SAdV-7 neutralizing antibody (NAb) titers ranged from undetectable (<5) to 640, with 11/17 macaques displaying a SAdV-7 titer of ≤10 (**Table 2.1**). All macaques had undetectable levels of HAdV-5 NAbs at baseline. Vaccination with either SAdV-7 or HAdV-5 vector occurred at weeks 0, 17, and 31, while rectal biopsies and peripheral blood were collected at -2 (baseline), 2, 5, 9, 16, 19, 21, 25, 29, and 33 weeks after the first immunization. Baseline rectal biopsies from four rhesus macaques (04C010, 05C069, 05D007, and 05D079) were lost during analysis. Additionally, the LPL sample from week 21 (04C058) and peripheral blood sample from week -2 (baseline) (04C059) were unable to be processed. Cells were stained for multiple markers, with a particular focus on memory markers (CD28, CD95), activation markers (HLA-DR, CD25, CD69, Ki67) and cytokine functionality (IL-2, IFNγ, TNFα) for flow cytometric analysis (representative gating shown in **Figure 2.3**).

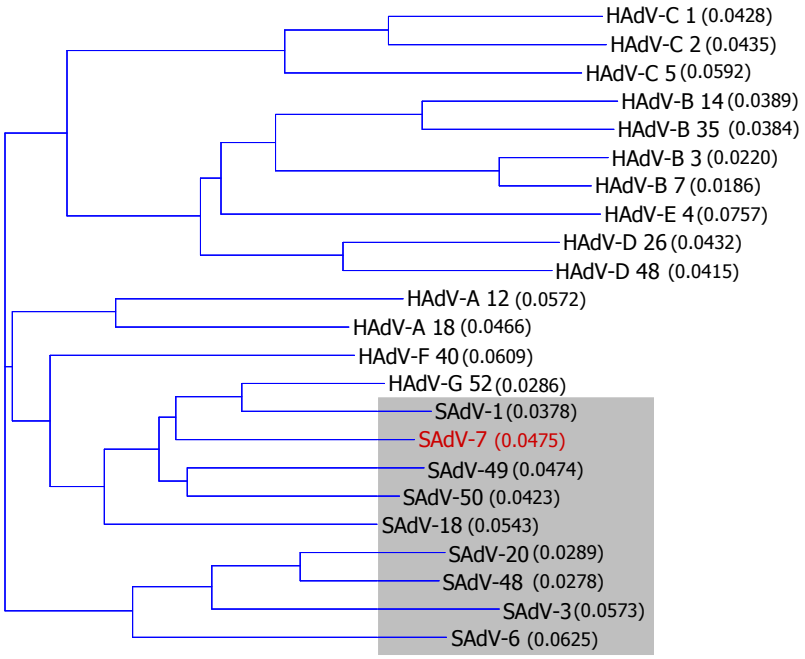

Figure 2.1. The phylogenetic relationships between hexon proteins from human and monkey adenoviruses are shown. (The amino-acid sequences were aligned using ClustalW version 2.0.3, and refined using Gblocks version 0.91b. The alignment was used to construct the phylogenetic tree using PhyML version 3.0 aLRT and rendered by using Treedyn 198.3. Branch support values are indicated as percentages. The scale bar indicates the number of substitutions per site). Representatives of human adenoviruses from species A through G (serotypes in parentheses, and including HAdV-5, bold font) and several monkey adenoviruses are shown. Monkey adenoviruses (including SAdV-7, which was used to construct vectors used in this study, highlighted in red) are seen to belong to a phylogenetically distinct group (grey box).

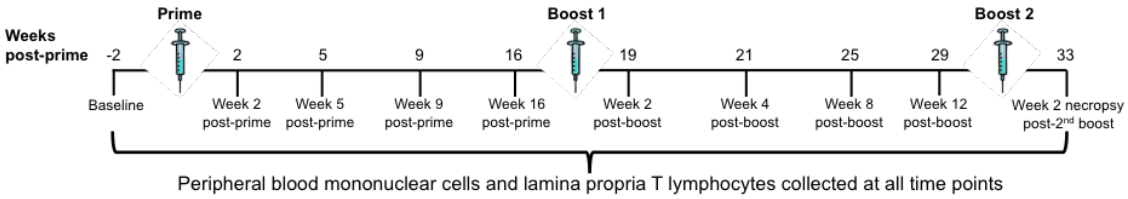

Figure 2.2. Vaccination and sample collection timeline. Macaque trial design with 10 tissue (PBMC and rLPL) collection time points, and either SAdV-7- or HAdV-5-vector vaccinations interspersed at weeks 0, 17 and 31.

Table 2.1. AdV-specific humoral immune response and transgene-specific cellular immune response

Macaque	Sex/DOB	Vaccine	Baseline SAdV-7 neutralizing Ab titer[a]	SARS Spike ELISpot: Baseline	SARS Spike ELISpot: Week 2
03C110	(M) 9/29/03	SAdV7	<5	+	+
04C068	(F) 6/9/04	SAdV7	<5	–	+
RQ6655	(M) 2/19/01	SAdV7	<5	–	+
04C010	(F) 4/14/04	SAdV7	5	–	+
04C058	(F) 5/26/04	SAdV7	5	–	+
05C041	(M) 5/23/05	SAdV7	5	–	+
04C066	(F) 6/8/04	SAdV7	10	–	+
05D007	(M) 3/7/05	SAdV7	10	–	+
RQ6529	(F) 3/20/00	SAdV7	20	–	+
03D330	(M) 8/4/03	SAdV7	40	–	+
04C078	(F) 6/15/04	SAdV7	160	–	–
05C069	(M) 6/14/05	SAdV7	640	–	–
02C015	(M) 4/17/02	HAdV5	<5	–	+
21606	(M) 5/22/00	HAdV5	<5	–	+
04C059	(F) 5/28/04	HAdV5	10	–	+
02C061	(M) 9/25/02	HAdV5	20	–	+
05D079	(M) 5/12/05	HAdV5	20	–	+

[a] Reciprocal of serum dilution

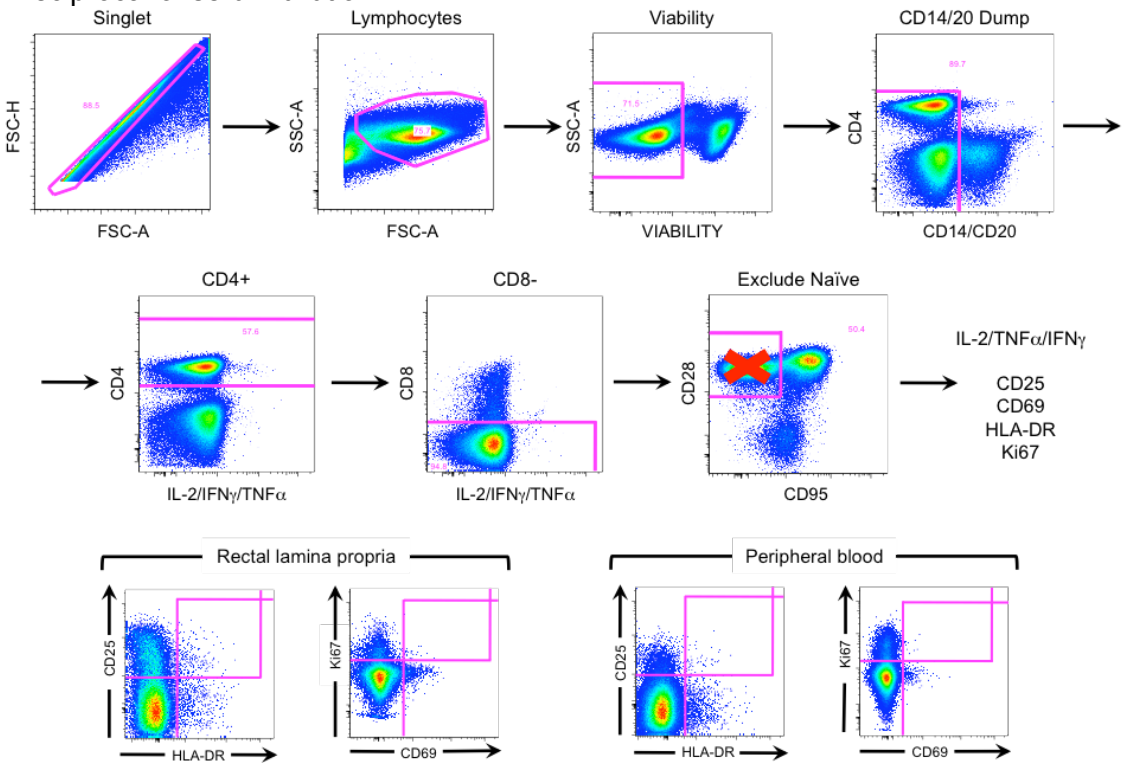

Figure 2.3. Gating strategy for identification of CD4+ memory T cells. First, to ensure only live single cells were collected from either PBMCs or rectal lamina propria lymphocytes (rLPL), FSC-H vs FSC-A and SSC-A vs. FSC-A plots were used to exclude doublets and focus on singlet lymphocytes. Dead cells were excluded by gating on the cells negative for the viability marker Aqua Blue. Monocytes and B-cells were excluded via the CD14/CD20 dump gate. To select for CD4+ T cells specifically, we included the CD4+ and CD8- T cells. To determine memory phenotype, we used CD28 vs. CD95, and excluded naïve cells (CD28+/CD95-) from analysis. From here, activation markers (CD25, CD69, HLA-DR, and Ki67) were gated as shown on the bottom plots on unstimulated memory CD4+ T cells, and cytokines (IL-2, TNFα, IFNγ) were analyzed. All gating is shown for rectal lamina propria lymphocytes, except where indicated otherwise.

Adenovirus-specific CD4+ T cells expand post-vaccination

We first examined AdV-specific CD4+ T cells at baseline and whether AdV vector

vaccination influenced these frequencies. We assessed cytokine expression on memory CD4+ T cells after stimulation with either SEB or SAdV-7 (or unstimulated) in the rectal lamina propria (rLPL) and peripheral blood (PBMCs) in vaccinated macaques **(Figure 2.4)**. Cells from HAdV-5-vaccinated animals were stimulated with SAdV-7 to determine whether vaccination boosted cross-reactive responses against the heterologous SAdV-7 vector. SAdV-7-specific CD4+ T cell responses were determined by the production of IFNγ, TNFα, and/or IL-2 at each collection time as shown in **Figure 2.4***.* Several animals had detectable AdV-specific CD4+ T cells (response > 0.05% was considered positive) in both rectal lamina propria and peripheral blood at baseline, prior to AdV vector vaccination [4/9 SAdV-7 (3 lost to testing) rLPL, 4/12 SAdV-7 PBMC; 3/4 HAdV-5 rLPL, 1/4 PBMC (1 lost to testing)]. Only one SAdV-7 immunized animal (04C068) had detectable AdV-specific CD4+ T cells within both tissues, and one HAdV-5 immunized animal (02C061) had AdV-specific CD4+ T cells within both tissues. We found no statistically significant correlation between baseline AdV-specific CD4+ T cell responses and pre-existing baseline SAdV-7-specific NAbs in PBMCs or rLPLs (data not shown). While pre-existing AdV-specific CD4+ T cells were present in both compartments, the magnitude of responses was consistently higher in the rectal mucosa, with 4 macaques exhibiting >1% expression in the rLPL (ranging from 0-1.26% SAdV-7, 0-1.5% HAdV-5), while 0.139% was the highest cytokine response in the PBMC (ranging from 0-0.095% SAdV-7, 0-.139% HAdV-5).

After both the first and second immunization, SAdV-7-specific CD4+ T cell cytokine responses increased substantially in many animals and reached statistical significance as a group relative to baseline, within the rectal mucosa at week 5 post-prime ($P < 0.01$) **(Figure 2.4)**. We found a general association between the peak of the

AdV-specific CD4+ T cell response and pre-existing AdV NAb titers in the rLPL of SAdV-7-vaccinated macaques, with the three highest responses in macaques with a NAb titer $\leq$ 10. No association between AdV-specific CD4+ T cell response and pre-existing AdV NAb was found in the blood or in HAdV-5-vaccinated macaques (data not shown). The highest SAdV-7-specific cytokine responses were seen in the rLPL of SAdV-7-vaccinated macaques, peaking at 14.42% (#04C058) and 16.84% (#04C066) at week 5 post-prime, and 15.62% (#05D007) at week 9 post-prime (**Figure 2.4)**. An increase in SAdV-7-crossreactive CD4+ T cells in the rLPL of HAdV-5-vaccinated macaques was also noted in 3 animals with a >3% AdV-specific CD4+ T cell response at week 5 post-prime. While increases in AdV-specific responses were also observed in PBMC, they were largely under 1.5%, with singular exceptions in SAdV-7-vaccinated macaques at isolated time points. In both SAdV-7- and HAdV-5-vaccinated macaques, rLPL that were stimulated with SAdV7 vector were predominantly central memory CD4+ T cells ($CD28^{+}CD95^{+}$), while PBMCs were a mix of naïve ($CD28^{+}$CD95-) and central memory phenotype (data not shown). These results indicate that pre-existing AdV-specific T cell responses exist in both the peripheral blood and rectal mucosa, and that AdV vector vaccination can strongly increase AdV-specific T cell frequencies in the rectal mucosa, with peak mucosal responses at least 3-fold on average above that seen in peripheral blood.

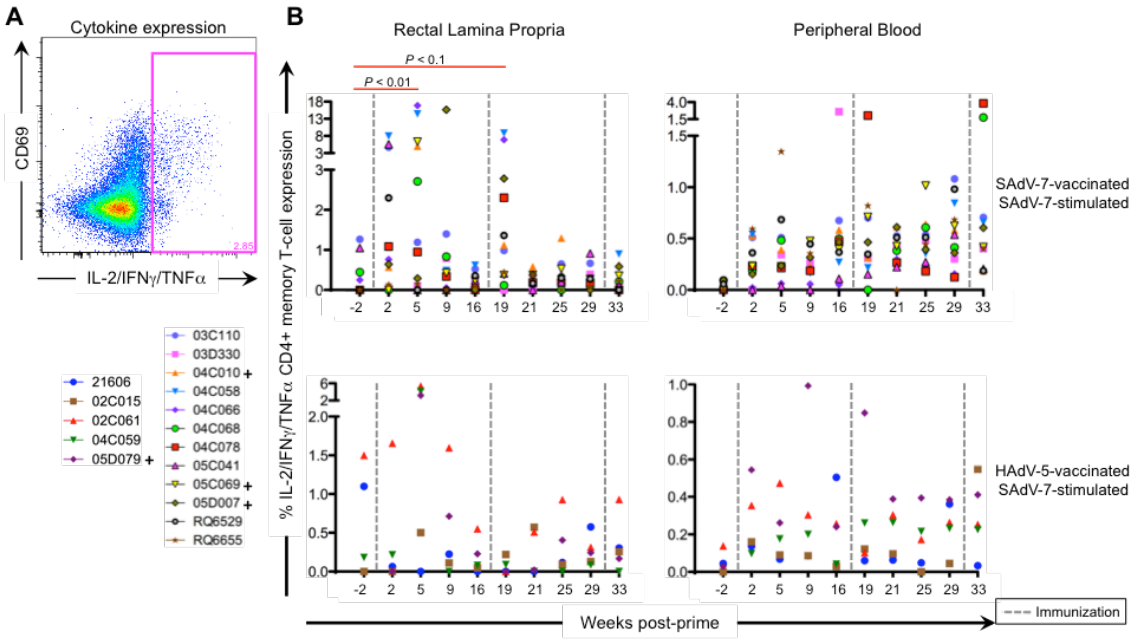

Figure 2.4. AdV vector vaccination increases memory AdV-specific CD4+ T cell percentage. (A) A representative memory CD4+ T cell IL-2/IFNγ/TNFα response, after stimulation with $1x10^{10}$ pt/ml of whole SAdV-7 vector. The number in the lower right of the plot represents the proportion of cells that express IL-2/IFNγ/TNFα. (B) Percent CD4+ memory T cell IL-2/IFNγ/TNFα expression in response to whole SAdV-7 vector stimulation (SAdV-7-stimulated) overnight after background (NS) subtraction. IL-2, IFNγ, and TNFα production were measured on the same fluorochrome. Peripheral blood and rectal lamina propria T cells from 12 SAdV-7-vaccinated and 5 HAdV-5-vaccinated rhesus macaques (symbols for each macaque are listed in the legend) were collected at ten time points including baseline (week -2) interspersed with three AdV vector immunizations (vertical dotted lines). Significance at any time point was calculated as a percent increase relative to baseline, and was determined as described in Material and Methods. Statistically significant increases relative to baseline are indicated above the red bars, (week 19 approached statistical significance). (+) Animals with no rectal biopsy data at baseline time point.

Adenovirus vector vaccination increases the frequency of activated memory CD4+ T cells in the rectal mucosa

To determine whether SAdV-7 vaccination affected the activation state of total CD4+ T cells in the rectal lamina propria or in blood, we assessed the expression of four different activation markers simultaneously (CD25, CD69, Ki67 and HLA-DR) on unstimulated CD4+ T cells from these sites **(Figure 2.3, bottom panels)**. At baseline, rectal lamina propria lymphocytes from SAdV-7-vaccinated macaques had a slightly higher percentage of activated memory CD4+ T cells (range 16.8-29.5% and 11.7-19.6% in SAdV-7- and HAdV-5-vaccinated macaques, respectively) compared to PBMCs (range 7.1-20.4% and 9.8-14% in SAdV-7- and HAdV-5-vaccinated macaques, respectively; **Figure 2.5**). We found no association between baseline SAdV-7 NAb titers and the frequency of activated memory CD4+ T cells over the immunization period (data not shown). Similarly to the rLPL of unstimulated cells of SAdV-7-vaccinated macaques at baseline, the rLPL of SAdV-7 vector stimulated cells **(Figure 2.6)** from SAdV-7-vaccinated macaques had the highest expression of activation markers on memory CD4+ T cells, if compared to the blood or HAdV-5-vaccinated macaques. Stimulating *ex vivo* with whole SAdV-7 vector overnight noticeably increased the percent of activated cells at baseline, relative to unstimulated cells.

Following vaccination, rectal LPL CD4+ T cell activation in unstimulated cells increased in several macaques at week 2 **(Figure 2.5)**. In two SAdV-7-vaccinated macaques (#04C010, #04C066) these levels reached nearly 50%, and SAdV-7 vaccinated macaques averaging a 1.42-fold increase from baseline at week 2 ($P < 0.01$). These macaques maintained heightened activation for several weeks, peaking up to a 1.5-fold group average increase from baseline at week 16. Interestingly, we also observed an increase in mucosal activation levels following prime and boost in some

HAdV-5-vaccinated animals, with one macaque (#05D079) reaching nearly 40% total activated CD4+ memory T cells at week 2. At week 9, HAdV-5-vaccinated macaques had a 1.7-fold average increase from baseline, with average values ranging between 1.35- to 1.7-fold throughout the duration of this study. We did not find a significant increase of PBMC activation levels in unstimulated cells of SAdV-7- or HAdV-5-vaccinated macaques at any study time point (**Figure 2.5**).

For the SAdV-7-stimulated condition **(Figure 2.6)** at week 2, over 40% of the rLPL consisted of activated CD4+ memory T cells in 7/12 SAdV-7-vaccinated macaques and 2/5 HAdV-5-vaccinated macaques. Changes in mucosal SAdV-7-specific activation averaged consistently above 1.49-fold (SAdV-7-vaccinated) and 1.18-fold (HAdV-5-vaccinated) during weeks 2-16. Thus, after the SAdV-7 prime, a marked increase in activation levels of both resting and SAdV-7-specific memory CD4+ T cells was noted in the rLPL, that was maintained at a heightened level relative to baseline for several weeks. In contrast a significant reduction in the number of activated CD4+ memory T cells was observed in PBMCs **(Figure 2.6)**. These data indicate that immunization with an AdV vector, whether of macaque or human origin, can cause heightened activation marker expression in rectal lamina propria CD4+ T cells not observed in the peripheral blood.

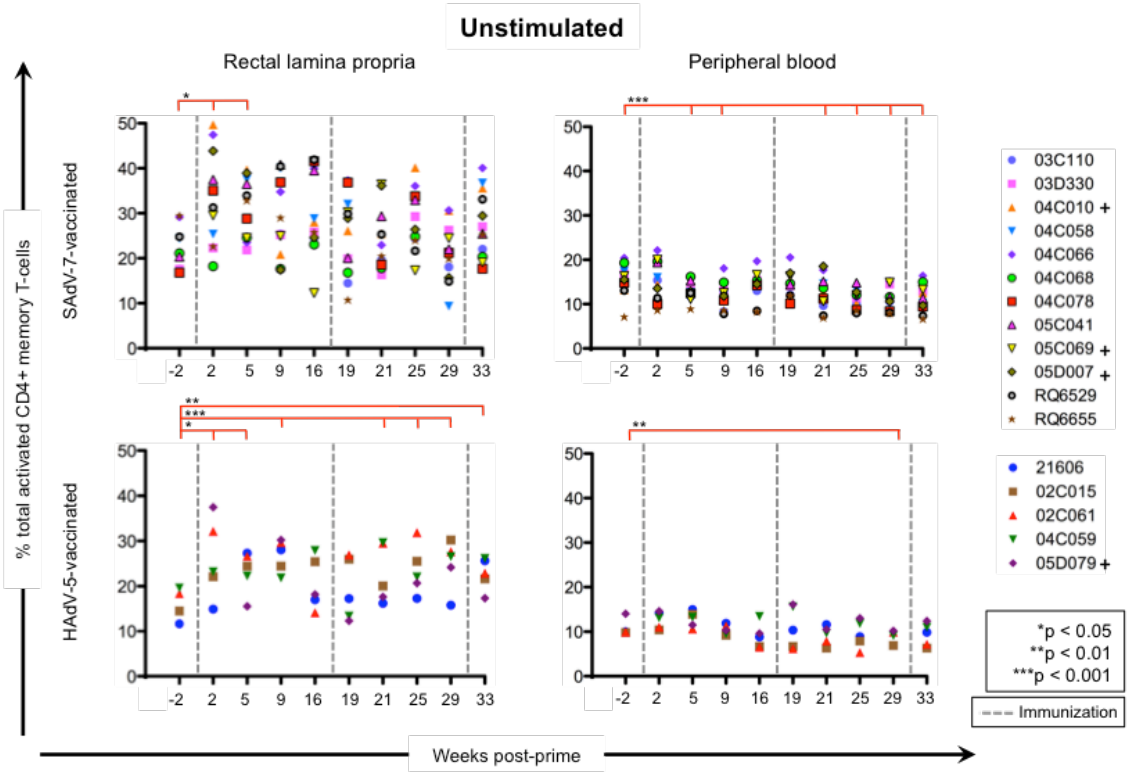

Figure 2.5. Heightened activation marker expression on total unstimulated memory CD4+ T cells in rectal mucosa after AdV vector vaccination. Percent total unstimulated memory CD4+ T cells expressing at least one of four activation markers (HLA-DR, CD25, Ki67, CD69). Peripheral blood and rectal lamina propria T cells from 12 SAdV-7-vaccinated and 5 HAdV-5-vaccinated rhesus macaques (symbols for each macaque are listed in the legend) were collected at ten time points including baseline (week -2) interspersed with three AdV vector immunizations (vertical dotted lines). Significance at any time point was calculated as a percent change relative to baseline and is shown by red lines above each plot (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), and was determined as described in the Material and Methods section. (+) Animals with no rectal biopsy data at baseline time point.

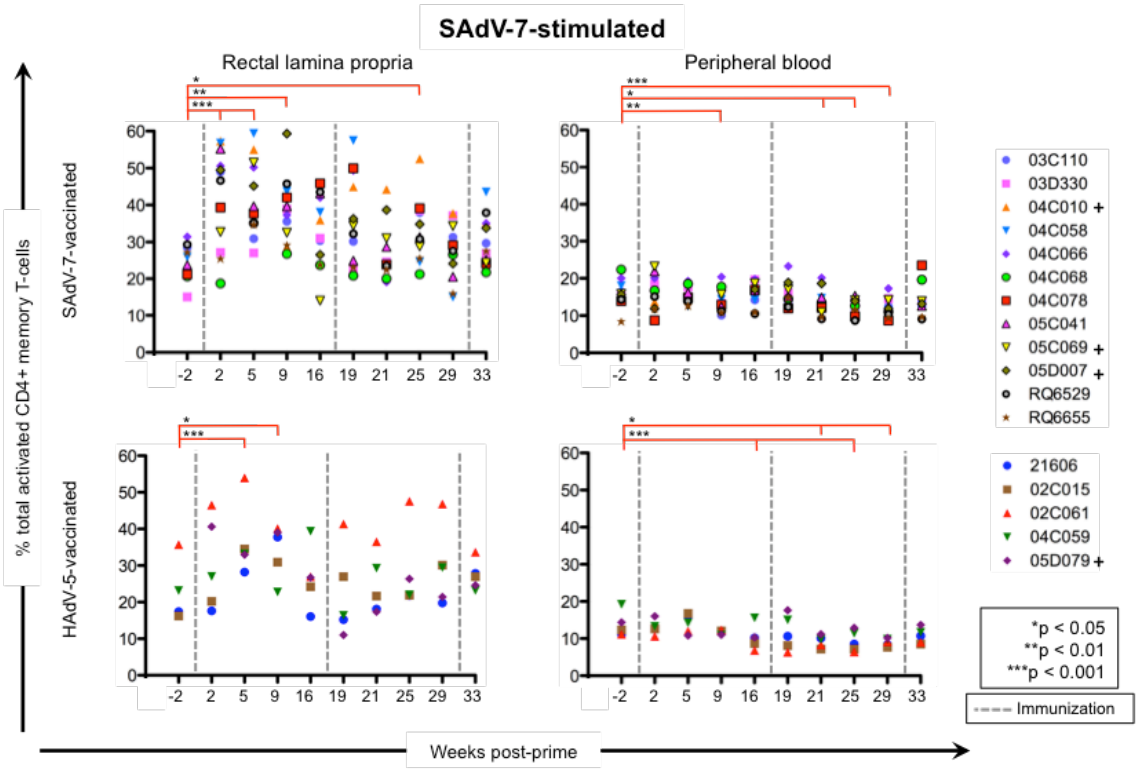

Figure 2.6. Heightened activation marker expression on total SAdV-7-stimulated memory CD4+ T cells in rectal mucosa after AdV vector vaccination. Percent total overnight whole SAdV-7 vector-stimulated memory CD4+ T cells expressing at least one of four activation markers (HLA-DR, CD25, Ki67, CD69). Cells are from the same rhesus macaques and time points as described in **Figure 5**, as well as statistical significance. (+) Animals with no rectal biopsy data at baseline time point.

Increase in rectal mucosal naive CD4+ T cells after adenovirus vector vaccination

Given the increase in both activated and cytokine-expressing memory CD4+ T cells in the rectal mucosa after vaccination, we examined whether there were overt changes in the naïve CD4+ T cell population **(Figure 2.7)**, defined as CD28+ CD95- **(Figure 2.7a),** in the peripheral blood or rectal lamina propria following vaccination. We found a pronounced decrease in the relative frequency of naïve CD4+ T cells within the lamina propria of SAdV-7-vaccinated macaques up to week 16, after which these frequencies slowly returned to around baseline level by week 33 **(Figure 2.7b)**. In contrast, the frequency of naïve CD4+ T cells within the PBMC of SAdV-7-vaccinated macaques increased steadily above baseline levels as a group average post-vaccination. At baseline, PBMCs had a higher naïve CD4+ T cell population ranging between 18-44.4% and 17.4-39.7% for SAdV-7- and HAdV-5-vaccinated macaques, respectively, while the rLPL varied from 1.5-24% and 1.6-9.6% for these groups. There was no evidence for an association between baseline SAdV-7 NAb titer and percentage of naïve CD4+ T cells over time (data not shown). No such overall decrease was noted in naïve rLPL CD4+ T cells after HAdV-5 vaccination in the control animals.

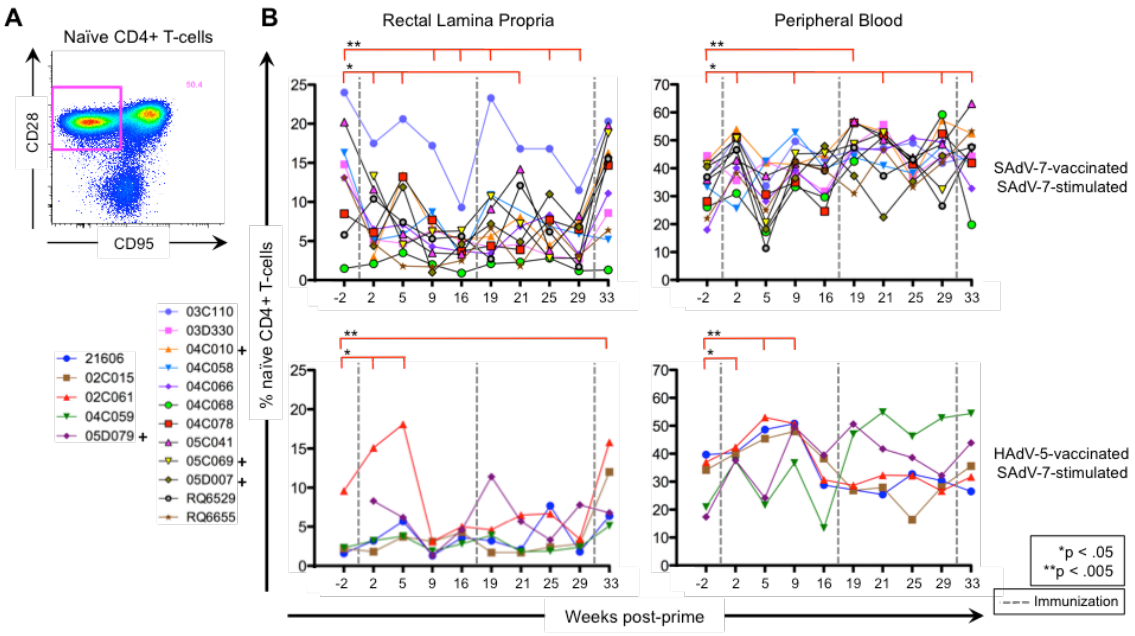

Figure 2.7. Naïve CD4+ T cell percentage at baseline and after AdV vector immunization. (A) Representative example of a flow cytometric plot gating on overnight SAdV-7-stimulated CD4+ T cells using memory markers CD28 and CD95, where naïve = $CD28^{+}$, CD95-; central memory = $CD28^{+}$, $CD95^{+}$; effector memory = CD28-, $CD95^{+}$; and effector = CD28-, CD95-. The number in the top right of the plot represents the percent of naïve CD4+ T cells. (B) Percent of naïve CD4+ T cells stimulated overnight with whole SAdV-7 vector. Samples were collected from macaques and immunizations administered as described in **Figure 5**. Significance at any time point was calculated as a percent change relative to baseline and is shown by red lines above each plot (**p* < 0.05, ***p* < 0.005) and was determined as described in the Material and Methods section. (+) Animals with no rectal biopsy data at baseline time point.

Discussion

The importance of T cell responses in HIV control is well documented [215,299,300] and HAdV-5 vector-based vaccines have been shown to be highly immunogenic for inducing cell-mediated immunity [220,301]. The Step and Phambili studies were able to generate HIV-specific T cell immunogenicity in many participants, but did not accomplish their goal of either preventing HIV-1 infection or lowering viral load setpoint [221], a finding mirrored in the recently discontinued HVTN 505 trial. Perhaps more troublingly, is the clear evidence of a vaccine-mediated enhancement effect for HIV acquisition reported from the Step and Phambili studies. The underlying mechanism for this AdV vaccine-mediated enhancement of infection has remained controversial. It has been speculated that the presence of both cross-reactive AdV-specific T cells and pre-existing antibodies to AdV, due to previous natural exposure to any adenoviral serotype, had a direct causal effect on increased HIV infection, though it is unclear yet if these may be surrogates for a different mechanism. Other proposed theories target the inability of the HAdV-5 vaccine to elicit a sufficiently broad immune response to counteract HIV-1 strain diversity, or that the AdV vector induced a response that was of an altered, undesirable quality [222,302].

In this study, we used a rhesus macaque model to investigate the hypothesis that the rectal mucosa is a site for increased T cell activation, either global or antigen-specific, after AdV vector vaccination. Furthermore, while we did not test it here, persistent adenoviral presence (17, 27) is a driver for T cell migration to the gut, possibly increasing the population of activated T cells for SIV to infect. Our data show that vaccination with AdV vector, whether of human or macaque origin, increased the percentage of total and AdV-specific activated CD4+ memory T cells into the rectal mucosa, an initial site of HIV/SIV transmission. This effect was not observed in the

blood. Additionally, our analyses reveal AdV vector vaccination selectively increases AdV-specific memory CD4+ T cell responses in the rectal mucosa above already existing levels. In contrast, little change in CD4+ T cell activation was found in the peripheral blood. We do not expect that SAdV-7-specific CD4+ T cells are any more or less susceptible to SIV infection compared to any other activated CD4+ T cell, but it is clear from our results that there is an increase in activated mucosal CD4+ T cells in vaccinated animals relative to baseline. Peripheral blood Ad-specific CD4+ T cells within these animals (n=3) did not appear to express different levels of CD4, CCR5, or MIP-1b upon stimulation compared to non-Ad-specific CD4+ T cells (activated or resting, data not shown). Future studies will be necessary to determine whether mucosal Ad-specific CD4+ T cells express differential levels of these markers after vaccination. Furthermore, our results showed that priming with SAdV-7 vector decreased the percentage of naïve CD4+ T cells in the rectal lamina propria, but not in blood, prior to boosting. Together, these findings emphasize the value of sampling from mucosal sites in the context of vaccination with putative HIV vaccine platforms. Indeed, mounting evidence for unique phenotypes between the peripheral blood and gut mucosa [303,304] indicates that analyzing one compartment alone is insufficient for guiding further vaccine development.

Previous studies prior to the Step Trial had examined the impact of HAdV-5 vector vaccination followed by SIV infection in macaques, but these models consistently failed to predict adverse effects of vaccination [188,215,219,305]. When we compared the immunological effects of SAdV-7 to HAdV-5 vector vaccination, we anticipated increase in activated and AdV-specific CD4+ T cells specifically in SAdV-7-vector vaccinated macaques. Instead, we were surprised to find that HAdV-5-vector vaccinated macaques showed significant responses as well, suggesting a cross-reactivity of memory AdV-specific T cell epitopes between species-specific AdV serotypes. With 65 known strains

of human adenovirus, and at least 25 non-human primate strains [203-206], it is likely that AdV-specific T cell memory and cross-reactivity may affect more rare serotypes, in addition to common AdV serotypes [207]. We have demonstrated AdV T cell cross-reactivity in monkeys and humans in this current study as well as previously [297], and recognize that changes in activation level or trafficking patterns depend on the specific Ad vector used, as well as the degree of cross-reactivity of the humoral and cellular immune responses. Furthermore, we have also shown that human intestinal tissue, particularly intestinal lymphocytes, can frequently be found to harbor adenoviral DNA [206] and that captive macaques are chronically infected by adenoviruses [208]. Indeed, AdV has been shown to persist for years in humans, and replication-defective HAdV-5 vectors can persist over a year in mice [185,209], potentially resulting in prolonged antigen presentation and stimulation of T cells at a common site of entry for HIV. Notably, AdV vector-specific T cells have been shown to be permissive for HIV infection due to their high proliferative potential and expression of the HIV coreceptor CCR5 [295]. We did not measure CCR5 in this study, since most mucosal CD4+ T cells express CCR5 at sufficient levels to be infected by SIV, and CCR5 expression through flow cytometry may be below the limit of detection [273]. These observations would explain how peripheral vaccination with an AdV-based vector may cause increased activation in the gut mucosa. The combination of a complex adenoviral flora naturally found in the gut together with continuous AdV shedding results in persistent antigen presence acting as a driver for migration of cells to the gut. In addition to AdV-specific activation, global activation may occur as a bystander effect where the influx of AdV-specific T cells perturbs an existing balance leading to generalized inflammation in the intestinal mucosa.

In the Step trial, when participants were divided into those who were HAdV-5

NAb negative (titer ≤18) or HAdV-5 NAb positive (titer >18) at baseline, statistically significant differences were seen in rates of HIV acquisition [221]. Additionally, individuals with pre-existing HAdV-5 NAb had a lower percentage of activated AdV-specific CD4+ T cells in the blood [232], which may indicate AdV-specific CD4+ T cells had homed to the mucosa or that AdV-specific antibodies blocked AdV infection. We did not find an association between pre-existing SAdV-7 NAbs and increased CD4+ T cell mucosal activation in this study confirming early work showing no link between HAdV-5 seropositivity and the magnitude of the Ad-specific T cell response in humans after infection [231], it is possible that other macaque AdV strains could be stimulating the immune system and influencing the expression of activation markers. AdV-directed cellular immune responses seen in SAdV-7 seronegative macaques likely accumulate from exposure to multiple AdVs [231,232] and not solely to SAdV-7. Since we have shown before that HAdV-5 vector vaccination in humans expands HAdV-5-specific $CD8^+$ T cells [306], we believe that intercurrent adenoviral infections in macaques and humans are likely modeling each other, as they both exhibit increases in AdV-specific T cells. Further, one unresolved observation was the downward trend of naïve CD4+ T cells in the rectal lamina propria of SAdV-7-vaccinated macaques following the vaccine prime, which appeared to return to baseline following the vaccine boosts. The initial decrease in naïve T cells may indicate trafficking of activated memory AdV-specific CD4+ T cells into the gut mucosa. Alternatively, our observations may reflect loss of naïve cells, expansion of memory cells, transition of naïve to memory, or altered trafficking of naïve cells out of the gut mucosa. Regardless of the underlying mechanism, the net result of this effect is a temporary increase in activated memory CD4+ T cells in the mucosa, creating a potentially favorable dynamic for increased susceptibility to HIV infection.

We have shown that vaccination with both an endogenous macaque, as well as a human, adenoviral vector can lead to significant increases in AdV-specific and activated CD4+ T cells in the rectal lamina propria of macaques, but not in the peripheral blood. While it is unclear at this time whether this induction of mucosal CD4+ T cell activation is sufficient to increase susceptibility to SIV infection, these results clearly suggest that adenovirus vaccination might indeed create such an environment. Previous adenovirus vaccine platform studies have not indicated an increase in SIV susceptibility, but, importantly, SIV challenge in these models has not taken place within the vaccine-induced mucosal CD4+ T cell activation window that we found here. Ultimately a low-dose SIV challenge study following AdV-based vector vaccination within the activation period we have found will offer a proof-of-concept for the effects of such activation. In the meantime, our findings raise a note of caution regarding the development of HIV vaccine vectors based upon mucosal pathogens, and suggest that greater consideration should be placed upon the induction of vector-specific immunity and more generalized activation effects within appropriate tissue sites. More broadly, the effects on mucosal CD4+ T cell activation that we have observed may not be restricted to adenovirus, and may be difficult to circumvent for the induction of effective mucosal immune responses by vaccination.

CHAPTER 3

Development of a Low-Dose Intra-Rectal SIVmac251 Challenge Model After Adenovirus Vector Vaccination

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Abstract

Vaccination with the Merck human adenovirus serotype-5 (HAdV-5) vectored HIV-1 subtype B gag/pol/nef vaccine was unexpectedly associated with enhanced susceptibility to HIV-1 infection in uncircumcised HAdV-5 seropositive men by an as of yet unknown mechanism. It has been hypothesized that vaccination may have resulted in activated CD4+ T lymphocytes trafficking to mucosal sites thereby increasing targets for HIV infection. Recently, we found that AdV-vector vaccination in rhesus macaques resulted in an increase in the frequency of activated mucosal CD4+ T cells. However, whether this increase in activation is sufficient to increase susceptibility to HIV/SIV infection is unclear. To examine this scenario, we developed a preliminary, proof-of-concept vaccination-challenge model in order to examine vaccine-induced SIV susceptibility in rhesus macaques. Rhesus macaques (n=10/group) were vaccinated with a simian AdV-7 (SAdV-7)-vector encoding an irrelevant insert (human α-anti-trypsin) and challenged 5 weeks post-prime in an escalating dosing regimen starting with sub-infectious doses (1:10,000 or $2TCID_{50}$) of SIVmac251. Although not statistically significant, we found a trend towards increased SIV acquisition with 5/10 SAdV-7-vaccinated (versus 3/10 placebo-vaccinated) macaques becoming infected after repeated low-dose intra-rectal SIVmac251 challenge ($p < 0.2$). These results lay the groundwork for study designs to assess vaccine-induced SIV susceptibility studies in

rhesus macaques. Further larger-scale studies are necessary to confirm the AdV-vector vaccination associated trend towards increased SIV/HIV acquisition and clarify any associated mechanisms.

Introduction

Various aspects of HIV biology have complicated the pursuit of an efficacious vaccine. The global HIV pandemic, currently infecting an estimated 34 million people, continues to be a public health concern due to the lack of clear correlates of immune protection, viral heterogeneity, and difficulty fully eradicating viral reservoirs. In line with this, two main avenues for vaccine design have been pursued, including induction of broadly neutralizing antibodies, as well as vector-based vaccines that generate sustained and robust HIV-specific T cell responses [291,292]. For the latter, recent large-scale clinical trials including Merck's Step study [221], Phambili [224], and HVTN-505 [225] have all utilized recombinant replication-defective human adenovirus type-5 vectors (HAdV-5) vectors to decrease HIV viral load or prevent HIV infection altogether. Unfortunately, all three trials failed to protect from HIV acquisition, and were halted [223,225]. In fact, participants in the Step Trial revealed an enhanced susceptibility to HIV infection in HAdV-5 baseline seropositive, uncircumcised men [221,222].

One hypothesis for the Step study failure was that following vaccination of participants with pre-existing neutralizing antibodies (nAb) to HAdV-5, expansion and/or activation of memory CD4+ T cells that are already present in, and traffic to, the mucosa [307] – preferred target cells at the site of HIV transmission [61,308] – may have increased HIV susceptibility. However, only peripheral blood samples were collected in these clinical trials, obviating the ability to directly assess potential mucosal-based mechanisms [221]. To address this experimentally, we previously examined CD4+ T cell activation and AdV-

specific CD4+ T cell responses in the peripheral blood (PBMC) and rectal lamina propria (rLPL) after HAdV-5 and simian adenovirus type-7 (SAdV-7) vector vaccination using a rhesus macaque (RM) model. We found that the expression of activation markers on CD4+ T cells was heightened in the rLPL, but not the PBMC, after both HAdV-5 and SAdV-7 vaccination. Further, a statistically significant increase in the percentage of AdV-specific CD4+ T cells was found after AdV-vector vaccination in the rLPL [309]. Noting these differences between the peripheral blood and rectal mucosa, we initiated a proof-of-concept experiment to determine if AdV-based vector vaccination followed by SIV challenge during the activation window would increase SIV acquisition. Here, we assessed post-AdV vector vaccination induction of SIV susceptibility using an ultra-low dose SIV mucosal challenge. Unlike traditional vaccine studies where control animals are expected to become infected, we hypothesized that in order to model potential vaccination-induced SIV susceptibility enhancement we would need to challenge RMs with an extremely low dose of virus generally insufficient to infect the control animals. We therefore assessed susceptibility to dose-escalating intra-rectal challenges with SIVmac251 beginning at a minimally infectious dose ($2TCID_{50}$), 5 weeks after a single immunization with a SAdV-7-based vector compared to a sham vaccinated control. We found a trend towards increased SIV acquisition with 5 SAdV-7-vaccinated versus 3 placebo-vaccinated macaques becoming SIV+ ($p < 0.2$). Together, these studies establish a model system in which to monitor potential vaccine-induced enhancement of SIV susceptibility in the SIV-RM model.

Materials and Methods

Adenovirus vectors

Wild-type SAdV-7 was purchased from the ATCC (VR-201, originally isolated from rhesus monkey kidney cells). SAdV-7-based vectors were constructed as previously described [309,310].

Animals

Titration study: 15 healthy, SIV-uninfected Indian rhesus macaques (RMs) were used in the SIVmac251 titration study. All animals were housed that the Yerkes National Primate Research Center and in accordance with NIH guidelines. These studies were approved the Emory University Institutional Animal Care and Use committee (IACUC).

Ad-vector vaccination challenge study: Twenty captive bred five-year old male Indian origin rhesus macaques (*Macaca mulatta*) were purchased from Covance Research Products (Alice, TX) and enrolled in this study. All macaques were housed in accordance with the *Guide for the Care and Use of Laboratory Animals*, Public Health Service Policy, and Animal Welfare Act and Regulations in an AAALAC-accredited facility. All experiments were performed under protocols approved by The Children's Hospital of Philadelphia Institutional Animal Care and Use Committee.

Titration study rectal challenge with infectious simian immunodeficiency virus (SIVmac251)

All 15 macaques in the titration study were challenged intra-rectally every week with a low-dose of SIVmac251 (provided by Dr. Nancy Miller, DAIDS, NIAID, NIH, Bethesda,

MD: see **Supplemental Table 3.1**). Challenges were performed with a 1cc slip tip syringe, containing 1 ml SIVmac251 inserted ~4cm gently into rectum. A plunger was depressed to instill virus into the rectum. The macaque was then returned to its cage in a prone position. Challenge doses began at $4TCID_{50}$, which were repeated for 3 weeks, then increased to $20TCID_{50}$. This process was repeated for 15 weeks total, increasing by half a log every 3 weeks, until the animal became infected. The highest dose was $666TCID_{50}$. Macaques became infected at various doses over the course of the study, with >1000 copies/ml considered infected. Animals were followed until day 42 post-infection.

AdV-vector vaccination study rectal challenge with SIVmac251

A 3cc slip tip syringe lightly coated with lubrication jelly and containing the specific SIVmac251 challenge dose was gently inserted into the rectum ~4cm, and the dose was slowly instilled. The animal remained in prone position for at least 5 minutes after instillation of the virus. Animals were challenged weekly starting at week 5 post-vaccination, repeating the same dose for 3 weeks (1x/week) and increased half a log every 3 weeks until the animal became infected. Challenge doses began at 1:10,000 ($2TCID_{50}$) and the highest dose was $66.6TCID_{50}$.

Immunizations

Macaques in the vaccine group received a single, intramuscular (IM) injection of SAdV-7 at a dose of $1x10^{11}$ particles. Macaques in the placebo group similarly received a single IM injection of sterile saline as a control (see **Figure 3.1** for the immunization schedule).

Endoscopic sampling of macaque rectum and isolation of Lamina Propria Lymphocytes (LPLs)

All rhesus macaques were fasted the evening before the procedure, with free access to water at all times. NHPs were sedated and biopsies were obtained utilizing an alligator-jaw style endoscopic biopsy pinch held free hand. 20 biopsies were taken spaced far enough apart so as not to weaken the rectal wall. Biopsies were placed in RPMI medium and LPLs isolated using collagenase type II as previously described [297].

Blood collection and isolation of peripheral blood mononuclear cells

PBMCs were isolated from whole blood collected in Vacutainer CPT cell Preparation Tubes with Sodium Heparin (Ref 362753, Becton, Dickinson and Company**,** Franklin Lakes, NJ) following the protocol recommended by the vendor. Briefly, tubes were initially centrifuged at 1700 RCF at ambient temperature to isolate the mononuclear cells. After centrifugation, the mononuclear cells were resuspended into the plasma by tube inversion and all contents above the gel were pipetted into a separate 15mL conical tube. PBMCs were centrifuged at 300 RCF and washed in PBS following protocol.

AdV neutralizing antibody assay

Anti-SAdV-7 neutralizing antibody titers in serum samples were measured by assessing the ability of serum to inhibit transduction of the corresponding reporter vector SAdV-7LacZ into HEK 293 cells as previously described [309]. The NAb titer was reported as the highest serum dilution that inhibited AdV.CMV.LacZ transduction (β-gal expression) by 50%, compared with the serum control. Limit of detection of the assay is 1/5.

Antibody reagents

Antibodies used for surface staining included: anti-CD14 Qdot 655, anti-CD20 Qdot 655, anti-CD4 PeCy5.5, anti-CD8 Qdot605 (Invitrogen; Carlsbad, California), anti-CD14 BV650, anti-CD20 BV650 (Biolegend; San Diego, California), anti-CD28 ECD (Beckman Coulter; Fullerton, CA), anti-CD25 APC-Cy7, anti-CD95 PE-Cy5, anti-HLA-DR APC (BD Pharmingen; San Diego, California). Antibodies used for intracellular staining included: anti-interleukin-2 (IL-2) Alexa700 (Biolegend), anti-interferon-gamma (IFNγ) Alexa 700 (Invitrogen), anti-tumor necrosis factor-alpha (TNFα) Alexa 700, anti-CD3 Pac Blue, anti-CD69 PE, anti-Ki67 FITC (BD Pharmingen).

Determination of plasma viral RNA (viral load)

SIVmac251 loads were measured in plasma samples by real-time PCR as previously described [311].

Cell processing and stimulation

Rectal biopsies were processed within 6 hours of being collected. Rhesus macaque PBMC were cryopreserved in fetal bovine serum (FBS; ICS Hyclone, Logan, Utah) containing 10% dimethyl sulfoxide (DMSO; Fisher Scientific, Pittsburgh, Pennsylvania) and stored in liquid nitrogen until use. After washing fresh LPL cells or thawed PBMCs once in RPMI (Mediatech Inc; Manassas, Virginia), both PBMC and LPL cells were resuspended in complete medium [(RPMI supplemented with 10% FBS, 1% L-glutamine (Mediatech Inc) and 1% penicillin-streptomycin (Lonza; Walkersville, Maryland), sterile filtered] at a concentration of 1-2x10^6 cells/mL medium in FACS tubes. Cells were split into three stimulation conditions, at a volume of 1 mL each, with either: no stimulation, 1

μl Staphylococcus Enterotoxin B (SEB) at a concentration of 1 mg/mL (Sigma-Aldrich; St. Louis, Missouri) as a positive control, or $1x10^{10}$ particles/mL of the SAdV-7 vector. Cells were stimulated overnight at 37°C, 5% CO_2.

FACS Staining Assay

Stimulation tubes were removed from the incubator in the early morning to add monensin (0.7 μg/ml final concentration; BD Biosciences) and brefeldin A (1 μg/ml final concentration; Sigma-Aldrich; St. Louis, Missouri) and incubated for an additional 6 hours. Cells were then washed once with PBS and stained for viability with Aqua amine-reactive dye (Invitrogen) for 10 minutes in the dark at room temperature. A mixture of antibodies used for staining surface markers were added to the cells and kept at room temperature for 20 minutes. Cells were washed with PBS containing 1% bovine serum albumin (BSA, Fisher Scientific) and 0.1% sodium azide (Fisher Scientific) and permeabilized for an additional 20 minutes at room temperature using the Cytofix/Cytoperm kit (BD Pharmingen). Next, cells were washed in Perm/Wash buffer (BD Pharmingen). A mixture of antibodies used for staining intracellular markers was added to the cells and incubated in the dark for one hour at room temperature. Cells were again washed with Perm/Wash buffer and fixed with PBS containing 1% paraformaldehyde (Sigma-Aldrich). Fixed cells were stored in the dark at 4°C until collection.

Flow Cytometric Analysis

For each sample, between $3x10^5$-$1x10^6$ total events were acquired on a modified flow cytometer (LSRII; BD Immunocytometry Systems; San Jose, CA) equipped to detect up

to 18 fluorescent parameters. Antibody capture beads (BD Biosciences) were used to prepare individual compensation tubes for each antibody used in the experiment. Data analysis was performed using FlowJo version 9.0.1 (TreeStar, Ashland, Oregon). Percent expression is shown after background subtraction, where values are calculated as the difference between cells that were stimulated with SAdV-7 vector overnight minus those that were left unstimulated. AdV-specific percentages are reported as the population of CD4+ memory T cells, which express IL-2, IFNγ, and/or TNFα within each compartment. To separate naïve cells from memory, effector memory, and effector T cells, we stained with fluorochrome-conjugated antibodies for CD28 and CD95, where $CD28^{+}$ CD95- CD4+ T cells indicated the naïve subset, whereas all other cells were grouped as memory. Naïve CD4+ T cells were gated using FlowJo for each macaque at all time points in both compartments.

Figures

Prism software, version 5.0 (Graphpad; La Jolla, California) was used to create the figures.

Statistical Analysis

This study was designed to have sufficient power (80%) to detect a significant result (p<0.05) with group differences in SIV acquisition if 0/10 placebo group and 5/10 vaccine group RM became infected, or 1/10 placebo group and 7/10 vaccine group RM became infected. The groups were compared with respect to the probability of infection as a function of challenge dose using a logistic regression model. In order to account for the structure of the data, p-values were computed based on a permutation null distribution.

The permutation null distribution was created by randomly permuting the group labels between animals 1000 times and recomputing the statistic after each permutation; the observed statistic was then compared to this permutation null distribution.

Results

SIVmac251 low-dose titration

We recently described the development and use of the simian adenovirus type 7 (SAdV-7) vector in order to model natural adenovirus immunological responses in macaques compared to using a human AdV vector [309,310]. In order to assess whether the vaccine-induced mucosal CD4+ T cell activation we observed previously induced a heightened state of SIV infection susceptibility, we first needed to develop an ultra-low dose challenge system. Therefore, we first performed a low-dose intra-rectal titration of our SIVmac251 stock in 15 RM. We began this titration at a 1:5000 ($4TCID_{50}$) dilution that, in previous titration studies involving only a few macaques, appeared to produce little or no SIV infection. Two of fifteen macaques were infected at the lowest 1:5000 dose (**Supplemental Table 3.1**). As a result, we started our vaccination-challenge study at 1:10,000 ($2TCID_{50}$), in order minimize the chance of infection in the control group.

Simian adenovirus type 7 immunization/challenge study design

Twenty Indian-origin rhesus macaques were stratified into vaccine and placebo groups based on group distribution of baseline cytokine and activation marker expression, as well as baseline SAdV-7 neutralizing antibody (nAb) titers (**Supplemental Table 3.2**). SAdV-7 nAb titers ranged from <5 (undetectable) to 640 in one macaque, and did not correlate with SIV acquisition by the end of the study. We vaccinated 10 RM intramuscularly with $1x10^{11}$ viral particles (VP) of an E1-deleted replication-defective

SAdV-7 vector diluted in sterile saline (see Materials and Methods for construct description). As a control, we vaccinated 10 RM with sterile saline. Peripheral blood mononuclear cells (PBMCs) and rectal lamina propria T lymphocytes (rLPL) were obtained at two baseline time points (week -5 and week -3) and week 2 post-vaccination (**Figure 3.1**). Once rectal pinch biopsies were collected at week 2 post-vaccination, macaques were rested three weeks to allow healing of the rectal mucosa tissue before challenge. Based on our previous study, AdV-induced rLPL CD4+ T cell activation peaks at 5 weeks and decreases nearly to baseline by 16 weeks post-SAdV-7 vaccination. For this reason, we chose to structure the timeline such that the peak window of activation could overlap with the SIV challenge window. This would help determine if increased CD4+ T cell activation in the rectal mucosa due to SAdV-7 vector vaccination may increase the likelihood of SIV acquisition. Starting at week 5, macaques were intra-rectally challenged weekly with SIVmac251, starting at a 1:10,000 ($2TCID_{50}$) dose dilution. Macaques were challenged 3x/dose before increasing the dilution half a log, until SIV acquisition, as indicated by weekly plasma viral load assays. SIV challenges continued until week 16 (1:300 or $66.6TCID_{50}$), at which point 12 macaques total did not acquire SIV. Macaques were monitored for viral set-point once infected.

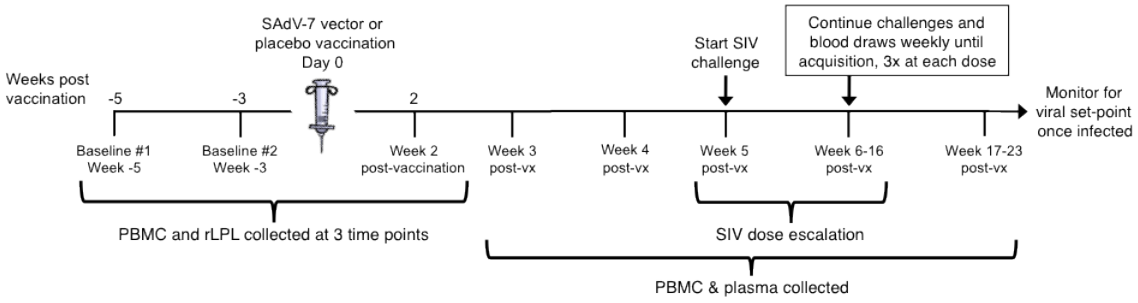
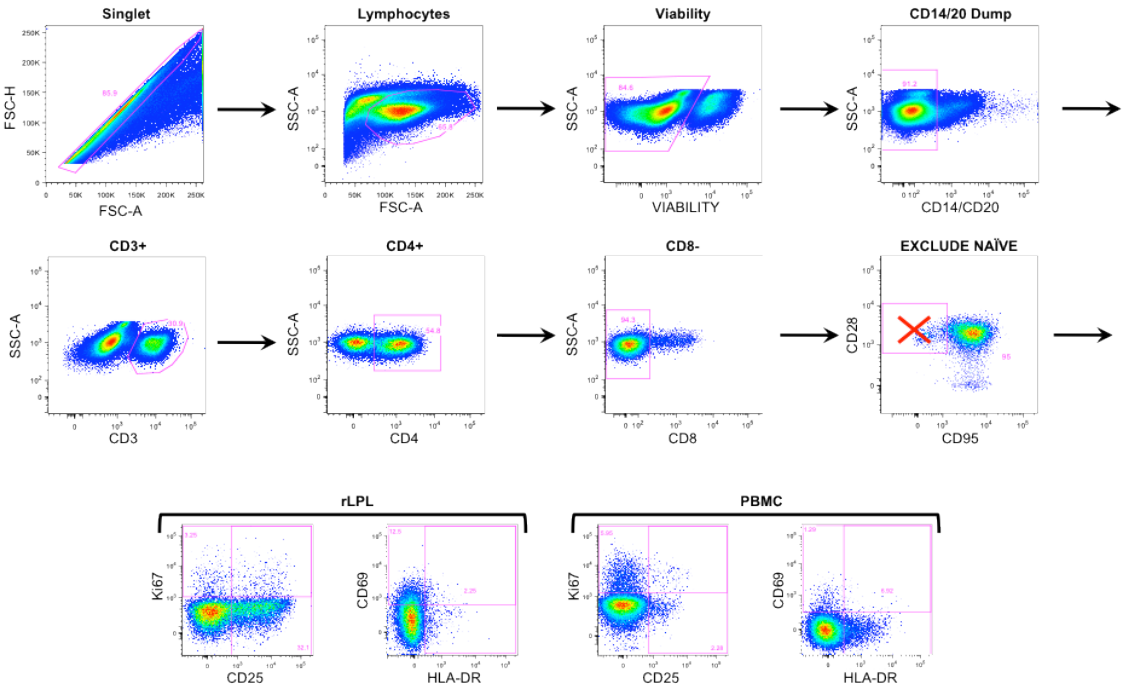


Figure 3.1. Study timeline. Overview of the three peripheral blood mononuclear cell (PBMC) and rectal lamina propria T lymphocyte (rLPL) collection time points interspersed around vaccination (day 0). Intra-rectal SIV challenges began at week 5 post-vaccination (post-vx) and continued until week 16.

Challenge dose causing infection	$TCID_{50}$ equivalent	# of RMs infected	Challenge #
1:5000	$4TCID_{50}$	2	3
1:1000	$20TCID_{50}$	2	4
1:1000	$20TCID_{50}$	2	5
1:1000	$20TCID_{50}$	1	6
1:300	$66.6TCID_{50}$	3	8
1:300	$66.6TCID_{50}$	1	9
1:100	$200TCID_{50}$	1	10
1:100	$200TCID_{50}$	1	12
1:30	$666TCID_{50}$	1	13
1:30	$666TCID_{50}$	1	14

Supplemental Table 3.1. SIVmac251 low-dose intra-rectal titration. 15 rhesus macaques were SIV challenged intra-rectally weekly and viral loads were monitored weekly until SIV acquisition. Challenge doses began at 1:5000 ($4TCID_{50}$) and was repeated 3 times before increasing by half a log every three weeks, until SIV infection occurred. All 15 macaques became SIV+ by the 14th challenge at 1:30 dilution.



Supplemental Figure 3.1. Representative flow cytometry gating. Rectal LPL and PBMCs were gated on single cells, including live lymphocytes and excluding CD14/20 cells. Within the CD3+ pool, CD4+/CD8- T cells further excluded naïve (CD28+/CD95-) T cells. Plots on the top two rows were obtained from the rLPL at baseline week -5. The four bottom panels represent activation marker expression in the rLPL (left) and PBMC

(right).

Monkey ID	SAdV-7 baseline NAb titer
Placebo group	
07D228*	80
08C015*	20
08C045*	5
08C001	40
08C004	<5
08C009	<5
08C020	<5
08C042	80
08D035	5
08D311	640
SAdV-7 vaccinated	
08C008*	80
08C025*	<5
08C026*	20
08C033*	80
08C046*	20
08C006	40
08C014	80
08C018	5
08C032	40
08P047	5

Supplemental Table 3.2. SAdV-7 baseline neutralizing antibody (nAb) titers. Titers are reported for placebo-vaccinated and SAdV-7-vaccinated macaques, the assay is described in the Materials & Methods. (*) indicates that macaque acquired SIV before the study completion.

Immunologic assessment of SAdV-7 vectored vaccine induced responses

Previously, we found that SAdV-7 vector vaccination preferentially induced strong AdV-specific T cell responses, heightened CD4+ T cell activation, and decreased the frequency of naïve CD4+ T cells in the rectal lamina propria of vaccinated RM (37). As such, we first sought to confirm that the SAdV-7 vector used here similarly induced these changes within the rectal lamina propria following immunization.

Baseline AdV-specific CD4+ T cells were detectable for all macaques in at least one compartment (**Figure 3.2;** representative data shown in **Supplemental Figure 3.1**), with 7/10 SAdV-7-vaccinated RMs and 9/10 placebo-vaccinated RMs showing responses in the peripheral blood, and 10/10 SAdV-7-vaccinated RMs and 9/10 placebo-vaccinated RMs showing responses in the rectal mucosa (response >0.05% was considered positive). However, while SAdV-7 vaccination resulted in a greater average percentage of AdV-specific CD4+ T cells in both PBMC (0.55%) and rLPL (0.28%) at week 2 relative to placebo vaccination **(**PBMC 0.09%**,** rLPL 0.16%**)**, this increase was not statistically significant.

We next assessed CD4+ T cell activation at baseline weeks -5 and -3, and week 2 post-vaccination (**Figure 3.3**) to determine whether vaccination with this SAdV-7 vector induced heightened mucosal CD4+ T cell activation. However, unlike our previous study, we found no significant changes in the percent of total activated CD4+ memory T cells (representing the summed percentages of any CD4+ memory T cell that expresses at least one (or more) of the four activation markers) before and after vaccination. As expected, the rectal lamina propria had, on average, consistently higher expression of activation markers compared to PBMCs (rLPL ~35% vs. PBMC ~16%). Moreover, the percent total CD4+ T cell activation marker expression was comparable between the

placebo and vaccine groups within each compartment at both baseline and week 2 post-vaccination.

Finally, we measured naïve CD4+ T cells (**Figure 3.4**), defined as CD28+ CD95-, in the peripheral blood and rectal lamina propria for cells stimulated with SAdV-7 overnight at all three time points. As expected, the average relative percentage of naïve CD4+ T cells was much higher in PBMCs (baseline placebo 45.62% vs. baseline SAdV-7-vaccinated 48.18%) (week 2 placebo 46.7% vs. week 2 SAdV-7-vaccinated 42.09%) than the rLPL (baseline placebo 7.15% vs. baseline SAdV-7-vaccinated 5.11%) (week 2 placebo 8.69% vs. week 2 SAdV-7-vaccinated 10%). Overall, the values did not fluctuate significantly before and after vaccination in any group, except for SAdV-7-vaccinated RM #08C014 displaying an unusually high naïve CD4+ T cell percentage at week 2 post-vaccination. Taken together, these results indicate that the SAdV-7 vector used in this study performed quite differently in this group of RM compared to that used in our previous study.

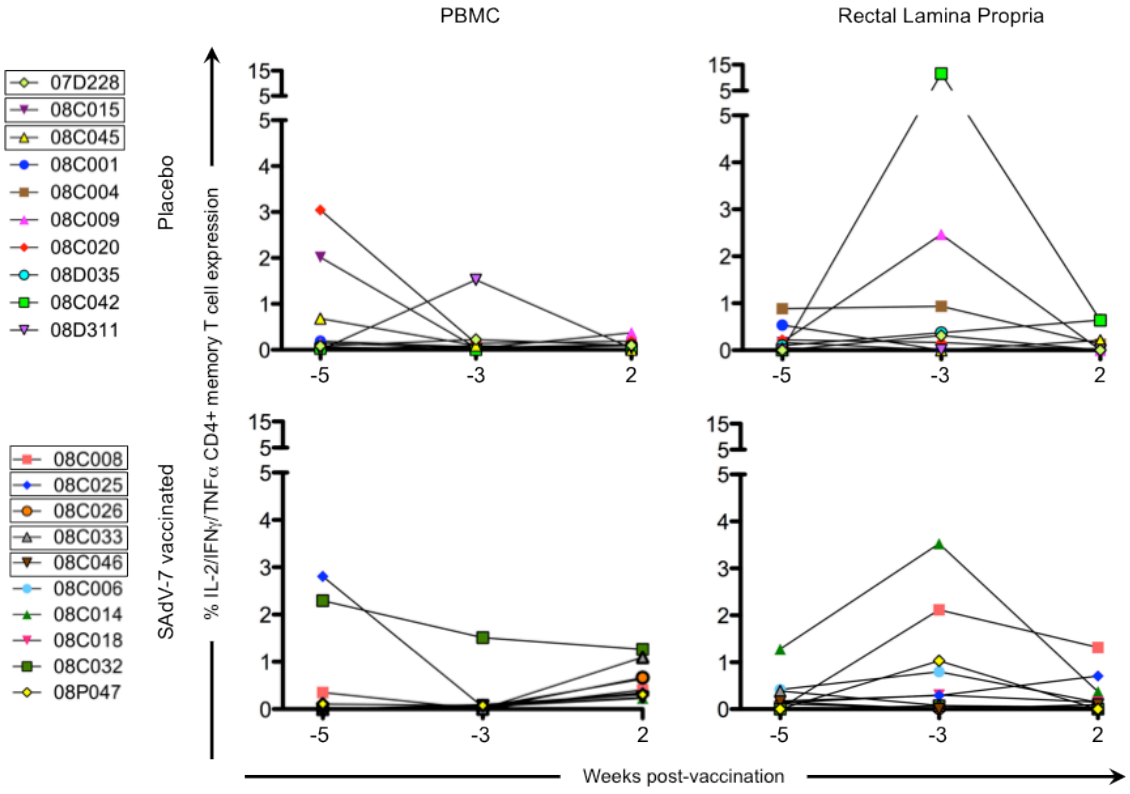


Figure 3.2. CD4+ memory AdV-specific T cell cytokine responses in the PBMC and rLPL. Cytokine responses (IL-2, IFNγ, TNFα) were combined for SAdV-7 overnight-stimulated CD4+ memory T cells and baseline subtracted from unstimulated cells. Values are shown for both baseline time points (weeks -5 and -3) and week 2 post-vaccination. Boxes around macaque numbers in the legend on the left indicate that animal became SIV+ before study completion.

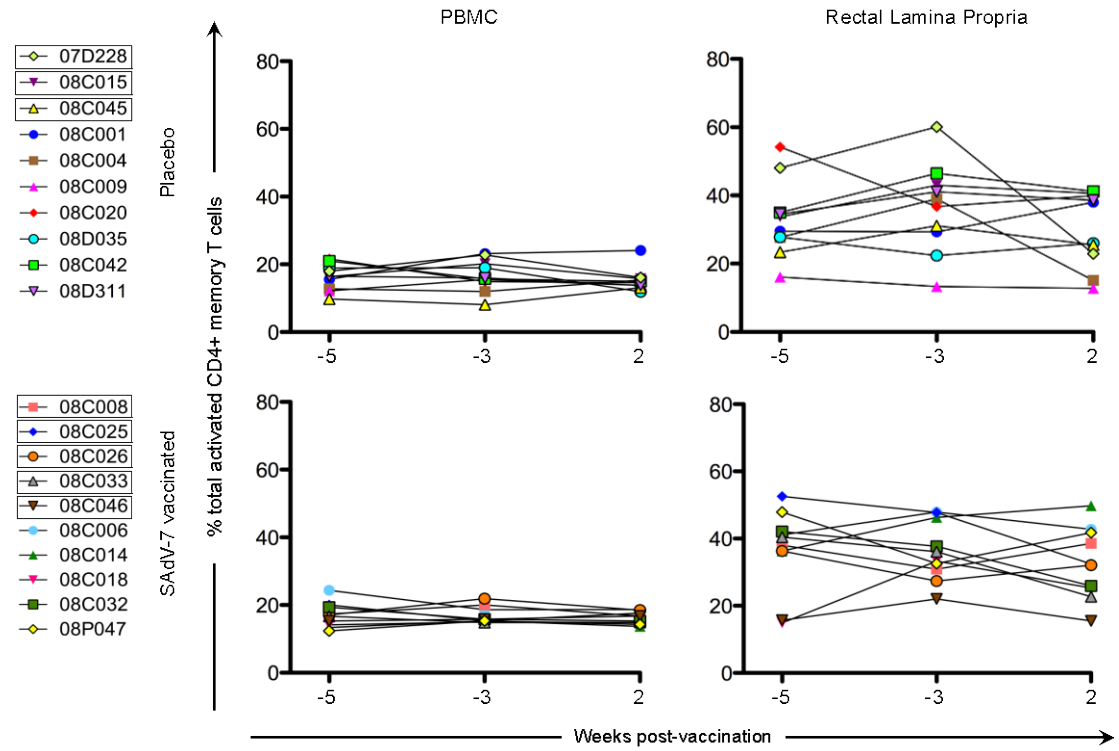


Figure 3

Figure 3.3. Percent of total activated CD4+ memory T cells with no overnight stimulation. Values are shown for both baseline time points (weeks -5 and -3) and week 2 post-vaccination in the peripheral blood (PBMC) and rectal lamina propria. Activation values for each macaque represent the summed percentages of any CD4+ memory T cell that expresses at least one (or more) of the four activation markers measured in this study (HLA-DR, CD25, CD69 and/or Ki67) within that time point. Boxes around macaque numbers in the legend on the left indicate that animal became SIV+ before study completion.

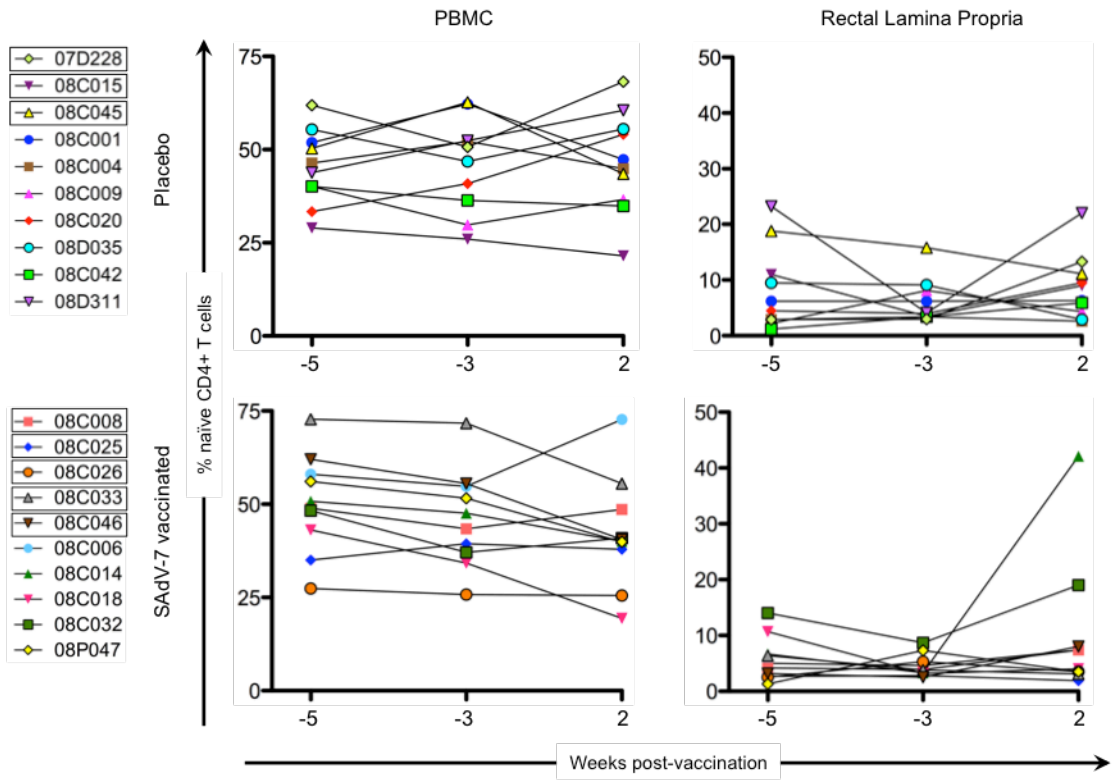


Figure 3.4. Percent of naïve CD4+ T cells at each time point. Naïve cells are CD28+/CD95- by flow cytometry (a representative plot can be found in Supp. Fig. 1). All cells were SAdV-7-stimulated overnight before fluorochrome staining. Values are shown for both baseline time points (weeks -5 and -3) and week 2 post-vaccination in the peripheral blood (PBMC) and rectal lamina propria. Boxes around macaque numbers in the legend on the left indicate that animal became SIV+ before study completion.

Increased trend of SIV acquisition after SAdV-7 vector vaccination relative to placebo

Starting at week 5 after either SAdV-7 vector or placebo vaccination, we began weekly intra-rectal low-dose SIV challenges at 1:10,000 ($2TCID_{50}$) for all 20 macaques (**Supplemental Table 3.3**). Although we did not see evidence of increased AdV-specific or activated CD4+ T cells at week 2 post-vaccination, the week 5 challenge time point was chosen based on the peak activation levels observed in our previous study, which peaked at week 5 and subsided through week 16 (37). As shown in **Figure 3.5**, 5 SAdV-7-vaccinated RMs and 3 placebo-vaccinated RMs became infected throughout the challenge course. The time of SIV acquisition did not occur with any preference towards vaccination group. While this value is not statistically significant between groups, it trends towards significance ($p < 0.2$). Peak viral loads for SAdV-7-vaccinated macaques ranged from $9.34x10^5$ (#08C026) – $9.53x10^6$ (#08C008) copies/mL and in placebo-vaccinated macaques ranged from $1.45x10^4$ (#08C045) – $2.96x10^7$ (#07D228) copies/mL. Although macaques were not always followed through to final viral load set-point, final viral loads prior to euthanasia were not consistently higher or lower for either vaccination group (range of set-points for SAdV-7-vaccinated RMs was $3.57x10^2$ (#08C026) – $2.24x10^5$ (#08C008) and for placebo-vaccinated RMs was $1.3x10^2$ (#08C045) – $1.49x10^5$ (#08C015)). Finally, we found no association between pre-challenge activation levels, AdV-specific CD4+ T cell frequency, or pre-vaccination AdV titers (data not shown) and infection status or infection rate within either the vaccine or placebo groups.

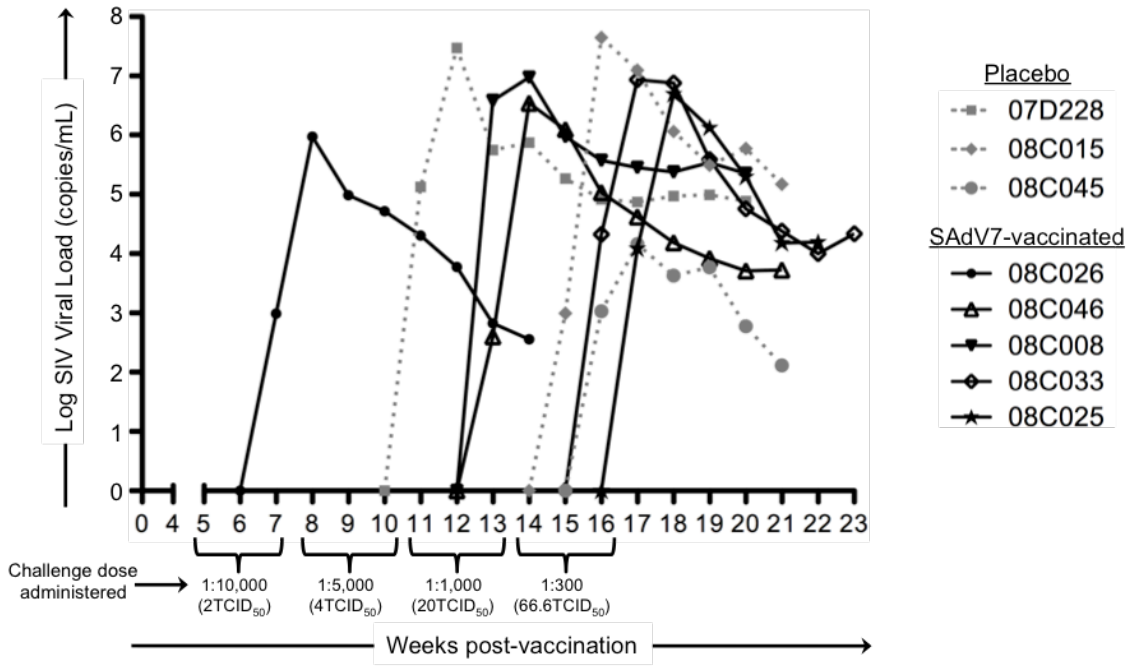


Figure 3.5. Viral loads in SIV-infected macaques after dose-escalating intra-rectal challenges. Three placebo-vaccinated macaques (gray dotted lines) and five SAdV-7-vaccinated macaques (black solid lines) acquired SIV as a result of weekly SIV challenges ($p < 0.2$). Challenge doses were administered 3x/dose before increasing to the next dose to all 20 macaques. SIV challenges began at 5 weeks post-vaccination and continued until 16 weeks post-vaccination.

1:10,000 (2TCID$_{50}$) 1:5,000 (4TCID$_{50}$) 1:1,000 (20TCID$_{50}$) 1:300 (66.6TCID$_{50}$)

	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12	Week 13	Week 14	Week 15	Week 16	Week 17	Week 18	Week 19	Week 20	Week 21	Week 22	Week 23
SAMPLE ID	7/8/13	7/15/13	7/22/13	7/29/13	8/5/13	8/12/13	8/19/13	8/26/13	9/3/13	9/9/13	9/16/13	9/23/13	9/30/13	10/7/13	10/14/13	10/21/13	10/28/13	11/4/13	11/11/13
Placebo																			
07D228	Undet	Undet	Undet	Undet	Undet	Undet	1.33E+05	2.96E+07	5.55E+05	7.41E+05	1.85E+05	8.25E+04	7.44E+04	9.32E+04	9.80E+04	7.72E+04	N/A	N/A	N/A
08C015	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	9.89E+02	4.44E+07	1.25E+07	1.15E+06	3.09E+05	5.87E+05	1.49E+05	N/A	N/A
08C045	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	1.07E+03	1.45E+04	4.25E+03	5.88E+03	5.94E+02	1.30E+02	Undet	N/A
08C001	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	N/A	N/A	N/A	N/A	N/A	N/A
08C004	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	N/A	N/A	N/A	N/A	N/A	N/A
08C009	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	N/A	N/A	N/A	N/A	N/A	N/A
08C020	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	N/A	N/A	N/A	N/A	N/A	N/A
08D035	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	N/A	N/A	N/A	N/A	N/A	N/A
09C042	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	N/A	N/A	N/A	N/A	N/A	N/A
09D311	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	N/A	N/A	N/A	N/A	N/A	N/A
SAdV-7 vx																			
08C026	Undet	Undet	9.81E+02	9.34E+05	9.72E+04	5.20E+04	2.01E+04	5.97E+03	6.64E+02	3.57E+02	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
08C046	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	3.96E+02	3.48E+06	1.25E+06	1.07E+05	4.15E+04	1.54E+04	8.35E+03	5.13E+03	5.28E+03	N/A	N/A
08C008	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	3.84E+06	9.53E+06	9.38E+05	3.74E+05	2.83E+05	2.37E+05	3.44E+05	2.24E+05	N/A	N/A	N/A
08C033	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	2.11E+04	8.60E+06	7.66E+06	3.87E+05	5.67E+04	2.39E+04	1.00E+04	2.16E+04
08C025	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	1.22E+04	4.95E+06	1.33E+06	1.98E+05	1.52E+04	1.57E+04	N/A
08C006	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	N/A	N/A	N/A	N/A	N/A	N/A
08C014	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	N/A	N/A	N/A	N/A	N/A	N/A
08C018	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	N/A	N/A	N/A	N/A	N/A	N/A
08C032	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	N/A	N/A	N/A	N/A	N/A	N/A
08P047	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	N/A	N/A	N/A	N/A	N/A	N/A

Supplemental Table 3.3. SIV viral loads in rhesus macaques (RMs). Numerical presentation of data shown in Figure 5. Three placebo-vaccinated RMs and five SAdV-7-vaccinated RMs became SIV+ and were sacrificed when viral loads started to reach set-point. Dose causing infection is administered 1 week prior to first detectable viral load. Undet = undetectable, assay sensitivity is 60 copies/mL.

Discussion

Numerous follow-up studies have aimed to understand the mechanisms of HAdV-5-based vector vaccination clinical trial failures, without any definitive answer. Some of these studies have attempted to elucidate the outcome of the Step Trial through examination of the peripheral blood only [231,232,306], leading to subsequent reports citing the value of both peripheral blood and gastrointestinal mucosa analyses. Our study was designed to clarify if increased frequencies of AdV-specific CD4+ T cells and CD4+ T cell activation levels in the gut mucosa after AdV-based vector vaccination that were evident in our previous study would lead to increased SIV susceptibility after challenge. We saw a trend of increased SIV acquisition after intra-rectal low-dose challenges in SAdV-7-vaccinated macaques (5/10) compared to placebo-vaccinated macaques (3/10) ($p < 0.2$), but we cannot at this time attribute this trend to mucosal CD4+ T cell activation or AdV-specific T cell responses. With a small sample size of ten macaques per group, and only one post-vaccination sample collection time point, we believe that additional exploration is necessary to determine whether AdV vectored vaccination results in increased SIV infection susceptibility.

The clear effects of AdV vectored vaccination we were able to show in our previous study, which displayed significant increases after vaccination relative to baseline in both AdV-specific CD4+ T cells and activated CD4+ T cells in the rectal mucosa, were not evident in our results here. Although we did verify that all the SAdV-7 vector vaccinated macaques (except #08C006) did seroconvert by week 2 post-vaccination (data not shown), macaques, like humans, show substantial variation, and this can be seen in immunological responses. One caveat of this study is that the genetic diversity (including MHC class I alleles, TRIM5α) can make smaller studies, such

as these, difficult to perform. Additionally, these animals came from a different source and with different housing conditions than our previous study, both of which may be a factor in the baseline and post-vaccination responses. We saw higher baseline levels of AdV-specific CD4+ memory T cell responses, albeit insignificantly, than previously. Also, at week 2 post-vaccination, none of the macaques had AdV-specific CD4+ T cell responses above ~2%, whereas in our previous study, 5 macaques had elevated AdV-specific CD4+ T cell responses in the rLPL. AdV-specific CD4+ memory T cells reached statistically significant increases only starting at week 5 post-vaccination in our last study, so it is possible that if we were able to wait longer to assess these responses, we may have seen changes here as well. Further, the percent of total activated CD4+ memory T cells at baseline in the rectal mucosa was substantially higher in the current study (~15-60% vs. ~10-30% previously), although levels of activation in the peripheral blood were similar. It is unclear what the cause of this large difference is, but it is possible that macaques in this study were exposed to more antigens at baseline or had a substantially different gut microbiome leading to differential antigen-induced CD4+ T cell activation. A larger study may have been able to produce a statistically significance outcome, rather than a trend towards SIV acquisition, as it would have balanced out genotypes and immune responses.

There are a few main factors that influenced the design of this study and are important to consider with regards to the data. The primary focus of our study design was to synchronize the SIV challenges with the peak of SAdV-7 vector vaccination-induced mucosal CD4+ T cell activation, which (from our first study) occurred at approximately 2-5 weeks post-vaccination, and gradually subsided by week 16. We hypothesized that exposure to SIV during this time period would lead to increased SIV acquisition in the SAdV-7 vector vaccinated group relative to the placebo group. As

such, we were only able to obtain a single post-vaccination week 2 rectal biopsy to assess vaccine-induced effects in order to provide a long enough time for the rectal mucosa to heal prior to SIV challenge at week 5. Based on our data before where several macaques showed peaks of activation after week 2 [309], we recognized that immunological dynamics might be slightly different in this group of macaques and we could miss sampling the rectal mucosa activation peak. Finally, we were limited by relatively small groups (20 macaques split evenly into vaccine and placebo recipients), which limited our statistical power. While we saw a trend towards increased SIV acquisition in the SAdV-7 vector vaccinated group, this was unfortunately not enough to reach statistical significance. The conclusion from the Step Study that the Merck HAdV-5 HIV-1 subtype B gag/pol/nef vaccine may have increased HIV acquisition was based on 49/914 male vaccine recipients versus 33/922 male placebo recipients acquiring HIV [221]. Therefore, this is a difference of 16 infections in 1,836 men (0.87%) between vaccine and placebo groups – a relatively marginal increase in HIV acquisition, but significant due to the large size of the study population.

Unifying the HIV vaccine community towards an appropriate non-human primate model to illustrate immunological and virological outcomes from clinical efficacy trials using AdV-based vectors and other vector-based HIV vaccines will be critically important as the field progresses forward. The failure of the Step Trial, Phambili, and HVTN-505 were disappointing, but were fairly consistent with previous SIV vaccine studies using HAdV-5 vectors [188,215,216]. Although the mechanism for the failure of the Step Study has yet to (and may never) be resolved, the developmental path of any HIV vaccine should thoughtfully employ the NHP model in such a way to directly assess potential vaccine enhancement effects at relevant challenge sites. The studies we have described here provide an initial strategy upon which future studies can build to improve our ability to

properly assess potential detrimental effects of suboptimal HIV vaccine strategies.

CHAPTER 4

Discussion

Significance

Although HIV was found to be the causative agent of AIDS over 25 years ago, there is still no effective cure. There have been numerous vaccine trials, but HIV treatment still relies on a combination of three or more medications (to decrease the likelihood of HIV developing resistance) called highly active antiretroviral therapy (HAART). There are several classes of drugs that target different stages of the HIV life cycle via inhibition of HIV enzymes, leading to suppressed viral burden and thus improved morbidity and mortality. HAART consists of a “cocktail” of medicines that include nucleoside/nucleotide and non-nucleoside reverse transcriptase inhibitors, protease inhibitors, entry inhibitors, and integrase inhibitors. Although HAART has allowed HIV-infected individuals to live decades more than before the advent of these drugs, there are still difficulties with high cost, availability, side effects, drug adherence and the potential for drug-resistant viruses. For these reasons, research has continued to search for a vaccine that affords reliable protection and/or is able to eliminate HIV from infected individuals.

Strategies for a vaccine have ranged from the classically empirical, such as the first HIV vaccine candidate in 1987, to increasingly refined approaches utilizing structural biology and virally vectored vaccines. Nearly 100 clinical vaccine trials have taken place, many of them first tested in a NHP model and challenged with SIV or SHIV (chimeric SIV/HIV). Vaccination and subsequent challenge of NHPs has aimed to elucidate the immunogenicity and efficacy of vaccines with the goal of inducing either T cells or antibodies, or both. The NHP challenge model is continuously undergoing evolution and

refinement, in order to more effectively simulate the outcome in humans. What started as a field that challenged NHPs with large doses of cloned viruses, is now more appropriately also using lower-dose, swarm-based mucosal challenges – closer to reflecting human exposure during transmission. Nonetheless, the "ideal" HIV model is still disputed, and it is possible that this debate will continue until clear correlates of protection for HIV emerge.

There are several benefits of the NHP model over other small-animal models for studying HIV. SIV/SHIV infection in macaques closely resembles HIV in humans based on the likeness in: susceptible cell types to infection, depletion of CD4+ T cells with a particularly rapid decline in the gastrointestinal mucosa, and the progression to AIDS [109,312]. Anatomically and physiologically, macaques and humans are similar, most importantly at the sites of SIV/HIV infection and dissemination. Furthermore, due the close phylogeny, many of the genes that control HIV immune responses are either directly orthologous or, often, at least very similar [reviewed in [104]]. For these reasons, using an NHP model to test HIV vaccines offers an opportunity to reveal, with a fair degree of predictable accuracy, whether the vaccines will succeed.

In this dissertation, our goal was to understand the virological and immunological mechanisms that led to the failure of the Step Trial using a rhesus macaque model. We hypothesized that immunizing macaques with a species-specific SAdV-7 vectored vaccine would lead to reactivation of cross-reactive T cell responses, as well as nAb responses in RMs that had been previously exposed to SAdV-7. AdV-specific CD4+ memory T cells already present in the gut mucosa are activated, as well as activation of AdV-specific CD4+ T cells in the peripheral blood which traffic to the original mucosal site of infection (due to AdV fecal-oral route of transmission). The establishment of increased activated CD4+ T cells in the mucosa, the preferred targets of SIV, resulting

from AdV-vectored vaccination would create a favorable dynamic for a greater likelihood of SIV acquisition. We vaccinated using both a human (HAdV-5) and simian (SAdV-7) adenovirus-based vector, intra-rectally challenged macaques with low doses of the swarm-based SIVmac251 virus, and obtained peripheral blood mononuclear cells and rectal lamina propria T lymphocytes. We believe that the combination of methods we have employed is as close to "ideal" as we are able to come, currently, in the NHP model to understand the Step Study and other virally vectored HIV vaccines. I argue that when investigating a mucosally transmitted viral vector, such as adenovirus, in combination with a mucosally transmitted pathogen, such as HIV, analysis of samples from mucosal, as well as peripheral blood, compartments are critical to predict and understand the vaccine's mechanisms of action. Our successful development of a system to model AdV vectored vaccination followed by low-dose intra-rectal challenge, which trends towards CD4+ T cell activation-induced SIV acquisition in rhesus macaques, can be further modified and improved upon in future studies.

Understanding the consequences of mucosal CD4+ T cell activation after AdV vectored vaccination

After the Step Trial revealed an enhanced susceptibility to HIV infection in HAdV-5 vector vaccinated individuals that were uncircumcised and HAdV-5 seropositive at baseline [221], studies began to investigate the mechanism for this unfortunate outcome. Post-hoc analyses focused on examination of peripheral blood from Step Study participants, leading to results that, while valid based on PBMC analyses, were not necessarily indicative of gastrointestinal mucosal cell phenotype and functionality. Previous work by Hutnick et al. from our lab has investigated PBMC samples from the Step Study. It was shown that following HAdV-5 vector vaccination, both CD4+ and

CD8+ AdV-specific T cells from PBMCs transiently expand, but there was no durable change in polyfunctionality, phenotype or homing marker expression [306]. Subjects who were either HAdV-5 seropositive or seronegative at baseline, prior to HAdV-5 vector immunization, had similar baseline and post-vaccination HAdV-5-specific CD4+ T cell responses [231]. This suggested, from peripheral blood analyses, that HAdV-5-specific CD4+ T cells did not lead to the increased HIV susceptibility seen in HAdV-5 baseline seropositive Step Trial participants. These findings are valuable, but the work presented in this dissertation further delves into CD4+ T cell phenotypes in the rectal mucosa – the site of HIV infection and dissemination in the Step Study.

It was not possible to obtain rectal biopsies from the Step Trial, since they were not collected [221]; therefore using the macaque model to replicate the AdV-based vector prime-boost-boost regimen was the best alternative. The main concern to address was the presence of pre-existing HAdV-5 neutralizing antibodies (from baseline seropositive individuals), which can limit vaccine efficacy by targeting and eliminating the AdV vector as well as transgene products [313]. Approximately half of the adult US population is HAdV-5 seropositive, yet outside of the US, >90% of individuals have pre-existing nAb [247]. In order to successfully model the Step Study in rhesus macaques and determine if pre-existing nAb responses were a factor in the outcome, we developed a vector using macaque-specific simian adenovirus type 7 (SAdV-7) for immunization [310]. Since nAb are serotype-specific, we believed it was appropriate to use a macaque-specific AdV-based vector in a macaque analogously to the way that a human-specific AdV-based vector was used in humans in the Step Trial.

After baseline PBMC and rLPL samples were collected, we challenged macaques with either SAdV-7 or HAdV-5 three times (day 0, week 17, week 31), and obtained samples at ten time points throughout the immunization period. Examination of

total CD4+ T memory cell activation (based on CD25, CD69, HLA-DR and Ki67) in the rLPL revealed statistically significant increases in both HAdV-5 and SAdV-7 vector vaccinated macaques, and AdV-specific CD4+ memory T cell percentage in the rLPL was statistically increased in SAdV-7 vector vaccinated macaques. This implied that using seroprevalence to determine the presence of T cell responses was not appropriate. Previous studies have shown that AdV-specific T cells are cross-reactive [202,314], even in subjects that have never been exposed to the vector serotype used for immunization. AdV-specific CD4+ memory T cells can either traffic from the blood to the mucosa, or cells already present in the mucosa can become reactivated [203,234]. We also did not find a correlation between baseline SAdV-7 nAb responses with any of the CD4+ T memory phenotypes we were investigating (as expected, HAdV-5 nAb responses were uniformly not present). Taken together, these results indicated that AdV-based vector vaccination, whether using SAdV-7 or HAdV-5 vector, increased the percent of AdV-specific and activated CD4+ memory T cells in the rectal mucosa, but not in the blood, and can thus establish a scenario of an increased risk of SIV/HIV acquisition due to the greater availability of target cells at the site of infection. We used this outcome to move forward with a proof-of-concept SIVmac251 intra-rectal low-dose challenge study after SAdV-7-based vector vaccination.

Our AdV-based vector vaccination followed by SIVmac251 challenge study was only designed to investigate the effects of SAdV-7 as a vector. We believe, though, that the AdV-specific T cell cross-reactivity previously seen with HAdV-5 vector vaccination in macaques may imply that these results will translate to rare vectors that are in development as well. Our goal was to first titrate SIVmac251 to a sufficiently low-dose such that any SIV acquisition that occurred after SAdV-7-based vector vaccination could be attributed to rectal mucosal CD4+ memory T cell activation-induced susceptibility.

This titration study was unique in that it rigorously tested 15 macaques with an intra-rectal low-dose SIV to determine activation-induced SIV susceptibility. We are unaware of any previous studies that have demonstrated low-dose SIVmac251 infection with such a dependable quantity of animals upon which to base future challenge experiments. Unfortunately, in the challenge study that followed, after we collected PBMC and rLPL at baseline and week 2 post-vaccination, neither the vaccine or placebo groups showed evidence of a significant change in AdV-specific CD4+ memory T cells or CD4+ memory T cell activation in the peripheral blood or rectal mucosa. We did show evidence for a trend towards SIV acquisition in the SAdV-7-based vector vaccine group, and recognize that the small size of the study was a limiting factor. Another obstacle was weighing the cost of obtaining a single week 2 post-vaccination rectal biopsy sample versus the benefit of initiating weekly SIV intra-rectal challenges earlier within the predicted window of increased CD4+ memory T cell activation in the rLPL. It is certainly possible that with an increased number of RMs, a similarly designed study may be able to reach statistical significance, although determining a more optimal SIV challenge and rLPL sample collection timeline may be worth investigating.

As I previously alluded to in this thesis, macaques are not without faults and the outcome of studies are not always foreseeable. There is substantial variation between macaques, as there are in humans, and this variation can be seen in SIV infection. This is a positive attribute in the sense that the differences seen in humans worldwide will be modeled in RM experiments, but the genetic diversity (including MHC class I alleles) can make smaller studies difficult to perform. The MHC genes in RMs are highly polymorphic and encode molecules that present virus-derived peptides on the surface of infected cells for T cell recognition. These MHC polymorphisms can profoundly influence the ability of the immune system to control SIV, since some MHC class I alleles are

associated with containment of viral replication [212,315,316]. This is one caveat of our SIV challenge study, which had one vaccine and one placebo group of 10 macaques each. These RMs were not evaluated for the presence of protective MHC class I alleles between placebo and control groups, and thus the protective effects of these alleles may have been overrepresented in one of the groups and could confound our interpretation. Additionally, these animals came from a different source and were housed in a different facility than our first SAdV-7-based vector vaccination study. Thus, the differences that we saw in CD4+ T cell activation status and AdV-specific CD4+ T cell responses between our two studies could be a consequence of this animal-to-animal variation. A larger study may have been able to produce a statistically significance outcome, rather than a trend towards SIV acquisition, as it would have balanced out genotypes and immune responses.

Concurrent with our the ongoing search for a mechanism to explain the failure of the Step Study, AdV-based vector vaccines for HIV are still continuing to be developed. AdV-specific T cell cross-reactivity is important to consider as the field of AdV vaccines moves forward with pre-clinical and Phase I trials using vectors based on rare AdV serotypes with low seroprevalence. Our lab has shown that AdV-reactive T cells have an effector and effector memory-like phenotype, indicating that these CTLs produce functions that make them particularly efficient at eliminating vector-infected cells [202]. The limitations of pre-existing nAb should hypothetically be avoided in rare AdV serotypes, but cross-reactive AdV-specific CD8+ T cells will still likely hinder transgene and vector responses. Furthermore, using heterologous AdV serotypes during a series of vaccinations will similarly be susceptible to targeting by cross-reactive AdV-specific CD8+ T cells.

Future directions

A few years after HIV was confirmed as the cause of AIDS, the US Health and Human Services Secretary promised an HIV vaccine within two years, based on recent successes in the vaccine field at the time. Unfortunately, HIV's rapid ability to escape from neutralizing antibodies, establishment of a latent viral reservoir, and other biological aspects of HIV, have illustrated that the traditional vaccine approach will not be sufficient. Starting from the first Phase I HIV vaccine trial in 1987 using a gp160 subunit vaccine to the most recent halting of HVTN 505, due to a lack of efficacy [225], many HIV trials have failed (these are more thoroughly reviewed in the Introduction). Throughout these past 25 years, though, NHP models have been used as the basis for preliminary results and largely with predictable efficacy. What have we learned from these clinical trials? First, a vaccine should optimally combine a strong induced T cell response, together with breadth, activation, and sufficient duration. Ideally, T and B cell responses should each effectively target vulnerable parts of HIV previously shown to be useful (i.e. CD8+ T cells specific to Gag p24 rather than towards Env). Also, for HVTN 505, the difference in HIV acquisition between vaccine and control groups was not significant [225]. This may indicate that when removing the risk factors seen in the Step Study (seropositive HAdV-5 baseline status and uncircumcised individuals), there might still be a chance that the strong immune responses AdV vectors induce may be beneficial when using rare AdV serotypes. Of note, the SIVmac251 challenge model tested in NHPs prior to HVTN 505 predicted a failure to prevent SIV acquisition and any control was modest and short-lived [317-319]. Finally, HAdV-5 vectored vaccines were not originally designed to induce virus-neutralizing antibodies. Induction of broadly-neutralizing antibodies is a difficult task, but may be a direction future vaccine trials can aim for in combination with

effective T cell responses.

Of the six large-scale HIV-1 vaccine trials, RV144 was the only one to show decreased transmission in the vaccine relative to the placebo group, with a modified intent to treat (mITT) efficacy of 31%. What was it about this vaccine trial compared to the others that led to this protection and what can we learn going forward in future HIV-1 vaccine studies? First, there were two humoral immune measurements that correlated with HIV-1 infection risk. IgG antibodies to the V1/V2 region of HIV-1 gp120 correlated with a decreased HIV-1 infection risk, and plasma HIV-1 Env-specific IgA score correlated with an increased risk of infection in the vaccine arm. Further studies also indicated that the vaccine-induced immune response in RV144 may have been due to the breadth of the V1/V2-specific response, as it cross-reacted to multiple HIV-1 clades. Also, within the factors that correlated with HIV infection risk were other humoral responses and host genetics (including both HLA Class I and II alleles), indicating that study of these factors may provide clues for potential mechanisms of vaccine efficacy. In addition, sieve analysis has delineated a site in the V2 region of Env that was a critical site of immune pressure. If the HIV strain the individual was infected with matched this site on the vaccine, then vaccine efficacy jumped up to 48%. Thus, future trials could include additional Envs or differing V2 sequences to enhance protection. Tomaras and Haynes believe that if multiple antibody specificities and anti-viral functions are evaluated, and combined with identifying correlates of HIV infection risk, then an efficacious vaccine strategy may be determined. Finally, the RV144 vaccine had a 60.5% efficacy 12 months post-vaccination, which declined to 31.2% at 42 months post-vaccination. It may be that the antibody response or cellular immune response was of a higher level or quality initially and waned over time. Future vaccine strategies should attempt to increase their durability with certain adjuvants in an attempt to maintain

efficacy[320].

The Step Trial has been called a failure numerous times due to the increased risk of HIV acquisition in the vaccine group [221], but post-hoc studies directly resulting from the Step Trial, such as the ones presented in this thesis and others, have moved scientific knowledge forward. In this sense, future vaccine studies for mucosally transmitted pathogens and mucosally targeted vaccine vectors are now better aware of the value of examining different anatomical compartments. The unique phenotypic combination of the mucosal surface as the site of HIV transmission, the greatest and most rapid depletion of CD4+ T cells due to the high availability of activated CCR5+ CD4+ T cells for viral replication, and structural disruption of the mucosal architecture, all advocate for careful investigation of this site [reviewed in [104]]. An effective HIV vaccine would likely have to stop viral replication between the small window of mucosal transmission and dissemination of HIV infection throughout the body. A vector-based vaccine, or any other modality, may need to induce a high frequency of HIV-specific immune responses locally in the mucosa, in addition to, or instead of, just systemically. Future work will probably have to determine the duration of this window and induce highly effective local responses, which also necessitates establishing correlates of protection for HIV (with a range of factors including cell functionality, differentiation status, migration patterns, proliferative potential, etc.). These responses certainly cannot be evaluated solely from the peripheral blood, although researchers are unlikely to have access to mucosal samples from all participants in a large-scale human vaccine trial. Therefore, the value of developing a dependable NHP model for HIV (and other vaccines) is of utmost importance.

In addition to the rectal mucosa, vaginal and penile mucosal tissues are important sites of HIV transmission to investigate in future studies. The unexpected

outcome of the Step Trial led to the realization that mucosal sampling and specimen analysis could help to explain the lack of efficacy, and also enhancement of HIV susceptibility. The foreskin is the principal site of heterosexual HIV-1 infection in men[321] but there is little known about HIV-1-specific immune responses in foreskin. Specifically, exploring the effects of vaccination on the induction or phenotype of SIV/HIV-specific immune responses in foreskin has not been extensively performed. Like we show in the rectal mucosa in this dissertation, CD4+ T cells in foreskin in humans are highly activated, potentially contributing to the risk of HIV-1 infection by increased HIV-1 target cells[322]. Other studies have shown that in rhesus macaques, vaccination with AdV26 vectored SIV vaccines elicited robust and durable SIV-specific CD4+ and CD8+ T cell responses of a largely transitional memory or effector memory phenotype at gastrointestinal and cervicovaginal mucosal sites[323]. Further, a recent study by Balandya and colleagues demonstrated the induction of SIV-specific cellular immune responses in foreskin of AdV26 and AdV35 vector vaccinated macaques, showing that foreskin T cells were more activated than peripheral blood T cells[324]. In sum, though, it still remains unclear whether rectal, vaginal, or penile mucosal tissue T cell activation or other immune responses at baseline or after AdV vectored vaccination may affect SIV/HIV infection, and therefore further research into these areas is certainly warranted.

As a whole, HIV vaccine studies have, until recently, been restricted to use of either a single or related vaccine vectors, such as AdV vectors derived from different serotypes, or different poxvirus vectors, or even combinations with DNA vaccines. Combining these different platforms in novel varieties may be useful to induce desired HIV responses. Research has even moved towards use of replication-competent viral vectors (as opposed to the overwhelmingly predominant use of replication-incompetent AdV vectors thus far), which would preferably include some features of live-attenuated

viral vaccines. These include adequate replicative capacity to generate a mild infection to induce pathogen recognition, provide prolonged exposure to antigens, and employ the adaptive immune system. For adenovirus, which naturally infects mucosal surfaces, the maintenance of its replicative capacity is preferential and well suited for HIV vaccine strategies, but also may risk establishing persistence and potential vaccine transmission [reviewed in [325]].

I previously mentioned the use of AdV vectors for malaria, hepatitis C and tuberculosis vaccines. These diseases can occur at high rates in HIV endemic areas, therefore care should be taken so as to not potentially induce increased risk of HIV acquisition due to AdV vectored vaccination for another pathogen, especially since there is still no definite clarity as to why the Step Trial failed. Recently, the Bill & Melinda Gates Foundation provided a large grant for the development of vaccines against tuberculosis, HIV, and malaria, to enable the large-scale production of multiple novel chimpanzee adenovirus vector constructs. Since AdV vectors of various serotypes have been shown to differ with respect to their subtype, seroprevalence, cell-binding receptors, tropism, as well as inducing different gene expression profiles and T cell and antibody phenotypic functionality, this has provided validation for some researchers to continue to evaluate rare serotype AdV vectors. While one cannot rule out this possibility, cross-reactive T cell responses, even to rare AdV vectors, may hinder this progress. Notably, plans for a large efficacy trial of an AdV35-based tuberculosis vaccine candidate – Crucell AdV35/AERAS-402 – have been significantly scaled back due to the observation that the approach is less immunogenic than preliminary results had indicated. It seems feasible, though, that if tuberculosis, malaria, hepatitis C and other potential AdV vectored vaccines are geared towards immunization in children, then risk for HIV infection in that age bracket might not be applicable, assuming that this risk

would wane over time. In summary, whether AdV vectors might enhance HIV acquisition risk in some individuals is not yet completely discerned, and more work is needed to understand how AdV vectored immunization for any pathogen affects interactions between AdV-specific CD4+ T cells and persistent adenovirus infections at mucosal sites of HIV exposure.

Besides AdV vectors, another very promising vector is cytomegalovirus (CMV), which persists indefinitely after infection since it is a member of the herpesvirus family. This persistence leads to constant, low-level immune stimulation that results in a mass of effector T cells. Picker et al. have pioneered the use of a rhesus CMV (RhCMV) vector for use in a multivalent RhCMV-SIV vaccine that covers much of the SIVmac239 proteome. When tested in CMV-seropositive RMs, pre-existing CMV infection did not affect the immunogenicity of the vector; a significant bonus compared to AdV-based vectors. Studies are ongoing and expected to continue into human trials due to their success thus far, as Picker's group has shown protection from SIVmac239 infection in 50% of RMs with early and durable control [128,300]. Although going in this direction is hopeful, there are a few challenges that remain with use of this CMV vector. First, studies thus far have used RhCMV, which, like all CMV vectors, is species-specific. Therefore, human CMV vectors will need to be developed that exhibit comparable results. Second, CMV is a pathogen as well, and currently even has vaccine strategies geared towards it. While it is generally nonpathogenic in humans, those who are pregnant or immunocompromised may still be exhibit a significant risk. Further CMV vector advances in humans will likely necessitate genetic modifications, including a reduced ability to spread, limited vector tropism, or the option of vector elimination for any adverse outcomes (as CMV is persistent). Finally, it will be interesting to definitively determine whether SIV control is governed by CD8+ T cell targeting of unconventional

epitopes. Why 50% of challenged of challenged macaques did not show evidence of viral load reduction is still unclear, and hopefully future studies will elucidate that question[326].

Regardless of which vaccine platform will prove to thrive, its design will need to improve upon the magnitude and breadth seen from vaccine trial immune responses thus far. Most optimally, this vaccine will be able to induce both broadly neutralizing antibodies as well as cytotoxic CD8+ T cell responses, while finding a suitable balance for CD4+ memory T cell activation levels. The most effective NHP model for HIV would mimic the salient features of initial viral infection and dissemination. Although a perfectly parallel system is not feasible, one alternative for future SIV challenge experiments is evaluating the dose which humans are exposed to during sexual HIV transmission and accurately determining that dose for different SIV challenge viruses. In our titration study, we began with 1:5000 ($4TCID_{50}$), which led to two RM infections, and we also had one RM infected in our challenge study at 1:10,000 ($2TCID_{50}$). During the challenge study, we escalated the dose ½ a log every 3 weeks until infection, since we did not want to potentially miss an activation-induced SIV susceptibility window. Additionally, we were not completely certain on what dose would truly elucidate a difference in SIV susceptibility, since our titration was the only previous study we could trust with an adequate sample size. If, in future challenge studies, a certain dose of SIV challenge stock is established to be analogous to the dose of typical sexual HIV exposure, this dose can be used consistently instead of dose-escalation. Notably, the macaque infected at the lowest dose of $2TCID_{50}$ during our challenge study belonged to the SAdV-7 vaccine group. It would be interesting to see if a difference in activation-induced SIV susceptibility would occur in a similarly designed study, but using the same SIV challenge dose.

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