INTERACTIONS WITH INNATE IMMUNITY DETERMINE THE IMMUNOGENICITY OF ADENOVIRUS VECTORS TYPES 5, 28, AND 35: IMPLICATIONS FOR VACCINE DESIGN

A dissertation
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By

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Recombinant adenovirus vectors (rAd) have proven to be very effective at inducing antigen-specific, polyfunctional T cell responses. Recombinant adenovirus serotype 5 (rAd5)-based vectors have been extensively studied as potential HIV/AIDS vaccines and tested in phase I and phase II clinical trials. The results of these trials, in conjunction with studies in rhesus macaques, have revealed that pre-existing immunity against the rAd5 vector can reduce the immunogenicity of the vaccine and limit the memory response to the HIV-antigen insert. Since 40-80% of the world’s population is seropositive for Ad5, the usefulness of a rAd5-based vaccine may be compromised. To circumvent preexisting immunity, alternative adenovirus vectors from serotypes with much lower seroprevalence, such as Ad28 and Ad35, are under development. However, some vectors constructed from low-seroprevalence adenoviruses have shown poor immunogenicity in vivo. This presents a paradox whereby rAd5, which induces a good immune response, is limited due to widespread preexisting immunity while rAd28 and rAd35, to which there is low pre-existing immunity, are inherently less immunogenic.

My results show that, in stark contrast to rAd28 and rAd35, rAd5 fails to induce significant changes in DC mRNA expression or maturation. These changes are independent of infectivity
and primarily involve the activation of innate immunity, particularly type I interferon signaling. The production of type I IFN and the activation or innate immunity by rAd28 and rAd35, but not rAd5 correlates well with the loss of the insert expression due to upregulated apoptosis of insert positive cells.

This paradigm was consistent in mouse DCs, with rAd28 and rAd35, but not rAd5 inducing significant IFNα production and that intact IFNα signaling reduces vector expression during vaccination. The induction of type I interferon in mice vaccinated with rAd28 or rAd35 lead to a decrease in the magnitude, but an improvement in the long-term memory potential (CD127 expression) and cytokine polyfunctionality of the insert-specific CD8 T cell response. The presence and impact of innate immune activation, type I interferon signaling, and insert loss should therefore be taken into account when designing future rAd vectors.
This dissertation will be divided into four chapters. The first chapter will introduce terms and concepts related to immunology and vaccination that may not be familiar to readers outside the specific subject area. It will conclude by summarizing and explaining current paradigms within the fields of vaccination in general, and HIV vaccination in particular. The second chapter will present original research regarding the use of rare serotype recombinant adenovirus vectors as platforms for potential HIV vaccine. I will compare the interactions of rAd5, rAd28, and rAd35 vaccine vectors with cells of the innate immune system and explain how these interactions impact the development of T cell memory in response to each vector. The third chapter will present original research from a study examining the use of an adenoviral vector vaccine in rhesus macaques that were challenged with SIV. This study involved a large multisite collaboration between several institutes with several broad aims. I will therefore focus primarily on my contributions to the study, focusing on the correlates of protection associated with load viral load after breakthrough infection, and summarizing the study’s other findings. The fourth chapter will discuss the broader implications of this work on vaccine development and outline future avenues of research.
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CHAPTER 1

1 Introduction

1.1 History of Vaccination

Traditional vaccination strategies against viruses involve the use of either live but attenuated strains of the virus or physically or chemically inactivated versions of the virus to stimulate protective memory immunity without exposing the individual to the harmful effects of a natural pathogenic infection (1, 2). The very first vaccines were constructed from related, but avirulent strains, of viruses. More recently, researchers have grown wildtype viruses in tissue culture for many passages or specifically altered the viral genome using molecular techniques, to yield a mutated, significantly less pathogenic version of the virus, often missing large sections of genome (3, 4). These mutated viruses have the advantage of producing a similar infection and immune response to the wildtype virus, but without harmful clinical symptoms (5). One potential concern with this method is that vaccines constructed from attenuated strains have the potential to revert back to wildtype virus and cause disease (6). Vaccines constructed from inactivated wildtype virus avoid problems with reversion and are generally easy to produce, however the inactivation process generally destroys all of the metabolic and infective properties of the virus and generally leaves the vaccine far less immunogenic (7, 8). With inactivated vaccines high doses, adjuvants, and multiple exposures are often required to prime and then boost a protective immune response (9, 10).
1.2 Emergence of HIV and need for new mechanisms of vaccination

Human immunodeficiency virus (HIV) emerged as a serious global health issue during the late 1980s and early 1990s (11). From its discovery in 1981 to 2006 acquired immunodeficiency syndrome (AIDS), resulting from late stage HIV infection, has killed over 25 million people (12). Currently an estimated 34 million people are infected with HIV and another 2.7 million become infected each year (13). Highly active antiretroviral therapy (HAART) has been shown to reduce the viral load of infected individuals and significantly delay progression to AIDS, but treatment is expensive and, given HIV prevalence in poorer areas, difficult for many to afford (14-16). Education and counseling has been partially successful in reducing the spread of HIV, but their effectiveness is incomplete (17-20).

An effective HIV vaccine would be able to further reduce the spread of HIV, limit viral load and disease once infected, and be inexpensive enough to be use in poorer endemic areas. Early attempts at developing HIV vaccines using heat-killed or chemically inactivated virus were not immunogenic (21). Potential live-attenuated HIV vaccines pose serious risks. In addition to the risk of reversion associated with all live-attenuated vaccines, a live-attenuated HIV vaccine would be at risk of recombining with wildtype virus (22, 23). HIV’s retroviral lifecycle means that any live attenuated vaccine will incorporate into the host genome and will not be cleared completely from the host (24). This allows live-attenuated vaccine and wildtype virus to interact within the host for
years, and potentially to recombine, potentially leading to more aggressive and pathogenic disease (23).

Alternative approaches to vaccination against HIV have been proposed. One approach, subcomponent vaccines, would use structural components of HIV to produce non-infectious virus like particles (VLPs) to generate an immune response, similar to the human papillomavirus (HPV) vaccines (25, 26). While using individual HIV proteins as part of a prime/boost strategy has shown promise, the construction of strongly immunogenic VLPs has proven more difficult (27, 28). Another possible approach to vaccination is the use of DNA vaccines, where DNA encoding specific HIV proteins is inject directly into the host (29, 30). This method has shown promise as a prime (as part of prime/boost strategies) but functions poorly as a boost (31-34). A third potential approach, the use of viral vectors to deliver antigen into host cells, is the focus of this dissertation.

1.3 Viral vector vaccines
Viruses have the unique ability to transmit genetic information into a select set of target cell types in vivo and in vitro (35). Researchers have taken advantage of this unique characteristic to design nonpathogenic viral vaccine vectors to transfect nucleic acids encoding antigen proteins from an unrelated pathogenic virus (36). These vaccines allow the host to develop memory immunity to antigen from the pathogenic virus without risk of pathogenic infection. These vectors have the additional benefit of delivering the antigenic protein intracellularly, similar to a natural infection (35). Certain vectors act as
their own adjuvant, stimulating innate immunity and upregulating costimulatory molecules, potentially increasing immunogenicity (35, 37).

Many vaccines vectors are replication-incompetent, able to undergo a single round of infection, but unable replicate to their genome, produce progeny, or infect any other cells after the initial infection (38). Many of these vectors are also transcription and translation-deficient, unable to produce any genes in the host other than the antigen insert (38). While this improves the safety of the these vectors, the fact that they only allow a single round of infection limits the magnitude and duration of the antigen exposure to the immune system, which may limit the immunogenicity of these vectors (39).

Alternatively, several vaccine vectors are replication-competent, meaning they can replicate within the host (39). Notably these vaccines express vector–specific proteins necessary for replication so they may function better as a boost, in a prime/boost vaccination strategy, because the host immune response may primarily target the vector proteins unless previously primed for the insert.

Vaccine vectors could potentially be constructed from almost any virus, but certain characteristics can make specific viral species more useful (38-41) (Table 1). Tissue tropism, viral lifecycle, and ease of vector construction must all be taken into consideration when selecting a base virus from which to construct a vaccine vector (42, 43). Viral tropism can impact on the immunogenicity of vectors and is partially determined by viral ligands for specific host cellular surface receptors (43-46). These
surface receptors may be expressed universally, by every host cell, or by distinct tissues or cell types. Here I explore the use of vectors constructed from the viral family Adenoviridae as platforms for an HIV vaccine.

<table>
<thead>
<tr>
<th>Viral vector</th>
<th>Type</th>
<th>Genome</th>
<th>Insert</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>Non-replicating</td>
<td>dsDNA</td>
<td>7-8kb</td>
<td>Safe, No integration, Physically and genetically stable, Targets mucosal inductive sites, Long history of use for gene therapy, Multiple serotypes, Highly immunogenic</td>
<td>Preexisting immunity</td>
</tr>
<tr>
<td>Adeno-associated virus</td>
<td>Non-replicating</td>
<td>dsDNA</td>
<td>&gt;5kb</td>
<td>Resistant to acid, Infects DCs</td>
<td>Difficult production, Possible integration</td>
</tr>
<tr>
<td>Alphavirus</td>
<td>Non-replicating</td>
<td>+ssRNA</td>
<td>8kb</td>
<td>No integration, No anti-vector immunity, Infects DCs, Highly immunogenic</td>
<td>Difficult production, Safety concerns</td>
</tr>
<tr>
<td>Herpesvirus</td>
<td>Non-replicating</td>
<td>dsDNA</td>
<td>50kb</td>
<td>Infects many cell types, Durable immunity</td>
<td>Difficult production, Preexisting immunity, Low immunogenicity</td>
</tr>
<tr>
<td>Measles virus</td>
<td>Replicating</td>
<td>-ssRNA</td>
<td>&gt;5kb</td>
<td>Durable immunity, Infects DCs, No integration, Physically stable</td>
<td>Preexisting immunity</td>
</tr>
<tr>
<td>Poxviruses</td>
<td>Non-replicating</td>
<td>dsDNA</td>
<td>10kb</td>
<td>Highly immunogenic</td>
<td></td>
</tr>
<tr>
<td>Vesicular stomatitis virus</td>
<td>Replicating</td>
<td>-ssRNA</td>
<td>&gt;5kb</td>
<td>No integration, No preexisting immunity, Safety concerns, Low immunogenicity</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Viruses commonly used to construct viral vectors and their properties. Adapted from Robert-Guroff, 2007.
2  Aims of Dissertation

The overall aim of this dissertation is to explore the use of viral vectors, specifically viral vectors constructed from adenoviruses, as platforms for vaccination against HIV or other diseases. Adenovirus-based vectors were chosen because of the large amount of preexisting research into their construction and because their mixed success in numerous human trials highlights the necessity of greater understanding of their interactions with the immune system. The focus of this dissertation is the development of an HIV vaccine, but much of the aims and information could easily be applied to vaccines against other diseases. The aims were as follows:

- To study the interactions of Ad vectors constructed from different serotypes with the innate immune system.
- To determine the impact of innate immunity on insert expression by the rAd vectors.
- To explore the impact of innate immunity against the rAd vectors on the development of immunological memory.
- To test the efficacy of a rAd vaccine in a vaccine challenge model in vivo
- To determine what characteristics of the vaccine-induced immune response are associated with protection.
3 Adenovirus

3.1 Virus and viral lifecycle

Adenovirus virions are non-enveloped icosahedral particles approximately 70-100 nm in size surrounding a dsDNA genome approximately 36kb in length (47-50). The Adenoviridae family, consisting of five distinct clades, is widespread in nature having been isolated from fish, reptiles, birds, and mammals (51, 52). Fifty-four known serotypes have been isolated from humans and are further classified into seven distinct subgroups based on genetic sequence (53-56).

The 54 serotypes of adenovirus cause a variety of human diseases (43) (Table 2). Subgroup C adenoviruses, which include adenovirus serotype 5 (Ad5), primarily cause upper respiratory tract infections (57). Other serotypes, such as Ad28, a subgroup D Ad, or Ad35 subgroup B2 Ad, can cause ocular, enteric, or urinary tract diseases depending on the strain (43, 58).
Table 2. The serotypes of Adenovirus known to infect humans and their receptor usage, tropism, and seroprevalence. Adapted from Arnberg, 2009.

Regardles of serotype all Ad virions are made up of 11 structural proteins (48, 58) (Table 3). The icosahedral capsid consists primary of hexon proteins with a penton base protein at each vertex and a fiber protein extending from each penton base (47, 48, 59).

The specific host cell surface receptor used for viral adhesion and cellular entry is large dependant on the serotype’s viral fiber protein, though several serotypes also use the hexon and penton to bind host cellular proteins (45, 60).
Table 3. The predicted number of each polypeptide in the currently accepted model of the icosahedral virion of Ad versus the experimentally observed

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>Size (kDa)</th>
<th>Measured number per virion</th>
<th>Predicted number per virion</th>
</tr>
</thead>
<tbody>
<tr>
<td>II (hexon)</td>
<td>109</td>
<td>720 ± 7 240 Trimmers</td>
<td></td>
</tr>
<tr>
<td>III (penton base)</td>
<td>63</td>
<td>56 ± 1 12 Pentamers</td>
<td></td>
</tr>
<tr>
<td>Illa</td>
<td>63</td>
<td>68 ± 2 60 Monomers</td>
<td></td>
</tr>
<tr>
<td>IV (fiber)</td>
<td>62</td>
<td>35 ± 1 12 Trimmers</td>
<td></td>
</tr>
<tr>
<td>V (core)</td>
<td>42</td>
<td>157 ± 1</td>
<td>-</td>
</tr>
<tr>
<td>Terminal protein</td>
<td>~55</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>VI</td>
<td>22</td>
<td>342 ± 4 60 Hexamers</td>
<td></td>
</tr>
<tr>
<td>VII 9 (core)</td>
<td>19</td>
<td>833 ± 19</td>
<td>-</td>
</tr>
<tr>
<td>VIII</td>
<td>15</td>
<td>127 ± 3</td>
<td>-</td>
</tr>
<tr>
<td>IX</td>
<td>14</td>
<td>247 ± 2 80 Trimers</td>
<td></td>
</tr>
<tr>
<td>μ (core)</td>
<td>~4</td>
<td>~104</td>
<td>-</td>
</tr>
</tbody>
</table>

Adenoviral genomes encode 5 early transcripts, 3 delayed transcripts, and one late transcript that is processed into 5 families of mRNA (58, 61-64) (Figure 1). The early transcripts, labeled E1A, E1B, E2, E3, and E4, are responsible for forcing the host cell into S phase, inhibiting the host innate immune system, and synthesizing the viral proteins needed for efficient viral DNA replication. Importantly E1A, initially controlled by a constitutively active promoter, is the first transcript synthesized (65, 66). E1A proteins bind host cellular proteins and modify their function to allow for transcription of the other viral genes (67-69).
E1A proteins and the E4 proteins E4orf1 and E4orf4 perform the critical task of forcing the host cell to cycle into S phase (70, 71). Host cellular proteins and other factors necessary for replication are normally sequestered or absent from the nucleus during non-S phase periods of the cell cycle, partially for host antiviral defense (72). Therefore adenovirus and other viruses contain elements that force the host cell into S phase so the host cellular machinery required for viral genome replication are available to be modified by viral proteins (73-78).

The inhibition of normal host innate immune responses is also critical for efficient adenoviral infection. The mechanisms the virus uses to accomplish this can be divided into three general categories: inhibition of apoptosis, inhibition of cellular DNA damage response, and interference with the type I interferon response. E1B-55K and E4orf6 both independently inhibit p53-dependent apoptosis and E1B-19K directly binds to the proapoptotic BCL-2 family members BAK and BAX to prevent them from initiating apoptosis (79-82). The E1B and E4 genes both prevent the activation of the cellular

---

Figure 1. Adenovirus genome and transcription map. Most late mRNAs originate at the MLP. Adapted from Stone et al, 2003.
DNA damage response pathways by inhibiting MRN complex formation (83-86). Type I interferon signaling is inhibited by E1A proteins which bind directly to STAT1 and by VAI RNA which non-productively binds to host PKR (87-91).

5 to 8 hours after initial infection, after a significant amount of early proteins have accumulated, DNA synthesis begins as part of a two step process (58, 92). Replication can begin at either end of the genome due to identical inverted terminal repeats located at both ends (93). The first step of replication begins with 5’ to 3’ DNA synthesis of a daughter strand using one strand of the genome as a template and displacing the other strand (94). New daughter strands can, in turn, displace the initial daughter allowing simultaneous replication of several daughter stands from one template. The inverted terminal repeats at ends of the displaced signal stranded DNA (ssDNA) molecules hybridize to form a panhandle molecule to begin the second stage of replication. DNA synthesis starts at one end of the DNA panhandle molecule and continues until the full double stranded copy of the genome is completed.

Late gene expression, starting soon after the initiation of DNA replication, begins through activation of the adenoviral major late promoter (MLP) (95-98). The MLP controls expression of all 5 late mRNA families (99). Theses 5 families encode all 11 structural proteins, including the major capsid proteins: penton, hexon, and fiber.

Once the virions are assembled, primarily by L1, L3, and L4 proteins, the adenovirus protease, encoded by L3, interrupts cell filament formation rendering the cells more
susceptible to lysis and the E3 11.6kd protein, also called the adenovirus death protein, accumulates and lyses the infected cell (100-107). Progeny virus are then released and are able infect new cells (108).

3.2 Use as vaccine vectors

Adenoviral vectors have long been studied as potential vectors for both gene therapy and vaccination (109-111). Most studies using adenoviral vectors have been conducted using a rAd5 EA1– vector with the inserted gene, controlled by a CMV or other constitutively active promoter, in the place of E1A (112-114). These vectors are generally considered to be replication deficient as the loss of E1A severely down-regulates the transcription of all other viral genes, though limited transcription of viral mRNA does occur in highly permissive cell lines (115, 116). In order to propagate these vectors the missing E1A proteins must be supplied in trans by the feeder cell line (117).

rAd5 vectors initially showed significant promise as vaccines because they induced strong and persistent insert-specific immunity (118-120). rAd5 vaccines have been shown to induce protective immunity in a variety of infection (121-126). Importantly, rAd5 vectors are especially useful for generating strong CD8 T cell responses.

However safety concerns have slowed rAd5 development. In 1999, during a pilot study examining the safety of rAd5 treatment for gene therapy, an 18-year-old male died following treatment with very high inoculum (6x10^{11} VP/kg) of E1E4– rAd5 vector (127). His death was determined to be due to strong activation of innate immunity
caused by the very high inoculum leading to intravascular coagulation and multiple organ system failure. This stopped all human trials involving rAd vectors for years while the death was investigated and as a result current studies now use a much lower maximum inoculum.

More recently an E1\(^{-}\)E3\(^{-}\) rAd vector expressing HIV-1 gag, pol, and nef was used in a phase IIb clinical trail, called the STEP trial, designed to test the efficacy of a rAd HIV vaccine in high-risk populations (128, 129). The vaccine, which consisted of three intra-deltoid injections of rAd5 on weeks 0, 4 and 26, was able to induce CD8 T cell responses against HIV-1 (130). However the study was stopped early when no protection against infection or reduction in viral load in individuals with breakthrough infection was observed (Figure 2). The STEP study was considered a major setback for the use of vaccine vectors and considerable effort was spent determining the mechanistic causes of the vaccine’s poor performance (131-136).
3.3 Preexisting Immunity

Posthoc analysis of the STEP trial and subsequent experiments in non-human primates (NHPs) have determined that the presence of pre-existing immunity against rAd5 can limit the immunogenicity of rAd5 vaccines (136-138). As approximately 40-60% of Europeans and Americans and over 90% of Sub-Saharan Africans are Ad5 seropositive due to wildtype Ad5 infection acquired environmentally, the future utility of rAd5 as an HIV vaccine vector is uncertain (139-142).
Additionally, a statistically insignificant increase in HIV-1 acquisition was detected in vaccinated Ad5-seropositive individuals compared with Ad5-seropositive placebo controls (129, 135). The cause of that increase in HIV vaccination is currently unknown. One study has shown that rAd5 treated with anti-rAd5 antibodies form rAd5 immune complexes (ICs) and that rAd5 ICs induce more DC activation and maturation than rAd5 alone. Furthermore they show that DC-T cell cocultures treated with rAd5 ICs are more permissive to HIV infection than cocultures treated with rAd5 vector alone (143). It has also been suggested that this observation may be caused by increased migration of Ad5-specific and HIV-1 specific CD4 T cells towards the mucosal surfaces, which would increase the presence of targets cells for HIV-1, but this hypothesis has not been proven (131).

3.4 Alternative serotype vectors

Given the issues regarding the used of rAd5-based vaccines in populations with preexisting immunity, other serotypes of adenovirus with lower seroprevalences have been considered as alternates to rAd5 vectors (144-148). The seroprevalence of Ads varies greatly between serotypes and between regions (149-152). Ad28 has a seroprevalence below 5% in North America and Europe, but over 40% in parts of Sub-Saharan Africa. Other serotypes, such as Ad35, have a low seroprevalence worldwide (149, 150, 153, 154).

Vectors have been constructed from dozens of different serotypes of human Ads including Ad28 and Ad35 (Table 4) (150). More recently vectors constructed from
simian or chimpanzee adenoviral strains, such as recombinant chimpanzee adenovirus serotype 63 (ChAd63) or simian adenovirus serotype (SAdV7), have been constructed and tested (155-158). These vectors have no or extremely limited pre-existing immunity in human populations (159).

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Species</th>
<th>Receptor(s)</th>
<th>Tropism</th>
<th>Seroprevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>C</td>
<td>CAR</td>
<td>Respiratory</td>
<td>40-80</td>
</tr>
<tr>
<td>28</td>
<td>D</td>
<td>Unreported</td>
<td>Enteric</td>
<td>3-44 (rare)</td>
</tr>
<tr>
<td>35</td>
<td>B2</td>
<td>CD46</td>
<td>Renal</td>
<td>1-3</td>
</tr>
</tbody>
</table>

Table 4. Vectors studied in this dissertation.

In general, rAd vectors constructed from alternative adenoviruses have proven to be poorly immunogenic when compared with rAd5 (150). The low seroprevalent vectors all compare favorably with rAd5 at high inoculum, but lose immunogenicity as vector inoculum is reduced. Some vectors, including rAd28, retain limited immunogenicity at intermediate doses, but lose immunogenicity at lower doses. Other vectors, including rAd35, are not immunogenic except at very high inocula (Figure 3).
Figure 3. The immunogenicity of rare serotype rAd vectors. The percent of tetramer binding CD8 T cells (A) or number of SFC (B) after vaccination with 6 different rAd vectors expressing SIVgag protein at 3 different inoculums. Adapted from Abbink et al, 2007 and used with approval from the publisher.
4 Antiviral immunity

4.1 Immunity and vaccination

The immune system can be divided into two broad categories: innate and adaptive (1, 2). The innate immune system recognizes pathogen associated molecular pathways (PAMPS), danger associated molecular patterns (DAMPS), and alarmins, which are molecules including nucleic acids, proteins, and polysaccharides generally associated with pathogenic infection and tissue damage (160, 161). It has a limited number of invariant targets by which to identify and eliminate pathogens. The innate immune response is very quick to detect and respond to these signals, often reacting in minutes or hours (Table 5). Additionally innate immunity is non-specific, unable to determine anything other than broad differences between pathogens and having no antigen-specific killing mechanism. There is no memory component to innate immunity, meaning the innate immune response does not differ between primary infection or re-infection with the same pathogen.

Table 5. Differences between innate and adaptive immunity

<table>
<thead>
<tr>
<th>Innate immunity</th>
<th>Adaptive immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responds quickly (minutes to hours)</td>
<td>Slower responses (days and weeks)</td>
</tr>
<tr>
<td>Invariant</td>
<td>Variable</td>
</tr>
<tr>
<td>Recognizes PAMPS and DAMPS</td>
<td>Numerous specificities</td>
</tr>
<tr>
<td>No development of memory</td>
<td>Generates memory immunity</td>
</tr>
</tbody>
</table>

By contrast the adaptive immune response is much slower to respond, taking 7 to 10 days to generate a primary response (1, 2). Additionally its recognition mechanisms are extremely variable, pathogen-induced, and pathogen-specific. Adaptive immunity is able
to develop a very specific, targeted immune response to antigen expressed by a specific pathogen. After an adaptive immune response, the host will develop immunological memory to the specific pathogen, which will allow the host to generate a powerful, secondary immune response quickly upon re-exposure to the same or similar pathogens.

The ability of the adaptive immune system to develop powerful, protective, and pathogen-specific memory immunity allows for the effectiveness of vaccination (1, 2). By allowing the adaptive immune system to encounter antigen and develop memory in a non-pathogenic scenario a vaccine ensures that upon subsequent exposure to the pathogen there will ensue a rapid response and not a less powerful, slower primary response.

Vaccination has no long-term impact on innate immunity, but an innate immune response during vaccination can have important impact on the development of memory immunity (162-165). Additionally, side effects following vaccination such as fever, increased pulse, and headache are due to innate immune activation and the systemic release of cytokines such as type I interferon (166-170).

4.2 Type I interferon and innate immunity

Type I interferon has been called the master regulator of the innate anti-viral immune response and is responsible for setting the “anti-viral state” in affected host cells (171-174). Type I interferon, consisting of 14 isoforms of IFNα and one isoform of IFNβ in humans, is one of the very first cytokines released during anti-viral immunity (175).
Type I interferon pathways, activated through binding to the interferon alpha-beta receptor (IFNAR), upregulate the expression of over 200 antiviral genes (176-179). Type I interferon signaling causes host cells to limit viral replication, increase MHC class I expression and antigen presentation, and active NK cells to destroy viral-infected cells (1). The antiviral actions of type I interferon and its downstream effector molecules are so effective that the many viruses have evolved mechanisms to avoid or interrupt them (180-183). These genes have a variety of effects, ranging from local autocrine and paracrine antiviral effects to endocrine effects manipulating and altering the eventual adaptive immune response (184-186). Importantly one of the major autocrine and paracrine effects of type I interferon signaling is the upregulation of additional type I interferon (187, 188). This ensures that all cells near the site of infection sense type I interferon and are switched to an antiviral state.

The two classical autocrine antiviral actions of type I interferon are the activation of RNase L and Protein Kinase R (PKR) (1, 2). RNase L, once activated by type I interferon, will dimerize in response to viral dsRNA in the host cytoplasm and degrade all cellular RNA, effectively blocking all translation (189, 190). PKR functions in an analogous manner; once activated by type I interferon it will dimerize in response to cytoplasmic viral dsRNA (191-193). Once dimerized PKR will phosphorylate the translation initiation factor EIF2A, preventing it from functioning thereby effectively stopping all protein synthesis in the cell. Both molecules also increase cell apoptosis after dimerization, killing infected host cells and preventing viral spread (194-196).
More recently, the effects of type I interferon on adaptive immunity have been well characterized using IFNAR$^{-/-}$ mice (197, 198). Type I interferon signaling on T cells leads to increased expression of IL2 and a greater fraction of multifunctional, antigen specific CD8 T cells, both of which are associated with higher quality T cell function. Furthermore type I interferon signaling during innate immunity increases the ratio of antigen specific T cells expressing CD127, a surface marker associated with long-lived memory cells, during adaptive immunity.
5 Dendritic Cells

5.1 General Function

Dendritic cells (DCs), named after the long dendrites that characterize their unique morphology, are the primary professional antigen presenting cells (APCs) involved in the anti-viral immune response (199-201). They are often described as having a dual role, being involved in both innate and adaptive immunity, and represent an important link between the two halves of the immune system (202-204). They play an important role in the initiation of an innate immune response, by secreting cytokines and chemokines at the site of infection and they are the primary cell responsible for presenting antigen to naïve and memory T cells (205-213). DCs are also reported to have a secondary role in the uptake and destruction of extracellular viral particles during certain viral infections.

During non-inflammatory conditions DCs located in the tissue have an immature phenotype and a high uptake capacity allowing them to sample the local microenvironment (199). These DCs present self-antigen on MHC class I and class II (214, 215). This self-antigen, presented to T cells without co-stimulation, will induce tolerance in self-antigen specific T cells driving them into a quiescent state. This mechanism helps prevents potential autoimmunity (216).

DCs express a large variety of pattern recognition receptors (PRRs) to detect the PAMPS, DAMPS, and alarmins associated with infection and inflammation (217). These receptors include toll-like receptors (TLRs), which detect surface and endosome PAMPS,
rig-like receptors (RLRs), which detect foreign nucleic acids in the cytoplasm, and nod-like receptors (NLRs), which sense a variety of microbial products and inflammatory signals in the cytoplasm (Table 6) (218). Ligation of these PRRs will activate the DC.

<table>
<thead>
<tr>
<th>PRR</th>
<th>ligand(s)</th>
<th>ligand associated with</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLRs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR1</td>
<td>lipopeptides</td>
<td>bacteria</td>
</tr>
<tr>
<td>TLR2</td>
<td>lipoteichoic acid</td>
<td>gram positive bacteria</td>
</tr>
<tr>
<td>TLR3</td>
<td>dsRNA</td>
<td>viruses</td>
</tr>
<tr>
<td>TLR4</td>
<td>Lipo polysaccharide</td>
<td>gram negative bacteria</td>
</tr>
<tr>
<td>TLR5</td>
<td>flagellin</td>
<td>bacteria</td>
</tr>
<tr>
<td>TLR6</td>
<td>lipopeptides</td>
<td>mycobacteria</td>
</tr>
<tr>
<td>TLR7</td>
<td>ssRNA</td>
<td>viruses</td>
</tr>
<tr>
<td>TLR8</td>
<td>ssRNA</td>
<td>viruses</td>
</tr>
<tr>
<td>TLR9</td>
<td>unmethylated CpG</td>
<td>viruses and bacteria</td>
</tr>
<tr>
<td>RLRs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIG-I</td>
<td>5′-triphosphate RNA, short dsRNA</td>
<td>viruses</td>
</tr>
<tr>
<td>MDA5</td>
<td>long dsRNA</td>
<td>viruses</td>
</tr>
<tr>
<td>LGP2</td>
<td>ends of dsRNA</td>
<td>viruses</td>
</tr>
<tr>
<td>NLRs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NLRc</td>
<td>meso-diaminopimelic acid</td>
<td>gram negative bacteria</td>
</tr>
<tr>
<td>NLRP</td>
<td>unmethylated CpG, dsRNA</td>
<td>viruses and bacteria</td>
</tr>
</tbody>
</table>

Table 6. PRR, including TLRs, RLRs, and NLRs, their ligands and pathogens the ligands are associated with.

Once activated DCs will increase antigen uptake and presentation, upregulate expression of costimulatory molecules, and migrate to the site of infection or to secondary lymphoid tissues (219). These changes allow DCs to execute their dual role in immunity. Activated DCs at the site of infection secrete cytokines and recruit additional innate and adaptive immune cells. Other activated DCs located in the secondary lymphoid tissue encounter naïve and memory T cells. Costimulatory molecules, such as CD40, CD80, and CD86 bind to CD40 ligand, CD28, or CTLA4, respectively and provide costimulatory signals along with antigen specific MHC/TCR binding (220, 221).
dual signal will cause the T cell to become activated, as opposed to quiescent, and to proliferate and mature.

5.2 DC subsets

DCs represent a large heterogeneous collection of cell types, possibly deriving from several different progenitors (222). Subsets vary in tissue residence, motility, function, and surface molecule expression. Most DC subsets are derived from the myeloid lineage, and function to capture and take up antigen before traveling the secondary lymphoid tissues where they present the antigen and activate naïve and memory T cells (223). Other subsets have less ability to present antigen but instead secrete large amounts of cytokine, driving innate and adaptive immune cell recruitment and activation.

It is important to note that the characterization of DC subsets, including their surface receptor expression, function, development, and categorization continually being refined and updated in the literature. No single classification scheme has been completely accepted and this is particularly true when comparing DC subsets between species. It therefore remains important to clearly state methods used in isolating and characterizing different DC subsets in studies.

5.2.1 Blood DC subsets

DC subsets in human blood can be broadly divided into plasmacytoid DCs (pDCs) and three distinct myeloid DC subsets (mDCs) based on surface molecule expression and function (Table 7) (222-226). In steady state conditions, pDCs express CD123
(interleukin 3 (IL3) receptor a), CD303 (Blood Dendritic Cell Antigen-2 (BDCA-2)), CD304 (BDCA-4) and human leukocyte antigen-DR (HLA-DR). All mDC subsets express surface CD11c and HLA-DR. Certain rare mDC subsets express transient CD14 upon activation, but majority are CD14- (226). mDCs can be further divided into three subsets based on the expression of CD1c (BDCA-1), CD141 (BDCA-3), or CD16 (227).

<table>
<thead>
<tr>
<th>Name</th>
<th>Location</th>
<th>Surface markers</th>
<th>Known functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1c mDCs</td>
<td>blood</td>
<td>CD11c, HLA-DR, CD1c</td>
<td>Antigen presentation, cytokine</td>
</tr>
<tr>
<td>CD141 mDCs</td>
<td>blood</td>
<td>CD11c, HLA-DR, CD141</td>
<td>Crosspresentation</td>
</tr>
<tr>
<td>CD16 mDCs</td>
<td>blood</td>
<td>CD11c, HLA-DR, CD141</td>
<td>Cytokine, especially TNF</td>
</tr>
<tr>
<td>pDCs</td>
<td>blood</td>
<td>CD123, HLA-DR, CD303, CD304</td>
<td>Type I interferon, crosspresentation</td>
</tr>
<tr>
<td>LCs</td>
<td>epidermis</td>
<td>CD1a, HLA-DR, E cadherin, langerin</td>
<td>Activating CTLs</td>
</tr>
<tr>
<td>dDCs</td>
<td>dermis</td>
<td>DC-SIGN, CD1a, CD14</td>
<td>Inducing hormonal immunity</td>
</tr>
</tbody>
</table>

Table 7. Known human DC subsets, their location, surface markers, and known functions.

CD1c mDCs are generally considered the most proficient DC subset for presentation of antigen on MHC class I to driving CD8 T cell activation and proliferation (228, 229). This population represents roughly 10-20% of all mDCs (230). During non-activated conditions these cells reside primarily in the blood, but transiently will travel to different tissues to sample the microenvironment. CD1c mDCs express multiple PRRs, including TLRs 1-8, and TLR10 (228). Once antigen is encountered, these cells will transit to lymph nodes, activate naïve T cells, and express IL8, IL12p70 and TNF, generally favoring a Th1 response (229, 231).
CD141 mDCs, a rare DC subset representing less than 5% of all mDCs, are particularly effective at presenting exogenous antigen on MHC class I, a process called cross-presentation (230, 232, 233).

CD16 mDCs, a more recently defined cell population comprising 65-75% of all mDCs, may potentially migrate to the site of inflammation or infection and secrete MIP1a, MIP1B, IP10, GM-CSF, G-CSF, or particularly large amounts of TNF (228, 230). These cytokines are thought to recruit and activate components of both the adaptive and innate immune system. CD16 mDCs can present antigen, but generally less efficiently than either CD1c mDCs or CD141 mDCs (230).

pDCs are rare compared to mDCs, being about 10% as frequent as mDCs in the blood (229). They are unique for DCs as they likely originate from lymphocyte progenitors and are thought to emerge fully developed from the bone marrow (222). These cells specialize in producing very large amounts of IFNα rapidly in response to viral infection and express TLR7 and TLR9 (234-236). They are found solely in the blood during steady state, but quickly transit to sites of inflammation upon activation. They can present antigen to T cells, but generally less efficiently than mDCs (229, 237). They are more efficient at cross-presentation than CD1c mDCs, but have not been compared directly with CD141 mDCs (238).

5.2.2 Cutaneous DC subset
There are currently two distinct subsets of DCs known to reside primarily in the skin (239). Langerhans cells (LCs) reside in the epidermal layer and express CD1a, E-cadherin, langerin, and HLA-DR (201, 240, 241). LCs specialize in inducing potent cytotoxic lymphocyte (CTL) responses after activation (240, 242).

Dermal Interstitial DCs (dDCs) are another cutaneous DCs population, but reside in the dermal layer (240). These cells are less well defined and may represent several distinct subsets. dDCs express a combination of DC-SIGN, CD1a, and CD14 and are defined primarily by their residence in dermal tissues (239). In contrast to LCs, activated dDCs, particularly CD14 expressing dDCs, are potent initiators of hormonal immunity (240).

5.2.3 Monocyte derived DCs (MDDCs)

MDDCs, which may phenotypically resemble dDCs, can be generated in vitro by culturing monocytes with high levels of GM-CSF and IL4 for 7-9 days (243). These cells, which are capable of presenting antigen, have a very highly activated phenotype and can be useful for studying certain aspects of DC function (240). The main advantage of MDDCs is that, unlike primary DCs subsets, they are very easy to isolate and differentiate in high numbers. The disadvantages of using MDDCs for research is that they do not fully mimic dDCs or other known DC subsets, are necessarily highly activated, and do not exist in vivo.

5.2.4 Follicular DCs (FDCs)
FDCs express the long dendrites typical of DCs, but are otherwise functionally and developmentally distinct from other DC subsets. They are derived from mesenchymal origins and not from hematopoietic stem cells (HSC) like other DCs subsets (244). They do not express MHC class II and do not migrate (245). They are only found in the primary and secondary lymphoid tissues and play important roles in maintaining lymphoid architecture, germinal center development, antigen capture, B cell development, and removal of dead and apoptotic cells from the lymphoid tissues (246-249). Their role in vaccination is poorly understood.

5.2.5 Other mammalian DC subsets

Blood DC subsets in NHPs are similar to human, both within pDCs and mDCs (250, 251). However mDCs from NHPs are currently divided into CD1c mDCs and CD16 mDCs subsets. CD141 mDCs have not been detected in NHPs (data not shown). It is currently unknown if anti-human CD141 AB cross-reacts with NHP CD141, if CD141-like mDCs exist in NHPs, but do not express CD141, or if there is another functionally analogous, but developmentally distinct DC subset.

Blood DC subsets in mice can also be separated into pDCs and mDCs, but their surface receptor expression differs (226, 252, 253). Mouse pDCs can be identified by expression of CD11c and B220. Like human and NHP pDCs, they specialize in quickly producing large amounts of IFNα. Mouse mDCs are defined by expression of CD11c but not B220 or CD8, and they might be considered analogous to human CD1c mDCs. Mouse blood also contains a third DC subset called cDCs that express CD11c and CD8, but no B220.
These cells are very efficient at cross-presentation and might be considered functionally similar to human CD141 mDCs.
6 Other immune cells

6.1 T cells

T cells play a core role in adaptive immunity and the development of immunological memory to viruses and as such have been well researched in the context of vaccination, including potential HIV vaccines. T cells mature in the thymus and are distinguished by the expression of T cell receptor (TCR) on their surface (1, 2). There several ways to functionally and phenotypically divide T cells into subsets (254).

6.1.1 CD4 vs CD8

The primary way to divide T cells into subsets is by expression of CD4 or CD8 (255). Immature T cells express both CD4 and CD8, but mature T cells will only express one of the molecules. CD4 expressing T cells are often referred to as T helper cells because their primary mechanism of action is to alter the function of other immune cells through the secretion of cytokines (256). These cells are activated through their TCR by professional APC displaying cognate peptide on MHC class II (257). Once activated they rapidly proliferate and produce cytokine.

CD4 T cells can be further divided into T\textsubscript{H}1, T\textsubscript{H}2, T\textsubscript{H}17, T\textsubscript{FH} or T\textsubscript{reg} subtypes based on cytokine production and function (Table 8). T\textsubscript{H}1 CD4 T cells primarily drive cellular immunity and secrete cytokines such as IFN\textsubscript{g} and IL12 that help to activate and recruit macrophages, NK cells, and CD8 T cells (258-260). Conversely T\textsubscript{H}2 CD4 T cells secrete cytokines such as IL4, IL5, IL6, IL10, and IL13 that favor humoral immunity and B cell
development (258-260). T\textsubscript{h}17 CD4 T cells, identified by their ability to produce IL17, were first identified as having an important role in autoimmunity (261, 262). The natural function of T\textsubscript{h}17 is more controversial, but they have been implicated as having a role during infection by various extracellular bacterial and fungal species and a possible role in anti-tumor immunity (262). T\textsubscript{FH} CD4 T cells, also called Follicular B Helper T cells are found primarily in the secondary lymphoid tissues, specifically the B cell follicles, and help drive B cell antibody affinity maturation and class switching (263-265). T\textsubscript{FH} CD4 T cells have received renewed interest recently and it is unclear how they relate to T\textsubscript{h}2 CD4 T cells in function (266, 267). T\textsubscript{reg} CD4 T cells, also called regulatory T cells, play an important role in down regulating immune responses and maintain tolerance to self-antigen and non-pathogenic commensal bacteria (268-270). It is important to note that much of the work characterizing T helper cell subset was completed in mice and it is somewhat controversial how directly this paradigm transfers to human CD4 T cells (271, 272).

<table>
<thead>
<tr>
<th>Subset</th>
<th>Primary Function</th>
<th>Cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>cellular immunity</td>
<td>IFNg, IL12</td>
</tr>
<tr>
<td>Th2</td>
<td>humoral immunity</td>
<td>IL4, IL5, IL6, IL10, IL13</td>
</tr>
<tr>
<td>Th17</td>
<td>autoimmunity, extracellular pathogens</td>
<td>IL17</td>
</tr>
<tr>
<td>Tfh</td>
<td>B cell development</td>
<td>IL6, IL21</td>
</tr>
<tr>
<td>Treg</td>
<td>downregulating immunity, tolerance</td>
<td>TGFbeta, IL10</td>
</tr>
</tbody>
</table>

Table 8. Subsets of CD4 T helper cells, their function, and associated cytokines.

CD8 T cells, also called cytotoxic lymphocytes (CTL), function primary by directly killing target cells (273, 274). They become activated after encountering professional APCs displaying antigen on MHC class I along with costimulatory molecules (275). Once activated CD8 T cells will scan MHC class I molecules present on all nucleated
host cells for their cognate antigen and kill all positive cells with a variety of effector molecules. These cytotoxic effector molecules include soluble factors such as perforin and granzymes, or through signaling through death receptors such as Fas (276). Perforin, once released, functions analogously to complement component 9 by nonspecifically forming pores in the cell membrane of target cells, causing them to lyse (277). Granzymes are serine proteases that enter the target cell through pores formed by perforin and activate caspases and other proteins that force the target cell into apoptosis (278, 279). Fas, expressed on the surface of activated CD8 T cells, can also bind Fas ligand on the surface of target cells inducing apoptosis through FADD and caspase dependent pathways (280). Because of their ability to detect intracellular pathogens and kill infected host cells, CTLs are especially effective combating intracellular pathogens (281).

6.1.2 Stages of differentiation

Both CD4 and CD8 T cells undergo several stages of development each with different proliferation potential, surface molecule expression, and effector ability (282). Naïve T cells are antigen-inexperienced and can differentiate into central memory (CM), effector memory (EM), and terminal effector (TE) subsets upon antigen encounter and activation (283, 284). CM cells are the least differentiated of the antigen-experienced subsets (285). They retain high proliferative ability and, importantly are capable of self-renewal in the absence of antigen (286, 287). These features may them an ideal goal of vaccination, as they can maintain a memory response for years or decades after immunization (288, 289). CM cells can further mature into EM or TE cells, but they are unable to become naïve cells. EM cells have a more limited proliferative potential, but function well as effector
cells. These cells are capable of antigen-dependant self-renewal and function well to control persistent infections, such as CMV (285, 290). EM can mature into TE cells, but are unable to become naïve or CM cells. TE cells are terminally differentiated and cannot proliferate (291, 292). They have strong effector function during an acute response, but have probably no role in memory. Interestingly, CD4 TE cells function well as cytolytic cells, a function not generally associated with the CD4 subset (293-295).

6.2 B cells
The primary function of the B cell branch of the adaptive immune system is to produce antibodies in response to pathogens (1, 2). Antibodies are small proteins (140-190 kDa) that specifically bind epitopes on the surface of pathogens with a very high affinity and are key to eliminating extracellular virus. The binding of antibody to the surface of the pathogen does not kill the virus directly, but does have several important anti-viral effects. Depending on the isoform of the antibody bound to the viral surface antibodies can activate the classical complement pathway, opsonize the virus for destruction by APC or CTL, or neutralize the virus through steric blocking of required cellular entry receptors.

B cells are able to achieve high diversity through somatic recombination (also called VDJ rearrangement), a process that rearranges sections of germline DNA sequence encoding the antibody (296). The Variability, Diversity and Joining (VDJ) sections encode the antigen-binding site of the antibody and are the most important section in determining antibody specificity. In a mature B cell, the antibody resultant from that cell’s particular
VDJ recombination is displayed on the cell surface in the form of a B cell receptor (BCR).

When a mature B cell, also called a naïve B cell, encounters its cognate antigen it will undergo somatic hypermutation to develop more high affinity antibodies (296). During this process the B cell will proliferate and insert random mutations in the VDJ region of the antibody. Periods of positive selection of higher affinity clones ensure that the hypermutation process proceeds towards higher affinity antibody clones. Also during this process, the conserved region of the heavy chain is switched in a process analogous to VDJ rearrangement to yield various isoforms of antibody.

After this process a B cell will further mature into a memory B cell or a plasma cell (297). Memory B cells retain the ability to mature into plasma cells, but plasma cells are terminally differentiated (297, 298). Plasma cells act to release large amounts of antibodies as part of an acute immune response and are replaced by other plasma cells at the conclusion of the immune response (298, 299). Memory B cells are retained for months or years after the primary immune response and have the ability to self renew (300, 301). They function to rapidly establish a secondary immune response upon re-exposure to cognate antigen. Because of these characteristics one goal of vaccination strategies is to establish a large population of memory B cells (32).

6.3 NK cells
NK cells, also called Natural Killer cells, are named for their ability to kill host cells in the absence of inhibitory signals (302, 303). These cells are derived from the lymphocyte lineage, can be identified by surface expression of CD56 in humans, and are generally considered to be part of the innate immune response (304, 305). NK cells can fill the same general role as CTLs, but don’t require clonal selection or clonal expansion allowing them to respond much more quickly than CTLs (306). Importantly, NK cells are generally thought not to require or detect specific antigen or develop memory, limiting their role in adaptive immunity.

NK cells kill any cell, host or foreign, they encounters in the absence of inhibitory signals (307). Inhibitory ligands, which when expressed by host cells prevent NK cell killing, include MHC class I and cadherins. One of the classic functions of NK cells is to detect the presence or absence of MHC class I on a target cells through a family of receptors called killer-cell immunoglobulin-like receptors (KIR). As CTL killing is dependant on MHC class I expression by the target cell, MHC class I expression is often down regulated by viral defense mechanisms, or genetic mutation in cancer cell to avoid CTLs. Thus one of the primary functions of NK cells is the killing of cells that CTLs cannot because of a lack of MHC class I expression, often referred to as the missing self hypothesis (308, 309).

NK cells also contain a broad set of activating receptors that can detect ligands associated with stressed, virally infected, or transformed host cells (307, 310, 311). Thus the decision of an NK cell to kill or not kill a target cell depends on the balance of both
activating and inhibitory signals from the target cell. Additionally, NK cells participate in antibody dependent cell mediated cytotoxicity (ADCC) through CD16, an Fc Receptor that detects antibody on opsonized cells (312-314).

Similar to CD8 T cells, NK cells kill target cells through the use of perforin and granzymes, and Fas (315-318). Additionally TRAIL, both secreted by and surface bound to activated NK cells, can bind its receptors on target cells, DR4 and DR5, activating NFkB and leading to expression of pro-apoptotic genes and killing the target cell (319, 320).

More recently NK cells have been implicated in playing a role in the development of adaptive immunity (321, 322). NK cells can influence an adaptive immune response through the production of cytokines, altering the microenvironment and affecting T cell differentiation (323, 324). Additionally, a subset of NK cells has been recently identified in CMV infected mice that express the virus specific receptor Ly49H (325). These cells proliferate extensively when activated, contract after infection, are capable of self-renewal, and rapidly degranulate and produce cytokines upon reactivation; all features typically associated with adaptive immunity.

6.4 Monocytes

Monocytes, considered part of the innate immune system, are derived from the myeloid lineage (326, 327). Monocytes are often considered precursors to macrophages and dendritic cells and in steady state conditions they are primarily responsible for
replenishing resident macrophages and dendritic cells (328). During inflammation and infection monocytes, activated by cytokines and chemokines, quickly travel to the site of infection and differentiate into macrophages and dendritic cells (326, 329). Monocytes are capable of phagocytosis, both via opsonised pathogens or directly using PRRs, and secrete proinflammatory cytokines, such as TNF, IL1, and IL12 (326). Monocytes can also present antigen to T cells, but are generally considered poor APCs compared to DCs (330).
7 Current HIV Vaccine Research

7.1 Sterilizing vaccines

It is currently not known what characteristics are necessary for an effective HIV vaccine. It is also unclear what host immunological features offer protection from HIV.

Historically vaccines provide sterilizing, protective immunity, and prevent the pathogen from establishing a productive infection through the elicitation of neutralizing antibodies (331, 332). In these vaccines the presence of neutralizing antibodies correlates well with sterilizing, protective immunity, thus they have been called a correlate of protection.

Vaccines that provide sterilizing immunity through the induction of neutralizing antibodies include rabies, polio, mumps, rubella, influenza, hepatitis A, hepatitis B, and HPV (Table 9) (333). Other vaccines, such as those for smallpox, measles, and varicella zoster, have both antibodies CTLs as a correlate of protection.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Type of vaccine</th>
<th>Vaccine-induced immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smallpox</td>
<td>Live</td>
<td>Antibodies, CTL</td>
</tr>
<tr>
<td>Rabies</td>
<td>Killed</td>
<td>Antibodies</td>
</tr>
<tr>
<td>Polio</td>
<td>Live or killed</td>
<td>Antibodies</td>
</tr>
<tr>
<td>Measles</td>
<td>Live</td>
<td>Antibodies, CTL</td>
</tr>
<tr>
<td>Mumps</td>
<td>Live</td>
<td>Antibodies</td>
</tr>
<tr>
<td>Rubella</td>
<td>Live</td>
<td>Antibodies</td>
</tr>
<tr>
<td>Varicella zoster</td>
<td>Live</td>
<td>Antibodies, CTL</td>
</tr>
<tr>
<td>Influenza</td>
<td>Surface Protein</td>
<td>Antibodies</td>
</tr>
<tr>
<td>Hepatitis A</td>
<td>Killed</td>
<td>Antibodies</td>
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<tr>
<td>Hepatitis B</td>
<td>Surface Protein</td>
<td>Antibodies</td>
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<tr>
<td>HPV</td>
<td>VLP</td>
<td>Antibodies</td>
</tr>
</tbody>
</table>

Table 9. Successful vaccines and their mechanism of protection.

Complete sterilizing immunity against HIV may be a necessary feature of an HIV vaccine. The retroviral lifecycle of HIV allows the virus to integrate into the genome of
host cells, which makes the elimination of infection, once established, very difficult (58, 334). Additionally, infected cells can enter a dormant, latent stage, often for years, expressing little to no viral proteins, effectively hiding from host immunity (Figure 4) (333, 335). These features would favor the development of a vaccine that elicits neutralizing antibodies and prevents initial infection.

Figure 4. Neither arm of the adaptive immune response can target latently infected cells. Adapted from Pantaleo et al, 2004 and used with approval from the publisher.

However there are several specific problems associated with the development of broadly neutralizing antibodies through vaccination. Antibody must be present at the site of infection and present continuously at high titers (336). The surface of HIV is heavily glycosolated preventing most antibody binding (337-339). Much of the antibody produced in response to HIV will bind but not neutralize the virus (340, 341). Additionally, HIV has been shown to rapidly mutate to escape neutralizing antibody (342, 343). Finally, most vaccine-derived neutralizing antibodies that productively
neutralize HIV are strain specific and only protect against strains similar to the ones used in the construction of the vaccines (344, 345).

Recently, a subset of mAbs with the ability to neutralize a broad range of HIV strains have been identified (346, 347). These antibodies can be divided into three general categories based on their binding site. CD4 binding site (CD4bs) antibodies, which include antibodies such as b12, b13 and VRC01, bind directly to the CD4 binding site of HIV gp120 and prevent attachment and entry by preventing gp120/CD4 interaction (348-351). A second set of broadly neutralizing antibodies target the membrane proximal region (MPER) of gp41 and include 2F5 and 4E10 (352, 353). Other broadly neutralizing antibodies, such as PG9/16 and 2G12 bind to a combination of variable region 2 (V2), V3, and glycans (353-355).

It is estimated that approximately 30% of HIV infected individuals develop broadly neutralizing antibodies (356). However neutralizing antibodies and broadly neutralizing antibodies are quite altered from germline sequence and take months and years to develop (357-359). It has also not been shown that the development of neutralizing or non-neutralizing antibody has any impact on pathogenesis (360). It is possible that these antibodies are developed too late, after infection has been well established, to have a major impact on disease progression. However, studies challenging NHP with low dose SHIV after passive immunization with broadly neutralizing antibody have demonstrated that neutralizing antibodies can protect against SHIV acquisition if present before exposure (361, 362).
The elicitation of broadly neutralizing antibodies by vaccination remains a significant challenge. Broadly neutralizing antibodies are significantly mutated from germline sequences and likely take many rounds of somatic hypermutation to develop. More work need to be done to determine what immune and vaccine characteristics are necessary for driving antibody production and hypermutation.

7.2 Non-sterilizing vaccines

For most viruses, the components of protective immunity can be discovered by studying those how have recovered from natural infection. These individuals do not exist for HIV because the infection is never completely resolved due the integration of the HIV genome and the establishment of latently infected cells. However there is a population of HIV infected individuals who are able to suppress their viral load below detection limits for an extended period without highly active antiretroviral therapy (HAART) (363). These individuals, called long term non-progressors (LTNPs), maintain high CD4 T cell counts and do not progress to AIDS for years (364).

While the immunological mechanisms used by LTNPs to reduce viral replication are not fully understood, there are several features that correlate with the development of the LTNP state (365-367). Individuals with HLA-B27 or HLA-B57 MHC class IB are more likely to become LTNPs (368, 369). The development of a large HIV-specific CD8 T cell response also correlates with LTNPs (370-372). Polyfunctional CD8 T cells, CD8 T cells that have the ability to produce more than one cytokine, such as IL2, IFNγ, or TNF,
at once, are more protective than single cytokine producing cells (373). These findings suggest that a large, polyfunctional CD8 T cell response may be effective in controlling HIV viremia. Additional studies, showing that depletion of CD8 cells in SIV infected NHPs lead to loss of viral control, support this conclusion (374, 375).

There are still many unknowns associated with the development of a CD8 T cell-based vaccine. It was originally thought that the ideal vaccine would generate a population of CM CD8 T cells, as these cells are capable of self-renewal without continuous exposure to antigen and allow for long-term maintenance of immunity after the vaccine antigen has been cleared from the host (285, 376).

It has also been suggested that EM CD8 T cell may be more protective than CM CD8 T cells (377-380). However EM CD8 T cells require the presence of antigen to be capable of self-renewal. This is problematic for vaccination as current vaccines deliver antigen in one or more short pulses meaning that EM CD8 T cells are not maintained long term. A study utilizing a live cytomegalovirus vector that continuously expresses SIV antigens and maintains a large SIV specific EM CD8 T cell population has been to shown to reduce viral load in SIV-challenged rhesus macaques (381, 382). Although any live virus vaccine would have to address several safety concerns before clinical use, the study does offer excellent an proof of concept about the utility of EM CD8 T cells and T cell vaccines in general.
Even if completely ineffective against viral acquisition, a vaccine that mimics the low viral loads of LTNPs, by whatever mechanism, would represent a major advancement. Low viral loads correlate with decreased disease, decreased CD4 loss, delayed progression to AIDS, and significantly decreased rates of transmission (383-389).

7.3 An ideal vaccine

As any vaccine is unlikely to offer complete sterilizing protection against all viral challenges, a vaccine that generates both strong neutralizing antibody responses and a large CD8 T cell population may be ideal (390, 391). The neutralizing antibodies would protect against infection and, if vaccine breakthrough infection occurs, the CD8 T cell response would reduce viral load, improve disease prognosis, and reduce subsequent transmission to other individuals.
CHAPTER 2

8 Innate immunity impacts immunogenicity of viral vaccine vectors

8.1 Introduction

HIV vaccines constructed from Ad5 vectors have been extensively researched and have undergone several phase I and phase II clinical trials (129). The results of these trials, in conjunction with extensive murine and monkey studies, have revealed that pre-existing immunity against the Ad5 vector will greatly reduce the immunogenicity of the vaccine and will inhibit the memory response to the HIV antigen insert (136, 139). This revelation, combined with the observation that 40-80% of the world’s population is seropositive for Ad5 due to natural infections with the wildtype virus, has limited the potential usefulness of any Ad5 vector vaccine.

An obvious alternative to circumvent the issue of preexisting immunity is the development of an adenovirus vector using an adenovirus serotype that has a much lower seroprevalence such as rAd28 and rAd35. Unfortunately, research in my mentor’s lab and elsewhere has repeatedly shown that vectors constructed from lower-seroprevalent adenoviruses consistently induce a low immune response against the insert (150, 229). This presents a paradox where the Ad5 vector, which induces strong memory immunity, is ineffective because of widespread preexisting immunity while alternative, lower-seroprevalent vectors are ineffective because they do not induce strong memory immunity.
It was my hypothesis that the poor immunogenicity of the low-seroprevalent vectors is a direct result of differences in how rAd5 and rare-serotype vectors interact with and activate innate immunity. Here I show rAd28 and rAd35 are more infectious and activate greater innate immunity, as evidenced by DC maturation and IFNα production, than rAd5. Microarray analysis of infected DCs cultures confirms that rAd5, compared to rAd28 or rAd35, differs in how they activate innate immunity, specifically the induction of IFNα production.

Furthermore I observe an increase in cell death and apoptosis in cell cultures treated with rAd28 or rAd35, but not in rAd5 treated cultures. This increases in apoptosis correlates well with a loss of insert expression. Insert expression in rAd5 treated cultures is maintained at a high level for several days while insert expressed by rAd28 or rAd35 is rapidly eliminated after 24 hours as insert expression is lost almost immediately after the beginning of apoptosis. I have indentified IFNα induced activation of NK cells as a potential mechanism responsible for elimination of insert protein.

Finally, I explore the in vivo impact of IFNα signaling on mice vaccinated with rAd5, rAd28, and rAd35. I find that IFNα signaling reduces the magnitude, but increases the quality of the insert-specific CD8 T cell response in mice vaccinated with rAd28 and rAd35. Combined, these results provide a clear understanding of the impact of innate immune activation and IFNα production on vaccination with rAd vectors.
8.2 Materials and Methods

*PBMC isolation*

Human PBMCs from normal, healthy donors were obtained by automated leukapheresis and isolated by density gradient centrifugation using Ficoll-Plaque PLUS (GE Healthcare). PBMCs were cultured in RPMI supplemented with 2 mM L-glutamine (Invitrogen), 1% streptomycin and penicillin (Invitrogen), and 10% FCS (Gibco) at a density of 1x10^6 cells/ml. Signed informed consent was obtained from all donors in accordance with the Declaration of Helsinki and the study was approved by the relevant Institutional Review Board.

*Isolation of primary DC subsets from blood*

The sorting processes for direct isolation of CD11c^+ myeloid DCs (mDCs) and CD123^+ plasmacytoid DCs (pDCs) from blood have been described previously (229, 392, 393). Briefly, PBMCs collected from healthy blood donors were further enriched for monocytes/DCs by semiautomatic counterflow centrifugation (Elutra Cell Separation System). CD1c and BDCA4 isolation kits (Mitenyi Biotec) were used in conjunction with an AutoMACS magnetic cell sorter (Miltenyi Biotec) to isolate mDCs and pDCs, yielding highly enriched populations for both (>85%) (229). Flow cytometry revealed the contaminating cells to be CD14^+. For microarray experiments, the respective DC populations were further purified into CD11c^+CD14^- populations (for mDCs) and CD123^+CD14^- populations (for pDCs) using a FACSARia cell sorter (BD Biosciences). This process yielded highly pure cell populations (>99.9%). Sorted cell populations were
cultured in RPMI supplemented with 2 mM L-glutamine, 1% streptomycin and penicillin, and 10% FCS at a density of 1x10^6 cells/ml.

Construction of rAds
Replication-deficient rAd5, rAd28, and rAd35 vectors expressing SIV Gag or eGFP were provided by GenVec Inc. The construction of the E1/E3/E4-deleted rAd5 and rAd35 (394, 395) and the E1-deleted rAd28 (154) has been described previously.

rAd infection and stimulation
Cells were exposed to multiple MOIs of rAd5, rAd28, or rAd35 encoding for eGFP for 24 hours. TLR7/8-ligand (1 µg/ml) (3M) was used to induce DC maturation.

CD46 blocking
Isolated mDCs were treated with anti-CD46 neutralizing antibody (clone M177, Hycult Biotechnologies) or an isotype-matched control (clone P3, eBiosciences) for 15 minutes at room temperature prior to rAd exposure.

DC infection and phenotype assessment by polychromatic flow cytometry
Isolated and rAd-infected DCs were washed with PBS and incubated with a viability marker (ViViD; Molecular Probes) and FcR blocking reagent (Miltenyi Biotec) for 10 minutes at room temperature. Cells were then washed with PBS containing 0.5% BSA and stained with fluorescently labeled antibodies to CD11c, CD123, CD14, and CD80 for 15 minutes at room temperature. Similarly, PBMCs were washed and incubated with
ViViD and FcR blocking reagent before the cells were stained with fluorescently labeled antibodies to CD3, CD4, CD8, CD14, CD19, CD56, and HLA-DR for 15 minutes at room temperature. All samples were analyzed using a LSR II flow cytometer (BD Biosciences). Data analysis was performed using FlowJo version 9.3.2 (Tree Star).

**Measurements of human and mouse IFNα release**

The supernatants from human and mouse DC cultures as well as serum collected from mice 6 hours post-immunization were analyzed using VeriKine Human or Mouse Interferon-Alpha ELISA Kits (PBL Laboratories), respectively, in accordance with the manufacturer’s instructions.

**Microarrays**

Purified DC populations were either left unexposed or incubated with rAd5, rAd28, rAd35, or TLR7/8-ligand for 24 hours. The cells were then harvested and placed at -80°C in RLT buffer. Total RNA was isolated using RNeasy Micro Kits (QIAgen) according to the manufacturer’s protocol, and checked for quantity and quality using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) and an Experion Automated Electrophoresis System. Samples with an RQI classification ≥7.0 were normalized at 150 ng for tRNA input and amplified using Illumina TotalPrep RNA amplification kits (Ambion) according to the manufacturer’s protocol. Microarray analysis was conducted using 750 ng of biotinylated cRNA hybridized to Human RefSeq-8 V3 BeadChips (Illumina) at 58°C for 20 hours. The hybridized BeadChips were washed, blocked, stained and scanned according to the manufacturer’s protocol. The arrays were scanned
using an Illumina BeadStation 500GX scanner and quantified using GenomeStudio software (Illumina). Analysis of the GenomeStudio output data was conducted using the R statistical language (R Development Core Team) (396) and various software packages from Bioconductor (397). Quantile normalization was applied, followed by a log2 transformation. The LIMMA package from Bioconductor (398) was used to fit a single linear model to each probe and to perform (moderated) t-tests on the incubation effects for a given DC population. Specifically, the fitted linear model assigned mock samples as DC population-specific and individual-specific intercepts (multivariate analog of paired-samples). The list of interferon-regulated genes (IRGs) employed to filter genes was defined in previous work (399, 400). Ingenuity Pathway Analysis (IPA; Ingenuity Systems, www.ingenuity.com) was used to test for over-representation of canonical pathways in the lists of genes differentially expressed upon incubation. For this purpose, statistical significance for gene differential expression was defined as |FC|>1.5, P<0.01 at FDR<30%, while statistical significance for over-representation (Fisher’s exact test) was defined at FDR<5%. The expected proportions of false positives (FDR) were estimated from the unadjusted P values using the Benjamini and Hochberg method. The microarray data are available through the National Center for Biotechnology Information Gene Expression Omnibus (GEO), accession number: GSE37128.

Labeling monocytes with violet dye

CD14+ microbeads (Miltenyi Biotec) were used in conjunction with an AutoMACS magnetic cell sorter (Miltenyi Biotec) to isolate CD14+ monocytes from PBMCs, yielding very highly enriched populations (>90%). The monocytes were then stained
violet using CellTrace Violet Cell Proliferation Kit (Invitrogen) by incubating monocytes at the concentration of $1 \times 10^6$ cells/mL with 5 µM CellTrace Violet Solution at 37°C for 7 minutes, in accordance with the manufacturer’s instructions.

**Sorting insert-expressing cells**

GFP+ cells from infected violet-labeled monocytes were sorted 24 hours post-exposure to rAd vectors using a FACSARia cell sorter (BD Biosciences). This process yielded very highly pure cell populations (>99.9% GFP+).

**Polychromatic flow cytometry and measurements of cell death and apoptosis**

rAd and mock-infected PBMCs were washed with PBS and incubated with a viability marker and FcR blocking reagent (Miltenyi Biotec) for 10 minutes at room temperature. Cells were then washed with buffer containing .5% BSA and 25µM CaCl and stained with fluorescently labeled antibodies to CD3, CD11c, CD14, CD123, and HLA-DR for 15 minutes at room temperature. For measurements of apoptotic markers, cells were additionally stained with fluorescently labeled Annexin V (Invitrogen), and an antibody to fluorescently labeled anti-active Caspase 3 (BD Pharmingen) for 15 minutes at room temperature. All samples were then fixed with 1% PFA analyzed using a LSR II flow cytometer (BD Biosciences). Data analysis was performed using FlowJo version 9.3.2 (Tree Star).

**Mice**
C57BL/6 mice purchased from The Jackson Laboratory were maintained in the Vaccine Research Center Animal Care Unit. IFNAR knockout (IFNAR⁻⁻) mice were provided by Brian Kelsall (NIH), and bred and maintained in the animal facility at the NIH. All experiments were conducted following the guidelines of, and with approval from, the Vaccine Research Center Animal Care and Use Committee.

*Murine DC isolation*

Splenocytes were harvested from wildtype mice. Total DCs were isolated to >50% purity using a pan-DC microbead kit (Miltenyi Biotec) and an AutoMACS cell sorter according to the manufacturer’s protocols.

*Immunizations*

Mice were immunized with rAd5, rAd28, or rAd35 in a total volume of 50 μL by subcutaneous injection in each of the hind footpads.

*Polychromatic flow cytometric analysis of SIV Gag-specific responses*

Blood was collected from mice into heparin-containing tubes and treated for 3 minutes at room temperature with ACK buffer (Lonza) to lyse the red blood cells. The cells were then pelleted and washed twice with PBS. Cells were transferred to 96-well plates and incubated with a viability marker (OrViD; Molecular Probes) and FcR blocking reagent for 10 minutes at room temperature. After a further wash with PBS containing 0.5% BSA, cells were stained for 15 minutes at room temperature with fluorescently labeled AL11 (AAVKNWMTQTL) SIV Gag tetramer, constructed around H2-Dᵇ using protocols
described previously (401), and antibodies to CD8, CD27, CD44, CD62L, and CD127. After fixation, cells were analyzed using an LSR II flow cytometer (BD Biosciences). Viable CD8 T cells were selected within a small lymphocyte gate based on CD8 expression and lack of staining with OrViD. The fraction of viable CD8 T cells binding the AL11 tetramer was used to determine the percentage of SIV Gag-specific CD8 T cells. Data analysis was performed using FlowJo version 9.3.2.

*Intracellular cytokine staining*

Splenocytes isolated from mice 82 days after immunization were transferred to a 96-well plate. Cells were exposed to AL11 peptide for 30 minutes and treated with brefeldin A (10 µg/ml; Sigma Aldrich) and monensin (0.7 µg/ml; eBiosciences) for 6 hours. Cells were then washed twice with PBS and incubated with a viability marker (ViViD) for 10 minutes at room temperature. After a further wash in PBS containing 0.5% BSA, cells were stained with fluorescently labeled antibodies to CD4 and CD8 for 15 minutes at room temperature. Cells were then permeabilized with Fix/Perm (BD Biosciences) for 20 minutes at room temperature, washed with Perm/Wash (BD Biosciences) and stained with fluorescently labeled antibodies to CD3, IFNγ, IL2, and TNF for a further 20 mins at room temperature. Cells were analyzed using an LSR II flow cytometer (BD Biosciences). Viable CD8 T cells were selected using the viability marker, a small lymphocyte gate, and expression of CD3 and CD8. Frequencies of cytokine producing cells were calculated within the viable CD3⁺ CD8⁺ T cell populations. Data analysis was performed using FlowJo version 9.3.2.
**Exogenous rIFNα treatment**

5ng/mL of exogenous mouse rIFNα (PBL Interferon Source) was added to cultures immediately before infection with the rAd vectors.

**Statistical analyses**

Statistical analyses were performed using the Wilcoxon’s signed-rank test or the Mann-Whitney U-test with GraphPad Prism version 5.0c software (GraphPad Software). Analysis and presentation of distributions was performed using SPICE version 5.1, downloaded from http://exon.niaid.nih.gov (402). Comparison of distributions was performed using a Student's T-test and a partial permutation test as described previously (402). Error bars shown represent standard deviation in all cases.
8.3 Results and discussion

8.3.1 Infectivity of rAd5, rAd28 and rAd35 vectors

8.3.1.1 Infectivity on sorted DCs subset

I began the study by comparing the in vitro infectivity of rAd5, rAd28 and rAd35 on isolated human DCs. rAd28 and rAd35 efficiently infected mDCs (59%±21 and 59%±32 eGFP⁺ cells at MOI 100, respectively). In contrast, rAd5 required a 10-fold higher virus inoculum to show similar levels of infectivity in mDCs (43%±15 eGFP⁺ cells at MOI 1000) (Figure 5). Furthermore, rAd28 and rAd35 infected over 25% of pDCs at MOI 100, whereas rAd5 exposure did not show detectable infection at any inoculum. These differences in infectivity between rAd5 and rAd35 have been described previously (154-156). These data suggest that the infectivity of rAd28 is much closer to that of rAd35 than rAd5. To keep the rate of infectivity consistent between vectors, an MOI of 1000 will be used for rAd5 and an MOI of 100 will be used for rAd28 and rAd35 for all subsequent in vitro humans studies.

Figure 5 – Infection frequencies of mDCs and pDCs treated with rAd5 vector, rAd28 vector, or rAd35 vector (n=5).
8.3.1.2 Infectivity on PBMCs

To test the ability of rAd5, rAd28, and rAd35 to infect other human immune cells, I performed similar infections on unfractioned PBMCs. At an MOI of 100, rAd28 and rAd35 infected mDCs (31%±24, 31%±26), pDCs (24%±21, 20%±13), monocytes (28%±21, 28%±22), and NK cells (7%±5, 6%±5) (Figure 6). rAd5 infected mDCs (22%±20), monocytes (27%±14), and NK cells (6%±5) at a similar levels. As expected, rAd5 did not infect pDCs. The rate of infection of B cells, CD4 T cells, and CD8 T cells was negligible with all rAd vectors. Importantly, the amount of insert expressed by each individual cell appears consistent between vectors and independent of the overall rate of infection, as measured by insert mean fluoresce intensity (MFI) of infected cells (data not shown).

Figure 6. Infection frequencies of mDCs, pDCs, monocytes, NK cells, B cells, CD4 T cells, and CD8 T cells from PBMCs treated with rAd5 vector (MOI1000), rAd28 vector (MOI100), or rAd35 vector (MOI100), each with an uninfected control (n=8).
It has been speculated that one possible mechanism for rAd5’s increased immunogenicity is better infectivity of rAd5 than rAd28 or rAd35, but this is not observed. rAd5 is less infectious than rAd28 or rAd35, but this does not impact the immunogenicity of rAd5.

8.3.2 Receptor usage

The entry receptors used by Ad5 and Ad35 are CAR and CD46, respectively (403). The entry receptor for Ad28 is less clear. An anti-CD46 antibody was unable to block infection of mDCs by rAd5 but efficiently blocked infection by rAd35 (Figure 7). In contrast, the anti-CD46 antibody only modestly reduced infection of mDCs by rAd28, and only at high concentrations. The isotype-matched control antibody showed no reduction in infectivity at any dose for any vector. While I cannot rule out that Ad28 may be using CD46 as one of several cellular receptors, I can conclude that Ad28 is not as dependent as Ad35 on CD46 for infection of human mDCs.

![Figure 7. Infection frequencies of mDCs treated with anti-CD46 antibody (solid line) or a matched isotype control (dashed line) (n=4).](image)

8.3.3 rAd vector-induced maturation

To determine the degree of maturation and activation induced by rAd vectors, I analyzed co-stimulatory receptor CD80 expression on mDCs and pDCs (Figure 8) 24 hours post-infection. In both mDCs and pDCs exposed to rAd28 and rAd35, CD80 expression was
upregulated similar to that induced by TLR stimulation. In contrast, expression of CD80 in either of the DC subsets exposed to rAd5 was unchanged compared to the uninfected control. The upregulation of other DC activation markers such as CD86 and CD40 in cultures exposed to rAd28 or rAd35, but not rAd5 (data not shown) confirm the activation of DCs by rAd28 and rAd35, but not rAd5 exposure.

Figure 8. CD80 expression in mDCs (A) and pDCs (B) treated with rAd5 vector (MOI1000), rAd28 vector (MOI1000), or rAd35 vector (MOI1000) (black line) compared to a TLR7/8-ligand-treated (grey line) controls and uninfected controls (grey background).

8.3.4 mRNA expression profile

To further characterize the effects of rAd exposure on human DCs I, with help from collaborators, performed gene array analysis on sorted populations of rAd-infected mDCs and pDCs. Microarrays of mDCs infected with rAd28 and rAd35 showed that the expression levels of 781 and 807 genes were either upregulated or downregulated as compared to donor-matched mock-infected controls, respectively (|FC|>2 at 5% FDR) (Figure 9). Conversely and unexpectedly, no genes were differentially expressed upon
infection with rAd5. This suggests that exposure to or infection with rAd5 has little to no impact on mDC function or activation. This difference in impact was also observed with pDCs, where expression levels of 202 and 198 genes were significantly altered upon infection with rAd28 and rAd35, respectively, compared with no changes after infection with rAd5. Many of the genes altered upon infection with rAd28 and rAd35 related directly to type I interferon and type I interferon signaling (Figure 10). Pathway analysis with IPA software confirmed that activation of type I interferon was a major effect of infection with rAd28 and rAd35 in both mDCs and pDCs (Figure 11). Additionally, 2D multidimensional scaling focusing on interferon related genes (IRGs) revealed that samples infected with rAd28 and rAd35 generally clustered together in mDC and pDC samples, away from the clusters formed by samples infected with rAd5 and mock-infected controls (Figure 12).

Figure 9. Number of genes differentially expressed relative to the uninfected control (|FC|>2 at 5% FDR).
Figure 10. Unsupervised heatmap representation and hierarchical clustering of the expression values for the 100 probes with the largest within-probe standard deviation across all samples.
Figure 11. Heatmap colors represent P values (Fisher’s exact test) for the over-representation of genes forming part of a given canonical pathway in the list of genes differentially expressed for a given incubation relative to the uninfected control. Statistical significance for the Fisher’s exact test is achieved at 5% FDR, and differential expression was defined as |FC|>1.5 at 30% FDR, together with P<0.01.

Figure 12. Two-dimensional multidimensional scaling. Genes were filtered for IRGs prior to applying the algorithm.

Many of the genes upregulated by infection with rAd28 and rAd35, but not by rAd5, are directly involved in the type I interferon response in both mDCs and pDCs (Figure 13). I therefore compared IFNα production induced by these vectors. pDCs infected with rAd28 and rAd35 produced large amounts of IFNα (25 ng/mL±19 and 29 ng/mL±19, respectively), while pDCs infected with rAd5 and uninfected controls did not produce IFNα (Figure 14). Notably, rAd28 and rAd35 infection led to levels of IFNα production
that were similar or higher than those induced by TLR7/8 ligation (19 ng/mL±16).

PBMCs cultures exposed to the rAd vectors showed a similar trend (data not shown).

![Figure 13](image)

**Figure 13.** Heatmap representation of (log2) fold-changes top most significant IRGs relative to the uninfected control from the same individual. Specifically, genes were selected as the top 10 upregulated and top 10 downregulated IRGs for each incubation separately, subsequently applying a cutoff for statistical significance at |FC|>5 at 5% FDR to limit the number of genes shown.

![Figure 14](image)

**Figure 14.** The level of IFNa, measured by ELISA, in the supernatant of pDCs treated with rAd5 vector (MOI1000), rAd28 vector (MOI100), rAd35 vector (MOI100), or TLR7/8-ligand, together with uninfected controls (n=5).
Similarly mDC cultures infected with rAd28 or rAd35 upregulated type I IFN production, as measured by ELISA, but not mDC cultures infected with rAd5 or in unexposed cultures (data not shown). However given the ability of pDCs to produce massive amounts of IFNα even a small amount of contaminating pDCs could be responsible for these results.

8.3.5 Loss of insert expression

To determine the impact of the innate immune response activated by rAd28 and rAd35, but not rAd5, I followed the expression level of GFP in exposed PBMC cultures for 4 days (Figure 15, top left panel). PBMC cultures exposed to rAd5 maintained high levels of insert expression throughout the experiment (Day 1 - 3.0% GFP+ ± 1.7, Day 4 - 3.5 GFP+ ± 1.5) while PBMC cultures exposed to rAd28 or rAd35 rapidly lost insert expression after 24 hours (Day 1 - 1.8% GFP+ ± 0.6, Day 4 - 0.4% GFP+ ± 0.2, and Day 1 - 3.9% GFP+ ± 1.5, Day 4 - 0.5% GFP+ ± 0.1, respectively). Gating on individual cell populations revealed that insert loss occurs in monocytes and mDCs infected with rAd28 and rAd35, but not in pDCs (Figure 15, top right and bottom left panels, respectively). No insert loss was detected in any cell population infected with rAd5.
Given that a strong majority of insert positive cells are located in the monocyte compartment for all three rAd vectors (data not shown) I choose to focus primarily on the loss of insert from monocytes.

8.3.6 Apoptosis of monocytes

In addition to the loss of insert expression, the percentage of monocytes was greatly reduced in PBMCs exposed to rAd28 or rAd35 for 48 hours (4.5% CD14⁺ ± 2.9 and 3.5% CD14⁺ ± 2.5, respectively) (Figure 16, left panel). The remaining monocytes in these cultures had upregulated Aqua viability marker staining (48.8% Aqua⁺ ± 22.7 and 50.8% Aqua⁺ ± 29.8, respectively) (Figure 16, right panel). This loss of monocytes or
upregulation of cell death was not observed in rAd5-infected cultures or unexposed controls (11.4% CD14\(^+\) ± 9.7, 19.5% Aqua\(^+\) ±12.9 and 11.4% CD14\(^+\) ± 9.7, 14.9% Aqua\(^+\) ± 15.7, respectively). Further staining for Annexin V and active caspase 3 confirmed that monocytes infected with rAd28 or rAd35 were undergoing apoptotic cell death (data not shown).

Figure 16. Frequency of CD14+ monocytes from PBMCs treated with rAd5, rAd28, or rAd35 for 48 hours and an unexposed control (left panel). Viability staining of CD14+ monocytes from PBMCs treated with rAd5, rAd28, or rAd35 for 48 hours and an unexposed control (right panel). (n=4)

Interestingly no increase in insert loss, cell death, or apoptosis was observed from sorted monocytes cultures infected with any of the rAd vectors when compared with unexposed controls (Figure 15 and data not shown). This suggests that other cell populations may be involved in insert loss and increased apoptosis of monocytes treated with rAd28 or rAd35.

8.3.7 Insert expression is lost during apoptosis

The simultaneous loss of insert and upregulation of apoptosis in rAd28 or rAd35-exposed monocytes suggests they are related. To determine the impact of apoptosis on insert loss the expression of Aqua viability makers and active caspase 3 was compared between insert expressing and non-insert expressing monocytes from PBMCs cultures infected
with the rAd vectors. Strikingly, no insert positive cells costained for either Aqua viability marker or active caspase 3 in any rAd-exposed samples (Figure 17A). All Aqua viability marker or activate caspase 3 positive cells in the PBMC cultures were insert-negative for all rAd vectors. Consistent with previous data, PBMCs exposed to rAd28 or rAd35 had increased apoptosis compared to rAd5-exposed samples or unexposed controls, but this increase was limited to insert negative cells.

These observations can be interpreted two ways; either insert positive cells contain a large survival advantages over insert negative cells or insert expression is lost very early during apoptosis, before caspase 3 is activated. To determine which of these options occurs CD14\(^+\) insert\(^+\) cells were sorted from PBMCs infected with each rAd vector. These sorted cells were labeled with a fluorescent dye and mixed back with PBMCs. After 24 hours in culture levels of insert expression in labeled CD14 cells were measured. Cultures infected with rAd28 or rAd35 lost significantly more insert expression than rAd5-exposed samples (34.7\% GFP\(^+\) ± 6.3 and 40.1\% GFP\(^+\) ± 5.2 vs 61.3\% GFP\(^+\) ± 13.5) (Figure 17B). Importantly, all cells that remained GFP\(^+\) were negative for Annexin V or Aqua viability marker staining (Figure 17C). Conversely, cells that had lost insert expression had high levels of Annexin V or Aqua viability marker staining indicating they were dead or dying. Combined these observations strongly support the hypothesis that insert expression is lost early during apoptosis and that apoptosis of monocytes leads to reduced insert expression and persistence.
Figure 17. (A) Viability (upper panels) and active Caspase 3 (lower panels) staining of PBMCs gated on CD3, CD14+ GFP+, or CD14+ GFP- cells after exposure to rAd5, rAd28 or rAd35 and an uninfected control after 48 hours (data are representative of four independent experiments). (B) GFP+ cells sorted from violet-labeled CD14+ cells after 24-hour exposure to rAd5, rAd28, or rAd35 are co-cultured with PBMCs. The percentage of violet-labeled CD14+ cells remaining GFP+ after 24 co-culture plotted
(n=4). (C) Viability staining of cells remaining GFP+ (upper panels) and cells that lose GFP expression (lower panels) (data are representative of four independent experiments).

8.3.8 NK cell activation

A pair of studies, one in vitro in human cells and one in vivo in mice, have explored the role of NK cells in the removal of insert positive cells following exposure to rAd vectors. One study found that NK cells in rAd5 vaccinated mice were involved in the removal of insert from the liver (404). Another study found that IFNα produced by pDCs exposed to rAd35, but not pDCs exposed to rAd5 was sufficient to activate human NK cells (405). Given the ability of NK cells to kill virally infected cells I hypothesized that NK cells activated by IFNα and other soluble factors such as IL12 and IL18 were preferentially killing monocytes infected with rAd vectors (406).

8.3.8.1 NK cells activation in rAd exposed PBMCs

To explore the role of NK cell in monocyte killing PBMCs were exposed to the rAd vectors and incubated for 24 hours. The level of CD69 expression in CD56⁺ CD3⁻ NK cells was then used to quantify the level of activation. NK cells in PBMC cultures exposed to rAd28 and rAd35 strongly upregulated CD69 compared to rAd5-exposed cultures or unexposed controls (Figure 18). This confirms the previous finding that NK cells from PBMCs exposed to rAd35, but not rAd5 are strongly activated and shows that rAd28 exposure also activates NK cells. Importantly this finding further demonstrates that rAd28 and rAd35 activate innate immunity, while rAd5 does not.
Figure 18. Expression level of the activation marker CD69 on NK cells from PBMCs cultures exposed to rAd vectors.

8.3.8.2 NK cell activation is induced by soluble factors.

Previous work has shown that treatment with IFNα and exposure to target cells will activate NK cells (407, 408). To determine if this occurs in the context of rAd infection, I first exposed NK cells to media, media supplemented with IFNα, conditioned media from PBMCs exposed to the rAd vectors, or conditioned media from pDCs exposed to the rAd vectors for 18 hours. Cultures were then co-cultured for 6 hours with monocytes previously infected with rAd vectors, and unexposed monocytes or K562 cells, a known NK cell targets, as controls. As expected all NK cells exposed to IFNα supplemented media upregulated CD69 (Figure 19A) and CD107a (Figure 19B), a marker of degranulation, while all NK cells cultured with unsupplemented media showed no upregulation of CD69 or CD107a. NK cells treated with conditioned media from PBMCs or pDCs exposed to rAd28 or rAd35 strongly upregulated CD69 or CD107a. Conversely NK cells exposed to conditioned media from rAd5 exposed PBMCs or pDCs or untreated PBMCs or pDCs had no upregulation of CD69 or CD107a.
Figure 19. Percentages of NK cells positive for CD69 (A) or CD107 (B) staining after coculture with rAd infected monocytes in media (R10), media conditioned from rAd-infected PBMCs, media conditioned from rAd-infected pDCs, or IFNα-supplemented media.

Combined these finding confirm that exposure to IFNα and target cells is sufficient to activate NK cells. Additionally, they prove that soluble factors, including IFNα induced by rAd28 and rAd35, but not rAd5, in combination with target cells will activate NK cells. Interestingly NK cells treated with conditioned media from rAd28 and rAd35 exposed PBMCs exhibited significantly greater upregulation of CD69 and CD107a than NK cells from conditioned media from pDCs exposed to rAd28 and rAd35. This finding was unexpected as conditioned media from pDC exposed to rAd28 or rAd35 contain significantly more IFNα than PBMCs exposed to rAd28 or rAd35. This suggest that
other cytokines, in addition to IFNa, may be present in PBMC supernatant and contributing to the activation of NK cells. Cytokines such as IL12, IL15, IL18, and RANTES are all known to activate NK cells, particularly in combination (406). More work needs to be completed to determine the role these cytokines may play in the activation NK cells in PMBC cultures exposed rAd28 or rAd35.

8.3.9 Impact of IFNα on insert expression
Given the ability of IFNα to activate NK cells and the ability of activated NK cells to remove rAd vector-infected cells, I then determined the impact of exogenous IFNα on PBMC cultures exposed to rAd5, rAd28, and rAd35. PBMC cultures exposed to rAd5 and treated with exogenous IFNα had over a 2 fold down regulation of insert expression when compared with PBMCs exposed to rAd5, but no exogenous IFNα (Figure 20, top panel). Cultures exposed to rAd28 or rAd35 showed no difference in insert expression in response in IFNα treatment.

Given the reduction in insert expression in response to treatment with IFNα I then explored the effects of blocking IFNα signaling with a monoclonal antibody on vector expression in rAd28 and rAd35-exposed PBMCs. As expected no differences were detected in rAd5-exposed PBMCs in response to IFNAR neutralizing antibody (Figure 20, bottom panel). However, treatment with IFNAR neutralizing antibody increased insert expression in rAd28 exposed cultures over 2 fold. Treatment of rAd35-exposed PBMCs had no effects on insert expression.
These observations show that IFNα signaling can reduce insert expression. Treatment with IFNα reduced insert expression in rAd5-exposed cultures and blocking IFNα increased insert expression in rAd28-exposed cultures. The observation that the addition of exogenous IFNα to rAd28 and rAd35-exposed DCs does not further decrease the fraction of insert positive cells suggests that the amount of vector-induced IFNα produced is already maximally suppressive and that this suppression is limited to a twofold reduction of insert expression in vitro. This observation, combined with my observation that NK cell activation is already maximal in PBMCs exposed to rAd28 and rAd35 may hint at a potential mechanism. It may be that the addition of extra IFNα to rAd28 and rAd35 cultures has no impact because the NK cells are already maximally activated and cannot be further activated. This hypothesis could also explain why treatment of PBMCs exposed to rAd35 with IFNAR neutralizing antibodies has no effect.
rAd35 may induce the production of other NK cell activating cytokines that compensate for the loss of IFNα signaling. While the hypothesis that activation of NK cells by IFNα reduces insert expression remains an interesting possibility, more research must be done before it can be confirmed or disproven.

8.3.10 Induction of IFNα in mice
Given the conclusion that the ability to induce type I interferon represents the major difference between rAd5 and rAd28 or rAd35 in vitro, I wished to examine the potential impacts of Ad-induced IFNα on vaccination in vivo. To do this I examined the immune responses in both wildtype and IFNAR−/− mice vaccinated with the rAd. However I first needed to confirm that the results observed in vitro in human cells also occurred in mice.

8.3.10.1 In vitro
The expression of the receptors for Ad5 (CAR) and Ad35 (CD46) differs between humans and mice (409-411). I therefore evaluated the ability of rAd5, rAd28, and rAd35 to infect and induce IFNα by exposing sorted mouse DCs to the rAds. As expected higher inocula of rAd28 and rAd35 were required to achieve infection and all three vectors were able to infect murine DCs (Figure 21A). Interestingly rAd5 is able to infect murine pDCs, but not human pDCs. The reason for this discrepancy is not currently known, but likely involved differing expression of entry receptors between species.

Importantly, the pattern of IFNα induction by these vectors observed in human DC cultures held in mouse DC cultures. As observed in human DCs, only rAd28 (352
pg/ml±121) and rAd35 (417 pg/ml±161) were able to induce IFNα production in mouse DCs, while neither rAd5 nor an uninfected control produced detectable IFNα (Figure 21B). Even when the same inocula of all three vectors were used, rAd28 and rAd35, but not rAd5, induced IFNα production (data not shown).

8.3.10.2 In vivo

To confirm the differential induction of IFNα production in vivo, I injected mice subcutaneously with the rAd vectors (1x10^9 VP/mouse). Mice injected with rAd28 and rAd35 vectors had serum IFNα levels of 153 pg/ml±88 and 206 pg/ml±121, respectively, 6 hours post-injection (Figure 21C). Serum IFNα levels in the rAd5-treated, PBS-treated, and naïve mice were all below the detection limit of the kit.
Figure 21. (A) Infection frequencies of sorted murine CD11c+ splenocytes treated with rAd5 vector (MOI1000), rAd28 vector (MOI10,000), or rAd35 vector (MOI10,000), together with uninfected controls (n=5). (B) Levels of IFNα, measured by ELISA, in the supernatant of sorted murine CD11c+ splenocytes treated with rAd5 vector (MOI10,000), rAd28 vector (MOI10,000), or rAd35 vector (MOI10,000), together with uninfected controls (n=5). (C) Serum levels of IFNα, measured by ELISA, in mice immunized with rAd5 vector (1x10^9 VP/mouse), rAd28 vector (1x10^9 VP/mouse), or rAd35 vector (1x10^9 VP/mouse), together with PBS-injected and naïve controls, at 6 hours post-vaccination. (n=5)

8.3.11 Impact of IFNα on insert expression in mice

Given the impact of exogenous IFNα treatment on insert expression in human primary cells, the impact of exogenous IFNα in mice was assayed. I first compared the infectivity of each vector in wildtype mouse DCs exposed or not exposed to exogenous IFNα. rAd5-infected DCs treated with IFNα were found to have a 1.8-fold decrease in GFP
expression compared with untreated rAd5-infected DCs (Figure 22, top panel). No difference in GFP expression was detected between IFNα treated or untreated DCs infected with rAd28 or rAd35.

I also compared the infectivity of the rAd vectors in DCs isolated from IFNAR−/− mice to DCs from wildtype mice. In rAd5-infected samples I found no difference in GFP expression between wildtype and IFNAR−/− DCs (Figure 22, bottom panel). In rAd28 and rAd35-infected samples DCs from IFNAR−/− mice expressed GFP at over twice the rate of wildtype DCs. These results are mostly consistent with studies performed in humans. However in IFNAR−/− mice rAd35 insert expression is increased where no increase is observed in human cells treated with an IFNAR neutralizing antibody. This discrepancy may be caused by incomplete neutralization of IFNAR in the presence of high IFNα associated with rAd35 infection in humans, by additional cytokines associated with rAd35 infection in humans, but not in mice, or additional unknown mechanisms.
8.3.12 Impact of IFNα on rAd vaccination

8.3.12.1 IFNα signaling reduces the magnitude of CD8 T cell responses following low-dose vaccination

Since the data generated both with human and murine cells showed that high levels of IFNα were induced by rAd28 and rAd35, but not by rAd5, I investigated the impact of this differential IFNα induction on vector immunogenicity. Previous studies have shown that rAd5 is significantly more immunogenic at lower doses than either rAd28 or rAd35 (412). I therefore immunized both wildtype and IFNAR−/− mice with a low dose (5x10^7 VP/mouse) of each rAd vector encoding a SIV Gag insert and compared the magnitude of the induced T cell response by quantifying the number of tetramer+ CD8 T cells specific for a dominant epitope derived from SIV Gag (AL11) (413). Only rAd5 immunization
was able to induce a large AL11-specific T cell response in both wildtype (peak 21.0%±7.4) mice and IFNAR−/− (peak 25.3%±4.5) mice (Figure 23A, top panel). Both rAd28 (peak 22.5%±7.4) and rAd35 (peak 18.0%±5.8) immunization induced a frequency of AL11-specific T cells similar to that induced by rAd5 in the IFNAR−/− mice, but not in the wildtype mice (Figure 23A, middle and bottom panels, respectively). Wildtype mice developed a small AL11-specific CD8 T cell response after immunization with rAd28 (peak 6.4%±2.2), and no measurable response was generated by rAd35 immunization. This difference in immunogenicity between wildtype and IFNAR−/− mice immunized with rAd28 and rAd35 could be overcome by increasing the immunization dose. At a 20-fold higher inoculation (1x10⁹ VP/mouse), all three vectors showed similar immunogenicity in wildtype and IFNAR−/− mice (Figure 23B).
Figure 23. Frequencies of AL11-specific CD8 T cells, quantified by tetramer staining, after low dose (5x10^7 VP/mouse) (A) (n=5) and high dose (1x10^9 VP/mouse) (B) (n=4) vaccination of wildtype and IFNabr^-/- mice with rAd5 vector, rAd28 vector, or rAd35 vector, all encoding SIV Gag.

Here I show that increased innate immune activation, notably IFNα signaling, by rAd vectors can have detrimental effects on the immunogenicity of the insert, reducing the magnitude of the resulting CD8 T cell response. IFNAR^-/- mice, when vaccinated with rAd28 and rAd35, could not respond to the IFN, and developed more robust CD8 T cell responses compared to wildtype mice, and similar to those observed after rAd5 vaccination. It is important to note that the detrimental impact of IFNα signaling is only observed at low inoculum vaccination. At a high inoculum no differences were observed
between wildtype and IFNAR−/− mice. This again may support the idea that reduced insert load and duration, caused by IFNα signaling, may be limiting the immunogenicity of rAd vectors. At the higher vaccine inoculum the amount of vector present in the mice may be enough to overcome detrimental effects of IFNα-induced insert loss.

8.3.12.2 IFNα signaling alter CD8 cell phenotype after high dose vaccination

Next, I determined the impact of excess IFNα production on long-term memory differentiation as defined by CD127 (IL-7 receptor) expression (414) in the high-dose immunized mice. Groups with either no induction of IFNα (rAd5-wildtype, rAd5-IFNAR−/−) or groups with interrupted IFNα signaling (rAd28-IFNAR−/−, rAd35-IFNAR−/−) failed to establish high percentages of AL11+ CD8 T cells that expressed CD127 (32.4%±15.4). In contrast, groups with intact IFNα induction and signaling (rAd28-wildtype, rAd35-wildtype) maintained a much higher percentage of AL11+ CD8 T cells that expressed CD127 (59.1%±8.8) (Figure 24A).

To determine cytokine production profiles in SIV Gag-specific CD8 T cells from high-dose immunized mice, I measured the frequency of IFNγ, IL2, and TNF producing CD8 T cells by flow cytometry after stimulation with the AL11 peptide. Overall, mice vaccinated with the rAd5 vector had slightly greater numbers of cytokine-producing cells than mice vaccinated with the rAd28 or rAd35 vectors (Figure 24B, upper left panel). This observation, although not statistically significant, is consistent with the larger AL11-specific CD8 T cell populations detected after high-dose vaccination. No significant
differences in the frequency of cytokine-producing CD8 T cells between wildtype and IFNAR\(^{-/-}\) mice were observed in any of the groups (Figure 24B).

When measured as a fraction of the total AL11-specific CD8 T cell response, wildtype mice vaccinated with rAd28 or rAd35 exhibited higher proportions of IL2-producing cells (57.2\(\pm\)6.3 and 60.6\(\pm\)10.0, respectively) compared to their IFNAR\(^{-/-}\) counterparts (29.22\(\pm\)2.9 and 32.7\(\pm\)13.2, respectively) (Figure 24B, lower panels). Both wildtype and IFNAR\(^{-/-}\) mice vaccinated with rAd5 had low percentages of IL2 producing cells (21.7\(\pm\)15.2 and 30.3\(\pm\)10.7, respectively) (Figure 24B, upper right panel). Wildtype mice vaccinated with rAd28 and rAd35 had a higher proportion of their response dedicated to the co-expression of three cytokines (IFN\(_{\gamma}\)\(^{+}\) TNF\(^{+}\) IL2\(^{+}\)) than IFNAR\(^{-/-}\) mice (Figure 24C). This difference was mostly due to the higher proportion of IL2-producing cells in the wildtype mice vaccinated with rAd28 and rAd35. Both wildtype and IFNAR\(^{-/-}\) mice vaccinated with rAd5 displayed a relatively lower proportion of triple cytokine positive AL11-specific CD8 T cells.
Figure 24. (A) Frequencies of CD127+ AL11-specific CD8 T cells from mice vaccinated with a high dose of rAd5 vector, rAd28 vector, or rAd35 vector (n=4). (B) Top left panel: frequencies of AL11 peptide-stimulated CD8 T cells producing IFNg, IL2, or TNF after vaccination of wildtype or IFNabr−/− mice with rAd5 vector, rAd28 vector, or rAd35
vector (n=4). Top right and lower panels: frequencies of IFNg+, IL2+, or TNF+ cells as a percentage of the total AL11-specific response for each vector (n=4). (C) SPICE plots illustrating the functionality of AL11-specific CD8 T cells from wildtype and IFNabr−/− mice vaccinated with rAd5 vector, rAd28 vector, or rAd35 vector (n=4).

At a high inoculum of rAd28 and rAd35, I observed that vaccine-elicited CD8 T cells from wildtype mice were more polyfunctional and had a more pronounced long-term memory phenotype than the vaccine-elicited CD8 T cells from IFNAR−/− mice or mice vaccinated with rAd5. These findings are consistent with prior observations showing an increased long-term memory phenotype in CD8 T cells stimulated with IFNα (415). It is also feasible that CD8 T cell expansion in the presence of IFNα leads to a predominantly central memory-like phenotype, which has previously been associated with enhanced polyfunctionality due to the retention of IL2 production (416). Alternatively, separate mechanisms may be operating to mediate these distinct effects. IFNα has also been shown to either promote or suppress T cell proliferation and survival depending on the timing, level, and duration of exposure. The effects of IFNα signaling on CD8 T cell proliferation and survival after vaccination with rAd vectors are not fully understood. However, the inherent paradox of my results is that vector-induced IFNα production can lead to a reduction in the magnitude of the CD8 T cell population while simultaneously increasing the quality and longevity of the response.
8.4 Conclusions

My results show that, in stark contrast to rAd28 and rAd35, rAd5 fails to induce significant changes in DC mRNA expression or maturation. These changes are independent of infectivity and primarily involve the activation of innate immunity, particularly IFNα signaling. The production of type I IFN and the activation of innate immunity by rAd28 and rAd35, but not rAd5 correlates well with the loss of the insert expression due to upregulated apoptosis of insert positive cells. Interestingly, NK cells, which have been shown to be responsible for clearance for vector-infected cells in vitro and vivo, are strongly activated by soluble factors, such as IFNa, associated with rAd28 or rAd35 exposure, but not exposure to rAd5.

I found that this paradigm was consistent in mouse DCs, with rAd28 and rAd35, but not rAd5 inducing significant IFNα production and that intact IFNα signaling reduces vector expression during vaccination. The induction of IFNα in mice vaccinated with rAd28 or rAd35 leads to a decrease in the magnitude, but an improvement in the long-term memory potential (CD127 expression) and cytokine polyfunctionality of the insert-specific CD8 T cell response. The presence and impact of IFNα production and signaling should therefore be taken into account when designing future rAd vectors. Furthermore the impact of NK cell activation and insert loss on vaccination must be further researched.
9 Impact of adenovirus vector vaccination on SIV challenge

9.1 Introduction

Recently, a study testing the effectiveness of a DNA prime/rAd5 boost vaccination of 19 rhesus macaques expressing the MHC class I allele Mamu-A*01 was conducted (417). A 50% reduction in SIV acquisition was observed in vaccinated monkeys compared with a non-vaccinated control group. Protection from viral acquisition was strongly associated with monkeys that expressed two TRIM5 alleles known to restrict SIV replication compared with monkeys that expressed at least one permissive TRIM5 allele. Additionally, neutralizing antibody and virus specific CD4 T cell responses were shown to play a role in protection against viral acquisition. Innate immunity or CTL responses were not associated with protection from acquisition.

Of the 19 vaccinated, animals challenged with SIV 7 acquired infection. Of these 7 monkeys, 3 were able to partially or completely suppress set point viral loads (Figures 25A and B). Interestingly suppression of viral load correlates well with the post-vaccination, pre-challenge viral inhibitory activity (VIA) of EM CD8 T cells (Figures 25 C and D). This suggests that preexisting EM CD8 T cells with high VIA help to suppress viral loads following vaccine breakthrough infection. As potential HIV vaccines may offer incomplete protection from acquisition, it is important to determine the mechanisms involved in viral suppression. My role in this study was to determine the post
vaccination, pre-challenge functional profiles of SIVgag CM9-specific EM CD8 T cells from vaccinated monkeys and correlate those profiles with VIA.

Figure 25. (A) Comparison of infection outcomes to prechallenge VIA. Red dots represent infected macaques that exhibited weak VIA. (B) Longitudinal viral load measurements in macaques that acquired infection. Red lines represent macaques with weak VIA. Black lines represent macaques with strong VIA. (C-D) Simple linear regression predicting mean (C) set-point and (D) peak viral loads by prechallenge VIA.
9.2 Materials and methods

Assessment of cytokine production and degranulation by polychromatic flow cytometry

Approximately $2 \times 10^6$ PBMCs were cultured in RPMI supplemented with 2 mM L-glutamine (Invitrogen), 1% streptomycin and penicillin (Invitrogen), and 10% FCS (Gibco) containing monensin (0.7 µg/ml; BD Biosciences) and brefeldin A (10 µg/ml; Sigma-Aldrich) in the absence or presence of CD107a MAbs and peptides (15mers overlapping by 11 residues) corresponding to full-length SIVmac239 Gag (2 µg/ml for each peptide at 5 µl/ml; National Institutes of Health AIDS Research and Reference Reagent Program) for 6 h. Following washing, cells were surface stained for CD4, CD8, CD28, and CD95 and Aqua viability marker. Cells were then permeabilized (Cytofix/Cytoperm kit; BD Biosciences) and stained for CD3, IFNγ, IL2, MIP-1β, and TNF. All samples were analyzed using a LSR II flow cytometer (BD Biosciences). Data analysis was performed using FlowJo version 9.3.2 (Tree Star). Cell aggregates, CD14+, CD19+, and Aqua positive cells were excluded from the analysis.

Assessment of production of killing molecules by SIV-specific CD8 T cells

PBMCs were stained with a fluorochrome-labeled peptide-Mamu-A*01 (pMamu-A*01) SIV Gag CM9-specific tetramer constructed as previously described (401) for 15 mins. Following washing, cells were surface stained for CD4, CD8, CD28, CD95, and PD1 and Aqua viability marker. Cells were then permeabilized (Cytofix/Cytoperm kit; BD Biosciences) and stained for CD3, perforin, granzyme A, and granzyme B. All samples were analyzed using a LSR II flow cytometer (BD Biosciences). Data analysis was
performed using FlowJo version 9.3.2 (Tree Star). Cell aggregates, CD14+, CD19+, and Aqua positive cells were excluded from the analysis.

Statistical analyses

For statistical analysis monkeys with EM CD8 T cells with greater than a 1 log reduction in viral load during VIA assays were considered to have strong VIA while cells with less than 1 log reduction were consider to have weak VIA. Correlations were performed using the nonparametric Spearman Rank test with GraphPad Prism version 5.0c software (GraphPad Software). Analysis and presentation of distributions was performed using SPICE version 5.2, downloaded from http://exon.niaid.nih.gov (402). Comparison of distributions was performed using a Student's T-test and a partial permutation test as described previously (402). Error bars represent median values and p values <0.05 were considered significant.
9.3 Results and discussion

9.3.1 Cytokine production and degranulation

EM CD8 T cells from rhesus macaques with strong VIA mobilized higher, although not statistically significant (p = 0.0823) level of CD107a, a marker of lymphocyte degranulation, than monkeys with weak VIA (Figure 26A). This increase was largely due to an increase in the fraction cells expressing CD107a, but no cytokines, though there was a slight increase of CD107a in other populations as well (Figure 26B). No differences in cytokine production were observed between EM CD8 T cells with strong or weak VIA.

9.3.2 Antigen specific EM CD8 T cells

Similarly perforin expression in antigen-specific EM CD8 T cells was higher in strong VIA populations than populations with weak VIA, but the difference was not statistically significant (p = 0.0823) (Figure 26C). No differences were detected in granzyme A or granzyme B expression. Generally, most of the increase in perforin expression was due to an increase in single positive cells, but an increase in double and triple positive cells was also observed (Figure 26D).

9.3.3 CD107a and perforin correlate with VIA

However the level of VIA did significantly correlate with both an increase in the activity of CD107a in antigen-specific EM CD8 T cells and an increase in the percentage of
perforin expressing EM CD8 T cells (CD107a, p = 0.074, r = 0.7727; perforin p = 0.0336, r = 0.6545) (Figure 26E).

Figure 26. Functional profiles of SIV-specific CD8 T cells responses. (A and B) CD107a mobilization, and the production of IFNg, IL2, MIP1b, and TNF by gag-specific EM CD8 T cells. (A) The production of individual cytokines by gag-specific EM CD8 T cells from macaques with strong or weak prechallenge VIA. (B) SPICE plots illustrating the functionality of gag-specific EM CD8 T cells from macaques with weak and strong VIA (n=4). (C and D) Production of perforin, granzyme B and granzyme C by CM9-specific EM CD8 T cells (C) The production of individual killing molecules by CM9-
specific EM CD8 T cells. (D) SPICE plots illustrating the functionality of gag-specific EM CD8 T cells from macaques with weak and strong VIA. (E) Simple linear regression predicting the percentage of CD107a+ gag specific EM CD8 T cells (left panel) and the percentage of perforin+ CM9-specific EM CD8 T cells (right panel) by prechallenge VIA.

Collectively these results suggest that while the functionality of EM CD8 T cells with strong or weak VIA is generally similar there are important differences. The increase in perforin expression in strong VIA cells may suggest that EM CD8 T cells with strong VIA may have increased cytolytic ability allowing them to suppress viral proliferation. Similarly the increase in CD107a expression suggests an increased ability to degranulate in response to virally infected cells.
9.4 Conclusions

These results are generally consistent with previously observed findings suggesting that increases in cytokine polyfunctionality and the production of killing molecules are associated with ability to suppress viral loads. They also may explain, in part, the mechanisms used by strong VIA cells to reduce viral load. Microarray data comparing the post vaccination, pre-challenge mRNA profiles of monkeys with strong VIA to monkeys with weak VIA confirms the increase in cytokine and killing molecule production (Figure 27A-D).
Figure 27. CM9-specific EM CD8 T cells with strong VIA exhibit a distinct transcription profile from that of cells with weak VIA. (A) Heat map of the top 50 genes that are differentially expressed between non-stimulated and stimulated CM9-specific EM T
cells. (B) Expression levels of relevant genes (non-stimulated vs stimulated). (C) Heat map of the top 50 genes that are differentially expressed between stimulated CM9-specific macaques with strong VIA and macaques with weak VIA. (D) Expression levels of relevant genes (strong VIA vs weak VIA).

An additional finding of this study was that monkeys with strong VIA generally expressed more commonly observed clonotypes, called public clonotypes, than monkeys with weak VIA (Figure 28). Monkeys with poor VIA were more likely to express uncommon or unique TCR sequences, referred to as private clonotypes. Previous work has shown that public clonotypes, which are well represented in the population, are associated with lower setpoint viral loads, than private clonotypes (418). These observations are consistent with those findings.
Figure 28. Clonotypic architecture of Gag CM9- specific CD8 T cells (A and B) TRBV and TRBJ usage, CD3Rbeta amino acid sequences and frequencies of CD T cells clonotypes for CM9 for macaques with strong (A) and weak (B) VIA. Of Simple linear regressions predicting the prechallenge VIA of (C) EM CD8 T cells or (D) CM CD8 T cells by the number of public clonotypes and frequency of public clonotypes.
It has been reported that antigen-specific EM CD8 T cells generated and maintained by vaccination with a replication competent CMV vector can control SIV viral loads after viral acquisition from repeated low-dose challenge. The results that the VIA of EM CD8 cells, but not the VIA of CM cells correlate with lower viral load would support this observation.

Overall the larger study establishes a paradigm where TRIM5 allele expression is the key to preventing SIV acquisition in rhesus macaques. In animals with permissive TRIM5 alleles the presence of neutralizing antibody correlates partially with prevention of viral acquisition. In vaccinated animals that acquired SIV infection, the presence of EM CD8 T cells with strong VIA correlates well with lower setpoint viral loads.

This suggests that a dual function vaccine may be ideal in protecting against HIV. One obvious function of the vaccine would be to protect against HIV acquisition by inducing neutralizing antibodies at mucosal surfaces. Given the likelihood of incomplete protection against acquisition the vaccine would also induce and maintain a population of antigen-specific EM CD8 T cells to reduce viral load after vaccine breakthrough acquisition. This vaccine would reduce the spread of HIV; both directly by preventing viral acquisition through neutralizing antibodies and indirectly by reducing the infectivity of infected individuals, by reducing their viral load. Additionally by reducing viral loads this vaccine would delay CD4 loss and progression to AIDS in infected individuals.
10 Conclusions

10.1 Impact of innate immunity on vaccination

rAd vectors have been studied for potential use in vaccination strategies because of their ease of construction and ability to infect large numbers of cells. Vectors constructed from Ad5 are efficient at eliciting strong T cell responses, but are limited clinically by extensive preexisting immunity in the general population (124-127). Rare serotype adenoviral vectors, such as rAd28 and rAd35, circumvent issues related to preexisting immunity, but are less immunogenic than rAd5 (150). The goal of this study was to determine the factors responsible for the differing immunogenicity of the rAd vectors.

DCs play an important role in both the initiation of innate immunity and the development of adaptive immunity (419). This dual function, combined with the important role that DCs play in anti-viral immunity, led us to investigate the impact of rAd exposure on two blood DC subsets, mDCs and pDCs (420). Notably, both rAd28 and rAd35 exerted substantial effects on both mDCs and pDCs, particularly with respect to IFNα production and maturation. In striking contrast, rAd5 did not infect pDCs and had almost no detectable impact on either DC population, including any effects on IFN-related pathways. It has been proposed that vectors that induce innate immunity might perform better as vaccines due to potential adjuvant effects (403). Alternatively, vectors that do
not alter the host cell or activate innate immunity were considered ideal for gene therapy, where immunity can prove detrimental (421, 422).

In my thesis I show that increased innate immune activation, notably IFNα signaling, by rAd vectors can have detrimental effects on the immunogenicity of the insert, reducing the magnitude of the resulting CD8 T cell response. IFNAR−/− mice, incapable of responding to type I interferon, when vaccinated with rAd28 and rAd35 developed more robust CD8 T cell responses compared to wildtype mice, and similar to those observed after rAd5 vaccination.

10.2 Strong VIA correlates with CD107 and perforin expression
In a separate study I have found that antigen specific EM CD8 T cells with strong VIA generally exhibit more degranulation, as evidenced by CD107a expression, and increased perforin production than EM CD8 T cells. These cells have been shown to be more polyfunctional in monkeys with strong VIA than monkeys with weak VIA. Therefore strong VIA, the ability to degranulate and the production of perforin from EM CD8 T cells at the time of viral acquisition should be considered a correlate of protection associated with increased viral control.
11 Future directions

The use of rAd vectors as part of an HIV vaccine remains intriguing. The failure of the STEP trial using rAd5, which has widespread preexisting immunity, promoted the development rAd vectors from rare serotype Ads. However vaccines constructed from rare serotypes have so far proven to be less immunogenic than rAd5. Here I shown that innate immune activation, particularly IFNα production, induced by the rare serotypes but not rAd5, is at least partially responsible for the differing immunogenicity. These observations raise several interesting possibilities regarding the future of rAd vaccines.

11.1 Innate immune activation by rAd28 or rAd35

Given the negative implications of innate immune activation, it is necessary to determine what characteristics of rAd28 and rAd35 lead to the activation of innate immunity. The mechanism(s) underlying the differential induction of IFNα by rAd5 and the lower seroprevalence vectors, rAd28 and rAd35, is currently unknown. All three vectors used in this study are E1-deleted, rendering them replication-deficient and greatly reducing the transcription of the viral mRNA (423). Importantly, expression of two non-translated viral RNA sequences, Viral Associated-RNA (VA-RNA) I and VA-RNA II, transcribed by host RNA polymerase III, are only partially downregulated (424). These sequences are potentially transcribed in infected host cells. All Ad serotypes contain a VA-RNA I sequence, and most encode a VA-RNA II (425). rAd5 VA-RNA I has been shown to inhibit RNase L and PKR function, as well as alter gene expression by functioning as microRNA (90, 426, 427). The function of Ad5 VA-RNA II is not well understood but it
may play a compensatory role in VA-RNA I-deleted vectors (428). The sequences of VA-RNAs vary greatly between Ad5, Ad28, and Ad35, and it is unknown if VA-RNAs from rAd28 or rAd35 function in a completely similar manner to VA-RNA from Ad5. It is known, however, that rAd5 VA-RNAs induce moderate IFNα production at very high doses (MOI 10,000) by stimulating host PPRs (429). It is possible that rAd28 and rAd35 stimulate IFNα production through a similar mechanism at lower MOI due to differences in VA-RNA sequence or expression levels, causing them to more efficiently induce IFNα. If VA-RNA sequences or transcription levels are involved in innate immune activation it may be possible to alter the sequence or swap the VA-RNA sequences between vectors.

Alternatively, surface receptor engagement may play a role the differing activation of innate immunity. While little research has been done into the effects of CD46 crosslinking by binding to rAd35 fiber protein in DCs, significant work has shown that crosslinking CD46 on CD4 T cells with rAd35 fiber protein can have massive effects on signal transduction, immune activation, and cytokine production (430, 431). Given these findings it is possible that the crosslinking of CD46 by rAd35, but not rAd5 on DCs may alter their activation. More research needs to be done to determine what, if any, affects this has on DC function and activation.

Additionally, Ad5 and Ad35 have been shown to take different intracellular routes to the nucleus following receptor engagement (432). Ad5 rapidly exits the endosome following endocytosis and travels free in the cytoplasm to the nucleus. Ad35, by contrast, remains in the endosome longer, for over 2 hours, utilizing endosomal movement towards the
nucleus to travel within the cell. This difference in length of time in the endosome could potentially be important as TLRs 3, 7, 8, and 9 are all located in the endosome compartment (433). Altering the route of intracellular tracking may reduce innate immune activation and increase the immunogenicity of rAd28 and rAd35. Several chimeras combining rAd5 and rAd35 have been developed and were tested by me and in other labs. A chimeric virus expressing rAd5 virion with rAd35 fiber protein was shown to mimic the intercellular pathway of rAd35 indicating that interactions between viral fiber protein and host cellular receptor determine intracellular pathway (432).

Interestingly, the CpG content of rAd5 differs from that of rAd28 and rAd35 (434). rAd28 and rAd35 have a much lower ratio of CpG in their genome than rAd5. One study has suggested that this difference is due to evolutionary pressure on rAd28 and rAd35 due to their exposure to TLR9 (435). The genome of rAd5 is less exposed to TLR9 because of the reduced time in the endosome so there is reduced evolutionary pressure to reduce the frequency of CpG motifs. I attempted to measure the innate immune activation induced by this, and several other chimeric vectors, but their infectivity is very low making the results difficult to interpret (data not shown). It would be useful to study a chimera constructed from a rAd35 virion with rAd5 fiber. The chimera would have limited preexisting immunity and could potentially exhibit rapid endosome escape and low innate immune activation of rAd5.

Another study measured the ratio of the ideal TLR9 agonist sequence, GTCGTT, and found that it was more frequent in rAd5 than in the low seroprevalent rAds (436).
However, in this study rAd5 activated DCs more strongly than rare serotype rAds, contradicting results presented here and in many other studies. The reason for this discrepancy is not clear. Both siRNA and a dominant negative protein have been shown to interfere with the type I interferon.

It may be possible to design a rare-serotype rAd vector that co-expresses siRNA that interferes with IFNα mRNA or a dominant negative protein that bind to IFNAR antagonistically with the insert antigen. This could potentially interrupt innate immune activation and increase vector immunogenicity. Both anti-IFNα siRNA and dominant negative protein have been shown to disrupt and reduce interferon signaling (REFs). However several issues need to be addressed. The stoichiometric ratio of siRNA or dominant negative protein to insert protein would need to be determined. Additionally the kinetics of siRNA or dominant negative protein expression may be important to consider. Because IFNα is one of the initial cytokines produced quickly after infection, it may be critical that it’s signaling in interrupted early during infection.

11.2 Insert persistence

Alternatively, it may not be necessary to reduce the innate immune activation induced by rAd28 or rAd35 if a mechanism can be developed to increase insert persistence in the presence of innate immune activation. It may be possible to design inserts that are resistant to innate immune induced degradation and retain the correct conformation necessary for immunization.
The mechanisms involved in insert loss are not at all understood. It has been shown that NK cells in human cultures are activated by rAd35, but not rAd5. It has also been shown that NK cells are responsible for removing insert from the liver of rAd5-vaccinated mice. Potentially, the increased activation of NK cells by rAd35 vaccination could be increasing the rate of insert loss, possibly due to apoptosis of insert positive cells, but more work is needed to confirm this mechanism.

Additionally the two classical functions of IFNα signaling, inhibition of translation via PKR, and inhibition of transcription via RNAse L may by stopping insert synthesis and limiting antigen duration. More work needs to be done to determine the relative impact of these mechanisms, and other unknown mechanisms, on insert persistence and the effects of insert persistence on vaccination.

11.3 Additional rAd vectors
My studies presented here examine the immunogenicity of three extensively well-researched rAd serotypes used in many clinical trials. While both rAd5 and rAd28 or rAd35 are limited because of extensive preexisting immunity or innate immune activation, screening of additional serotypes may yield a vector with both low preexisting immunity and low innate immune activation. Such a vector would be ideal and would allow for easy construction. Additionally, more recent research has examined the used of Simian or Chimpanzee Ad viruses as vaccine vectors. These vectors have limited or no preexisting immunity in humans. These vectors can also be screened quickly for innate immune activation and IFNα production.
11.4 Impact of innate immunity on other viral vectors

While the induction of IFNα in the setting of rAd vaccination clearly has a negative effect on long-term immunogenicity it is unknown if this reduction is universal, affecting all viral vectors, or limited to rAd vectors. Notably rAd vectors are replication-incompetent so the duration of antigen available is already quite limited compared to infection with wildtype virus.

It is possible that the adaptive immune system ignores antigen that is successfully cleared by innate immunity. Responding to antigen regardless of duration would force the adaptive immunity to respond to all antigens, regardless of need and would waste host resources. Therefore it may be possible that innate immune clearance of insert would limit the success all replication incompetent vectors. Notably, replication competent vectors would likely avoid this rapid clearance due to their ability to infect additional cells and continuously produce antigen. More research needs to be completed to fully determine the impact of innate immune activation on all viral vectors.

11.5 Use of rAd vectors in vaccines strategies

Research presented here and elsewhere has suggested that a combination of neutralizing antibody and EM CD8 T cells are required for a protective HIV vaccine. Fortunately rAd vectors have been shown to induce both antibody and CD8 cell responses (437). However, any potential HIV vaccine will likely consist of multiple immunizations as part of a prime/boost vaccination. It is therefore necessary to determine the ideal use of rAd
vectors during vaccination to generate maximal neutralizing antibody and EM CD8 T cells.

While the use of rAd vectors during vaccination remains promising due to their unique ability to induce strong CD8 T cell response. However more research is required to determine their immunological mechanisms of vaccination and interactions with immunity. Here I show that increased innate immune activation may be detrimental to vaccination, but there are likely many other aspects of rAd vaccination that are yet to be fully understood. Once these processes are better understood it may be possible to design an ideal rAd vector capable of inducing strong protective immunity without complications from preexisting immunity.
Appendix I: Abbreviations

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<td>antibody</td>
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<td>Ad</td>
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<td>rAd</td>
<td>recombinant adenovirus vector</td>
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<td>blood dendritic cell antigen</td>
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<td>DC</td>
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<tr>
<td>dDC</td>
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<tr>
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<td>double-stranded deoxyribonucleic acid</td>
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<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
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<td>enzyme linked immunosorbent assay</td>
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<td>interferon response factor</td>
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<td>KIR</td>
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<td>LTNP</td>
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<td>NLR</td>
<td>nod like receptor</td>
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<td>NHP</td>
<td>non human primate</td>
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<tr>
<td>mAB</td>
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<td>mDC</td>
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<td>MDDC</td>
<td>monocyte derived dendritic cell</td>
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<tr>
<td>microRNA</td>
<td>mirco ribonucleic acid</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MLP</td>
<td>major late promotor</td>
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<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
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<tr>
<td>PBS</td>
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<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
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<td>PKR</td>
<td>protein kinase R</td>
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<tr>
<td>RLR</td>
<td>rig like receptor</td>
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<tr>
<td>RPMI</td>
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<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
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<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TE</td>
<td>terminal effector</td>
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<td>Th</td>
<td>helper T cell</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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<td>TLR</td>
<td>toll like receptor</td>
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<tr>
<td>VA-RNA</td>
<td>virus associated ribonucleic acid</td>
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<td>VDJ</td>
<td>variable, diversity, and joining</td>
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<td>VLPs</td>
<td>viral like particles</td>
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<td>WBCs</td>
<td>white blood cells</td>
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Bibliography


reduce risk for sexual transmission of HIV among men who have sex with men. *Cochrane Database Systemic Reviews* 3.


clinical evaluation of the Plasmodium falciparum blood-stage antigen MSP1 in ChAd63 and MVA vaccine vectors. *Molecular Therapy* 19:2269-2276.


immunodeficiency virus type 1 envelope during recent HIV infection. *Proceedings of the National Academy of Sciences* 102:18514.


and CD8 T cell depletions on the roles of cellular and humoral immunity in the
control of an SHIV-89.6 P challenge in DNA/MVA-vaccinated macaques.  
*Virology* 343:246-255.

375.  
Genescà, M., P. J. Skinner, J. J. Hong, J. Li, D. Lu, M. B. McChesney, and C. J.  
Miller. 2008. With minimal systemic T-cell expansion, CD8+ T cells mediate  
protection of rhesus macaques immunized with attenuated simian-human  
immunodeficiency virus SHIV89. 6 from vaginal challenge with simian  

376.  
Champagne, P., G. S. Ogg, A. S. King, C. Knabenhs, K. Ellefsen, M. Nobile,  
V. Appay, G. P. Rizzardi, S. Fleury, M. Lipp, R. Föster, S. L. Rowland-Jones, R.  

377.  
Haase, A. T. 2010. Targeting early infection to prevent HIV-1 mucosal  

378.  

379.  
*Nature Reviews Immunology* 5:783-792.

380.  
Koff, W. C., P. R. Johnson, D. I. Watkins, D. R. Burton, J. D. Lifson, K. J.  
Hasenkruk, A. B. McDermott, A. Schultz, T. J. Zamb, R. Boyle, and R. C.  
*Nature Immunology* 7:19-23.

381.  
Hansen, S. G., C. Vieville, N. Whizin, L. Coyne-Johnson, D. C. Siess, D. D.  
Drummond, A. W. Legasse, M. K. Axthelm, K. Oswald, C. M. Trubey, M. J.  
responses are associated with protection of rhesus monkeys from mucosal simian  

382.  
Hansen, S. G., J. C. Ford, M. S. Lewis, A. B. Ventura, C. M. Hughes, L. Coyne-  
Johnson, N. Whizin, K. Oswald, R. Shoemaker, T. Swanson, A. W. Legasse, M. J.  
Chiuchiolo, C. L. Parks, M. K. Axthelm, J. A. Nelson, M. A. Jarvis, M. J.  

383.  
Khoury, Y. F., K. McIntosh, L. Cavacini, M. Posner, M. Pagano, R. Tuomala, and  
viral load and plasma levels of CD4 binding site anti-gp120 antibodies. *Journal of  
Clinical Investigation* 95:732.

384.  
Chakraborty, H., P. K. Sen, R. W. Helms, P. L. Vernazza, S. A. Fiscus, J. J. Eron,  
burden in genital secretions determines male-to-female sexual transmission of  

385.  
Relation between HIV viral load and infectiousness: a model-based analysis. *The  

386.  
Shah, I. 2006. Correlation of CD4 count, CD4% and HIV viral load with clinical  
manifestations of HIV in infected Indian children. *Annals of Tropical Paediatrics:  
International Child Health* 26:115-119.


