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Induction Of Antibodies To Hiv-1 Envelope Using Simian Adenovirus Vaccines

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Abstract
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Several approaches can be employed to improve upon vaccination strategies: heterologous prime-boost regimens, immunogen design, and alternative adjuvants. To enhance Env-specific antibodies, I tested simian-derived adenovirus (SAdV) vectors expressing Env proteins and administered them in heterologous prime-boost regimens with recombinant Env in adjuvant. In the first study, I tested the SAdV vectors in prime-boost regimens with trimeric Env protein in aluminum salt. Priming with SAdV vector was advantageous over protein prime and inclusion of the trimeric protein induced neutralizing antibodies.

It has been suggested that a vaccine designed to elicit greater V2-specific antibody responses than that seen in the RV144 trial may be more effective at preventing HIV-1 infection. The variable 1 and 2 (V1/V2) region of Env has been grafted onto a protein scaffold and shown to exhibit a native conformation. In the second study, I sought to enhance the V2-specific antibody response using SAdV vectors expressing scaffolded V1/V2. SAdV vectors expressing scaffolded V1/V2 elicited greater V2-specific antibodies only transiently. However, SAdV vectors expressing either HIV-1 gp140 or scaffolded V1/V2 induced sustained response rates of V2 conformational antibodies more than one year after vaccination. This contrasts with the RV144 trial, where V2-specific antibody responses declined quickly after vaccination.

In the third study, I tested calcium phosphate (CaP) as an adjuvant to a recombinant Env protein compared to alum adjuvant. Alum, the most commonly used adjuvant, induces suboptimal immune responses. CaP has demonstrated preferential immune responses as an adjuvant in animals and humans. CaP did not elicit greater antibody responses in this study.

These studies demonstrate SAdV vectors as advantageous vaccine vector platforms and that novel immunogen design can improve HIV-1 vaccines.

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INDUCTION OF ANTIBODIES TO HIV-1 ENVELOPE USING SIMIAN ADENOVIRUS VACCINES

Kristel Lucie Emmer

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DEDICATION

This dissertation is dedicated to my parents, James and Annedore Emmer, for all the support and encouragement they have provided my entire life.
I would like to thank my family and friends for all the support they have provided me throughout the years, especially my boyfriend Don. I would also like to thank the support of my dissertation committee: Mike Betts, Ron Collman, Dave Weiner, and Jean Boyer.
ABSTRACT

INDUCTION OF ANTIBODIES TO HIV-1 ENVELOPE USING SIMIAN ADENOVIRUS VACCINES

Kristel Lucie Emmer
Hildegund C.J. Ertl, M.D.

Human immunodeficiency virus type 1 (HIV-1) has infected 76 million people since the beginning of the epidemic. The first evidence that an HIV-1 vaccine could prevent infection in humans was provided in the RV144 vaccine efficacy trial. RV144 demonstrated 31.2% efficacy and immune correlate analyses indicated that antibodies targeting the variable 2 (V2) region of HIV-1 envelope (Env) correlated with decreased risk of infection. However, significant improvements are needed to develop a globally effective vaccine against HIV-1.

Several approaches can be employed to improve upon vaccination strategies: heterologous prime-boost regimens, immunogen design, and alternative adjuvants. To enhance Env-specific antibodies, I tested simian-derived adenovirus (SAdV) vectors expressing Env proteins and administered them in heterologous prime-boost regimens with recombinant Env in adjuvant. In the first study, I tested the SAdV vectors in prime-boost regimens with trimeric Env protein in aluminum salt. Priming with SAdV vector was advantageous over protein prime and inclusion of the trimeric protein induced neutralizing antibodies.

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In the third study, I tested calcium phosphate (CaP) as an adjuvant to a recombinant Env protein compared to alum adjuvant. Alum, the most commonly used adjuvant, induces suboptimal immune responses. CaP has demonstrated preferential immune responses as an adjuvant in animals and humans. CaP did not elicit greater antibody responses in this study.

These studies demonstrate SAdV vectors as advantageous vaccine vector platforms and that novel immunogen design can improve HIV-1 vaccines.
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CHAPTER 1

INTRODUCTION
HIV CLASSIFICATION

Acquired immune deficiency syndrome (AIDS) is the most extreme case of immune suppression caused by a pathogen\(^1\). The causative agent, human immunodeficiency virus (HIV), was first discovered in 1983. Without treatment, infection with HIV results in a gradual loss of immune competence eventually leading to AIDS. Since the beginning of the epidemic, 76 million people have become infected with HIV and 35 million people have died from AIDS-related illnesses\(^2\). Opportunistic infections and certain types of cancer are hallmark signs of AIDS. There are two closely related types of HIV, HIV-1 and HIV-2. HIV-1 is more virulent and found worldwide. Evidence suggests that HIV-1 was introduced into humans through at least three independent transmission events from chimpanzees and one transmission event from gorillas\(^3\). HIV-2 was transferred to humans by the sooty mangabey and is less prevalent than HIV-1. Due to the prominence of HIV-1 globally, it will be the focus of this dissertation.

HIV-1 is classified into four major groups based on nucleotide sequence, M (main), O (outlier), N (non-M, non-O), and P, each representing a separate crossover event. The global AIDS epidemic is dominated by a set of diverse viruses of the HIV-1 M group, which is classified into nine subtypes, or clades: A, B, C, D, F, G, H, J, and K. In addition, up to 77 circulating recombinant forms (CRFs) of group M exist, which are mosaic genomes consisting of two or more sections of different subtypes\(^4,5\). Subtype C accounts for the majority of all HIV-1 cases worldwide\(^6\). In the beginning of the twentieth century, HIV-1 M group began its expansion in humans\(^7,8\). Since then it has diversified rapidly and its diversity continues to grow\(^5\). This genetic variability is caused by a combination of the high mutation rate of the reverse transcriptase enzyme along with high rates of virus replication\(^6\).

HIV-1 GENOME AND LIFECYCLE

HIV-1 is a member of a group of retroviruses of the family Retroviridae called lentiviruses\(^1,9\). Lentivirus is derived from the Latin word lentus, meaning slow. These viruses are associated with
long incubation periods resulting in a slow, gradual course of disease. Lentiviruses are more complex than other retroviruses and therefore have relatively large genomes. The genome encodes three structural polyproteins common to all retroviruses, group-specific antigen (Gag), polymerase (Pol), and envelope (Env). Gag consists of the capsid (p24), matrix (p17), and nucleocapsid (p7) proteins. Pol comprises the enzymes of reverse transcriptase (p64), protease (p10), and integrase (p32). Env consists of two glycoproteins, gp120 and gp41, essential to viral attachment and entry. In addition to these structural proteins, lentiviral genomes encode several auxiliary proteins. Regulatory functions required for viral replication are performed by Tat and Rev. The accessory proteins Nef, Vif, Vpr, and Vpu modulate virus replication through a variety of mechanisms. Despite being necessary for efficient virus production in vivo, the accessory proteins are not needed for in vitro reproduction in certain cell lines.

The enveloped HIV-1 virion contains two copies of positive single-stranded ribonucleic acid (RNA) genome and the Pol enzymes necessary for initial infection and replication. After infection, reverse transcriptase reverse transcribes the RNA viral genome into deoxyribonucleic acid (DNA). Integrase then facilitates the integration of this DNA into the host-cell genome. Production of RNA transcripts from the integrated viral DNA ensues and these transcripts serve to produce viral proteins and RNA copies for new virions. The virions bud from the host-cell plasma membrane enclosed in a membrane envelope containing heterodimer complexes of transmembrane gp41 and non-covalently associated gp120.

The env gene produces a 160 kilodalton glycoprotein precursor, gp160, which is cleaved by the host cell protease furin into gp120 and gp41. The complex of these two glycoproteins is assembled into the viral envelope as trimers. For entry into the host cell, the gp120 component of Env binds to the receptor cluster of differentiation 4 (CD4), which is expressed on the surface of several cells of the immune system, including T cells, dendritic cells (DCs), and macrophages. A conformational rearrangement in the gp120 trimer occurs due to CD4 attachment resulting in
exposure of a co-receptor binding site on gp120\textsuperscript{10}. Typically, the co-receptor is either CC-chemokine receptor 5 (CCR5), expressed on memory CD\textsuperscript{4} T cells, DCs, and macrophages, or CXC-chemokine receptor 4 (CXCR4), expressed on activated T cells. After binding of the receptor and co-receptor, gp41 triggers the fusion of the viral envelope with the cell’s plasma membrane\textsuperscript{10}. This allows the contents of the virion to enter the host cell cytoplasm. Activated CD\textsuperscript{4}\textsuperscript{+} T cells are the preferred targets for infection, but other cells may be infected, including resting CD\textsuperscript{4}\textsuperscript{+} T cells, monocytes and macrophages, and DCs\textsuperscript{3}. One founder virus usually establishes transmission of HIV-1 across mucosal membranes.

**HIV-1 INFECTION**

Infection with HIV-1 normally happens when an infected person transfers bodily fluids to an uninfected person. This can occur through sexual intercourse, mother-to-child transmission (through birth or breast milk), contaminated needles used for intravenous drugs, or therapeutic use of contaminated blood or blood products. The dominant sites of primary infection are the gastrointestinal and genital mucosae. A small number of mucosal CD\textsuperscript{4}\textsuperscript{+} T cells harbor the initial infection before it spreads to draining lymph nodes. Memory CD\textsuperscript{4}\textsuperscript{+} T cells, the preferential target of HIV-1, predominantly populate the lymphoid compartment of mucosal tissues where viral replication is in turn favored. The virus subsequently disseminates via the bloodstream to the rest of the body, particularly to the location of the highest number of CD\textsuperscript{4}\textsuperscript{+} T cells, the gut-associated lymphoid tissues (GALT).

Viremia in the peripheral blood and a sharp decrease in circulating CD\textsuperscript{4}\textsuperscript{+} T cells characterize the acute phase of HIV-1 infection. Approximately 50-80\% of infected individuals experience an influenza-like illness during this phase. As virus replicates rapidly, the adaptive immune response activates cluster of differentiation 8 (CD\textsuperscript{8}) T cells targeting HIV-infected cells and antibody production from B cells. A significant depletion of CD\textsuperscript{4}\textsuperscript{+} T cells occurs via direct viral cytopathic effects or indirect activation-induced apoptosis, mainly in the GALT. As the anti-HIV-1 response
by CD8⁺ T cells and antibodies advances, HIV-1 mutates swiftly to escape the adaptive immune response and a diverse population of viruses develops. Virus levels peak and then decline to a persistent level, or viral set point. After 3-4 months of infection, the disease enters an asymptomatic phase of clinical latency despite persistent replication of the virus. The number and function of CD4⁺ T cells gradually decline until opportunistic infections begin to appear, which can occur anywhere between 6 months and 20 years or more after infection. This onset of AIDS will occur in almost all HIV-infected people, unless antiretroviral drugs are administered. Viral replication can be suppressed by combination antiretroviral therapy regimens with typically three of the more than 25 licensed drugs against HIV. Antiretroviral treatment decreases plasma viral load and increases CD4⁺ T cell counts, which can make HIV-1 infection a chronic, manageable disease. However, antiretroviral therapy does not eliminate the viral reservoir and therefore cannot eradicate the virus from the body.

**ADAPTIVE IMMUNE RESPONSE TO HIV-1**

HIV-1-infected individuals develop an adaptive immune response to the virus that includes CD8⁺ cytotoxic T cells, CD4⁺ T cells, and antibodies, but this response ultimately fails to control the virus. Significant depletion of CD4⁺ T cells during the acute phase of infection is generally accompanied by the development of CD8⁺ cytotoxic T lymphocytes (CTLs) against the virus. The CTLs become dysfunctional over time after chronic exposure to antigen¹¹,¹². Control of the virus is additionally lost due to CTL escape mutations¹³,¹⁴. Rare exceptions to this immune response occur in individuals with specific genetic variations in human leukocyte antigen (HLA) alleles. For instance, individuals with alleles HLA-B57 and HLA-B27 can develop an effective immune response and have a better disease prognosis. However, immune dysfunction occurs in most people and lifelong reservoirs of viral DNA are established.

Similar to T cell responses, antibody responses are raised against the virus in HIV-1-infected individuals, but are not able to clear the infection. Approximately 3 months after infection,
neutralizing antibodies (nAbs) develop, which again are outpaced by viral escape mutations. About 20% of patients develop broadly neutralizing antibodies (bnAbs) that can neutralize diverse HIV-1 subtypes. BnAbs usually take years to develop and are characterized by a high frequency of somatic mutations\textsuperscript{15}. Non-neutralizing antibody responses are also elicited and can function in a variety of ways, including antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cell-mediated viral inhibition (ADCVI), viral capture, and complement activation\textsuperscript{16}.

**HIV-1 ENVELOPE**

The HIV-1 Env proteins gp120 and gp41 are the primary targets of the HIV-1-specific antibody response. Env is displayed as a trimeric spike of three gp120/gp41 heterodimers on the surface of the viral membrane. The gp120 component interacts with the cellular receptors, while the transmembrane gp41 component mediates fusion of the viral and target cell membranes. The portion of gp120 that is closer to the inner region of the trimeric spike is called the “inner” domain, while the portion closer to the outer region is called the “outer” domain. HIV-1 gp120 is composed of five variable regions (V1-V5) and five constant regions (C1-C5). Although entry into the host cell occurs through the trimeric Env, different forms can exist on the viral membrane (e.g. monomers, dimers, gp41 stubs). Several potential sites of N-linked glycosylation are present in gp120 and about half of the molecular mass of gp120 is from N-linked glycans. Variations in the sequence, forms, glycosylation, and conformation of Env all contribute to viral evasion of the immune system. Genetic variation is largely seen in the variable regions of Env; however, these regions retain sites of conservation among viral isolates. Two of the most variable regions are the variable 1 and 2 regions (V1/V2); these regions vary in sequence, length, and N-linked glycosylation. Disulfide bridges form the V1/V2 loop that can be considered as a single topological entity and is localized to a membrane-distal cap of gp120. Variable regions 3 and 4 (V3 and V4) form independent loops, while V5 is the only variable region that does not form a loop. V1/V2 forms a bridging sheet between the outer and inner domains of gp120 and forms the
chemokine-receptor binding site along with residues in the V3 loop and the C4 region. Binding to the CD4 receptor is facilitated by several discontinuous residues in the constant regions. The V1/V2 loop is not required for viral entry, but its deletion renders the virus highly neutralization-sensitive\textsuperscript{17-20}. Some studies demonstrate that early in HIV infection, the V1/V2 region is targeted by the immune system and nAbs develop to autologous virus\textsuperscript{21-24}. Other studies show that only 20-45% of HIV-1-infected individuals develop an immune response to the V2 region\textsuperscript{25}. In contrast, immune responses to the V3 region develop in most patients\textsuperscript{26}. Despite a variety of immune responses that develop against HIV-1, no natural protective immune responses exist that could provide an example of what a prophylactic vaccine would need to elicit.

**IMMUNE RESPONSE TO VACCINATION**

Vaccination has played a significant role in the markedly decreased deaths from infectious disease in the past century. Since 1924, it is estimated that vaccines prevented a total of 103 million cases of childhood diseases\textsuperscript{27}. The aim of vaccination is to stimulate long-lasting, protective immunity without causing disease. Initial vaccines consisted of either attenuated organisms with reduced pathogenicity or killed organisms. Strategies advanced to utilize purified components of organisms that would elicit an effective immune response. Most current vaccines have been empirically developed and there is no great understanding on their immune correlates of protection.

The purpose of vaccination is to induce long-term protection from the adaptive immune system. This requires maintenance of antigen-specific immune effectors, induction of immune memory cells that may be quickly reactivated, or both. The type of immune effectors elicited are directly influenced by the nature of the vaccine. Upon immunization, vaccine antigens and/or adjuvants provide danger signals to trigger an inflammatory reaction that is mediated by the innate immune system\textsuperscript{28}. Antigen presenting cells (APCs), most commonly DCs, activate antigen-specific B and T cell responses by providing both antigen-specific and co-stimulation signals. In the case of viral
vaccines, the innate immune system is activated through recognition of pathogen associated molecular patterns by pattern recognition receptors (PRRs)\textsuperscript{29}. After administration of a vaccine, DCs are activated, migrate toward the draining lymph nodes, and trigger multiple foci of T and B cell activation. Most inactivated vaccines (proteins, polysaccharides, glycoconjugates, or inactivated microorganisms) require formulation with adjuvants to include the danger signal in order to induce sufficient activation of the innate immune system. Adjuvants function by either prolonging the antigen deposit at the site of injection and recruiting more DCs into the reaction or providing differentiation and activation signals via immune modulators to monocytes and DCs\textsuperscript{30}.

Vaccine antigens reach the lymph nodes by diffusion or association with DCs and activate B cells. Antigen-specific B cells then proliferate and differentiate into antibody-secreting cells (ASCs) or memory B cells. B cell differentiation into immunoglobulin (Ig) secreting plasma cells is driven by T cell help\textsuperscript{31}. Ig class-switch recombination occurs through the upregulation of the activation-induced deaminase (AID) enzyme, during which IgM switches to IgG, IgA or IgE. CD4\textsuperscript{+} T cells, of the subtypes T helper 1 (T\textsubscript{H1}) and T helper 2 (T\textsubscript{H2}), exert essential helper functions and may skew the class-switch recombination to specific Ig subclasses. Follicular DCs (FDCs) and CD4\textsuperscript{+} T follicular helper (T\textsubscript{FH}) cells help antigen-specific B cells differentiate into plasma cells in specialized lymph node structures called germinal centers\textsuperscript{32}. Intense proliferation takes place in germinal centers along with class-switch recombination and affinity maturation of B cells for their specific antigen. The variable-region segments of Ig genes undergo a somatic hypermutation process that causes the maturation of B cell affinity. In addition to driving the differentiation of B cells into ASCs, FDCs and T\textsubscript{FH} cells also drive the differentiation toward memory B cells. The peak antibody response from a vaccine is determined by the following: nature of the vaccine antigen, its intrinsic immunogenicity, dose of antigen, and adjuvant. The duration of the antibody response is proportional to the number of long-lived plasma cells produced by vaccination. Antibody responses that persist for decades in the absence of subsequent antigen exposure or reactivation of immune memory are induced only by live attenuated viral vaccines. This is most
likely due to \textit{in vivo} persistence of viral antigens that continuously trigger B cell responses. Memory B cells do not produce antibodies until re-exposure to antigen drives their differentiation into ASCs. These memory recall responses are characterized by rapid response of high titer antibodies with greater affinity for antigen (compared to antibodies of the primary immune response)\textsuperscript{33}.

Vaccines elicit T cell responses through interactions with activated DCs in parallel to B cell responses. The nature of the initial inflammatory reaction generally controls the induction of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. Most vaccines induce CD4\textsuperscript{+} T cells that support the differentiation of B cells. Cytotoxic CD8\textsuperscript{+} T cells capable of killing infected cells are induced by live vaccines and vaccines that insert antigen-encoding DNA into host cells. Vaccine antigens are taken up by DCs, processed into small fragments, and displayed on the cell surface on major histocompatibility complex (MHC) molecules. Usually peptides from antigens produced within infected cells are presented by MHC class I (MHC I), while phagocytosed antigens are presented on MHC class II (MHC II) molecules\textsuperscript{34-36}. DCs possess the ability to cross-present both MHC I and MHC II. MHC II-antigen complexes are recognized by CD4\textsuperscript{+} T cells, while MHC I-antigen complexes are recognized by CD8\textsuperscript{+} T cells. HLA is the human equivalent of MHC\textsuperscript{37}. HLA molecules differ among individuals and populations and specific HLA may only be bound by certain antigen-specific T cell receptors. Therefore, T cell responses may vary considerably within a population. To eliminate intracellular pathogens, T\textsubscript{H1} CD4\textsuperscript{+} T cells produce interferon (IFN)-\gamma and tumor necrosis factor \alpha (TNF-\alpha), which support macrophage activation and CD8\textsuperscript{+} T cell differentiation\textsuperscript{38}. To defend against extracellular pathogens, such as helminths, T\textsubscript{H2} CD4\textsuperscript{+} T cells produce interleukin (IL)-4, IL-5, and IL-13\textsuperscript{39}. B cell activation and differentiation is supported by both T\textsubscript{H1} and T\textsubscript{H2} cells outside of the germinal centers. T\textsubscript{FH} CD4\textsuperscript{+} T cells provide help to B cells in germinal centers\textsuperscript{32}. CD4\textsuperscript{+} T cell differentiation is mainly determined by DC activation by the immune system\textsuperscript{40}. Accordingly, CD4\textsuperscript{+} responses may be skewed towards T\textsubscript{H1} or T\textsubscript{H2} responses by the inclusion of specific adjuvants. The induction of CD8\textsuperscript{+} T cell responses is elicited by vaccines that introduce...
antigens within the cell cytosol, due to their presentation on MHC I molecules. Effector T cell responses are short-lived, while memory T cells may persist life-long. Therefore, T cell vaccine efficacy is dependent on immune memory.

Most vaccines generate protective immunity through the induction of antibodies against the pathogen; however, for some pathogens humoral immunity may not be sufficient for protection. Vaccines must be able to also induce strong and durable cell-mediated immunity. New approaches to generate cellular immunity from vaccination include viral vector-based vaccines and DNA vaccines. Viruses being considered as vaccine vectors include adenoviruses (Ads), mammalian and avian poxviruses, alphaviruses, paramyxoviruses, lentiviruses, and arenaviruses. This discussion will focus on Ads as vaccine vectors.

ADENOVIRUS

In 1953, Ads were first isolated and characterized from adenoid tissue in a laboratory. Ads isolated from mammals, including human Ads (HAdV) and simian Ads (SAdV), belong to the genus *Mastadenovirus* of the family *Adenoviridae*. Naturally occurring HAdV serotypes, 57 in total, are classified into seven subgroups (A-G) based on neutralization, hemagglutination, oncogenic properties, DNA homology, and more recently genetic sequencing. SAdV is classified into 27 serotypes. Ads are non-enveloped, double-stranded DNA viruses with an icosahedral capsid. The genome of Ad is 36 kilobase linear, double-stranded DNA with inverted terminal repeats (ITRs). The genome consists of five early units (E1A, E1B, E2, E3, and E4), four intermediate units (IVa2, IX, VAI, and VAII), and one late unit that is subdivided into L1-L5. After the virus enters the cell nucleus the first viral transcriptional unit to be expressed is E1A. The E1A proteins act as the main regulators of viral transcription by *trans*-activating other viral genes. E1B proteins allow survival of infected cells by inhibiting p53-dependent apoptosis. E2A encodes DNA polymerase and other proteins important for replication of the viral genome. E3 proteins allow persistence of infected cells by suppressing the host immune response. E4 proteins influence cell
cycle control and transformation, while the late transcription unit encodes structural capsid proteins.

Ad is composed of a capsid surrounding a DNA-containing core. The capsid is formed by hexon and penton complexes with fibers projecting from the icosahedron vertices. Hypervariable regions of hexon are the major site for neutralization and therefore determine serotype specificity. The initial stages of Ad infection involve the penton base and fiber. The fibers can also vary by serotype. The knob region of the fiber protein facilitates binding to cellular receptors. Almost all Ad serotypes utilize the coxsackie and adenovirus receptor (CAR) as the primary receptor for attachment\(^48\). After binding the CAR receptor, viral particles are internalized by endocytosis, or more rarely by some serotypes using macropinocytosis\(^49\)\(^54\). Ad is then released from the endosome and moves toward the nucleus via dynein and microtubules. The capsid of Ad attaches to the nuclear pore complex and a nuclear histone attaches to the hexon\(^55\). Finally, the microtubule motor protein kinesin-1 disassembles the hexon and disrupts the nuclear pore complex allowing the viral genome to enter the nucleus\(^56\).

**ADENOVIRUSES AS VACCINE VECTORS**

Ads intrinsically stimulate host immune responses upon infection and can efficiently introduce foreign DNA into target cells; thus, making them desirable candidates as vaccine vectors. Portions of the adenoviral genome can be deleted allowing a relatively high capacity for transgene insertion. The antigen of interest for vaccination can be inserted into the genome in E1, E3, or the short region between E4 and the right ITR. E1 generally renders Ad replication-deficient, but low level transcription can still occur\(^57\)\(^58\). E1-complementing producer cell lines, such as HEK-293 or PER.C6, can be used to propagate E1-deleted virus\(^59\)\(^60\). Several characteristics provide advantages for Ad vectors. Both quiescent and actively dividing cells can be transduced by recombinant Ad vectors. Ad vectors can be produced at very high titers and
high expression of the recombinant protein can be achieved. In addition, Ads do not integrate into the host genome; therefore, insertional mutagenesis is not a safety concern.

Ads are exceedingly immunogenic and induce both innate and adaptive immune responses. Infection with Ad induces a variety of cytokines and chemokines, including TNF-α, interleukins 6, 1, 12, and 8, type I IFNs, RANTES, and monocyte chemoattractant 1 (MCP-1). These activated molecules modulate the initiation of inflammation. Interactions of the virus with PRRs or cell surface receptors can trigger these immune responses. Toll-like receptors (TLRs) can recognize adenoviral DNA after internalization into the endosome. Ad also activates the NOD-like receptor protein 3 (NLRP3) inflammasome from penetration of the endosome membrane. Thus, the virus itself acts as a natural adjuvant during vaccination. The adaptive immune responses against Ad consist of B cell, CD4⁺, and CD8⁺ T cell responses. These responses target the early and late adenoviral antigens. The hexon protein dominates the CD4⁺ and CD8⁺ T cell responses and are elicited quickly after vector administration. CD4⁺ and CD8⁺ T cells have been shown to cross-react between different Ad serotypes. NAbs develop predominantly against the surface loops of the hexon capsid protein and to a lesser extent against the penton base and fiber knob. The nAb response to human Ad serotype 5 (HAdV5) has been shown to differ depending on whether induced by immunization or natural infection. Immunization induces HAdV5 nAbs to hexon and other capsid proteins, while natural infection induces nAbs to fiber and hexon. Importantly, no cross-reactivity of nAbs is seen between different Ad serotypes. However, pre-existing immunity to the most common HAdV serotypes can be highly prevalent presenting a significant issue for successful vector delivery. High levels of nAbs can potentially decrease gene transfer efficiency or blunt vaccine potency. In the developing world, pre-existing immunity against HAdV5 can be found in 50-90% of the population. Alternative Ad serotypes that occur at low prevalence in human populations can circumvent the issue of pre-existing immunity. SAdV vectors derived from chimpanzees are similar to HAdV, in that they induce potent immune responses and are easily grown in human cell lines, but have no or low seroprevalence in
humans. The frequency of nAb to SAdV in humans in the United States and Europe ranges from 0-4% and in developing countries it reaches up to 20%. Several different SAdV serotypes have been isolated and sequencing has demonstrated that they are closely related to HAdV. SAdVs do, however, vary from HAdVs in the hexon hypervariable regions, therefore avoiding cross-neutralization. Immunological studies have demonstrated that SAdV vectors are just as immunogenic as their HAdV counterparts and persist at low levels. SAdV vectors are thus attractive candidates as vaccine vectors.

It has been demonstrated that viral vectors are best utilized in heterologous prime-boost vaccination strategies. A heterologous prime-boost vaccine involves immunizing first with one type of vaccine and antigen followed by at least one more immunization with a different type of vaccine and/or antigen. SAdV vectors can be combined with other types of vaccines (protein subunit, DNA, or other viral vector) in a heterologous prime-boost regimen to preferentially elicit both humoral and cell-mediated immunity.

**HIV-1 VACCINE EFFICACY TRIALS**

The first HIV-1 vaccine efficacy trials were designed to induce humoral immunity against the HIV-1 envelope glycoprotein, gp120, and were based on a monomeric, recombinant gp120 (rgp120) protein, AIDSVAX. The first trial, VAX004 (phase III), was comprised of AIDSVAX B/B, two subtype B rgp120 immunogens from strains MN and GNE8, and was conducted mostly in high-risk men who have sex with men (MSM) in North America and the Netherlands. VAX003, another phase III trial, evaluated AIDSVAX B/E, the subtype B strain MN rgp120 and a CRF01_AE rgp120 from strain A244, in injection drug users in Thailand. Both VAX trials elicited only weak nAb responses and did not demonstrate efficacy against acquisition of HIV-1. The next HIV efficacy trials were designed to stimulate T cell responses from a recombinant viral vector expressing HIV-1 antigens, MRKAd5. The MRKAd5 vaccine was a highly immunogenic recombinant HAdV5 expressing genes for Gag, Pol, and Nef from subtype B viruses and was
tested in two phase IIb trials, Step (HVTN 502) and Phambili (HVTN 503). Step was conducted primarily in high-risk MSM in North and South America, the Caribbean, and Australia, while Phambili was conducted primarily in low-risk heterosexuals in South Africa. CD8+ T cells were induced by MRKAd5 in a majority of vaccinees in Step, however this did not protect against virus acquisition or alter the course of virus progression upon breakthrough infections\textsuperscript{88,89}. The Step trial was stopped for futility and long-term follow-up indicated that men who were both uncircumcised and HAdV5 seropositive had a statistically significant increased risk of HIV infection\textsuperscript{90}. This risk waned with time from vaccination and the causal mechanism remains unclear\textsuperscript{90,91}. Due to the halting of the Step trial, enrollment and vaccination in Phambili was also stopped early. The early termination of the trial may limit the conclusions that can be drawn, but a trend was observed toward higher rate of infection in vaccinees versus placebo recipients\textsuperscript{92}.

The vaccination strategy then changed to a heterologous prime-boost in attempt to induce both humoral and cellular immunity. This was tested in the phase III RV144 trial, which evaluated a canarypox viral vector prime expressing Env, Gag, and Pol, followed by a rgp120 (subtype B and CRF01_AE) boost in a community-based population in Thailand. This remains the first and only trial to date to demonstrate statistically significant vaccine efficacy. The RV144 trial showed a relatively modest and transient reduction of HIV-1 acquisition (31.2% vaccine efficacy at 42 months follow-up)\textsuperscript{93}, suggesting that vaccine-induced immunity can prevent infection. A large-scale collaborative endeavor to elucidate the correlates of HIV-1 risk in RV144 was undertaken. Two primary immune correlates of risk were identified; IgG antibodies to the V1/V2 region of Env correlated with decreased risk of infection\textsuperscript{94-96}, while plasma IgA antibodies to Env correlated with increased risk of infection\textsuperscript{94,97}.

The most recent HIV vaccine efficacy trial, phase IIB HVTN 505, also drew on a prime-boost strategy. HVTN 505 evaluated a DNA vaccine prime expressing Gag, Pol, Nef (subtype B), and Env (subtypes A, B, and C), followed by an HAdV5 vector boost expressing Gag, Pol (subtype B),
and Env (subtypes A, B, and C). This heterologous prime-boost vaccine was evaluated in MSM in the United States, and was redesigned due to Step/Phambili to include only men who were circumcised and HAdV5 seronegative. Again, this trial lacked efficacy and was stopped prematurely for futility to reduce either the rate of HIV-1 acquisition or the viral-load set point$^{98}$. Follow-up studies do not support increased risk of infection by DNA/HAdV5 vaccination$^{99}$. Further study of HIV-1 vaccines are currently underway in clinical trials.

**VACCINE ADJUVANTS**

Several different strategies are utilized for vaccination: live-attenuated vaccines, inactivated vaccines, recombinant viral vector vaccines, and subunit vaccines. The former three have inherent immune-stimulating capacity. An attenuated vaccine is a weakened virus or bacterium that can replicate within the host, albeit with limited capacity. A recombinant viral vector can be either replication-competent or replication-deficient, however still must enter the host’s cells to produce antigen. Both these vaccines contain sequences that induce an inflammatory reaction through activation of innate immunity that is essential to elicit adaptive immune responses. Both types of vaccines subsequently elicit robust humoral and cell-mediated immunity. Inactivated vaccines consist of whole pathogens that have been either heat-inactivated or chemically inactivated and are therefore biologically inactive. Inactivated vaccines, despite being safe and non-infectious, elicit weak, mainly humoral, immune responses. Subunit vaccines consist of purified or recombinant particles of the pathogen instead of the entire microbe. Subunit vaccines are usually proteins that are very safe and relatively easy to produce, however they are poorly immunogenic in part for they fail to induce an innate immune response. To overcome poor immunogenicity, inactivated and subunit vaccines often require assistance to stimulate protective immunity.

An adjuvant, derived from the Latin term *adjuvare* meaning “to help” or “to aid”, is a compound added to vaccine formulations to enhance the immune response to antigens. The concept of
adjuvants was first introduced by Ramon in the 1920s, as “substances used in combination with a specific antigen that produce more immunity than the antigen alone”. In 1926, Glenny et al. demonstrated the adjuvant activity of aluminum salts (alum) by precipitating diphtheria toxoid with aluminum potassium sulfate. They discovered that the adjuvanted toxoid induced a stronger antibody response as compared to soluble toxoid alone. Over 90 years later, alum remains the most commonly used adjuvant in vaccine formulations and has been administered in hundreds of millions of doses.

The goal of any adjuvant is to improve the potency of a vaccine while minimizing toxicity, however, unfortunately, strong adjuvanticity is often correlated with increased toxicity. Despite having a reasonably good safety record, alum has some limitations as an adjuvant; it is relatively weak, induces mainly T\textsubscript{H}2 responses, and rarely induces cellular, or T\textsubscript{H}1-linked, immune responses. In addition, alum can induce IgE antibodies and has been associated with some allergic reactions in humans. Over the years, many other types of adjuvants have been proposed, but have failed due to toxicity, stability, bioavailability, and cost. Until 2009, the only adjuvant licensed in a vaccine for use in humans in the United States was alum. Currently, the following adjuvants are licensed for use as part of a vaccine in the United States: alum, MF59 (a squalene-based oil-in-water emulsion), AS03 (another squalene-based oil-in-water emulsion), and AS04 (monophosphoryl lipid A [MPL] and alum). An important aspect to consider for vaccine licensing is that antigenic components and adjuvants are not approved individually; they are only approved for the vaccine with which they are formulated. The discovery and testing of potential new adjuvants is still a very active field of vaccinology. Table 1.1 lists some benefits of adjuvants as rationale for including them as components of vaccines.
Table 1.1. Rationale for use of adjuvants in vaccine formulations. (adapted from Lee and Nguyen)

<table>
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<th>The benefits of adjuvants</th>
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<tr>
<td>1  Decrease the dose of antigen needed (dose sparing)</td>
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<tr>
<td>2  Decrease the number of vaccine doses needed</td>
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<tr>
<td>3  Enhance vaccine efficacy in infants, the elderly, and immunocompromised people</td>
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<tr>
<td>4  Increase functional antibody titer</td>
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<td>5  Induce more rapid and long-lasting immune responses</td>
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<td>6  Induce robust cell-mediated immunity</td>
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<td>7  Provide broad protection (cross-reactivity)</td>
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<tr>
<td>8  Facilitate mucosal immunity</td>
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<td>9  Overcome antigen competition in combination vaccines</td>
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Adjuvants utilize several mechanisms to enhance the immune response to antigens. They can enhance immunogenicity by making antigens more readily ingested by antigen-presenting cells or by providing more effective presentation of antigen to the immune system. Adjuvants can facilitate this by transforming soluble protein antigens into particulate material. Alum particles adsorb antigen, while oil-in-water emulsifies the antigen to form particulate. This also allows for delayed release of antigen which enhances the immune response. Adjuvants can also enhance immunogenicity by directly activating innate immune receptors, which subsequently promote the adaptive immune response. Some adjuvants may provide both delivery and immunostimulatory mechanisms. Several adjuvants are being tested to improve the performance of vaccines.

Dissertation Aims

A vaccine to prevent HIV-1-infection would alleviate a significant disease burden worldwide. However, no natural protective immune response is available to guide vaccine design. Passive infusion of nAbs has been shown to be protective in animal models, but eliciting them through vaccination has not been effective. The most promising results that might indicate what a protective immune response against HIV-1 would consist of come from the RV144 trial. The RV144 trial suggests that non-nAbs against gp120, in particular the V1/V2 region, may have provided protection against infection. Despite the usefulness of the RV144 trial for elucidation of correlates of protection, the RV144 vaccine regimen was not sufficient for a successfully effective vaccine. Therefore, substantial improvement of vaccines for HIV-1 is needed.

In this dissertation, I examine several different strategies for improved HIV-1 vaccines through heterologous prime-boost regimens, immunogen design, and vaccine-adjuvant formulation. All strategies focus on the induction of antibody responses to HIV-1 Env using SAdV vectors for delivery of vaccine antigen as they are potent inducers of immune responses, easily modified, and safe. These SAdV vectors, based on chimpanzee-derived serotypes 24 (SAdV24) and 23 (SAdV23), encoded an E1-located HIV-1 Env antigen.
Heterologous prime-boost with trimeric Env protein

In the first study, I sought to test heterologous prime-boost vaccine regimens using recombinant viral vectors and a recombinant protein subunit. The SAdV vectors expressed an HIV-1 gp140 protein and the protein subunit consisted of a novel trimeric Env protein immunogen formulated with alum adjuvant. The trimeric Env protein was designed to mimic the native Env spike on the HIV-1 virion. The study assessed the vaccine-elicited antibody response from: (i) protein prime-vector boost, (ii) vector prime-protein boost, and (iii) vector prime-heterologous vector boost. I hypothesized that priming with viral vector would be advantageous over priming with protein and that trimeric protein would enhance the quality of the immune response, as assessed by antibody function.

Enhancing the V2-specific antibody response

The RV144 trial correlated antibodies targeting the V2 region of HIV-1 gp120 with decreased risk of infection\(^{93,94}\). Follow-up studies indicated this region of Env as a potential site of vulnerability for the virus\(^{26,95,117-124}\). Recent crystal structures demonstrated that grafting the V1/V2 loop onto a specific heterologous protein scaffold presented this domain in a native conformation\(^{125}\). My lab and I developed an SAdV vector encoding V1/V2 on the protein scaffold to elicit a more focused vaccine-induced immune response to the V2 region. I hypothesized that immunization with an SAdV vector expressing the scaffolded V1/V2 protein would elicit a greater V2-specific antibody response than an SAdV vector expressing a larger Env protein.

Calcium phosphate as a vaccine adjuvant

Alum was first utilized as a vaccine adjuvant over 90 years ago and until recently, it was the only adjuvant approved for use in a vaccine in humans in the United States\(^{100-104}\). Alum induces an immune response skewed toward Th2 cells and IgE antibody production\(^{106-108}\). A more desirable vaccine adjuvant would induce a more balanced response between Th1 and Th2 cells and functional IgG antibody responses. Calcium phosphate (CaP) is a naturally occurring compound
in the body; moreover, CaP has shown to induce a more balanced \( T_{H1}/T_{H2} \) response and minimal to no IgE production as a vaccine adjuvant\(^{126,127}\). I compared the immunogenicity of CaP formulated with a recombinant HIV-1 Env protein to alum formulated with the same protein. Additionally, I tested if CaP could synergize with alum to further enhance the antibody response over either adjuvant used individually. I hypothesized that CaP would induce a greater antibody response than alum adjuvant and that when combined CaP might synergize with alum.

This dissertation examines several approaches to improving the immunogenicity of HIV-1 vaccines.
CHAPTER 2

ANTIBODY RESPONSES TO PRIME-BOOST VACCINATION WITH AN HIV-1 GP145 ENV ENVELOPE PROTEIN AND SIMIAN ADENOVIRUS VECTORS EXPRESSING HIV-1 GP140
ABSTRACT

Over two million individuals are infected with HIV-1 each year, yet an effective vaccine remains elusive. The most successful HIV-1 vaccine to date demonstrated 31% efficacy. Immune correlate analyses associated HIV-1 Env-specific antibodies with protection, thus providing a path toward the development of more effective vaccines. I sought to test the antibody response from a novel prime-boost vaccine regimen with an SAdV vector expressing a subtype C Env gp140 combined with either a serologically distinct SAdV vector expressing gp140 of a different subtype C isolate or an alum-adjuvanted, partially trimeric gp145 from yet another subtype C isolate. Three different prime-boost regimens were tested in mice: SAdV prime-protein boost, protein prime-SAdV boost, and SAdV prime-SAdV boost. Each regimen was tested at two different doses of SAdV vector in a total of 6 experimental groups. Sera were collected at various time points and evaluated for Env-specific antibody binding, isotype, and avidity. Antibody functionality was assessed by pseudovirus neutralization assays. Priming with SAdV followed by a protein boost or sequential immunizations with two SAdV vectors induced HIV-1 Env-specific binding antibodies including those to the V2 region, while priming with protein followed by an SAdV boost was relatively ineffective. Antibodies that cross-neutralized tier 1 HIV-1 from different subtypes were elicited with vaccine regimens that included immunizations with protein. This study warrants further investigation of SAdV vector and gp145 protein prime-boost vaccines and their ability to protect against acquisition in animal challenge studies.

Portions of this chapter adapted from:

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INTRODUCTION

Thirty-five million people are living with HIV-1 and over 2 million become infected each year. Despite advances in therapy, HIV causes over 1.5 million deaths annually. A vaccine to prevent HIV-1 infection could drastically reduce the global burden of AIDS. A desirable immune target is the trimeric HIV-1 Env glycoprotein that facilitates entry into susceptible host cells, which can be blocked by antibodies.

The complexity of HIV-1 presents challenges for an efficacious vaccine. There have been six HIV-1 vaccine efficacy trials to date. Initial efforts involved homologous prime-boost strategies with recombinant HIV-1 gp120 (rgp120) monomeric proteins (AIDSVAX B/B and AIDSVAX B/E). The first two trials with rgp120, VAX004 and VAX003, resulted in only weak nAb responses and failed to confer protection. Therefore, the field turned to vaccines that induce cell-mediated immunity using homologous prime-boost with a recombinant Ad vector of HAdV5 expressing HIV-1 Gag, Pol, and Nef. Again, two trials testing the HAdV5 vector, MRKAd5, failed to show protection and even showed enhanced risk in a certain subgroup of vaccinees.

Following these failures, a heterologous prime-boost strategy was proposed involving vaccination with a canarypox vector and one of the previously tested rgp120 proteins.

Heterologous prime-boost vaccines for HIV-1 originally developed, because recombinant Env glycoproteins could elicit nAbs but not cytotoxic T cells, while recombinant vaccinia virus expressing HIV-1 antigens could elicit good T cell responses but only suboptimal antibody titers. Thus, the principle of heterologous prime-boost immunization was invoked to elicit both cellular and humoral immune responses against HIV-1. Modalities of heterologous immunization include recombinant protein subunits, DNA encoding antigens, and viral vectors expressing antigenic transgenes. A DNA prime-protein boost approach proved effective at eliciting cross-reactive anti-Env IgG responses and polyfunctional T cell responses in humans. Another study demonstrated that a DNA prime-poxviral vector (NYVAC) boost was more immunogenic than the
NYVAC vector alone. The fifth HIV-1 vaccine efficacy trial, RV144, capitalized on the heterologous prime-boost strategy by priming with a canarypox vector expressing Gag, Pol, and Env (ALVAC-HIV [vCP1521]) and boosting with a monomeric rgp120 protein (AIDSVAX B/E). The RV144 trial resulted in 31.2% efficacy, which offered the first indication that a vaccine could prevent HIV-1 infection, although the level of protection against virus acquisition was modest and transient and the vaccines failed to reduce peak or set point viral loads in individuals with breakthrough infections. Heterologous prime-boost vaccination has the advantage of eliciting higher quality immune responses with greater breadth over homologous prime-boost vaccination, as was shown in an animal study of influenza where a DNA prime-inactivated influenza virus boost was more immunogenic than homologous prime-boost with either DNA or inactivated influenza virus alone. Including either viral vectors or DNA as one component of a prime-boost strategy may be of great importance, because these vaccines produce antigens in vivo. This may allow for antigens to be presented to the immune system in their native conformations. Choice of antigen, vaccines, and order of vaccination are all critical to the success of an immunization strategy, as exemplified by the sixth efficacy trial, HVTN 505, which failed to show efficacy with a DNA prime and HAdV5 boost. Clearly, improvements in vaccine vector choice and immunogen design are still needed.

One alternative for vaccine vector choice is to utilize a different type of viral vector for prime-boost applications. Ads have been highly utilized as vectors in heterologous prime-boost vaccination. HAdV5 vectors are potent inducers of T and B cell responses, but failed in HIV-1 vaccine trials in part due to pre-existing immunity to the vaccine vector. Vaccines based on alternative Ad viruses, with lower seroprevalence in humans such as those derived from chimpanzees (SAdV), might be more advantageous. Several SAdV vectors have been shown to be highly immunogenic in mice and non-human primates (NHPs). In addition, prime-boost regimens based on SAdV24 and SAdV23 demonstrate induction of potent and sustained transgene-specific T and B cell responses.
Another way to potentially improve upon existing strategies is to utilize an immunogen that better mimics the antibody target in its native state. Trimeric HIV-1 Env may elicit improved immune responses compared to monomeric gp120, as it may be more representative of a functional Env trimer on the surface of the virion\textsuperscript{142}. Several trimeric Env proteins have recently been shown to elicit nAbs in preclinical studies\textsuperscript{143}. The CO6980v0c22 gp145 Env, isolated from an acute, subtype C infection, represents a tier 2 HIV-1 Env with an intact membrane-proximal external region (MPER) extended by 3 lysine residues. Soluble gp145 was enriched for trimers displaying a “fan blade” motif when visualized by cryoelectron microscopy (cryo-EM). The HIV-1 neutralizing monoclonal antibodies (mAbs) 4E10, PG9, PG16, and VRC01 react with CO6980v0c22 gp145. Notably, in the RV144 trial, IgG antibodies, specifically IgG3 targeting the V2 region of gp120, correlated with decreased risk of infection\textsuperscript{94,117,144}. The V1/V2-specific mAbs PGT121, 697, 2158, and 2297 also react with CO6980v0c22 gp145 and it binds to the integrin α4β7 whose binding site is located within V2. Immunization of rabbits with the gp145 Env elicited binding antibodies to Env, including V1/V2 and V3 specificities, and cross-reactive nAbs\textsuperscript{143}.

I tested SAdV vectors and recombinant trimeric protein in prime-boost vaccine strategies in mice, utilizing replication-defective SAdV vectors expressing subtype C Env without the transmembrane domain (gp140), and subtype C gp145 protein, CO6980v0c22\textsuperscript{143}. I assessed the immunogenicity of six prime-boost regimens, categorized as three prime-boost strategies tested at two SAdV vector doses. The first strategy involved priming three times with gp145 protein followed by boosting with SAdV vector, the second primed once with SAdV vector then boosted three times with gp145. The third primed once with SAdV vector then boosted once with a heterologous SAdV vector.

Boosting SAdV vector-primed mice with the heterologous SAdV vector or alum-adjuvanted gp145 protein significantly enhanced Env-specific antibody responses including V2-specific binding antibodies. Priming with protein followed by SAdV vector boost was relatively ineffective.
Induction of nAbs that cross-reacted between different subtypes required inclusion of protein into the vaccine regimen. This study warrants further investigation of SAdV vector and gp145 protein prime-boost vaccines.
RESULTS

Study design and vaccines
The study design is shown in Figure 2.1A. Groups 1 and 2 tested protein prime at day 0, week 4, and week 8 followed by a boost with \(10^{10}\) or \(10^9\) viral particles (vp) of SAdV24 at week 12. Groups 3 and 4 tested SAdV prime at the 2 doses followed by protein boosts at weeks 8, 12, and 18. Groups 5 and 6 tested priming with \(10^{10}\) or \(10^9\) vp of SAdV24 at day 0 and boosting with heterologous SAdV23 using the same doses at week 8. Sera were collected at the indicated time points.

Magnitude and kinetics of antibody responses
Ad vectors are very immunogenic and induce both B cell and T cell responses\(^{140}\). These responses can be boosted by heterologous vaccines, such as a protein subunit or an Ad vector from a different serotype. I sought to determine which of the six immunization regimens elicited the highest and most sustained HIV-1 Env-specific antibody response. In addition, the study was designed to assess if any of the regimens allowed for dose sparing of the SAdV vector.

Binding antibody responses to gp140 Env strain Du172 subtype C are shown for individual mice (Figure 2.1B and C) and as mean response by group (Figure 2.1D and E). I sought to determine (i) whether the prime-boost regimen increased antibody titers over baseline response (wk 0), (ii) whether boosting increased antibody titers over that induced by the prime, and (iii) whether antibody titers were sustained. For this analysis, antibody titers were compared within each group at all time points (weeks 0 through 24). In the SAdV24/protein \(10^{10}\) group there was a significant increase in antibody titers from baseline beginning at week 4 and all the time points thereafter (\(p\) values reported in Table 2.1). In the SAdV24/protein \(10^9\) group, a significant increase in titers from baseline was first seen after the protein boost (wk 12) and was maintained thereafter. For both doses of SAdV24/protein there were significant increases from responses 4 and 8 weeks after SAdV24 prime and all time points after the protein boosts. However, for both
SAdV24/protein groups there were no significant differences among antibody responses at weeks 12, 18, 20, and 24, indicating that the second and third protein boosts did not increase titers. For both doses of SAdV24/SAdV23 significant increases in titers from baseline were first seen after the SAdV23 boost. There were significant increases from the response 4 weeks after SAdV24 prime to all time points following the SAdV23 boost. In addition, the $10^9$ vp SAdV24/SAdV23 group showed a difference between week 8 after SAdV24 prime, the time point immediately prior to SAdV23 boost, and week 24. In the protein/SAdV24 $10^{10}$ group a significant increase in titers from baseline was seen after the SAdV24 boost. An increase in titers from week 4 was seen following the SAdV24 boost. There were no significant differences in antibody titers over baseline or among time points in the protein/SAdV24 $10^9$ group.

I sought to determine if one type of prime-boost regimen offers an advantage over the others. I compared the different regimen types at each dose of SAdV. When comparing the three regimens at the $10^{10}$ vp dose of SAdV (Figure 2.1D), the SAdV24/protein regimen elicited the highest titers of binding antibodies. Antibody responses to the SAdV24/protein regimen were significantly greater than those to the protein/SAdV24 regimen by 8 weeks after priming ($p=0.0204$) and significant differences were maintained through week 24. SAdV24/protein induced higher responses than SAdV24/SAdV23 by 4 weeks after the first boost, which were sustained through week 24. There was a marginally higher response in SAdV24/protein compared to SAdV24/SAdV23 at week 8 ($p=0.0451$) despite receiving the same prime, but after boosting the difference was highly significant (wk 12 $p<0.0001$). There were no significant differences between the protein/SAdV24 and SAdV24/SAdV23 regimens. When comparing the three regimens at the $10^9$ vp dose of SAdV (Figure 2.1E), the SAdV24/protein-induced antibody response was significantly greater than that elicited by protein/SAdV24 from weeks 12 through 24. The SAdV24/SAdV23 response was greater than the protein/SAdV24 response only at week 24. There were no significant differences in responses when comparing the SAdV24/protein and SAdV24/SAdV23 regimens at the $10^9$ vp dose.
I was interested in ascertaining whether dose sparing of the SAdV vector is possible. Thus, I compared the two doses ($10^{10}$ vp SAdV and $10^9$ vp SAdV) for each regimen type. In the SAdV24/protein regimens, antibody responses were significantly higher in the $10^{10}$ vp group as compared to the $10^9$ vp group at 8 weeks post-prime ($p=0.0191$), differences observed at other time points were not significant. For both the protein/SAdV24 regimens and the SAdV24/SAdV23 regimens, there were no differences between the $10^{10}$ and $10^9$ vp groups.

It has been shown previously that antibody responses induced by Ad vectors are extremely stable, which may reflect the low level in vivo persistence of Ad vectors$^{83,112,145}$. Some of the mice were tested 46 weeks after priming and their titers were comparable to those tested earlier at week 18. Using a different monomeric gp140 for a single boost of an SAdV-induced antibody response showed that responses, once they peaked around week 18, remained stable through week 46 (data not shown).

Overall these results show that boosting of an SAdV vector with a serologically distinct SAdV vector or an alum-adjuvanted gp145 protein significantly enhances antibody responses. Interestingly, repeated immunizations with the gp145 protein did not significantly increase antibody responses nor did it seem to affect response longevity.
Groups of 5 outbred Crl:CD1 (ICR) mice were bled and immunized at the time points indicated in A. On immunization days, mice were bled prior to vaccine administration and sera were isolated. Protein (P): gp145 protein strain CO6980v0c22 10 μg in 1% aluminum hydroxide gel (alum) adjuvant; SAdV24 (V24): encodes gp140 strain Du172; SAdV23 (V23): encodes gp140 strain Du422. Responses over time for individual mice in each group (B and C). Mean responses ± standard error of the mean (SEM) for each group (D and E). B and D show responses to the $10^{10}$ vp dose of SAdV vectors, C and E show those to the $10^9$ vp dose. Magnitude of IgG binding responses at each time point were determined by ELISA, calculated using background-adjusted optical density dilution curves, and are reported as area under the curve. All statistical comparisons were made by two-way ANOVA and Holm-Sidak’s multiple comparisons test; significant multiplicity adjusted $p$ values are reported in Table 2.1.
<table>
<thead>
<tr>
<th>Group (Fig. 2.1D)</th>
<th>wk comparison</th>
<th>( p )</th>
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<tr>
<td>SAdV24/protein (10^{10})</td>
<td>wk 0 vs. wk 4</td>
<td>0.0009</td>
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<td></td>
<td>wk 0 vs. wk 8</td>
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<td>wk 0 vs. wk 18</td>
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<td>wk 0 vs. wk 24</td>
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<td>wk 8 vs. wk 12</td>
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<tr>
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<td>wk 0 vs. wk 18</td>
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<td>wk 8 vs. wk 18</td>
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<td>wk 8 vs. wk 20</td>
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The antibody responses shown in Figure 2.1 were analyzed to compare: (i) the response over time within each group, (ii) the different regimens at the same SAdV vector dose, and (iii) the SAdV dose for the same regimen. Table 2.1 lists each statistically significant comparison and its corresponding $p$ value. All statistical comparisons were made by two-way ANOVA and Holm-Sidak’s multiple comparisons test; multiplicity adjusted $p$ values are reported.
I further characterized isotypes of the vaccine-induced antibodies elicited by the different regimens (Figure 2.2A and B). For groups primed with the SAdV vector, the antibody response was primarily composed of IgG2a (55-65%) and IgG2b (25-36%) isotypes, indicative of responses driven by T\(_{\text{H}1}\) cells. I also detected IgG1 (3-11%) in these groups, an isotype that is promoted by T\(_{\text{H}2}\) cells, but little to no IgG3, IgM, or IgA. In mice that were primed with protein approximately half of the antibody response was comprised of IgG1 (44%), while IgG2a and IgG2b responses were comparable (22-30%) and I detected some IgG3, IgM and IgA (0.5-3%). Comparing the groups within a given SAdV vector dose showed significant differences in IgG1 and IgG2a between protein/SAdV24 compared to SAdV24/protein or SAdV24/SAdV23 \((p<0.0001)\). These data show that priming with the SAdV vectors induces predominantly a T\(_{\text{H}1}\) response and that this is not modified by boosting with alum-adjuvanted protein, which generally induces a T\(_{\text{H}2}\) response\(^{116,146}\). Priming with protein, on the other hand, favors induction of a T\(_{\text{H}2}\) response.
Isotyping was performed by ELISA following the last immunization: protein/SAdV24 groups at wk 18, SAdV24/protein groups at wk 20, and SAdV24/SAdV23 groups at wk 12. (A) At both SAdV doses, SAdV24/protein elicits a greater combined isotype response than protein/SAdV24 and SAdV24/SAdV23 ($10^{10}$ dose $p=0.0013$, $p=0.0034$, respectively; $10^9$ dose $p=0.0085$, $p=0.0442$, respectively). (B) Relative isotype frequency. Priming with protein versus SAdV24 elicits a different isotype response independent of dose. Protein/SAdV24 elicits predominantly IgG1 at a significantly greater frequency than both SAdV24/protein and SAdV24/SAdV23, $p<0.0001$. Conversely the SAdV24-primed regimens, SAdV24/protein and SAdV24/SAdV23, predominantly

FIGURE LEGEND CONTINUED ON PAGE 35
FIGURE LEGEND CONTINUED FROM PAGE 34

elicit IgG2a and at greater frequency than protein/SAdV24, $p<0.0001$. The symbols x/x over part B represent the significance differences between groups for the isotypes IgG1 and IgG2a, respectively (two-way ANOVA with Holm-Sidak’s multiple comparisons test).
**Antibody function**

I tested the avidity of the Env-specific antibodies induced by the vaccine regimens at the week following the last immunization (Figure 2.3A). The groups that received the protein immunizations first showed markedly lower avidity compared to the others and only retained 50% antibody binding at sodium thiocyanate (NaSCN) concentrations slightly above 1M. The other groups retained 50% antibody binding at 2M NaSCN or greater (Figure 2.3A). To determine the effect of additional protein boosts on antibody avidity in the SAdV24/protein $10^{10}$ and SAdV24/protein $10^{9}$ groups samples from week 12, 18, and 20 were evaluated (Figure 2.3B). There were no significant differences in the avidity after additional protein boosts.

To assess a virus inhibitory function of the antibodies, we performed tranzyme-β-galactosidase and luciferase (TZM-bl) neutralization assays against 4 tier 1 pseudoviruses (PVs), MW965 (subtype C), GS015 (subtype C), SF162 (subtype B) and TH023 (subtype CRF01_AE), and 1 tier 2 PV, TZBD 9/11 (subtype C) (Figure 2.4). A PV containing the Env from murine leukemia virus (MLV) was tested as a non-specific control. Sera were tested at different times after vaccination and the 50% inhibitory dose (ID$_{50}$) values were determined. Sera failed to neutralize MLV (not shown). All the vaccine regimes induced nAbs to MW965 PV, with the highest titers observed in the group receiving the $10^{10}$ dose of SAdV24 followed by protein. Response rates to this virus, shown as the ratio of seropositive mice over all mice in each group (Figure 2.5), were comparable between all 6 groups (0.8) by the end of the study. Regimens that included immunizations with protein also induced cross-subtype nAbs to other tier 1 viruses, although response rates were lower. Responses upon sequential immunization with the two SAdV vectors were less broad. None of the regimens induced nAbs to the tier 2 subtype C virus.
Figure 2.3. Avidity of Env-specific antibody binding.

NaSCN-displacement ELISA performed (A) following last immunization: protein/SAdV24 groups at wk 18, SAdV24/protein groups at wk 20, and SAdV24/SAdV23 groups at wk 12 and (B) on week 12, 18, 20 for SAdV24/protein 10^{10} and SAdV24/protein 10^{9} to determine effect of additional protein boosts on avidity. Results are reported as the concentration of NaSCN (mean ± SEM) at which IgG binding was reduced by 50% as compared to binding in the absence of NaSCN. (A) Avidity of SAdV24/protein 10^{10} is significantly greater than both protein/SAdV24 10^{10} and SAdV24/SAdV23 10^{10}, p=0.0009 and p=0.0439, respectively, while SAdV24/SAdV23 10^{10} is greater than protein/SAdV24 10^{10}, p=0.0339. At the 10^{9} dose, SAdV24/protein and SAdV24/SAdV23 avidities are significantly greater than that of protein/SAdV24, p=0.0030 and p=0.0164, respectively. (B) No significant change in avidity is seen in the SAdV24/protein groups with administration of additional protein boosts. (all statistics performed by one-way ANOVA with Holm-Sidak’s multiple comparisons test).
Figure 2.4. Breadth and potency of pseudovirus neutralization.

Sera were screened at a 1:40 dilution against six PVs: MW965 and GS015 PVs (tier 1, subtype C), TH023 PV (tier 1, CRF01_AE), SF162 (tier 1, subtype B), TZBD PV (tier 2, subtype C), and MLV PV (non-specific control) for all time points; 50% neutralization is the negative cut-off value (indicated by the dashed line). Statistical comparisons were made by two-way ANOVA and Holm-Sidak’s multiple comparisons test; significant multiplicity adjusted $p$ values are reported. The x/x/x symbols above the graphs indicate significant differences with – symbolizing $p$ values >0.05, * $p$ values between 0.01 – 0.05 and ** $p$ values between 0.001-0.01. The symbols are arranged from top to bottom to compare (top) protein/SAdV24 to SAdV24/protein, (middle) protein/SAdV24 to SAdV24/SAdV23, (bottom) SAdV24/protein to SAdV24/SAdV23.

More specifically the following differences reached significance with the following $p$ values:
MW965 PV: at wk 18 SAdV24/protein $10^{10} >$ protein/SAdV24 $10^{10}$ $p=0.0258$, SAdV24/protein $10^{10} >$ SAdV24/SAdV23 $10^{10}$ $p=0.0317$
GS015 PV: protein/SAdV24 $10^{10} >$ SAdV24/protein $10^{10}$ at wk

FIGURE LEGEND CONTINUED ON PAGE 39
20 $p=0.0340$, protein/SAdV24 $10^{10}$ > SAdV24/SAdV23 $10^{10}$ at wk 20 $p=0.0102$ and wk 24 $p=0.0424$, SAdV24/protein $10^9$ > protein/SAdV24 $10^9$ at wk 24 $p=0.0032$, SAdV24/protein $10^9$ > SAdV24/SAdV23 $10^9$ at wk 24 $p=0.0057$; TH023 PV: at wk 12 SAdV24/SAdV23 $10^9$ > protein/SAdV24 $10^9$ $p=0.0073$, SAdV24/SAdV23 $10^9$ > SAdV24/protein $10^9$ $p=0.0115$; SF162 PV: protein/SAdV24 $10^9$ > SAdV24/SAdV23 $10^9$ at wk 20 $p=0.0318$, protein/SAdV24 $10^9$ > SAdV24/protein $10^9$ at wk 12 $p=0.0397$, protein/SAdV24 $10^9$ > SAdV24/SAdV23 $10^9$ at wk 12 $p=0.0397$; TZBD PV: at wk 20 SAdV24/protein $10^9$ > protein/SAdV24 $10^9$ $p=0.0055$, SAdV24/protein $10^9$ > SAdV24/SAdV23 $10^9$ $p=0.0055$. 
Responders are classified as animals with a final titer (reported as the reciprocal of the dilution of serum necessary to achieve 50% neutralization [ID$_{50}$]) greater than the negative cut-off value. Positive response rates are reported as a ratio of responders per total animals per group.
**V2-specific antibody response**

I tested whether linear V2-specific antibodies, shown to be protective in the RV144 trial, were elicited by the vaccine regimens (Figure 2.6A). Anti-V2 antibodies were detected in all vaccine groups. In the SAdV24/protein $10^{10}$ and SAdV24/SAdV23 $10^{10}$ groups there were significant increases in V2-specific antibody titers from baseline ($p<0.0001$ and $p=0.0255$, respectively). I characterized the V2-specific response by isotyping (Figure 2.6B). The SAdV24/protein V2 response was dominated by IgG2a antibodies, while the other groups showed a more even distribution of IgG1, IgG2a and IgM. IgM was overall higher than in the response to whole protein (Figure 2.1), suggesting that the response to the V2 loop matures with a delay.
Figure 2.6. Induction of V2-specific antibodies.

(A) Antibody binding to an immunodominant linear epitope within the V2 region of subtype C strain Du172. Sera were analyzed after the last immunization for each group; 1:100 dilution reported, mean ± SEM. Using pre-immunization values as baseline, both the SAdV24/protein $10^{10}$ ($p<0.0001$) and SAdV24/SAdV23 $10^{10}$ ($p=0.0255$) regimens elicited significant titers of V2-specific antibodies (two-way ANOVA and Holm-Sidak’s multiple comparisons test). Statistical comparisons among groups were made by one-way ANOVA and Holm-Sidak’s multiple comparisons test. (B) Relative percentages of V2 Du172-specific antibodies of the different
isotypes at the week following last immunization using a serum dilution of 1:200. Overall the V2 antibody response trends toward a more pronounced frequency of IgM isotype as compared to the gp140-specific response. The symbols x/x over part B represent the significant differences between groups for the isotypes IgG2a and IgM, respectively (two-way ANOVA with Holm-Sidak’s multiple comparisons test). SAdV24/protein $10^9$ elicits a significantly greater frequency of IgG2a antibodies than protein/SAdV24 $10^9$, $p<0.0001$. At both $10^{10}$ and $10^9$, the percentage of IgG2a antibodies elicited by SAdV24/protein is greater than SAdV24/SAdV23, $p=0.0204$ and $p=0.0062$, respectively. IgM antibodies are elicited at a greater frequency by protein/SAdV24 $10^9$ as compared to SAdV24/protein $10^9$, $p=0.0345$. 
DISCUSSION

Ads isolated from several different species have been studied as alternative vaccine vectors to avoid the issue of pre-existing nAbs to human serotype Ad vectors. Chimpanzee-derived Ads, or SAdV, have demonstrated their usefulness as vaccine vectors due to their low seroprevalence in humans\textsuperscript{79-81}, ease of genetic modification and production\textsuperscript{78,110-112}, and induction of potent immune responses\textsuperscript{78,83,113-116}. SAdV have been developed in preclinical studies for several infectious diseases including HIV\textsuperscript{67,116,141,147,148}, malaria\textsuperscript{149-153}, Ebola\textsuperscript{154}, severe acute respiratory syndrome (SARS)\textsuperscript{155}, hepatitis C virus (HCV)\textsuperscript{156}, and rabies\textsuperscript{157}, as well as for cancer\textsuperscript{82}. SAdV vectors have also been tested in humans in clinical trials for malaria\textsuperscript{158-166}, HIV\textsuperscript{167,168}, HCV\textsuperscript{169,170}, Ebola\textsuperscript{171-173}, respiratory syncytial virus\textsuperscript{145}, and influenza\textsuperscript{174}.

Early HIV-1 vaccine efficacy trials utilizing monomeric rgp120 proteins failed to produce protective immune responses against infection\textsuperscript{84,85}. The RV144 trial highlighted a role for non-neutralizing antibodies, particularly those targeting the V2 region, in protection\textsuperscript{93}. Recently, broadly reactive antibodies targeting quaternary neutralizing epitopes (QNEs) present on trimeric forms of Env have been identified, some targeting epitopes in V1 and V2\textsuperscript{175-177}. These findings emphasize the need for improved immunogens to mimic the conformational forms of HIV-1 Env present on the surface of the virion. Since Env is present in a trimeric orientation, production of native-like trimers for structural studies and immunization has been undertaken. Previous strategies involved removing the cleavage site on the gp160 precursor to prevent dissociation of gp120 from gp41 and adding trimerization motifs such as leucine zippers or the T4 bacteriophage fibrin domain (foldon) to stabilize the subsequent gp140 trimers\textsuperscript{178,179}. However, these modifications impacted the quaternary structure in a way that it did not adopt a stable, native-like fold\textsuperscript{180,181}. Another strategy retained, and with mutations even enhanced the cleavage site, but incorporated a disulfide bond (SOS) between gp120 and gp41 to prevent dissociation. To assume the appropriate pre-fusion, ground state conformation an isoleucine residue in the N-terminal HR1 of gp41 was mutated to proline (IP). The resulting SOSIP gp140 trimer held gp120 and gp41
together while enhancing trimerization\textsuperscript{182,183}. The Polonis Lab developed trimeric gp140 molecules with an alternative strategy to minimally alter the CO6980v0c22 Env gp140 sequence. The following modifications were made: (i) cleavage of gp120 and gp41 was prevented by mutating the cleavage site, (ii) a full-length MPER was retained, and (iii) three lysine residues were added to the C-terminus (at amino acid 648) to increase the hydrophilicity and enhance exposure of the MPER domain\textsuperscript{184,185}. The resultant gp145 Env exists as a dynamic equilibrium of trimers, dimers, and monomers as determined by negative-stain cryo-EM, with the enriched trimeric fraction consisting of 60\% true physical trimers exhibiting a fan blade motif\textsuperscript{143}. The gp145 Env binds to the integrin α4β7 and several mAbs, including mAbs to the V2 region of gp120. This gp145 immunogen elicits advantageous antibody responses in rabbits and is undergoing GMP production for use in human clinical trials\textsuperscript{143}.

In this study, I tested the immunogenicity of novel vaccine regimens involving SAdV vectors expressing a subtype C gp140 and heterologous CO6980v0c22 gp145 protein in mice. The goal was to assess antibody responses elicited by three different prime-boost regimens (protein/SAdV24, SAdV24/protein, and SAdV24/SAdV23) at two different doses of SAdV vector. The SAdV24/protein regimen elicits significantly higher IgG titers compared to the protein/SAdV24 and SAdV24/SAdV23 regimens at certain doses. These results are consistent with other studies that demonstrate optimal immune responses from heterologous prime-boost vaccination when protein is used as the boost\textsuperscript{146,186,187}. The mechanism for higher antibody responses upon protein boost is not fully understood. I would assume it reflects that SAdV vectors are far more immunogenic than protein vaccines and are thereby better suited to drive activation of naïve B cells, which can then be expanded by a less immunogenic vaccine. However, further protein boosting did not significantly increase antibody titers nor did it ensure durability of the response, as observed in a concurrent study using a different gp140 protein for boosting (data not shown). This suggests that heterologous protein boosts may be required or
that a single protein boost may suffice after an SAdV vector prime. The latter would be highly advantageous considering the cost of protein vaccines and the complexity of multiple dose regimens.

All of the vaccine regimens induced Env-specific IgG2b which is the mouse isotype most closely related to IgG3 that was shown to correlate with vaccine-induced resistance to HIV-1 infection in the RV144 trial\textsuperscript{188}. These results differ from the IgG1-biased response in a protein and DNA immunizations protocol\textsuperscript{189}.

Antibody avidity was evaluated to determine the strength of antibody binding as an assessment of affinity maturation. Previous studies showed an inverse correlation between post-challenge viral titers and the avidity of antibodies induced by preventative vaccination\textsuperscript{190}. This study did not find significant differences in antibody avidity among the SAdV24/protein and SAdV24/SAdV23 regimens, but markedly lower avidity in mice that received the protein primes followed by an SAdV24 boost.

Induction of potent, broadly cross-reactive nAbs is fervently pursued by HIV-1 vaccine researchers. They face many challenges, including extraordinary diversity of circulating strains, a high degree of glycosylation, and new epitopes revealed upon receptor and co-receptor binding. Large-scale clinical vaccine trials have induced low-titer nAbs with limited cross-reactivity\textsuperscript{84,85,93}. The vaccine regimens that included trimeric protein immunizations induced antibodies that neutralized tier 1 viruses from subtype B, C, and CRF01_AE, although they failed to neutralize the one tier 2 virus tested. NAb responses to the SAdV24/SAdV23 immunization were less broad, which may reflect that the structure of viral transgene products is distinct from that of the trimeric protein immunogen. Notwithstanding, including an SAdV vector into a vaccine regimen has the added advantage that all Ad vectors induce potent cellular immune responses (most notably CD8\textsuperscript{+} T cells) to the transgene product, which may not prevent infection but allows for rapid viral clearance\textsuperscript{191}. Other pre-clinical vaccine studies have also elicited nAbs; tier 1 and tier 2 PV
neutralization was achieved with a DNA vaccine expressing gp160 given together with particles displaying the MPER of Env\textsuperscript{192}. Similar results were obtained with an adjuvanted DNA vaccine followed by a protein boost, as well as with a DNA prime-protein boost regimen using constructs that expressed diverse polyvalent heterotrimeric Envs\textsuperscript{193,194}. Careful design of immunogens and adjuvants thus allows for the induction of cross-reactive nAbs to HIV-1, which upon passive transfer have been shown to protect NHPs against challenge with an HIV-1/simian immunodeficiency virus (SHIV) chimera and to reduce viral loads in chronically infected individuals in human trials\textsuperscript{188,189,195}.

In the RV144 trial, protection was correlated with antibodies to the V2 region of gp120\textsuperscript{94}. Subsequent studies confirmed these results in a NHP low dose simian immunodeficiency virus (SIV) challenge study\textsuperscript{191}. The SAdV24 vector followed by SAdV23 or protein boost also induced V2-specific antibodies. This is in contrast to studies based on immunization with a monomeric gp120 protein\textsuperscript{192}.

Altogether, these results are very promising as they show that a simple vaccine regimen can elicit cross-neutralizing (albeit tier 1 responses), as well as V2-specific antibodies in animals. Further studies are warranted in NHPs to elucidate appropriate dosing of both the SAdV24 vector and gp145 protein and to assess if antibodies provide protection against viral challenge.
MATERIALS & METHODS

Vaccines

Ad vectors based on simian serotypes SAdV24 and SAdV23 were E1-deleted, rendering them replication defective. The E1 domain was replaced with a codon-optimized transgene encoding HIV-1 subtype C gp140, strain Du172 (for SAdV24) and Du422 (for SAdV23), under the control of the cytomegalovirus (CMV) promoter. Vectors were generated from recombinant viral molecular clones and rescued and expanded on HEK293 cells. Purification was performed using cesium chloride gradient centrifugation. The vectors were titrated for their contents of vp by spectrophotometry at 260 nm using the following formula: optical density at 260 nm (OD260) × dilution × 1.1 × 10^{12}. The content of infectious virus particles was measured by nested reverse transcriptase polymerase chain reaction (RT-PCR) with transgene- or Ad (hexon)-specific primers on RNA isolated from HEK 293 cells infected for 5 to 7 days with serial dilutions of vector. Batches were tested for endotoxin using the Limulus amebocyte lysate (LAL) gel clot method and a commercial kit. Genetic integrity and identity were assessed by isolation of viral DNA. The recombinant DNA, in parallel with the original molecular clones and shuttle plasmids used for generating molecular clones, was digested with a set of restriction enzymes and analyzed by gel electrophoresis.

Construction and production of gp145 CO6980v0c22 Env protein subtype C have been described. Briefly, CO6980v0c22 Env was expressed in CHO-K1 cells and purified by lectin affinity chromatography with *Galanthus nivalis* lectin agarose (Vector Laboratories, Burlingame, CA) followed by anion exchange on Q-Sepharose fast flow (GE Healthcare, Little Chalfont, UK). Protein was further concentrated, exchanged into phosphate-buffered saline (PBS) and sterile filtered. Oligomeric form was determined to be about 60% trimeric by negative stain electron microscopy.
Animals & vaccination protocol

Six groups of 5 female outbred Crl:CD1 (ICR) mice (Charles River Laboratories, New York, NY) were treated at the Wistar Institute Animal Facility following IACUC-approved procedures. Vaccines were administered intramuscularly in 200μL/dose. SAdV gp140 vectors were given in PBS; gp145 protein was given at 10 μg/dose in a 1% aluminum hydroxide gel suspension (alum; Alhydrogel®, Sigma-Aldrich, St. Louis, MO).

Serum isolation

Whole blood was collected by submandibular bleeding and allowed to coagulate undisturbed for 1 hour at room temperature. Samples were then centrifuged at 1600 rpm for 30 minutes at 4°C. Serum was isolated after centrifugation and frozen at -20°C until assayed.

HIV-1 Env-specific IgG enzyme linked immunosorbent assay (ELISA)

Sera were tested for HIV-1 gp140-specific and V2-specific antibodies by ELISA on plates coated with gp140 protein or linear V2 peptide, respectively. A recombinant protein of HIV-1 gp140 subtype C strain Du172 was produced in baculovirus as described previously. V2 peptide was based on a 19 amino acid immunodominant linear epitope within V2 of HIV-1 Env strain Du172: TTTEITDKKKKEYALFYKL (GenBank: ABD83638.1; Genscript, Piscataway, NJ). Gp140-specific IgG was tested in 2-fold serum dilutions from 1:1000-1:16,000. V2-specific IgG was tested using 2-fold serum dilutions from 1:100-1:6400. Briefly, 96-well plates (Nunc-Immuno™ Microwell™) were coated overnight at 4°C with (A) gp140 protein (200 ng/well) or (B) V2 peptide (400 ng/well). Wells were blocked overnight at 4°C with (A) PBS/3% bovine serum albumin (BSA)/0.1% Tween-20 or (B) PBS/8% BSA/0.1% Tween-20. Duplicate samples and a standard were incubated at room temperature for (A) 2 hours or (B) 90 minutes. Bound IgG was detected with goat anti-mouse IgG alkaline phosphatase conjugate (Sigma-Aldrich) at (A) 1:30,000 or (B) 1:15,000. Bound enzyme was detected with phosphatase substrate tablets (Sigma-Aldrich) in DEA substrate buffer (KPL, Gaithersburg, MD) by optical density (OD) at 405 nm. Gp140-specific
IgG is reported as area under the curve (AUC) for 1:2000-1:16,000 dilution curves. V2-specific IgG is reported as OD at 1:100 dilution.

**Sub-isotype ELISA**

Sub-isotyping of the gp140- and V2-specific antibody responses was performed similarly as above except bound antibody was detected using rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM (Calbiochem, San Diego, CA) followed by goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma-Aldrich) at 1:30,000 for (A) and 1:15,000 for (B). Gp140-specific and V2-specific sub-isotypes were tested at serum dilutions of 1:2000 and 1:200, respectively.

**Avidity ELISA**

Avidity of gp140-specific antibodies was assessed by NaSCN-displacement ELISA. Briefly, samples were diluted to 1:2000 in PBS/3% BSA/0.1% Tween-20 and incubated for 2 hours at room temperature on 96-well plates (Nunc-Immuno™ Microwell™) coated with HIV-1 gp140 strain Du172. Ascending concentrations of the chaotropic agent NaSCN were added to the wells (0-4 M). Plates were incubated for 15 minutes at room temperature then treated with goat anti-mouse IgG alkaline phosphatase conjugate (Sigma-Aldrich) at 1:30,000. Bound enzyme was detected as above by OD at 405 nm.

**Pseudovirus preparation**

PV was prepared as previously described\(^ {197}\). The following PVs were produced: GS015 and MW965, both tier 1 subtype C; TH023, tier 1 CRF01_AE; SF162, tier 1 subtype B; TZBD 9/11, tier 2 subtype C; and MLV (non-specific control).

**High-throughput PV neutralization assay**

NAb titers were determined using TZM-bl cells in a high-throughput assay utilizing robotic liquid handling. Serum was diluted 1:10 in growth medium and serially diluted using the Biomek NXP liquid handler (Beckman Coulter, Indianapolis, IN)\(^ {198}\). Titered serum (12.5 μL/well) was
transferred to 384-well culture plates and incubated with an equal volume of PV for 45 minutes at 37 °C. TZM-bl cells (3x10^3 cell/well) with DEAE-dextran (25 μg/mL) were added to each well and incubated for an additional 48 hours. Relative light units (RLU) were detected with the SpectraMax Paradigm Microplate Reader (Molecular Devices, Sunnyvale, CA) using the Bright-Glo Luciferase Assay System (Promega Corporation, Madison, WI). Percent neutralization (percent reduction of RLU in the presence of serum) was calculated for each serum dilution. Neutralization dose-response curves were fitted by non-linear regression using the LabKey Server, and the final titer is reported as the reciprocal of the dilution of serum necessary to achieve 50% neutralization (ID_{50}).

**Statistical analysis**

One-way analysis of variance (ANOVA) was used with Holm-Sidak’s correction for comparing three or more parameters for one group or one parameter for three or more groups. Two-way ANOVA was used with Holm-Sidak’s correction for comparing multiple parameters among three or more groups. Analyses were performed in GraphPad Prism 6.
CHAPTER 3

INDUCTION OF ANTIBODIES TO V2 OF HIV-1 ENVELOPE BY SIMIAN ADENOVIRUS VECTORS
ABSTRACT

An effective vaccine against HIV-1 remains a critical unmet need. The most successful HIV-1 vaccine to date, RV144, correlated antibodies against the V2 region of the HIV-1 Env protein with decreased risk of infection, giving rationale for focusing a vaccine-induced immune response on V2. V2 and the adjacent V1 region of HIV-1 Env exist together as a single topological entity. Recent advances in immunogen design have shown that expression of the V1/V2 region on a scaffold protein, 1FD6, presents this region in a native structural conformation. I hypothesized that presentation of the isolated, structurally accurate V1/V2 region to the immune system will focus the immune response to this particular region. Therefore, along with my lab, I developed SAdV vectors encoding the scaffolded V1/V2 region of HIV-1 Env. Mice were immunized with the vectors in prime-boost regimens; recombinant gp140 was administered to the boost the V2-specific prime response. The antibody response elicited by regimens containing vectors expressing V1/V2 was compared to regimens containing vectors expressing HIV-1 gp140. The V1/V2-expressing vectors and the comparison gp140-expressing vectors both induced a sustained antibody response to a conformational V1/V2 antigen. The V1/V2-expressing vectors elicited a greater antibody response to linear V2 epitopes briefly, however this occurred in only a fraction of animals and waned over time. Gp140-expressing vectors elicited a greater gp140-specific antibody response that was sustained through the duration of the study. The V2- and gp140-specific antibody responses are highly correlated in immunization regimens containing the V1/V2 vectors. In RV144, the V2 antibody response waned over time corresponding with waning vaccine efficacy. In contrast to the RV144 vaccines, this study demonstrated that V1/V2-expressing SAdV vectors induced durable antibody responses to V1/V2 conformational epitopes and sustained response rates.
INTRODUCTION

Anti-retroviral therapy protects HIV-1-infected individuals from the classic symptoms of AIDS, but there is still no cure and the pandemic continues to spread. A vaccine that could prevent HIV-1 remains elusive and vaccines that have undergone testing in large-scale clinical trials either lacked efficacy or only provided transient protection to a fraction of vaccine recipients. Initial attempts to design an effective HIV-1 vaccine involved immunization with rgp120 protein to induce Env-specific Abs. This approach failed to show protection in two efficacy trials, VAX004 (AIDSVAX B/B) and VAX003 (AIDSVAX B/E). The failure of the antibody-based vaccine resulted in a shift in vaccine strategy to a T cell-based vaccine. The Step trial, an HAdV5 replication-deficient vector encoding gag, pol, and nef, attempted to induce HIV-1-specific CD8+ T cells. This vaccine failed and also appeared to increase HIV-1 acquisition in uncircumcised male vaccinees with pre-existing immunity to the HAdV5 vector. The RV144 trial merged the T cell- and antibody-based approaches with a canarypox vector encoding gag, pol, and env, accompanied by a boost with rgp120 protein. The RV144 trial was the first indication that a vaccine could prevent HIV infection, albeit with modest efficacy. The 31.2% efficacy was not enough for licensure, but demonstrated a negative association between the presence of V1/V2-binding antibodies and the risk of infection. The most recent efficacy trial HVTN 505, a DNA prime with HAdV5 boost, was terminated early for futility. The V1/V2 IgG antibody response was found to be particularly low in HVTN 505, supporting the notion from RV144 that higher levels of these antibodies correlate with decreased risk of infection.

As a result of these studies, great emphasis has been placed on V1/V2 of HIV-1 Env. The V1/V2 region exhibits great diversity in both sequence and length. Recent crystal structures show that it forms a four-stranded β sheet domain with sequence diversity and glycosylation isolated to the strand-connecting loops. V1/V2 is located at the distal apex of the Env trimer and contributes to stabilization of this region. Further analysis of the V1/V2 antibody response induced by the RV144 vaccine regimen indicated that the antibodies specifically targeted the V2 region within
V1/V2\textsuperscript{26,117-119}. Genetic sieve analysis indicated a target of immune pressure within V2, highlighting a potential site of vulnerability for HIV-1 in this domain\textsuperscript{202}. V2 forms part of the co-receptor binding site with CCR5 and facilitates interactions with the integrin \( \alpha 4 \beta 7 \) expressed on gut homing T cells\textsuperscript{203-206}. It is a target for nAbs, although results from the RV144 trial indicated that non-neutralizing antibodies to V2 may also be protective\textsuperscript{207}.

To assess if I could selectively enhance antibody responses to the V2 loop of HIV-1 I developed Ad vaccine vectors based on simian serotypes SAdV23 and SAdV24. The vaccines expressed the V1/V2 region of a subtype C HIV-1 virus from a patient with a recent infection. The V1/V2 sequences were inserted into a scaffold that had been shown previously to preserve the structure of the loops so that they retained binding of nAbs\textsuperscript{125}. The vectors were tested for induction of antibody responses in mice in a prime-boost regimen in comparison to the SAdV23 and SAdV24 vectors expressing gp140 of subtype C HIV-1. In contrast to the RV144 regimen, the SAdV vectors elicited antibodies to conformational V1/V2 epitopes that were sustained. Antibody responses to linear V2 epitopes were less robust and were significantly greater at certain time points for regimens containing V1/V2-expressing vectors. Vectors expressing gp140 induced greater responses to the entire gp140 protein. The results presented herein warrant further investigation of the SAdV-V1/V2 vectors.
RESULTS

Construction of vectors expressing V1/V2 of a subtype C HIV-1

The crystal structure of V1/V2 of two subtype C viruses, ZM109 and CAP45, on the 1FD6 scaffold complexed to the antigen-binding fragment of the bnAb PG9 has been solved. Within V1/V2 the sequences based on subtype C Du172 and Du422 show 63% sequence homology to each other and 60% and 66% to CAP45, respectively (Figure 3.1A). The sequence homology is higher for the V2 loop with homologies ranging from 70% between Du172 and Du422, 67% between Du172 and CAP45 and 71% between Du422 and CAP45. All three carry a lysine in position 169, which is crucial for binding of PG9 and for ADCC activity of antibodies. Du422 unlike Du172 also carries a valine in position 172, which has been implicated in binding of cross-reactive V2-specific antibodies. Our efforts therefore focused on Du422 and we modeled its V1/V2 region (residues 119-205, HXB2 numbering) onto the 1FD6 scaffold. Despite sequence differences with V1/V2 of CAP45, the model indicates that the Du422 V1/V2 loops would complex similarly with PG9 thus assuming their native structure upon expression on the 1FD6 scaffold (Figure 3.1B and C). The model also suggests two sites of potential N-linked glycosylation at positions 156 and 160 (Figure 3.1D and E). A synthetic gene expressing V1/V2 of Du422 fused with 1FD6 was generated and replication-defective SAdV23 and SAdV24 vectors expressing this sequence were produced. To ensure that the inserted scaffolded V1/V2 sequences were expressed from the SAdV vectors, I performed V1/V2- or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific PCRs on cDNA from A549 cells that had been infected with SAdV24-V1/V2 or SAdV23-V1/V2. The reaction was controlled using cDNA from cells infected with an SAdV24 vector expressing an unrelated transgene, i.e., the nucleoprotein (NP) of influenza A/PR8 virus. Amplicons of the expected size of 137 base pairs (bp) were generated with the V1/V2-specific primers from cells infected with the scaffolded V1/V2-carrying vectors, but not from those infected with the control vector. GAPDH (128 bp amplicon) was amplified in all samples (Figure 3.1F).
**Immunization Regimen**

Groups of 10 outbred ICR mice were immunized intramuscularly with $10^{10}$ vp of the SAdV24-V1/V2 vector or for comparison with a SAdV24 vector expressing gp140 of Du422 (Figure 3.1G). SAdV24-V1/V2-immune mice were boosted eight weeks later with a higher dose of $10^{11}$ vp of SAdV23-V1/V2 vector while SAdV24-gp140-immune mice were boosted with $10^{10}$ vp of SAdV23-gp140 vector. SAdV23-V1/V2 was given at a higher dose based on its lower immunogenicity in a preliminary dose escalation pilot study (not shown). At week 16 of the study, all animals were boosted with an alum-adjuvanted baculovirus-derived gp140 protein of Du172. Controls included animals that only received one of the SAdV vectors and the protein boost or only the gp140 protein. Animals were bled before vaccination and then initially in 2-4 week intervals till week 24 after vaccination. They were bled again at week 59 after priming.
Figure 3.1. HIV-1 V1/V2 region is predicted to assume native conformation when grafted onto 1FD6 scaffold.

![Figure 3.1](image)

**FIGURE LEGEND CONTINUED ON PAGE 59**
FIGURE LEGEND CONTINUED FROM PAGE 58

A. V1/V2 sequence alignment for HIV-1 strains Du422, Du172, CAP45, and Consensus C (ConsC) using EMBL-EBI MUltiple Sequence Comparison by Log- Expectation (MUSCLE) software; conserved residues capitalized, unique residues lowercase. Strain HXB2 is provided as a residue numbering reference.

B. Model of HIV-1 strain Du422 V1/V2 (green) grafted onto 1FD6 scaffold (gold) in complex with the broadly neutralizing antibody PG9 (light chain, yellow; heavy chain, pink).

C. V1/V2-1FD6 protein of strain Du422 (orange) superimposed with strain CAP45 (blue).

D. Potential site of N-linked glycosylation at Asparagine (N) 156

E. Potential site of N-linked glycosylation at N160

F. PCR amplicons on 1% TAE gel show presence of V1/V2 mRNA (137 bp) for SAdV24-V1/V2 and SAdV23-V1/V2, but not the negative control SAdV-NP. GAPDH mRNA (128 bp) present in all samples.

G. Study timeline. Groups of 10 outbred Crl:CD1 (ICR) mice were bled and immunized at the time points indicated. Prime/boost SAdV vectors encoding sequences of HIV-1 strain Du422 and final boost composed of baculovirus-derived gp140 protein of HIV-1 strain Du172 in alum adjuvant. Schedule A-C, immunization with SAdV24 and/or SAdV23 encoding the V1/V2-1FD6 scaffold protein followed by a gp140/alum boost. Schedule D-F, immunization with SAdV24 and/or SAdV23 encoding gp140 protein followed by gp140/alum boost. Schedule G, immunization with gp140/alum only.
**Antibody responses to gp140**

Sera were tested for antibodies to gp140 by ELISA. Animals primed with SAdV24-gp140 developed a robust antibody response after priming that increased after the boost with either SAdV23-gp140 (Figure 3.2A) or gp140 protein (Figure 3.2B). The protein boost following two immunizations with the serologically distinct SAdV-gp140 vectors did not further increase antibody titers. After a single immunization with one SAdV vector antibody titers increased after the protein boost (Figure 3.2B and C). Protein given without SAdV prime elicited a small antibody response to gp140 (Figure 3.2D).

Priming with a single SAdV-V1/V2 vector induced low titers to gp140 (Figure 3.2A to C), which increased after the 2nd SAdV-V1/V2 immunization (Figure 3.2A) or upon protein boost (Figure 3.2B and C). Unlike immunization with the gp140-expressing vaccines, a protein boost following two SAdV-V1/V2 immunizations resulted in further increases in gp140 antibody titers. In all regimens, gp140 antibody titers were higher following immunizations with the gp140- than the V1/V2-expressing vaccines and these differences were highly significant at most time points.

All mice developed antibody titers to gp140 after vaccination, although kinetics differed. All mice primed with SAdV24-gp140 seroconverted and remained positive, while priming with SAdV23-gp140 achieved a 70% conversion rate (Figure 3.2E). Priming with SAdV24-V1/V2 was relatively ineffective with 10-20% of mice developing a positive response. This rate increased to 40% after the heterologous Ad vector boost and to 100% after the protein boost. The SAdV23-V1/V2 vector elicited a response in 30% of the mice, which again increased to 100% after the protein boost. The adjuvanted protein alone elicited a response in all mice. By week 59 all the mice remained seropositive.

Isotyping of gp140-specific antibodies at 18 weeks after priming (i.e., 2 weeks after the last boost) showed that the viral vectors expressing V1/V2 mainly induced IgG2a responses while upon priming with the gp140 vectors IgG2a and IgG2b responses were equally common. IgG1
responses, which were low after viral vector priming, dominated the gp140 antibody response in mice that had only been immunized with the protein vaccine.
Figure 3.2. Antibody responses to gp140.

A-D. Absorbance of IgG binding antibodies against gp140 strain Du172 protein by ELISA at a 1:200 serum dilution. Arrows indicate time of immunization. Geometric mean reported with 95% confidence intervals (CI) and two-way ANOVA performed on log transformed data. **** indicates \( p<0.0001 \), *** indicates \( p<0.001 \), ** indicates \( p<0.01 \)

E. Responsiveness after each vaccine. Responders classified as IgG absorbance greater than overall background mean (wk 0) plus 3 times the standard deviation of the mean. Percent responders of total mice per group reported as maximum after SAdV24 administration, wks 4-8, SAdV23 administration, wks 10-16, gp140/alum administration, wks 18-24, and at the end of the

FIGURE LEGEND CONTINUED ON PAGE 63
study, wk 59. Comparison by two-way ANOVA: wks 4-8 D>A \( p=0.025 \), D>B \( p=0.043 \), D>C \( p=0.014 \), D>F \( p=0.014 \), D>G \( p=0.014 \), E>A \( p=0.025 \), E>B \( p=0.043 \), E>C \( p=0.014 \), E>F \( p=0.014 \), E>G \( p=0.014 \); wks 10-16 D>B \( p=0.043 \), D>G \( p=0.014 \), E>B \( p=0.043 \), E>G \( p=0.014 \).

F. Antibody isotypes. Left, combined antibody isotype response for IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA after full regimen of vaccines, wk 18, at 1:200 dilution of pooled sera. Two-way ANOVA on cumulative responses results: D>G \( p=0.020 \), E>G \( p=0.035 \), F>G \( p=0.022 \). Right, relative frequency of each isotype per total response. Two-way ANOVA results: IgG1 G>B \( p=0.035 \), G>C \( p=0.026 \), G>D \( p=0.021 \), G>E \( p=0.023 \), G>F \( p=0.008 \); IgG2a B>G \( p=0.007 \), C>G \( p=0.004 \).
Antibody responses to the V1/V2 loop

Sera were tested for reactivity to V2 using three different reagents to assess binding, i.e., gp70-V1/V2, a structurally confined V1/V2 Consensus subtype C sequence on a scaffold protein composed of MLV glycoprotein, which was also used to assess antibody responses in the RV144 trial\textsuperscript{118}, and two peptides reflecting the V2 sequences of Du422 or Du172.

Screening with gp70-V1/V2 showed that priming with SAdV24 followed by SAdV23 expressing V1/V2 or gp140 elicited antibody titers following the 2nd immunization (Figure 3.3A), with 70% and 90% of mice developing a response, respectively (Figure 3.3E). Mean antibody titers did not increase upon boosting with gp140 protein, however response rates within 8 weeks after the protein boost increased to 100% in both groups. SAdV24 or SAdV23 given in single vector immunization regimens induced lower responses to gp70-V1/V2 and only 10% to 40% of animals developed a response (Figure 3.3B, C, and E). Antibody responses at week 10 were significantly greater in SAdV23-V1/V2- than SAdV23-gp140-immune mice. Titers increased in mice that received a single dose of a SAdV vector upon the protein boost with 90 to 100% of animals responding.

Responses to V2 of Du422 were low after priming with either the gp140 or V1/V2 loop-expressing SAdV vectors (Figure 3.4A to C). They increased after SAdV booster immunizations (Figure 3.4A) and in SAdV23 primed mice after the protein boost (Figure 3.4C). Upon SAdV24 priming followed by the SAdV23 and protein boosts, titers to V2 Du422 were higher four weeks after the protein boost in animals that had received the gp140 rather than the V1/V2 vaccines, however this difference disappeared after another four weeks. V2 Du422 titers were lower and overall less sustained than those detected on gp140- or gp70-V1/V2-coated plates.

Only 10 to 30% of mice developed antibodies to V2 Du422 after the SAdV24-V1/V2 prime and this was similar at 10 to 20% after the SAdV24-gp140 prime (Figure 3.4E). Response rates upon SAdV24-V1/V2 prime increased after the SAdV23-V1/V2 boost by 50% while the protein boost
was relatively ineffective after one or two doses of the SAdV-V1/V2 vectors causing an additional response in 0% to 30% of mice. The SAdV23-V1/V2 prime elicited a response to the V2 Du422 peptide in 40% of mice. After the protein boost SAdV23-V1/V2 primed mice tended to have better titers and response rates than mice primed with SAdV24 and boosted with protein. Response rates were lower after immunization with the SAdV-gp140 vectors; the triple immunization elicited a response in 70% of mice while two immunizations with one of the SAdV-gp140 vectors followed by a heterologous vector or a protein boost resulted in an antibody response in less than 50% of mice. The protein vaccine alone induced a V2 Du422 antibody response in only 20% of mice. Again, after the protein boost responses were higher in mice primed with SAdV23-gp140 than with SAdV24-gp140. The response to the V2 Du422 peptide was not as durable as the response to gp140 or gp70-V1/V2. By the end of the study 50% of mice that receive the two SAdV-V1/V2 vectors followed by protein remained seropositive, while 30% maintained their response upon SAdV24-V1/V2 prime followed by the protein boost or SAdV23-V1/V2 and protein. The response to the SAdV-gp140 regimens was less durable; 40% of mice maintained a response after triple immunization, 10 to 20% after vector immunization followed by a protein boost and 0% after a single protein vaccination.

Responses upon immunizations with the SAdV-V1/V2 vectors or prime-boosting with the SAdV-gp140 vectors were dominated by IgG2a antibodies (Figure 3.4F). Single doses of either of the SAdV-gp140 vector triggered mainly an IgG2b response. The protein given alone induced very low antibody titers to V2 Du422 with a mixed isotype profile including IgM.

Responses as well as response rates were similar upon their testing on the V2 Du172 peptide (Figures 3.5A to E), where again priming with the SAdV23-V1/V2 produced higher response rates compared to priming with the SAdV24-V1/V2 vector.
Figure 3.3. gp70-V1V2 Consensus C-specific antibody responses.

A-D. Absorbance of IgG binding antibodies against gp70(MLV)-V1V2 Consensus C protein by ELISA at a 1:200 serum dilution. Individual responses shown with line connected through the mean; two-way ANOVA performed on log transformed data. *** indicates $p<0.001$

E. Responsiveness after each vaccine. Percent responders of total mice per group reported as

FIGURE LEGEND CONTINUED ON PAGE 67
maximum after SAdV24 administration, wk 10, SAdV23 administration, wk 18, gp140/alum administration, wk 24, and at the end of the study, wk 59. Comparison by two-way ANOVA: wk 10 A>F p=0.013, A>G p=0.005, D>B p=0.013, D>C p=0.033, D>E p=0.013, D>F p=0.002, D>G p=0.0006; wk 18 A>G p=0.013, C>G p=0.013, D>E p=0.033, D>G p=0.002, F>G p=0.033.
Figure 3.4. Linear V2 Du422-specific antibody responses.

A-D. Absorbance of IgG binding antibodies against V2 Du422 peptide by ELISA at a 1:200 serum dilution. Arrows indicate time of immunization. Geometric mean reported with 95% CI and two-way ANOVA performed on log transformed data. * indicates $p<0.05$

E. Responsiveness after each vaccine. Percent responders of total mice per group reported as maximum after SAdV24 administration, wks 4-8, SAdV23 administration, wks 10-16, gp140/alum administration, wks 18-24, and at the end of the study, wk 59. Comparison by two-way ANOVA: wks 10-16 A>F $p=0.007$, A>G $p=0.007$, B>F $p=0.020$, B>G $p=0.020$, D>F $p=0.020$, D>G $p=0.020$;

FIGURE LEGEND CONTINUED ON PAGE 69
wks 18-24 C>E $p=0.020$, C>G $p=0.020$, D>E $p=0.020$, D>G $p=0.020$; wk 59 A>G $p=0.020$, D>G $p=0.038$.

**F.** Antibody isotypes. Left, combined antibody isotype response for IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA after full regimen of vaccines, wk 18, at a 1:200 dilution of pooled sera. Two-way ANOVA performed on cumulative responses. Right, relative frequency of each isotype per total response. Two-way ANOVA results: IgG2a B>E $p=0.033$, B>F $p=0.032$, C>E $p=0.032$, C>F $p=0.031$; IgG2b E>B $p=0.027$, E>C $p=0.028$, E>D $p=0.019$, F>B $p=0.027$, F>C $p=0.027$, F>D $p=0.019$. 
Figure 3.5. Linear V2 Du172-specific antibody responses.

A-D. Absorbance of IgG binding antibodies against V2 Du172 peptide by ELISA at a 1:200 serum dilution. Arrows indicate time of immunization. Geometric mean reported with 95% CI and two-way ANOVA performed on log transformed data. * indicates p<0.05

E. Responsiveness after each vaccine. Percent responders of total mice per group reported as maximum after SAdV24 administration, wks 4-8, SAdV23 administration, wks 10-16, gp140/alum administration, wks 18-24, and at the end of the study, wk 59. Comparison by two-way ANOVA: wks 10-16 A>F p=0.034, A>G p=0.034; wks 18-24 C>E p=0.034, C>G p=0.010; wk 59 C>E

FIGURE LEGEND CONTINUED ON PAGE 71
FIGURE LEGEND CONTINUED FROM PAGE 70

p=0.034, C>G p=0.034.

F. Antibody isotypes. Left, combined antibody isotype response for IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA after full regimen of vaccines, wk 18, at a 1:200 dilution of pooled sera. Two-way ANOVA performed on cumulative responses. Right, relative frequency of each isotype per total response. Two-way ANOVA results: IgG2a B>E p=0.007, B>G p=0.045, C>E p=0.004, C>G p=0.029, D>E p=0.015, F>E p=0.015; IgG2b E>A p=0.038, E>B p=0.006, E>C p=0.007, E>D p=0.020, E>F p=0.028.
**Correlation between responses**

Using Pearson correlation on log transformed data I tested if responses to gp140 or gp70-V1/V2 correlated to each other or to those to the two V2 peptides at week 10 after prime, week 18 after the protein boost and weeks 24 and 59 after completion of vaccination in mice that received one of the immunization regimens A-F. Regimen G did not induce a sufficiently strong V2-specific antibody response and was thus excluded from these analyses. After vaccinations with the SAdV vectors expressing the scaffolded V1/V2 loop of Du422 (Figure 3.6A) I saw at the early time points correlations between antibodies binding to gp140 or the three V2 loop-specific reagents especially in vaccine regimens that included the SAdV24 vector. These correlations waned over time. Correlations between gp70-V1/V2 and peptide binding antibodies were also observed; they tended to be more sustained. Responses induced by gp140 expressing SAdV vectors showed no correlations between antibodies binding gp140 or the three different V2 reagents although there were some weak correlations mainly early after the protein boost between gp70-V1/V2 and peptide binding antibodies (Figure 3.6B).
Figure 3.6. Antibody correlations.

Pearson correlation coefficient, r, for each pair of variables on log transformed IgG ELISA data for wks 10, 18, 24, and 59, for SAdV-V1/V2-containing regimens (A) and SAdV-gp140-containing regimens (B). *** indicates $p<0.001$, ** indicates $p<0.01$, * indicates $p<0.05$. 

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DISCUSSION

The RV144 trial, the only encouraging large scale HIV-1 efficacy vaccine trial to date, indicated that cross-reactive antibodies targeting conformational or linear epitopes within the V2 region of HIV-1 Env correlated with a decreased risk of infection\textsuperscript{117,118}. All RV144 vaccinees developed antibodies to HIV-1 gp120, and approximately 84\% developed V1/V2 antibodies\textsuperscript{208}. A high binding pattern to linear peptides in V1/V2 and V3 were observed and this reactivity was very different from patients who were infected with HIV-1. The highest binding antibody titers conferred greater protection upon vaccinees than those with lower titers. There was a linear association between peak concentration of antibody to V1/V2 and vaccine efficacy; as the response rate and magnitude of V1/V2 antibodies waned efficacy waned\textsuperscript{208}. Much of the antibody response to V1/V2 was mapped to a linear epitope interval with a lysine residue located at position 169 in the V2 region. This was further corroborated by the genetic sieve analysis of breakthrough viruses from RV144 subjects who became HIV-1-infected. Infected vaccinees were less likely to have viral isolates with a lysine at position 169 of V2 compared to infected placebo recipients\textsuperscript{202}. Thus, vaccine efficacy was significantly higher against viruses with K169 than against viruses with an alternative residue at position 169. This immunological and virological evidence taken together indicates the V2 region as a point of vulnerability for the virus and a target site for antibodies associated with vaccine efficacy.

The structure of the V1/V2 loops of HIV-1 has been resolved upon grafting of this sequence into the scaffold protein 1FD6\textsuperscript{125}. Scaffold proteins facilitate atomic-level characterization of parts of a complex protein sequence. Accordingly, McLellan et al. demonstrated that V1/V2 scaffolded onto 1FD6 maintains its native structure and binds to two bnAbs, i.e., PG9 and PG16. Scaffold proteins are also useful and efficient tools for delivery of polypeptides in a form that preserves the structure they assume within the entire protein and thus allows for induction of conformation-dependent antibodies\textsuperscript{209,210}. We capitalized on this concept by modeling the V1/V2 region of HIV-1 subtype C strain Du422 into the 1FD6 scaffold protein. Our model suggests that the Du422
V1/V2 assumes a structure that is very similar to that of 1FD6 scaffolded V1/V2 of CAP45 used in previous studies. To elicit a V1/V2-focused antibody response I developed and tested replication-defective SAdV vectors that express the scaffolded V1/V2 regions and compared antibody responses to those elicited by the same SAdV vectors expressing gp140 of Du422. As expected, the SAdV-gp140 vectors induced a stronger response to gp140 than the SAdV-V1/V2 vectors. Responses to the conformational epitopes of V2 were comparable upon immunization with either the gp140- or V1/V2-expressing SAdV vectors. They were sustained upon vector priming but contracted rapidly in animals that were only immunized with the protein vaccine. In RV144, response rates to gp120 were 100% at 2 weeks after final vaccination (week 26) and 79% at 52 weeks. In contrast, response rates to gp70-V1/V2 were 97% at 26 weeks, but dropped precipitously to 11% at 52 weeks. Similarly, in this study response rates to gp140 were 90-100% in regimens including SAdV vectors at 2 weeks after final vaccination (week 18) and week 59. In contrast to RV144, response rates to gp70-V1/V2 in SAdV-V1/V2-containing regimens were 60-80% at week 18 and 88-90% at week 59. Regimens containing SAdV-gp140 vectors responded to gp70-V1/V2 at 50-100% at week 18 and 78-89% at week 59. Thus, the SAdV vectors induced a more sustained antibody response to conformational V1/V2 epitopes than was seen in RV144.

Antibodies were also detected to linear V2 peptides. There was a trend of higher responses towards the Du422 than the Du172 V2 peptide potentially confirming a critical role for valine at position 172. Responses and response rates to peptides tended to be higher after the protein boost following a single vector immunization with SAdV23 than SAdV24. The SAdV23-V1/V2 vector was used at a 10-fold higher dose than the SAdV24-V1/V2 vectors. This is unlikely to have contributed to the better responses upon priming with the former, as the SAdV-gp140 vectors, which showed the same response pattern, were both used at the same dose. It is feasible that differences in timing of the protein boost, which in regimens B and E was given at 16 weeks after
SAdV24 vector priming and in regimens C and F at 8 weeks after SAdV23 vector priming, may have contributed as may have subtle differences in the biology of the two SAdV vectors\textsuperscript{211,212}.

Although both the SAdV-gp140 and SAdV-V1/V2 vectors followed by a protein boost induced comparable and durable antibody titers to V2, which were largely of IgG2a isotypes, features of the responses were not identical as indicated by differences in correlation between antibodies tested on the different antigens. Loss of initial correlations between gp140-binding antibodies and antibodies to conformational epitopes of V2 over time suggests that affinity maturation may have selected against cross-reactive antibodies. The more sustained or later onset of correlations between antibodies to conformational and linear V2 epitopes on the other hand indicate that antibodies with these specificities increased during B cell maturation.

A previous study in rabbits tested the immunogenicity of 3 doses of priming with a DNA vaccine expressing gp120 followed by booster immunizations with 3 doses of proteins including a 1FD6 scaffolded V1/V2 sequence\textsuperscript{213}. The vaccines were shown to elicit tier 1 nAbs that bound the V1/V2 loop in a fashion that interfered with binding of PG9. We were unable to demonstrate induction of nAbs to a Du422 Env virus due to high neutralizing background commonly seen in mouse sera (data not shown).

HIV-1 vaccines from other large-scale clinical trials that lacked efficacy such as VAX003, VAX004 and HVTN 505 induced antibody responses to gp70-V1/V2\textsuperscript{207}. V2 antibody titers were lower in participants of the latter two trials but comparable in those enrolled in VAX003. Differences in the mode of transmission, which was intravenous in VAX003 trial participants and by heterosexual contact in RV144 trial participants, may have affected protection by V2-specific antibodies.

Priming with a genetic vaccine as also shown here influences the selection of antibody isotypes\textsuperscript{122}, which in turn affects functions of non-neutralizing antibodies. VAX003, which only immunized with protein vaccines, elicited a dominant IgG4 response while RV144 induced IgG1 and IgG3 responses. V1/V2-specific IgG3 antibody response rates were higher in RV144 than in
VAX003 recipients. In RV144, the IgG3 antibodies to the V1/V2 region were associated with vaccine efficacy\textsuperscript{95}. Further analyses indicated that the selective induction of highly functional IgG3 in RV144 resulted in highly coordinated F\textsubscript{c}-mediated effector responses\textsuperscript{122}. In my experiments the protein vaccine induced mainly a mouse IgG1 response to gp140 which is related to IgG4 responses in humans while viral vectors stimulated IgG2a and IgG2b responses to gp140 and the V2 loop; these mouse isotypes are functionally similar to human IgG1 and IgG3\textsuperscript{214} again stressing that viral vectors should be incorporated into HIV-1 vaccines to assure induction of antibodies of protective isotypes.

While magnitude of antibody titers as well as response rates to V2 in mice immunized with the gp140- or the scaffolded V1/V2-expressing SAdV vectors were comparable, the more focused response achieved with the latter may provide clinical benefits. A number of studies showed that suboptimal titers of HIV-1 Env binding antibodies can increase HIV-1 infection in cell culture or in animal models\textsuperscript{215,216}. Although the relevance of such antibody-mediated enhancement of HIV-1 acquisition remains controversial for human vaccine recipients\textsuperscript{217,218}, one would assume that a very focused response to the V2 loop would lessen this potential threat.

In summary, this study shows immunization of mice with SAdV vectors expressing a scaffolded V1/V2 of HIV-1 Env followed by a gp140 protein boost induces antibodies of protective isotypes to V2. Most importantly, unlike in RV144 trial recipients, these responses were sustained as is typical after SAdV immunization\textsuperscript{83,219}.
MATERIALS & METHODS

Generating the 3D model of the Du422 V1/V2 – PG9 complex

The Du422 V1/V2 region bound to PG9 was generated using the software Protein Homology/analogY Recognition Engine V 2.0 (PHYRE 2), which produces a model of the protein of interest based on sequence alignment to known structures\(^\text{220}\). The 3D structure of DU422 V1/V2-PG9 complex was modeled of the x-ray crystal structure of the human Fab VRC38.01-HIV-1 V1V2-directed nAb (PDB ID: 5VGJ), to which it has >70% sequence identity. The presence of x-ray crystal structures with significantly high sequence identity between the two genes has allowed PHYRE to generate this model with 100% confidence. The model was further refined by applying geometry minimization in PHENIX\(^\text{221}\). Figures were generated in PyMOL\(^\text{222}\).

Vaccines

Ad vectors were based on simian serotypes SAdV23 and SAdV24 and each was generated with two distinct transgenes: SAdV23-V1/V2, SAdV24-V1/V2, SAdV23-gp140, and SAdV24-gp140. The V1/V2 transgene consisted of the V1/V2 region of HIV-1 gp120 fused with the scaffold protein 1FD6 and the gp140 transgene was HIV-1 gp140, both of subtype C strain Du422. Vectors were generated from recombinant viral molecular clones and the E1 gene was deleted rendering them replication defective. The E1 domain was replaced with a codon-optimized transgene under the control of the CMV promoter. Both vectors were developed with two different transgenes for a total of four different SAdV vectors. One transgene consisted of the V1/V2 region of HIV-1 with the scaffold protein 1FD6 and the second transgene was HIV-1 gp140, both of subtype C strain Du422. Vectors were rescued and expanded on HEK293 cells. Purification was performed using cesium chloride gradient centrifugation. The vectors were titrated for their contents of vp by spectrophotometry at 260 nm using the following formula: \(\text{OD}_{260} \times \text{dilution} \times 1.1 \times 10^{12}\). The content of infectious virus particles was measured by nested RT-PCR with transgene- or Ad (hexon)-specific primers on RNA isolated from HEK 293 cells infected for 5 to 7 days with serial dilutions of vector. Batches were tested for endotoxin using the LAL gel clot
method and a commercial kit. Genetic integrity and identity were assessed by isolation of viral DNA. The recombinant DNA, in parallel with the original molecular clones and shuttle plasmids used for generating molecular clones, was digested with a set of restriction enzymes and analyzed by gel electrophoresis. A recombinant protein of HIV-1 gp140 subtype C strain Du172 was derived in baculovirus as described previously.

Real Time PCR
V1/V2 vector expression was tested in A549 cells with SAdV24-V1/V2 and SAdV23-V1/V2 at $10^{11}$ vp and $10^{10}$ vp. SAdV24 encoding influenza NP A/PR8 was used as a negative control for V1/V2 expression. Briefly, cells were serum-starved in DMEM (Mediatech, Manassas, VA) for 1.5 hours with SAdV, fetal bovine serum (FBS) was then added and cells were incubated with virus for 2 days, all at 37°C. Cells were harvested and washed twice with PBS. Cellular RNA was isolated using the RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems by ThermoFisher). Real time PCR was performed using Fast SYBR™ Green Master Mix (Applied Biosystems by ThermoFisher) and the ABI Fast 7500 Real Time PCR System (Applied Biosystems by ThermoFisher). V1/V2 expression was detected using forward primer 5' TGCGTGACCCTGAACTGCAAG-3' and reverse primer 5' TTCTGCTTCTTGTGCGCAG-3'. The housekeeping gene GAPDH was tested as a control for normalization using forward primer 5' TGCCCGCATGTTGTGATGG-3' and reverse primer 5' AATGCCAAAGTTGTCATGGACC-3'. PCR amplicons were run on 1% agarose Tris-acetate-EDTA (TAE) gel and documented using a UVP GelDoc-It Imager (UVP, Upland, CA).

Animals & vaccination protocol
Seven groups of 10 female outbred Crl:CD1 (ICR) mice (Charles River Laboratories, New York, NY) were immunized intramuscularly in a total of 100 μL/dose. SAdV vectors were given in PBS; gp140 protein was given at 6.7 μg/dose in a 1% suspension of alum (Alhydrogel®, Sigma-Aldrich,
St. Louis, MO). Mice were treated at the Wistar Institute Animal Facility and the Wistar Institute Institutional Animal Care and Use Committee approved all procedures.

**Serum isolation**

Whole blood was collected by submandibular bleeding and allowed to coagulate undisturbed for 1 hour at room temperature. Samples were then centrifuged at 1600 rpm for 30 minutes at 4°C. Serum was isolated after centrifugation and frozen at -20°C until assayed.

**HIV-1 Env-specific IgG ELISA**

Sera were tested for HIV-1 gp140-, gp70-V1/V2-, or linear V2-specific IgG antibodies by ELISA on plates coated with (A) gp140 protein, (B) gp70-V1/V2 protein (Immune Technology Corp., New York, NY), and (C) linear V2 peptide (Genscript, Piscataway, NJ), respectively. The HIV-1 sequences were based on (A) strain Du172 (GenBank: ABD83638.1) and (B) Consensus subtype C. The (C) peptides were based on a 19 amino acid immunodominant epitope within the V2 region of HIV-1 strain Du172 (TTTEITDKKKKEYALFYKL) and strain Du422 (TTTELRDKKQKVYALFYKP; Genbank: ABD83641.1). Sera were tested at weeks 0, 4, 8, 10, 12, 16, 18, 20, 24, and 59 for (A) and (C) using 2-fold dilutions from 1:100-1:800, results are reported at 1:200. Sera were tested at weeks 0, 10, 18, 24, and 59 for (B) at a 1:200 dilution.

Briefly, 96-well plates (Nunc-Immuno™ Microwell™) were coated overnight at 4°C with (A), (B) (both 200 ng/well), or (C) (400 ng/well). Plates were blocked overnight at 4°C with (A) PBS/3% BSA, (B) PBS/15% BSA, or (C) PBS/8% BSA. Duplicate samples were incubated at room temperature for 2 hours for (A) and (C) and 90 minutes for (B). Standard samples were also applied to all plates to ensure uniformity of detection across several plates. Bound IgG was detected with a goat anti-mouse IgG alkaline phosphatase conjugate (Sigma-Aldrich) at 1:30,000 for (A) and 1:15,000 for (B) and (C). Bound enzyme was detected with phosphatase substrate tablets (Sigma-Aldrich) in DEA substrate buffer (KPL, Gaithersburg, MD) and OD was read by absorbance at 405 nm on a microplate reader.
**HIV-1 Env-specific sub-isotype ELISA**

Sub-isotyping of the gp140- and V2-specific antibody responses was performed at week 18 on pooled sera at 1:200 dilution. The ELISA was performed similarly as described above except bound antibody was detected using rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM (Calbiochem, San Diego, CA) followed by goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma-Aldrich) at 1:30,000 for (A) and 1:15,000 for (C).

**Statistical analysis**

Responders were classified as animals with OD values greater than the mean background plus three standard deviations of the mean. IgG ELISA OD values were background adjusted and log transformed for statistical comparison. Two-way ANOVA was performed without multiplicity corrections with Fisher’s Least Significant Difference (LSD) test. Correlations of log-transformed data were calculated by Pearson. Analyses were performed using GraphPad Prism 7.
CHAPTER 4

CALCIUM PHOSPHATE AS A VACCINE ADJUVANT
ABSTRACT

Adjuvants are included as components of vaccines to enhance the immune response to the antigens. Adjuvants can act through a variety of mechanisms and can influence the immune response in a variety of ways. Therefore, choice of adjuvant is a critical decision in vaccine formulation. Aluminum salts, or alum, have been used as adjuvants in vaccines for over 90 years and until 2009, were the only adjuvants approved for use in vaccines in the United States. Alum salts induce a T\textsubscript{H}2-biased immune response with a high IgE antibody response. Thus, the need for alternative adjuvant options in vaccine development remains. CaP is a non-toxic, biocompatible, and biodegradable compound that has potential as a vaccine adjuvant. CaP has been used successfully as an adjuvant in humans in France, however it is not approved as a component of a licensed vaccine yet in the United States. CaP has been shown to elicit a more balanced immune response, composed of both T\textsubscript{H}1 and T\textsubscript{H}2 cells, and induces little to no IgE antibodies. In the HIV-1 vaccine field, various combinations of HIV-1 antigens and adjuvants are being studied, as an effective vaccine to prevent infection remains an unmet need. In this study, I sought to determine if CaP combined with a HIV-1 antigen performed better as an adjuvant than alum when utilized in a heterologous prime-boost immunization with SAdV expressing another HIV-1 antigen. When administered with a high dose of protein antigen, CaP elicited a transiently greater IgG antibody response. In a lower dose of protein, CaP did not induce greater IgG responses than alum. When CaP was formulated with alum and the antigen, it did not enhance the antibody response. Further studies are needed to determine if the type or quality of the immune response elicited by CaP-adjuvanted vaccines is beneficial as compared to the current standard, alum.
INTRODUCTION

Adjuvants are compounds that can enhance and/or alter an antigen-specific, vaccine-induced immune response. Live-attenuated, recombinant viral-vectored, and inactivated vaccines contain particulate components that inherently contain natural adjuvants that are immune-stimulating. Some inactivated vaccines and particularly recombinant subunit vaccines may require formulation with an adjuvant to elicit sufficiently protective immune responses. Several parameters define the choice of adjuvant and formulation. These include, but are not limited to, physical and chemical natures of the antigen, preferred type of immune response, target population age, and route of administration\textsuperscript{223}. The goal of including an adjuvant in a vaccine is to enhance the efficacy of weak antigens and/or to induce immune responses not sufficiently induced by the vaccine alone\textsuperscript{223}. Adjuvants provide several advantages for vaccines. The amount of antigen needed to induce target levels of immune responses can be greatly reduced by appropriate pairing with an adjuvant. For example, the amount of recombinant influenza H5 protein required for 40\% seroconversion after one immunization was reduced by greater than 30-fold by addition of the adjuvant glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE) as compared to antigen alone\textsuperscript{224}. Adjuvants can also reduce the number of vaccine doses required in a regimen, therefore enabling a more rapid response to a potential pandemic outbreak. For instance, the doses required for successful immunization with hepatitis B antigen (Fendrix, GlaxoSmithKline) was reduced from three doses to two doses by inclusion of the adjuvant AS\textsubscript{04}\textsuperscript{225,226}. Adjuvants can help to broaden the antibody response, not only through increasing antibody titers, but also by increasing functional antibodies, eliciting higher affinity antibodies, and expanding the diversity of the B cell repertoire\textsuperscript{227-229}. The broadening of the antibody response by adjuvants has been shown by influenza and human papillomavirus (HPV) vaccines\textsuperscript{230-232}. Finally, adjuvants can aid in eliciting effective T cell responses that would otherwise not be engaged.

The oldest and most commonly used adjuvant in humans is alum. An alternative compound that is under consideration as a vaccine adjuvant is CaP. CaP has some characteristics related to
alum salts, yet it is extremely well tolerated as it is a natural compound of the human body. CaP, composed of the three elements calcium, phosphorus, and oxygen, is a naturally occurring compound in biological hard tissues, such as teeth and bones. CaP is therefore non-toxic due to its biocompatibility and it is easily biodegradable. As a vaccine adjuvant, this calcium salt can adsorb antigen and enhance delivery to the immune system. CaP has been used in humans for diphtheria-tetanus-pertussis vaccines in France. In contrast to alum, CaP does not induce production of IgE antibodies.

Substantial effort is being put forth to develop a HIV-1 vaccine that improves upon the 31.2% efficacy achieved in the RV144 vaccine trial. HIV-1 immunogen design and adjuvant formulations are being developed concordantly with the goal of combining them for a vaccine-adjuvant formulation with enhanced potency and durability. The RV144 trial tested a canarypox vector prime and a subunit gp120 protein boost adjuvanted with alum. Prior to RV144, two phase I/II trials were conducted, one testing the identical regimen to RV144 with the alum adjuvant (RV135) and the other testing the same canarypox prime with a different gp120 boost with the squalene-in-water emulsion adjuvant, MF59 (RV132). The MF59 adjuvant formulation in RV132 did not elicit higher IgG responses than the alum formulation in RV135, however it is possible that this was the result of the difference in the gp120 immunogen. Therefore, a phase IIb efficacy study (HVTN 702) is currently underway testing the RV144 regimen using MF59 in place of alum. Additional adjuvants, including AS01 and AS02 (both consisting of MPL and the saponin Quillaja saponaria fraction 21 [QS-21]) have been evaluated in phase I HIV-1 vaccine trials. Clearly, adjuvants for use in HIV-1 vaccines still require further evaluation.

In the study presented in this chapter, CaP is assessed as an adjuvant for an HIV-1 vaccine. CaP was formulated with a recombinant HIV-1 gp140 protein and combined in heterologous prime-boost regimens with an SAdV vector encoding HIV-1 gp140. The ability of CaP adjuvant to elicit antibody responses was examined in three parts. In the first part, CaP adjuvant was formulated
with 10 µg protein per dose and administered as a boost after SAdV prime. Secondly, the protein
dose was lowered to 1 µg, formulated with CaP, and given as a boost after SAdV prime. Finally,
CaP and alum adjuvant were both formulated with 1 µg protein and this time administered as a
prime immunization followed by SAdV boost. In all experiments, CaP was compared to the
standard adjuvant alum or protein alone. The resulting IgG antibody responses are reported.
RESULTS

Comparison of CaP adjuvant versus alum adjuvant when formulated with 10 μg/dose protein antigen

I sought to determine if CaP performs better as an adjuvant when formulated with protein as compared with the standard alum adjuvant. To test this, outbred CD-1 (ICR) mice were primed with SAdV24 expressing HIV-1 gp140 protein of strain Du422 at a relatively low dose, 10⁹ vp per dose, and then boosted with recombinant gp140 protein strain Du172 with adjuvant, at 10 μg protein per dose, or with adjuvant alone (Table 4.1). Previous studies have demonstrated (see Chapter 3) that priming mice with higher doses of SAdV, 10¹⁰ or 10¹¹ vp per dose, result in a robust antibody response that may not be increased by boosting with protein in adjuvant. Since the goal of this project was to be able to determine a potential difference in antibody response from the protein/adjuvant boost, the SAdV vector was administered at a lower dose to allow for enhancement of the antibody response upon protein/adjuvant boost.

All mice received SAdV24-gp140 at 10⁹ vp at week 0 and then eight weeks later were boosted with either gp140 at 10 μg in alum (Group A), gp140 at 10 μg in CaP (Group B), alum only (Group C), or CaP only (Group D) (Table 4.1). Antibody responses to gp140 protein were assessed by ELISA (Figure 4.1). Antibody titers were comparable through week 8, as expected because all mice received the same prime immunization. After the boost immunization at week 8, antibody responses increased significantly in groups that received gp140 protein in either CaP or alum adjuvant. Responses were higher in mice that received CaP-adjuvanted protein as compared to alum-adjuvanted protein at week 10. However, this difference contracted over time and responses were comparable between these two groups at week 18. Mice that received adjuvant alone at week 8 maintained nominal antibody titers through week 18.

One of the benefits of including an adjuvant as a vaccine component is dose-sparing of the antigen, i.e. because adjuvant increases the overall immunogenicity of the formulated vaccine,
less antigen is required to induce a protective immune response. It is possible that the high dose of protein used in this study (10 µg/dose) was saturating the antibody response and beneficial effects of the adjuvant were being lost. Therefore, I next sought to lower the dose of protein being administered and assess the resultant impact of the CaP and alum adjuvants.
Table 4.1. Study design for SAdV prime and protein/adjuvant 10 µg or adjuvant alone boost.

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<tr>
<td>A</td>
<td>SAdV24-gp140  10⁹ vp</td>
<td></td>
<td>gp140/alum  10 µg</td>
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<tr>
<td>B</td>
<td>SAdV24-gp140  10⁹ vp</td>
<td></td>
<td>gp140/CaP  10 µg</td>
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<tr>
<td>C</td>
<td>SAdV24-gp140  10⁹ vp</td>
<td></td>
<td>alum alone</td>
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<tr>
<td>D</td>
<td>SAdV24-gp140  10⁹ vp</td>
<td></td>
<td>CaP alone</td>
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Groups of five outbred Crl:CD1 (ICR) mice were immunized and bled at the time points indicated. On immunization days, mice were bled prior to vaccine administration and sera were isolated. SAdV24 encoding HIV-1 gp140 strain Du422 was administered on week 0 at 10⁹ viral particles (vp) per dose. Gp140, a recombinant baculovirus-derived protein of HIV-1 strain Du172, was administered on week 8 at 10 µg per dose in adjuvant; either 1% aluminum hydroxide gel (alum) or 0.3% calcium phosphate (CaP). Alternatively, alum or CaP was administered alone in PBS or water, respectively.
Figure 4.1. CaP adjuvant is not inferior to alum adjuvant.

(A) IgG antibody responses against gp140 protein strain Du172. Responses over time shown as mean ± standard error of the mean (SEM) of individual animal responses. Magnitude of IgG binding at each time point was determined by ELISA, calculated using background-adjusted optical density, and reported as area under the curve. (B) Comparisons between groups in part A were made at each time point by two-way ANOVA and Holm-Sidak’s multiple comparisons test; significant multiplicity-adjusted $p$ values are reported.
Comparison of CaP adjuvant versus alum adjuvant when formulated with 1 µg/dose protein antigen

To determine if the CaP adjuvant performed better than alum when administering a lower dose of protein, mice were primed with SAdV24-gp140 at $10^9$ vp and then six weeks later were boosted with gp140 protein at 1 µg formulated in either alum or CaP adjuvant (Table 4.2). Alternatively, mice were boosted with alum or CaP adjuvant alone or gp140 protein alone at 1 µg. Again, antibody responses to the SAdV prime were similarly low among all groups. Upon boost, responses were greatest in mice that received alum-adjuvanted protein. Mice that received the protein with CaP adjuvant showed lower titers than alum adjuvant, but greater titers than protein without adjuvant. Again, mice that received adjuvant without protein maintained nominal antibody responses. Based on this, the CaP adjuvant does not appear to induce a greater antibody response than the standard alum adjuvant, however it is possible that the CaP adjuvant has a different mechanism of action than alum. Thus, combining CaP adjuvant in the same vaccine formulation with alum adjuvant could have an additive effect on antibody responses.
Table 4.2. Study design for SAdV prime and protein/adjuvant 1 µg or adjuvant alone or protein alone boost.

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<th>Schedule</th>
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<tr>
<td>A</td>
<td>SAdV24-gp140 10⁹ vp</td>
<td>gp140/alum 1 µg</td>
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</tr>
<tr>
<td>B</td>
<td>SAdV24-gp140 10⁹ vp</td>
<td>gp140/CaP 1 µg</td>
<td></td>
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<tr>
<td>C</td>
<td>SAdV24-gp140 10⁹ vp</td>
<td>alum alone</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>SAdV24-gp140 10⁹ vp</td>
<td>CaP alone</td>
<td></td>
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<tr>
<td>E</td>
<td>SAdV24-gp140 10⁹ vp</td>
<td>gp140 alone 1 µg</td>
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<td>Bleed</td>
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Groups of five outbred Crl:CD1 (ICR) mice were immunized and bled at the time points indicated. On immunization days, mice were bled prior to vaccine administration and sera were isolated. SAdV24 encoding HIV-1 gp140 strain Du422 was administered on week 0 at 10⁹ vp per dose. Gp140 of strain Du172 was administered on week 6 at 1 µg per dose in adjuvant; either 1% alum or 0.3% CaP. Alternatively, gp140 was administered alone in PBS and alum or CaP was administered alone in PBS or water, respectively.
Figure 4.2. At lower protein dose alum is superior adjuvant to CaP.

(A) IgG antibody responses against gp140 protein strain Du172. Responses over time shown as mean ± SEM of individual animal responses. Magnitude of IgG binding at each time point was determined by ELISA, calculated using background-adjusted optical density, and reported as area under the curve. (B) Comparisons between groups in part A were made at each time point by two-way ANOVA and Holm-Sidak’s multiple comparisons test; significant multiplicity-adjusted \( p \) values are reported.
CaP adjuvant combined with alum adjuvant when formulated with 1 µg/dose protein antigen compared with either adjuvant formulated with 1 µg/dose protein antigen or 1 µg/dose protein antigen alone

To determine if CaP combined with alum had a synergistic effect, mice were primed with 1 µg of gp140 protein with either (i) CaP and alum, (ii) CaP, (iii) alum, or (iv) alone and boosted with SAdV-gp140 at $10^{10}$ vp four weeks later (Table 4.3). For this study, mice were primed with protein to determine if significant differences were seen from the CaP/alum combination initially after immunization. Two weeks after prime, the three different adjuvant formulations performed similarly and only gp140 in alum was slightly greater than gp140 alone (Figure 4.3). Four weeks after prime, gp140 in CaP and alum induced similar titers to gp140 in alum, while both these groups induced greater titers than gp140 in CaP. All three adjuvant groups showed a greater antibody response than gp140 protein without adjuvant. Two weeks after the SAdV-gp140 boost (week 6), mice receiving protein with both adjuvants had the highest antibody titers, while gp140 in either adjuvant were similar. Again, all three adjuvant groups were greater than protein without adjuvant. Finally, four weeks after the SAdV boost, protein with both CaP and alum elicited similar titers as protein with either adjuvant, however gp140 in alum was greater than gp140 in CaP. Once again, all three adjuvant groups were greater than protein alone. This study indicates that CaP combined with alum adjuvant can increase antibody responses slightly at certain time points, however this advantage does not appear to be durable.
Table 4.3. Study design for protein/adjuvant or protein alone 1 µg or adjuvant alone prime and SAdV boost.

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<th>Wk 4</th>
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<tbody>
<tr>
<td>A</td>
<td>gp140/CaP/Alum 1 µg</td>
<td></td>
<td>SAdV24-gp140 10^{10} vp</td>
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<tr>
<td>B</td>
<td>gp140/CaP 1 µg</td>
<td></td>
<td>SAdV24-gp140 10^{10} vp</td>
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</tr>
<tr>
<td>C</td>
<td>gp140/Alum 1 µg</td>
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<td>SAdV24-gp140 10^{10} vp</td>
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<tr>
<td>D</td>
<td>gp140 1 µg</td>
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<td>SAdV24-gp140 10^{10} vp</td>
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Groups of five outbred Crl:CD1 (ICR) mice were immunized and bled at the time points indicated. On immunization days, mice were bled prior to vaccine administration and sera were isolated. Gp140 of strain Du172 was administered on week 0 at 1 µg per dose in adjuvant; 1% alum with 0.3% CaP or 1% alum or 0.3% CaP. Alternatively, gp140 was administered alone in PBS. SAdV24 encoding HIV-1 gp140 strain Du422 was administered on week 4 at 10^{10} vp per dose.
Figure 4.3. CaP does not enhance adjuvanticity of alum.

(A) IgG antibody responses against gp140 protein strain Du172. Responses over time shown as mean ± SEM of individual animal responses. Magnitude of IgG binding at each time point was determined by ELISA, calculated using background-adjusted optical density, and reported as area under the curve. (B) Comparisons between groups in part A were made at each time point by two-way ANOVA and Holm-Sidak’s multiple comparisons test; significant multiplicity-adjusted p values are reported.
DISCUSSION

Since the discovery of alum as an adjuvant in the 1920s, hundreds of millions of doses of vaccines containing alum have been administered. Alum’s potential mechanisms of action are not yet completely understood, however some of them are clear. The mechanisms of action of alum include affecting antigen uptake, inducing danger signals, recruiting different types of immune cells, and eliciting T_{h}2 responses. Alum stimulates one of the innate immune system’s bacterial sensor mechanisms, NLRP3. This in turn activates the inflammasome and a set of inflammatory reactions that can lead to effective adaptive immune responses.\(^{242,243}\)

The mechanism of action for the CaP adjuvant is not yet fully understood. It is expected that as a particulate material, CaP creates an antigen depot that allows the slow release of antigen and extended presentation to the immune system.\(^{244,245}\) CaP may also promote antigen uptake and presentation by APCs.\(^{245}\)

In a mouse model of herpes simplex virus type 2 (HSV-2), a protein vaccine with CaP adjuvant was shown to be more potent than alum. The CaP adjuvant promoted high binding IgG2a antibody titers, high nAb titers, and a high rate of protection against a HSV-2 challenge.\(^{127}\) Notably, this study demonstrated a more balanced immune response of both T_{h}1 and T_{h}2 cell responses from the CaP adjuvant as compared to a predominantly T_{h}2 cell response from alum.\(^{127}\) Also, this study showed low IgE antibodies with use of CaP adjuvant. In a similar study, CaP proved to be a potent adjuvant for mucosal immunization.\(^{246}\)

The study described in this chapter assessed CaP as an adjuvant when combined with a high dose of protein antigen (10 µg), a low dose of protein antigen (1 µg), and alum with a low dose of protein antigen. This was a novel study using recombinant HIV-1 gp140 as the protein antigen combined in heterologous prime-boost regimens with SAdV encoding HIV-1 gp140. Taken together, this study indicates that at higher doses of protein CaP adjuvant induces similar or slightly greater antibody responses than alum. Upon dose-sparing, however, protein with CaP
adjuvant does not perform better than protein with alum adjuvant. CaP combined with alum does not significantly enhance overall antibody responses. Nevertheless, CaP is not necessarily an inferior adjuvant. Future studies may help elucidate whether other aspects of the antibody response are impacted by these different adjuvant modalities. Antibody avidity may be altered by use of different adjuvants. Alum tends to induce a T\(_{H2}\) cell-skewed immune response. As indicated by previous studies, it is possible that CaP may induce a more balanced immune response toward both T\(_{H1}\) and T\(_{H2}\) cell responses. This is more likely to be the desired immune response for a HIV-1 vaccine, as cell-mediated immunity plays an important role, in addition to antibodies, in an effective response against HIV-1.
MATERIALS & METHODS

Vaccines
The Ad vector was based on simian serotype SAdV24 and was generated with a gp140 transgene of subtype C strain Du422. The vector was generated from a recombinant viral molecular clone and the E1 gene was deleted rendering it replication-defective. The E1 domain was replaced with a codon-optimized transgene under the control of the CMV promoter. The vector was rescued and expanded on HEK293 cells. Purification was performed using cesium chloride gradient centrifugation. The vector was titrated for its content of vp by spectrophotometry at 260 nm using the following formula: OD260 × dilution × 1.1 × 10^{12}. The content of infectious virus particles was measured by nested RT-PCR with transgene- or Ad (hexon)-specific primers on RNA isolated from HEK 293 cells infected for 5 to 7 days with serial dilutions of vector. Batches were tested for endotoxin using the LAL gel clot method and a commercial kit. Genetic integrity and identity were assessed by isolation of viral DNA. The recombinant DNA, in parallel with the original molecular clone and shuttle plasmid used for generating the molecular clone, was digested with a set of restriction enzymes and analyzed by gel electrophoresis. A recombinant protein of HIV-1 gp140 subtype C strain Du172 was derived in baculovirus as described previously.

Adjuvant formulation with gp140 protein
CaP adjuvant was formulated with gp140 protein antigen for a final concentration of 0.3% CaP with 0.1 mg/mL gp140 or 0.01 mg/mL gp140. Briefly, equal parts gp140 antigen solution and 0.6% CaP (CaPtivate Pharma, Doylestown, PA) were mixed and incubated for 4 hours at room temperature by rotating on an end-to-end rotator. Alum adjuvant was formulated with gp140 protein antigen for a final concentration of 1% alum with 0.1 mg/mL gp140 or 0.01 mg/mL gp140 by mixing equal parts gp140 antigen solution and 2% alum (Alhydrogel®, Sigma-Aldrich, St. Louis, MO).
**Determination of antigen loading capacity**

Percentage of gp140 adsorption to CaP particles was estimated by Bradford’s method using a Bio-Rad Protein Assay Kit (Hercules, CA)\(^{247}\).

**Animals & vaccination protocol**

Groups of five female outbred Crl:CD1 (ICR) mice (Charles River Laboratories, New York, NY) were immunized intramuscularly in a total of 100 μL/dose. SAdV vector was given in PBS; gp140 protein and/or adjuvant was given at various doses as described above. Mice were treated at the Wistar Institute Animal Facility and the Wistar Institute Institutional Animal Care and Use Committee approved all procedures.

**Serum isolation**

Whole blood was collected by submandibular bleeding and allowed to coagulate undisturbed for 1 hour at room temperature. Samples were then centrifuged at 1600 rpm for 30 minutes at 4°C. Serum was isolated after centrifugation and frozen at -20°C until assayed.

**HIV-1 Env-specific IgG ELISA**

Pooled or individual sera were tested for HIV-1 gp140-specific IgG antibodies by ELISA on plates coated with gp140 protein based on strain Du172 (GenBank: ABD83638.1). Sera were tested at various time points using 2-fold dilutions from 1:1000-1:4000 (SAdV prime-protein boost at 10 μg), 1:400-1:6400 (SAdV prime-protein boost at 1 μg), or 1:200-1:6400 (protein prime at 1 μg-SAdV boost). Results are reported as AUC of background-adjusted OD values. Briefly, 96-well plates (Nunc-Immuno™ Microwell™) were coated overnight at 4°C with gp140 at 200 ng/well. Plates were blocked overnight at 4°C with PBS/3% BSA. Duplicate samples were incubated at room temperature for 2 hours. Bound IgG was detected with a goat anti-mouse IgG alkaline phosphatase conjugate (Sigma-Aldrich) at 1:30,000. Bound enzyme was detected with phosphatase substrate tablets (Sigma-Aldrich) in DEA substrate buffer (KPL, Gaithersburg, MD) and OD was read by absorbance at 405 nm on a microplate reader.
Statistical analysis

Two-way ANOVA was performed with multiplicity corrections using Holm-Sidak’s multiple comparisons test. Analyses were performed using GraphPad Prism 7.
Vaccines are the most effective tool in public health to prevent acquisition of infectious diseases. Vaccines have significantly reduced the global burden of many diseases, including poliomyelitis, diphtheria, tetanus, pertussis, rabies, measles, mumps, rubella, and Haemophilus influenza type b disease, and resulted in the complete eradication of smallpox worldwide\textsuperscript{248}. Yet some diseases, like malaria, tuberculosis, and HIV-1 remain severe threats to global health despite great efforts to develop effective vaccines against them. HIV-1 continues to spread, infecting two million people each year, with AIDS claiming more than 35 million lives since the beginning of the epidemic\textsuperscript{2}. Even though tremendous scientific efforts have been undertaken, a globally effective vaccine to prevent HIV-1 infection remains elusive. Here I will discuss the efforts undertaken in my lab toward developing more effective vaccines against HIV-1 and the future studies to further these efforts.

Several obstacles impede the progress toward an effective vaccine against HIV-1. The large viral diversity of HIV-1 presents an enormous challenge. Nine subtypes (A, B, C, D, F, G, H, J, and K) and over 35 CRFs of the virus make development of a universally effective vaccine a far-reaching goal. The amino acid sequence of HIV-1 Env can vary up to 20% within a subtype and 35% between subtypes\textsuperscript{5,249,250}. The high mutation rate during HIV-1 replication presents the compounding factor of immune escape\textsuperscript{251-253}. These complexities emphasize the importance of antigen selection and elicitation of broadly cross-reactive immune responses.

HIV-1-infected individuals develop natural immune responses against the virus, including CD8$^+$ T cell and nAbs, but these responses are usually strain-specific and the high mutation rate of the virus allows for rapid escape so these responses fail to eradicate the virus. A natural immune response to prevent HIV-1 infection could provide a mechanistic correlate of protection as a guidepost for vaccine strategy, but no such response exists. Therefore, immune correlates that would be necessary and sufficient to protect against HIV-1 acquisition need to be identified. HIV-1
prophylactic vaccine efforts have persisted despite these obstacles and some information regarding immune correlates of protection has been gleaned.

The first HIV-1 vaccine efficacy trials, VAX004 and VAX003, demonstrated that weak nAb responses elicited by recombinant proteins were not sufficient to confer protection\textsuperscript{84,85}. The Step trial showed development of antigen-specific T cells, yet no efficacy was demonstrated in the Step and Phambili trials\textsuperscript{88,89,92,128,254}. Furthermore, an increase of infections occurred in a subset of vaccinees in the Step trial, partially due to pre-existing immunity to the HAdV5 vaccine vector. HTVN 505, the first trial including a DNA vaccine component, showed no efficacy despite development of polyfunctional CD8\textsuperscript{+} T cell responses to Env\textsuperscript{98}. The most successful trial to date was RV144, which demonstrated 60% vaccine efficacy at 12 months after initial vaccination and 31.2% efficacy after the 42-month length of the study\textsuperscript{93,94,255}. In this trial, the vaccine reduced virus acquisition but did not reduce virus titers in individuals with breakthrough infections. The modest results from the RV144 trial provided the first setting for immune correlates of protection to be investigated with HIV-1 vaccine efficacy.

In the RV144 trial, two immune responses were significantly associated with vaccine efficacy: (i) the binding of IgG antibodies to the V1/V2 region of gp120 and (ii) the binding of plasma IgA to Env\textsuperscript{208}. In the presence of low plasma IgA responses, the avidity of IgG antibodies for Env and the magnitude of Env-specific CD4\textsuperscript{+} T cells were also significantly associated with vaccine efficacy\textsuperscript{208}. A follow-up study was undertaken to confirm the V1/V2 IgG immune correlate of decreased HIV-1 risk and to ensure reproducibility of the results with different labs, assays, and reagents\textsuperscript{96}. This study confirmed the V1/V2 immune correlate and also showed that the breadth of the V1/V2 IgG response correlated with decreased infection risk\textsuperscript{26,96,118}. Indeed, evidence of immune pressure on the V2 region of HIV-1 was demonstrated by a genetic sieve analysis that found significantly greater vaccine efficacy against HIV-1 strains matching the vaccine sequence in V2 (with a lysine at position 169)\textsuperscript{117}. Furthermore, V2-specific mAbs isolated from RV144
vaccinees were able to mediate ADCC, virion capture, neutralization, and antibody-mediated phagocytosis\textsuperscript{120,121,256,257}. The role of IgG subclass in protection was also investigated; Env- and V1/V2-specific IgG3 responses correlated with decreased risk of infection\textsuperscript{94,95}. IgG3 response rates to both Env and V1/V2 were significantly higher in RV144 as compared to VAX003, which both utilized the AIDSVAX B/E vaccine with only RV144 demonstrating efficacy\textsuperscript{95}. In RV144, both Env- and V1/V2-specific IgG3 antibodies were significantly associated with ADCC levels, indicating an antiviral functional role for IgG3\textsuperscript{95}. However, the IgG3 response in RV144 declined quickly after vaccination, corresponding with waning vaccine efficacy\textsuperscript{95}. Notably, VAX003 induced significantly greater response rates to IgG4 over time, while IgG3 responses declined quickly\textsuperscript{95}. A decline in IgG3 antibodies is also seen during acute HIV-1 infection\textsuperscript{258}. The rapid decline of IgG3 responses in these studies may indicate that a sustained IgG3 response will be difficult to attain.

Studies with different immunization strategies, improved immunogens, and alternative adjuvants may prove beneficial in maintaining a preferential IgG3 antibody response.

The RV144 trial provided the first evidence that vaccine-elicited immune responses could protect against HIV-1 infection. Several strategies to enhance or alter the RV144 regimen are currently being investigated. To prolong the protective responses in RV144, an extension of the boosting schedule of the ALVAC-HIV (vCP1521) and bivalent AIDSVAX B/E regimen is being studied in the RV305 trial in Thailand\textsuperscript{259}. Similar to RV144, no tier 2 nAbs were elicited in RV305\textsuperscript{259}. However, RV305 demonstrated increased somatic hypermutation of Env-specific antibodies and expanded pools of neutralizing B cell clonal lineages\textsuperscript{259}. To determine if the protection afforded by RV144 in Thailand can be transferred to a new region with different circulating strains, the ALVAC-HIV/AIDSVAX B/E regimen is being tested in South Africa in the HTVN 097 trial\textsuperscript{260}. In addition, the HVTN 100 trial is testing a similar strategy to RV144 in South Africa using locally circulating strains with ALVAC-HIV (vCP2438) and a bivalent subtype C gp120 in MF59 adjuvant\textsuperscript{260,261}. HVTN 100 will move forward into efficacy testing in the HVTN 702 trial. A phase I/IIa trial, HVTN 107, in Zimbabwe will assess using MF59 or alum as the adjuvant with bivalent...
subtype C gp120 as the boost to ALVAC-HIV (vCP2438) prime. Results from these studies may help elucidate mechanisms of protection against HIV-1 acquisition.

Until the immunological mechanisms required for protection against HIV-1 infection are clearly understood, progress can be made through continued investigation of alternative HIV-1 vaccine strategies. I have investigated improvements in HIV-1 vaccines using novel heterologous prime-boost vaccination strategies, immunogen design, and vaccine-adjuvant formulations. These investigations are described herein in three separate studies aimed at improving the induction of antibodies to HIV-1 Env.

Early HIV-1 vaccine studies indicated that heterologous prime-boost regimens can strengthen and broaden vaccine-elicited immune responses. Prime-boost vaccine strategies have subsequently been studied in more than 100 NHP and human clinical trials, including the moderately successful RV144 trial. Common heterologous prime-boost strategies include: (i) DNA prime and protein boost, (ii) DNA prime and viral vector boost, (iii) viral vector A prime and viral vector B boost, and (iv) viral vector prime and protein boost. These prime-boost strategies are employed as they generally elicit better immune responses than either vaccine alone. It has become clear from the vaccine trials to date, particularly for HIV-1, that eliciting only one arm of the adaptive immune system may not be sufficient for protection. Immunization with Env proteins alone typically induces strong binding antibodies, but nAb and CD4+ T cell responses are limited, and no CD8+ T cells are induced. Heterologous prime-boost strategies designed to elicit both humoral and cell-mediated immunity may be required for a successful vaccine strategy.

My lab sought to develop a novel heterologous prime-boost strategy utilizing SAdV in combination with an Env protein. Ad viral vectors induce potent T and B cell responses. To circumvent the issue of pre-existing nAbs to the human serotype Ad vectors, chimpanzee-derived SAdV vectors can be utilized. My lab developed SAdV vectors of serotype 23 and 24 to express HIV-1 gp140 of subtype C as an antigenic transgene. We collaborated with the Polonis...
Lab, who developed a partially trimeric HIV-1 Env protein immunogen, gp145, also of subtype C\textsuperscript{143}. The goal of utilizing a trimeric Env immunogen is to better mimic the conformational forms of Env present on the surface of the virion rather than monomeric Env proteins that have been used in the previous HIV-1 efficacy trials. The trimeric Env may be more representative of a functional, native Env trimer as it binds to several neutralizing mAbs that recognize field isolates and therefore may be a more effective immunogen\textsuperscript{143}. We combined the SAdV vectors and Env trimer in three heterologous prime-boost regimens: (i) gp145 Env prime and SAdV24-gp140 boost, (ii) SAdV24-gp140 prime and gp145 Env boost, and (iii) SAdV24-gp140 prime and SAdV23-gp140 boost\textsuperscript{267}. Each of these strategies were tested at two different doses (10\textsuperscript{10} and 10\textsuperscript{9} vp) of SAdV vector and I assessed the vaccine-elicited antibody responses. Boosting SAdV vector-primed mice with the heterologous SAdV vector or alum-adjuvanted gp145 protein significantly enhanced Env-specific antibody responses, including V2-specific binding antibodies. Priming with gp145 Env followed by SAdV vector boost was relatively ineffective, which is consistent with other studies that demonstrate optimal immune responses from heterologous prime-boost vaccination when protein is used as the boost\textsuperscript{146,186,187}. The mechanism for higher antibody responses upon protein boost is not fully understood. I surmise it reflects that SAdV vectors are far more immunogenic than protein vaccines and thereby better suited to drive activation of naïve B cells, which can then be expanded by a less immunogenic vaccine. Induction of nAbs that cross-reacted between different subtypes required inclusion of gp145 Env into the vaccine regimen, emphasizing the importance of this trimeric immunogen. Interestingly, repeated immunizations with the gp145 protein did not significantly increase antibody responses nor did it appear to affect response longevity. The SAdV prime regimens elicited predominantly IgG2a and IgG2b, which are the mouse isotypes most closely related to human IgG1 and IgG3, respectively\textsuperscript{214}. Notably, human IgG3 was shown to correlate with vaccine-induced resistance to HIV-1 infection in the RV144 trial\textsuperscript{95,188}. In contrast, the gp145 Env prime regimens elicited mainly an IgG1 response, which is most closely related to human IgG4, but also induced IgG2a and IgG2b\textsuperscript{214}. The isotype
profile shows that priming with the SAdV vectors induces a predominantly T\textsubscript{H}1 response and that this is not modified by boosting with alum-adjuvanted protein, which generally induces a T\textsubscript{H}2 response\textsuperscript{116,146}. On the other hand, priming with protein favors induction of a T\textsubscript{H}2 response. It has been shown previously that antibody responses induced by Ad vectors are extremely stable, which may reflect the low level \textit{in vivo} persistence of Ad vectors\textsuperscript{83,112,145}. Including an SAdV vector into a vaccine regimen has the added advantage that all Ad vectors induce potent cellular immune responses to the transgene product, most notably CD8\textsuperscript{+} T cells. CD8\textsuperscript{+} T cells may not prevent infection, but allow for rapid viral clearance\textsuperscript{191}.

Based on these initial findings, additional studies are warranted of SAdV prime-gp145 Env boost vaccination. Investigation of a regimen for SAdV24 prime-SAdV23 boost followed by a gp145 Env boost might provide further insight into the kinetics of the vaccine-elicited responses. Other considerations for investigation include elapsed time between immunizations and diversity of antigen strains. Examination of additional antibody functions such as ADCC, virus capture, and ADCVI, could provide useful information due to the IgG subclasses elicited. Studies are warranted in NHPs to elucidate appropriate dosing of both the SAdV24 vector and gp145 protein and to assess if the vaccine-induced antibodies provide protection against viral challenge.

HIV-1 vaccine immunogen design can be informed by results of previous efficacy trials. The RV144 trial highlighted the V2 region of HIV-1 Env as a potential site of vulnerability for the virus. Designing a vaccine immunogen to induce an enhanced, functional V2-specific antibody response over that afforded by the RV144 regimen may be advantageous and provide greater efficacy. I sought to capitalize on recent breakthroughs in the structural determination of Env, particularly in the V1/V2 region, to focus a vaccine-elicited immune response on V2. Structurally, the V1/V2 domain is considered to be a singular topological entity, thus a conformationally relevant immunogen should include the entire domain\textsuperscript{125}. In 2011, Dr. Peter Kwong and colleagues reported on the crystal structure of V1/V2 in complex with the bnAb PG9\textsuperscript{125}. The
V1/V2 region was grafted onto a protein scaffold, 1FD6, and shown to adopt a native-like conformation. With the assistance of my lab, I developed SAdV vectors expressing the scaffolded V1/V2 domain for use in heterologous prime-boost vaccination with recombinant gp140 protein. The antibody response to V1/V2-expressing SAdV vectors were compared to SAdV vectors expressing gp140 protein. The V1/V2-expressing vectors and the comparison gp140-expressing vectors both induced a sustained antibody response to a conformational V1/V2 antigen. The V1/V2-expressing vectors elicited a greater antibody response to linear V2 epitopes briefly, however this occurred in only a fraction of animals and waned over time. Gp140-expressing vectors elicited a greater gp140-specific antibody response that was sustained through the duration of the study. In immunization regimens containing the V1/V2 vectors, the V2- and gp140-specific antibody responses were highly correlated. In contrast, in the gp140-expressing vector responses V2 antibodies did not generally correlate with gp140 antibodies, indicating that these are independent responses presumably to other regions of Env outside of the V2 domain. The viral vectors stimulated IgG2a and IgG2b responses to gp140 and the V2 loop; these mouse isotypes are functionally similar to human IgG1 and IgG3 again stressing that viral vectors should be incorporated into HIV-1 vaccines to assure induction of antibodies of protective isotypes. Limiting immune responses to the V2 region may have benefits over immunization with the entire protein as IgA antibody responses to the C1 region of Env abrogated the favorable V2 response in the RV144 trial. The V2 antibody response in RV144 waned over time corresponding with waning vaccine efficacy. In contrast to the RV144 vaccines, V1/V2-expressing SAdV vectors induced sustained response rates and durable antibody responses to V1/V2 conformational epitopes.

While it is not yet clear whether the V2-specific antibody response associated with decreased risk of infection in the RV144 trial was a direct mechanistic correlate of protection or a surrogate correlate of protection, there is some evidence to suggest why the V2 domain may be an important target for vaccine-elicited antibodies. The V1/V2 region varies in sequence, length, and
N-linked glycosylation. This region is not required for viral entry into host cells, but plays a critical role in masking neutralizing epitopes of gp120, rendering the virus highly neutralization-sensitive when V1/V2 is deleted\textsuperscript{17-20}. HIV-1 must bind to two cell-surface receptors, CD4 and CCR5 or CXCR4, to initiate virus-host cell fusion via exposure of the gp41 fusion domain. Two conserved β strands in the V1/V2 region of gp120 form part of the chemokine receptor binding site\textsuperscript{203-205}. The V2 loop contains a tripeptide motif, LDI/V, at residues 179-181 that binds to the integrin α4β7, which forms a virological synapse. α4β7 is a gut mucosal homing integrin receptor that facilitates the migration of lymphocytes from the genital mucosa to the GALT\textsuperscript{268,269}. The interaction of V2 with α4β7 may increase the efficiency of viral transmission.

Several antibody responses to V1/V2, which are associated with decreased risk of infection in RV144, mapped to a specific region in V2. The common region of these responses is the V2 hotspot, spanning positions 166-178, identified from peptide microarrays. The V2 hotspot lies immediately adjacent to the α4β7 binding motif at positions 179-181. The role that the V2 hotspot antibodies play in protection and how these antibodies function to potentially confer protection is not known. These antibodies do not seem to function via neutralization of CD4-mediated infection, as indicated by the RV144 immune correlates analysis. There is evidence to show that the V2 antibodies function via non-neutralizing mechanisms, including Fc-mediated ADCC and complement activation. It is possible that there is steric hindrance of the viral interaction with α4β7 by these antibodies. There is also the possibility that these antibodies have some important interaction with α4β7. The presence of the α4β7 binding motif in V2 may provide insight into why protection was associated with antibodies targeting this region and into potential mechanisms of protection.

Further investigation of SAdV-V1/V2 vector immunization could assist in elucidating the role of V2 antibodies in protection from HIV-1. Future studies should examine several factors to possibly further enhance the V2-specific antibody response, including vector prime choice (SAdV23 vs.
SAdV24), elapsed time between immunizations, dose-escalation, and diversity of antigen strains. To illuminate antibody function, additional analysis should include avidity, ADCC, ADCVI, and neutralization. It would also be interesting to investigate the durability of the human IgG3 correlate, mouse IgG2b. Finally, assessment of these vectors in NHP challenge studies would determine if the SAdV-V1/V2 vectors induce protective immunity.

Another strategy that may enhance induction of HIV-1 Env-specific antibody responses would be the use of novel vaccine adjuvants. Adjuvants help to enhance the efficacy of weak vaccine antigens and/or to induce immune responses not sufficiently induced by the vaccine alone. Alum was first discovered as an adjuvant over 90 years ago and until recently was the only adjuvant approved for use in a vaccine in the United States. Despite its long history, alum has some limitations as an adjuvant, including being relatively weak, inducing mainly Th2 responses, and rarely inducing cellular, or Th1, immune responses. Alum has a relatively good safety record, but has been associated with some IgE-induced allergic reactions in humans. More recently, two oil-in-water emulsions, MF59 and AS03, and one MPL/alum combination, AS04, have been licensed for use as part of vaccines in the United States. The adjuvants MF59 and the MPL-based AS01 combined with recombinant proteins, including HIV-1 Env, have been shown to enhance the durability of immune responses as compared to alum-adjuvanted proteins.

CaP is a naturally occurring compound in the human body and has been utilized as an adjuvant in humans for diphtheria-tetanus-pertussis vaccines in France. CaP is non-toxic and does not induce production of IgE antibodies, unlike alum. It is suspected to function as an adjuvant by creating an antigen depot that allows the slow release of antigen and extended presentation to the immune system. CaP has been shown to elicit a more balanced immune response of both Th1 and Th2 responses as compared to a predominantly Th2 response from alum. I assessed CaP as an adjuvant for an HIV-1 vaccine by formulating it with a recombinant HIV-1 gp140 Env protein. IgG antibody responses elicited by the protein vaccine in CaP were
compared to those induced by the protein vaccine mixed with alum adjuvant. This study demonstrated generally similar antibody responses from CaP-adjuvanted vaccines and alum-adjuvanted vaccines. CaP also did not act synergistically with alum to enhance antibody responses. This is not to say that CaP is an inferior adjuvant. Future studies should examine whether CaP increases the durability or avidity of antibodies. CaP used in the context of a recombinant Env protein may induce a more balanced response of $T_{H1}$ and $T_{H2}$ cells, which could skew the antibody response to more desirable isotypes and invoke ADCC, ADCVI, or neutralization.

Despite significant advances in HIV-1 treatment, a vaccine to prevent infection from HIV-1 is still desperately needed. Six HIV-1 vaccine efficacy trials have taken place with only one demonstrating moderate efficacy. Several different strategies can be employed to enhance immune responses to HIV-1 vaccines. I have described three novel studies examining the following approaches: heterologous prime-boost strategies, immunogen design, and alternative adjuvant formulations. These studies demonstrated novel SAdV vectors that induce enhanced antibody responses to HIV-1 Env. These SAdV vectors are a desirable platform for vaccine development and are good candidates for prime-boost regimens. I also showed that protein immunogens can be improved by more accurately representing the protein as it is present on the whole pathogen. These studies have advanced the current knowledge of HIV-1 vaccines and future studies can further advance the field toward an effective HIV-1 vaccine.
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