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Andrew T. Jones Date

Novel Immunogens and Mucosal Vaccination For Improving Protection Against HIV-1

By

Andrew T. Jones

Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Immunology and Molecular Pathogenesis

Rama Rao Amara, Ph.D.
Advisor

Eric Hunter, Ph.D.
Committee Member

Andrew Neish, M.D.
Committee Member

Joshy Jacob, Ph.D.
Committee Member

Ifor Williams, M.D. Ph.D.
Committee Member

Accepted:

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

Date

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By

Andrew T. Jones

B.S., Northern Kentucky University, 2010

Advisor

Rama Rao Amara, Ph.D.

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Abstract

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By Andrew T. Jones

Despite continuous efforts since its initial discovery, an effective prophylactic vaccine against Human Immunodeficiency Virus (HIV) -1 has yet to be developed. Insights from vaccine efficacy trials in both animal models and humans, as well as basic research to understand the complexities of HIV-1, have greatly informed current efforts in HIV-1 vaccine design. Current efforts are focused on the design and characterization of novel immunogens that promote protective antibody responses. Many immunogens are designed to better recapitulate the trimeric structure of the native HIV-1 Envelope, composed of heterotrimers of gp120 and gp41, which mediates viral entry.

This dissertation will characterize the immunogenicity and efficacy of a novel trimeric HIV-1 gp120 immunogen, named cycP-gp120, in rabbits and rhesus macaques. We demonstrate cycP-gp120 to be robust vaccine immunogen, generating high titers of cross-reactive antibodies targeting the variable loops 1 and 2 (V1V2) of gp120, a key correlate of protection in the RV144 vaccine efficacy trial in humans. We show that cycP-gp120 in conjunction with priming with the poxvirus vector modified vaccinia Ankara (MVA-HIV) promotes both V1V2-directed antibodies and antibody-dependent cellular cytotoxicity (ADCC) in rabbits.

As HIV-1 is primarily transmitted across mucosal surfaces via sexual contact, generating mucosal immunity to HIV-1 is thought to be a crucial component to an effective HIV-1 vaccine. Here we describe a novel method of oral immunization in which we immunized the sublingual and buccal tissue of rhesus macaques with a needle-free injector. Needle-free immunization resulted in a large expansion of both systemic and mucosally localized vaccine-specific antibodies, demonstrating this route as an easy and effective method of mucosal vaccination.

To test the protective efficacy of MVA-HIV/cycP-gp120 immunization in rhesus macaques, as well as the needle-free oral vaccination route, we challenged immunized animals with a weekly low dose of pathogenic SHIV-SF162P3. We observed significant protection in rhesus macaques immunized with via needle-free oral immunization or intradermally/subcutaneously. Protective efficacy correlated with anti-gp120 binding IgG, anti-V2 peptide antibodies, and antibody-dependent cell-mediated viral inhibition (ADCVI). These studies thus describe cycP-gp120 as an attractive vaccine immunogen for further studies in non-human primates and humans, as well as the needle-free oral immunization route as mucosal vaccination technique.

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Chapter 1: Introduction

Since its initial discovery almost 40 years ago, the Human Immunodeficiency Virus-1 (HIV-1) pandemic has caused millions of deaths worldwide. As of 2017 there are approximately 37 million people living with HIV-1, and more than 1 million individuals became newly infected with HIV-1 in 2016 (1). Despite major successes at combating HIV-1 and acquired immune deficiency syndrome (AIDS), including the development of highly antiretroviral drugs (HART) which drastically improve the outcome of HIV-1 infected individuals, an effective prophylactic vaccine has yet to be developed. The need for an effective HIV-1 vaccine remains important as access to HART is limited to many HIV-1 infected individuals due to economical, technological, and societal factors (2). Numerous HIV-1 vaccine efficacy trials have been tested in humans, with the majority failing to confer any significant protection from infection, and none being licensed for further use (3). Lessons learned from these trials, as well as a continuous effort to understand the virology and pathogenesis of HIV-1, have greatly informed researchers of the requirements for a protective HIV-1 vaccine. This dissertation focuses on two areas of active HIV-1 vaccine research: the development of novel immunogens to serve as potential vaccine candidates, and the design and evaluation of a novel mucosal immunization route for HIV-1 vaccination. Implementing these concepts in future studies may potentially aid in the development of more efficacious HIV-1 vaccines. Additional background and context is provided in the following sections.

A. Virology and Pathogenesis of Human Immunodeficiency Virus-1 (HIV-1)

Structure and Virology of HIV-1

HIV-1 is an enveloped RNA virus within the *Lentivirus* genus and *Retroviridae* family. Other members of the *Lentivirus* genus include HIV-2, a more rare and less pathogenic virus in humans, and simian immunodeficiency virus (SIV), a *Lentivirus* found in non-human primates and thought to be the zoonotic origin of HIV-1. The genome of HIV-1 consists of three major genes, *gag* (structural proteins), *pol,* (polymerase), and *env* (viral glycoprotein) as well as number accessory genes *tat, rev, nef, vif, vpr, vpu,* all of which have various roles in viral replication and pathogenesis (**Fig. 1a**) (4).

Lentiviruses consist of a single-stranded positive-RNA genome, which is converted to double-stranded DNA upon entry to host cells by the viral enzyme reverse transcriptase. The viral DNA is then trafficked to the nucleus and integrated into the host genome by the viral enzyme integrase. The integrated viral DNA can then be transcribed by hose cell machinery and new viral RNA genomes and proteins can be packaged to form new virions and be released from the cell. Alternatively, the integrated DNA can become latent, silencing the production of new virions, forming a viral reservoir undetectable by the immune system (5). The ability of HIV-1 to form these viral reservoirs is a crucial component of its longevity in the host, and a major area of HIV-1 research is in the identification and elimination of the viral reservoir in infected individuals. A hallmark of HIV-1 is its immense genetic diversity, due largely to the error prone reverse transcriptase which can cause 0.2-2 mutations per genome per cycle

(6). HIV-1 can easily mutate away from immune pressure, especially once a viral reservoir has been established and the immune pressure is focused on a non-conserved region. The diversity of HIV-1 is so great that some studies have compared the diversity of HIV-1 isolates within one infected individual to an entire year of world-wide influenza isolates.

HIV-1 Envelope and Viral Entry

HIV-1 Envelope (Env), the viral glycoprotein responsible for binding and entry into host target cells, is the major target of antibody-based HIV-1 vaccines (7). HIV-1 Env is first expressed as a gp160 precursor peptide of approximately 850 amino acids, forming homotrimers before furin-mediated cleavage into gp120 (~480 amino acids) and gp41 (~345 amino acids) subunits, resulting in a non-covalently linked heterotrimeric spike (8). Native infectious HIV-1 Env exists in this trimeric state, a complex of gp41 stalks linked to the viral envelope and gp120 head units at the apex of the complex (**Fig. 1b**).

Viral entry occurs through a series of steps. Initially, HIV-1 Env will bind to the CD4-receptor on a target cell, primarily $CD4^+$ T-cells, via the CD4-binding site located in gp120. Additionally, viral entry requires HIV-1 Env binding to a co-receptor, primarily the CCR5 or CXCR4 chemokine receptors. There is evidence that gp120 also binds to $\alpha_4\beta_7$, an integrin expressed on CD4+ target cells, inducing a conformational change and activation of LFA-1, an integrin involved in the virological synapse between infected and non-infected cells (9). Binding of HIV-1 Env to CD4 induces a conformational change to gp120, exposing the co-receptor binding site, which can then bind to the CCR5 or

CXCR4 co-receptor. This binding to the co-receptor induces another conformational change in gp41, which inserts its fusion peptide into the host membrane, triggering a fusion of the host and viral membranes, and delivery of the HIV-1 capsid to the target cell.

HIV-1 Env is a heavily glycosylated and antigenically complex glycoprotein (**Fig. 2a, b**) (10), with each gp120 subunit containing approximately 25 N-linked glycans, accounting for up to half of its mass (6, 11). Initial studies characterizing the structure of HIV-1 Env were hindered by the general instability and flexibility of gp120 (12). The gp120 subunits of native HIV-1 Env will readily dissociate from the gp41 stalks as they are not covalently linked, and the hypervariable regions within gp120 rendered the protein highly flexible, making efforts difficult in solving its structure (13). Recently, studies on a modified BG505 clade A gp140 (a truncated gp160 lacking the trans-membrane domain of gp41) cleaved trimer, stabilized via disulfide bonds have allowed for the first time high resolution imaging of the native trimeric HIV-1 Env (14, 15). This modified gp140, named SOSIP, revealed native HIV-1 Env to resemble a pin-wheel or propeller structure, with the gp120 subunits as the wings of the propeller and gp41 a pillar-like structure in close association with the inner domains of the gp120 subunits (**Fig. 2c**). The CD4-binding site is located on the side of each gp120 subunit, forming a pocket within the outer domain. The variable loops 1, 2, and 3 (V1, V2, V3) are located near the head of the trimer, the V1 and V2 loops reside at the apex, and the V3 loops buried underneath the V1 and V2 loops. Residues within the V2 loops of one gp120 interact with the adjacent promoter, aiding in the formation and stabilization of the trimeric complex. Much of the inner domain of gp120 is buried within the trimer

complex, in close association with gp41 and inaccessible to the immune system. The glycan shield covers much of the trimeric surface, limiting access of antibodies to the protein backbone to much of the structure (**Fig. 2d**). This data shows that the native HIV-1 Env exists as complex and quaternary structure, distinctly different than monomeric gp120.

While HIV-1 Env is related to SIV Env, HIV-1 vaccine development, especially antibody-based vaccines, should primarily focus on antibodies generated toward HIV-1 Env epitopes. However, HIV-1 does not typically infect common non-human primates, such as rhesus macaques, used in vaccine trials. To overcome this, chimeric viruses encoding HIV-1 Env and with a SIV backbone (known as SHIV) can be used to effectively challenge and infect non-human primates with an HIV-1 Env containing virus (16). The use of SHIVs in animal challenge models is now crucial component to testing an HIV-1 vaccine's efficacy

Broadly Neutralizing Antibodies to HIV-1

The generation of broadly neutralizing antibodies (bNAbs) capable and binding to and inhibiting a wide array of HIV-1 strains is a long-sought goal for HIV-1 vaccine development. While these antibodies do naturally develop in up to 50% of infected people (17), these responses take multiple months and years to develop and are unable to clear the established and diverse chronic infection (18). Furthermore, these bNAbs are often highly somatically hypermutated and often contain longer HCDR3 regions, allowing the antibodies to extend into hard to reach areas on HIV-1 Env. The difficulty

of developing broadly neutralizing antibodies is due to multiple factors, mainly concerning the nature of HIV-1 Env. The low number of HIV-1 Env spikes on virions (8-14 spikes) hinders B-cell activation as the B-cells often requires multiple cross-linking of their receptors to activate (19, 20). The extensive glycosylation of HIV-1 Env, often referred to as the "glycan shield" obfuscate the protein backbone from antibody binding. Essential regions for HIV-1 Env infectivity, such as the CD4-binding site are in poorly accessible areas on HIV-1, surrounded by glycans or non-essential structures that can easily be mutated. Regions that are exposed, such as the variable loops, may be hypervariable (with some notable exceptions) and can easily be mutated under immune pressure.

The CD4-binding site (CD4bs) is an obvious target for neutralizing antibodies, as blocking CD4 binding by HIV-1 Env would prevent viral entry (**Fig. 3a, b**). However, the CD4bs is found in a hard to reach area on gp120, a recessed pit on the side of each gp120 subunit. Furthermore, the site is surrounded by numerous glycans that further conceal the binding site (21), such as glycans at position 276 and N197 (14). These features cause the CD4bs, while conserved and essential for viral infectivity, to be obscured from conventional antibody binding.

An additional feature of HIV-1 Env is its ability to naturally exist in different conformational states, a form of "breathing". In this "breathing" the HIV-1 Env spike moves between differing degrees of openness. In the closed conformation, HIV-1 Env is in its most shielded state, with neutralizable epitopes guarded by the glycan shield and the CD4bs in its native, recessed state. The "breathing" occurs between this closed state and a more relaxed state in which the apex of the trimer becomes somewhat open,

exposing some antibody epitopes that are not accessible on the closed conformation. The propensity of HIV-1 Env to transition between these two states is highly variable and depends on the subtype and strain, as well as determining the neutralization sensitivity a given HIV-1 strain (22). Upon receptor binding, the Env spike shifts to an open conformation, exposing the V3 loop which contains the co-receptor binding site (CCR5 or CXCR4), and co-receptor binding triggers the insertion of the fusion peptide from gp41 into the host membrane. This open state exposes the inner domains of the gp120 subunits as well as other neutralizable epitopes. It is hypothesized that many bNAbs, especially ones that target sites other than the CD4bs, act by stabilizing the closed conformation of HIV-1 Env, blocking the opening of the HIV-1 Env spike required for viral fusion (22).

The isolation and characterization of bNAbs is divided into two generations. The first generation occurred in the 1990s using phage display libraries and human hybridomas (12), and monomeric gp120 was used for antibody screening. First generation bNAbs isolated targeted the CD4bs (b12 (23)), a glycan on the outer domain of gp120 (2G12 (24)), and the membrane-proximal external region (MPER) on gp41 (4E10 and 2F5 (25)). These bNAbs, while not as potent and broadly-reactive as second generation bNAbs, demonstrated the high levels of somatic hypermutation and long heavy chain complementary determining region (HCDR3) that is characteristic of most HIV-1 directed bNAbs. Studies in non-human primates showed that passive immunization with first generation bNAbs can confer protection from infection, demonstrating that it is possible to achieve sterilizing immunity from HIV-1 infection (26, 27).

The second generation of bNAbs, beginning in the late 2000's, is marked by a much greater breath and potency, their discovery aided by improvements to the methodology of screening sera from infected individuals for neutralization activity, and technological advancements in the cloning of human monoclonal antibodies (28). Early second-generation bNAbs include PG9 and PG16, two bNAbs that target the apex of the Env trimer, within the V1V2 loop (29). Additionally, these bNAbs were found to preferentially bind to trimeric HIV-1 Env over monomeric gp120, demonstrating the unique conformational epitopes found on the native trimeric HIV-1 Env. Other isolated second-generation bNAbs include potent CD4bs-directed bNAbs such as VRC01 (30, 31), bNAbs against V3 high-mannose patch glycans such as PGT121 (32), V2-apex binding bNAbs such as PGT145 and PGDM1400 (33, 34), and gp120-gp41 interface binding bNAbs such as PGT151 (35). Many of these bNAbs, such as PGT145 bind to or accommodate glycans via their light chain and use a long HDCR3 region on the heavy chain to extend through the glycan shield and interact with the protein backbone. Second-generation bNAbs have been shown to also elicit sterilizing immunity when passive immunized to non-human primates against SHIV challenge (36), and can induce transient viral control when given to chronically infected non-human primates (37). These bNAbs are also being considered for use in humans as prophylactic therapies as well aiding in viral control in infected individuals (38). A major goal of HIV-1 vaccines, like most viral vaccines, is to induce these broadly neutralizing antibodies via vaccination. However, no vaccine candidate to date has induced these responses in humans. Our understanding of the intricacies and complexities of both bNAbs and the HIV-1 Env they target have grown substantially over the last ten years, and the

development of novel immunogens to induce bNAbs continues to be a major area of focus for HIV-1 vaccine development.

Pathogenesis of HIV-1

HIV-1 is primarily transmitted across the mucosal epithelium through sexual contact. Heterosexual transmission is the most common route of HIV-1 transmission globally, but this incidence varies by region. In the United States, for example, the most HIV-1 transmissions occur in men who have sex with men (MSM) (39). The relative risk of HIV-1 infection is highly dependent on the exposure sites. Receptive anal intercourse has an infection probability of 0.04%-3.0% risk of infection, while exposure in the male genital tract has a probability of 0.03% risk of infection in MSM (40). Exposure to the female genital tract has a probability of 0.5% - 0.30% risk of infection (41). Other factors heavily influence the risk of infection, such as high vs. low risk behaviors, the infection state of the transmitting partner, the immune activation state in the mucosal exposure site, and the viral characteristics of the HIV-1 clade being transmitted.

During the first week of infection HIV-1 replication is localized to the site of transmission before rapid dissemination in the subsequent weeks (42). This dissemination is marked by a massive depletion of CD4+ T-cells in the gut lamina propria, detection of HIV-1 in the plasma, and the generation of HIV-1 specific T-cell and antibody responses. After 6-12 weeks post-acute infection, viral loads within the plasma stabilized to a chronic level known as the set-point viral load. During chronic infection, in the absence of antiviral therapy, an infected individuals CD4+ T-cell levels

will steadily decline to the point of AIDS, once CD4+ T-cell counts are less than 200 cells per ul blood. The transition to AIDS marks a surge in plasma viremia, and the inability of the immune system to combat opportunistic infections, often leading in death.

A hallmark of chronic HIV-1 infection is the presence of chronic immune activation. The depletion of gut resident CD4+ T-cells, due to a majority of these cells expressing high levels of CCR5, results in a breakdown of the healthy gut barrier allowing microbial translocation from the microbiota into the lamina propria. This influx of bacteria results in a systemic increase in activation, which in term promotes the generation of additional target cells for HIV-1.

B. *Protective Immunity from HIV-1 infection. Lessons from Vaccine Trials*

History of HIV-1 Vaccine Development

When HIV-1 was first isolated and understood to be the cause of AIDS in the early 1980's, it was widely believed than an effective vaccine would be easily developed. The United States Secretary of Health and Human Services, Margaret Heckler stated in 1984 that "a vaccine (will be) ready for testing in approximately two years" (43). Researchers were optimistic about the prospects of an HIV-1 vaccine due to the success of other viral vaccines against pathogens such as smallpox and polio, and at the time there was seen success in generating vaccines against another retrovirus, feline-leukemia virus, which causes AIDS-like disease in cats (44). However, despite clinical trials in humans,

including five phase-IIb vaccine efficacy trials (45) , an effective prophylactic vaccine has yet to be developed (**Fig. 4**).

At the outbreak of the HIV-1 pandemic most viral vaccines consisted of either inactivated or live-attenuated viruses, and today still many vaccines such as the influenza vaccine and the MMR (measures, mumps, and rubella) vaccine are based on these concepts. This strategy was not pursued in HIV-1 vaccine development, however, due to safety concerns with attenuated viruses and their ability to potentially integrate into target cells (46). With the development of the yeast derived, protein-based hepatitis B vaccine in 1984 (47, 48), it became possible for researchers to express recombinant HIV-1 proteins and test them in humans as potential vaccines. The rapid advancement of the understanding of the structure and pathogenicity of HIV-1, as well as the development of animal models for HIV-1 infection has spurred about large number of vaccine candidates. Additionally, the consensus among the scientific community regarding what an effective prophylactic HIV-1 vaccine must provide has evolved over time between three different paradigms: (1) the induction of neutralizing antibodies, (2) the induction of cell-mediated immunity, (3) a combination of humoral and cellular responses (43), all leading to a variety of vaccine strategies.

Paradigm 1: Induction of neutralizing antibodies, the VaxGen trials

As the mechanism of protection in most viral vaccines is the generation of neutralizing antibodies that effectively bind to and inhibit viral entry into target cells, researchers initially thought this would be a simple and easily achievable goal using HIV-

1 Env subunit-based vaccines. The most obvious immunogen to use for an HIV-1 vaccine would be either gp120 or gp160, the full-length HIV-1 Env containing both gp120 and gp41. During the late 1980s and early 1990s, much effort was made in developing and testing gp120 and gp160 immunogens in animal models and humans. From this effort two potential gp120 vaccine candidates emerged to be tested in an efficacy trial in humans in 1994, with notable skepticism among some in the scientific community (49). Previous studies in chimpanzees have shown that immunization of chimpanzees with gp120 can induce protection when challenged with homologous strains of HIV-1 (50), and passive transfer of V3-loop specific monoclonal antibodies can also induce protection in chimpanzees (51). Initial phase-I trials in humans showed that gp120 subunit vaccines were safe, immunogenic, and induced neutralizing antibodies in the majority of immunized individuals (52). The controversy surrounding these results stemmed from the experimental models of infection and the use of laboratory-adapted strains of HIV-1, cultured and grown in human cell lines, in both the chimpanzee protection studies and in testing neutralizing antibody responses in humans. At the time, it was not fully understood the antigenic complexity of HIV-1 Env and primary isolates of HIV-1, which are grown only in fresh human peripheral blood mononuclear cells (PBMCs), but it was becoming clear that laboratory-adapted strains of HIV-1 have several biological differences from primary isolates. Researchers found that laboratory-adapted strains are highly sensitive to neutralizing antibodies *in vitro* (53) and while sera from gp120 immunized recipients could neutralize laboratory-adapted strains, the sera did not neutralize primary isolates of HIV-1 (49). The scientific community was met with a dilemma; did the urgency of HIV-1 pandemic [in 1993 the United States Congress

voted to codify the U.S. HIV immigration exclusion policy, barring entry of HIV-1 infected individuals into the United States (54)] justify the risks of a failed vaccine? After all, some researchers argued, vaccines for pathogens such as smallpox were developed, tested, and shown to be protective without fully understanding the mechanism of protection (49). Ultimately, in 1994 the governmental advisory groups within the National Institute of Allergy and Infectious Disease (NIAID) decided to not support the proposed efficacy studies in humans. However, the same year the World Health Organization (WHO) did make a recommendation to further pursue the trials (55), and four years later two efficacy studies were started by VaxGen, in North America (VAX004) and Thailand (VAX003) (56).

To address the growing knowledge about the genetic diversity of HIV-1 strains, VAX003 and VAX004 were designed to each include two gp120 immunogens: VAX004 (North America and Europe) contained AIDSVAX B/B and VAX003 (Thailand) contained AIDSVAX B/E. The AIDSVAX B/B consisted of gp120 proteins derived from the CCR5-tropic clade-B HIV-1_{GNE8} and the CXCR4-tripic clade-B HIV-1_{MN}. To account for the prevalence of the different clades of HIV-1 in Thailand, the AIDSVAX B/E consisted of clade-B HIV-1MN and the CCR5-tropic clade-E HIV-$_{CRF01\_AE}$ gp120. The vaccinated population differed between VAX003 and VAX004 as well, VAX033 was given to primarily to high risk men (men who have sex with men) and women, the VAX004 was directed towards intravenous drug users. The two trials took place between 1998 and 2003 and consisted of seven injections given over 30 months.

In 2003 the final results of the trials were announced, neither trial conferred any protection from infection despite both vaccines being immunogenic. In the VAX003

trial, HIV-1 incidence was 6.7% in vaccinated individuals and 7% in the placebo group (57). In VAX004, incidence was 8.4% in vaccinated individuals and 8.3% in the placebo group (58). Further analysis suggested that there was some protective benefit in the VAX003 trial when analyzing results based on race and gender, with women and African Americans showing some protection (59, 60), however these results were largely met with skepticism and confusion by the scientific community. The results confirmed to some the inherent flaws in previous assays to characterize the antibody responses to HIV-1, as sera from VAX003 and VAX004 vaccinated individuals failed to neutralize primary isolates of HIV-1(61). The failure of these trials demonstrated the complexity and difficulty of generating an effective antibody-based HIV-1 vaccine, leading researchers to pursue other avenues of vaccination, in particular, cell-mediated immunity based vaccines.

Paradigm 2: Cell-mediated Immunity and the STEP trial

The induction of cell-mediated immunity, primarily through strong cytotoxic lymphocyte (CTL) responses in infected individuals has been well established and was considered a possible strategy for vaccination (62, 63). This immunity stems from cytotoxic $CD8^+$ and $CD4^+$ T-cells recognizing and killing HIV-1 infected cells. While the CTL responses in infected people did not cause complete clearance of the virus, it was proposed that the CTL's did play a role in controlling viral replication. The possibility of pre-existing CTL-mediated immunity, generated via vaccination, protecting from HIV-1 infection was unknown at the time.

The development of CTL-based vaccines was primarily based around other viral vectors and DNA-vaccines. DNA-based vaccines consist of a DNA plasmid that is

injected into the skin or muscle, transfecting the local cell populations (such as fibroblasts) and expressing the engineered viral genes (64, 65), and initial studies in rhesus macaques with DNA-SIV alone immunization, while not inducing sterilizing immunity against SIV challenge, did induce some reductions in pathology and viral loads (66). To increase the immunogenicity in non-human primates, DNA-based vaccines have been used in conjunction with other viral vectors (such as Modified Vaccinia Ankara (MVA)) and recombinant proteins to boost the immune responses, resulting in much better control of viral replication (67, 68). DNA/MVA vaccines are still being developed with phase-I clinical trials showing immunogenicity in humans, and phase-II trials are currently being planned (69).

In addition to DNA-based approaches, research was also heavily focused on developing other viral vector-based vaccines such as poxvirus and adenoviruses. Most poxvirus vectors are based on various attenuated strains of vaccinia, engineered to express HIV or SIV antigens. Several vaccine candidates based on poxviruses have been developed and tested in humans, namely the NYVAC, ALVAC, and MVA strains (70-72). Adenoviruses have been studied heavily has vectors for inducing HIV-1 cellular immunity (73), and in 2001 Merck & Co, Inc. announced they were pursuing a replication-defective adenovirus 5 (Ad5) vector as a vaccine candidate for HIV-1 (74). Rhesus macaques immunized with Ad5 expressing SIV *gag* showed high viral control, but not sterilizing immunity, and low to no depletion of $CD4^+$ T-cells (75). To improve this vector for testing in humans, Merck developed a mixture of Ad5 vectors expressing HIV-1 clade-B *gag* (HIV_{CAM-1}), *pol* (HIV_{IIIB}) *and nef* (HIV_{JR-FL}) genes (MRKAd5), which was tested and found to be safe and immunogenic in a phase-I clinical trial (76).

In 2004 enrolling began for the STEP trial to test the protective efficacy of MRKAd5 in North America, Brazil, and Australia, mainly in high risk women and MSM populations. The immunization was given in three doses on weeks 0, 4, and 26, and initial immunogenicity studies showed that the majority (72%) of vaccinated individuals generated interferon (IFN)-g responses to peptides from at least two of the genes in the vaccines (76). However, the STEP trial was stopped in September 2007 when an interim analysis revealed that no protective efficacy was induced by vaccination [24 infections (3%) in vaccinated and 21 infections (3%) in the placebo-treated individuals] (77, 78). Further analysis revealed that in some cases, such as men who had pre-existing Ad5-directed neutralizing antibodies and/or were uncircumcised, vaccination even increased the risk of infection (79). These results caused much disappointment in the scientific community, which expected the trials to at least confer some degree of viral control in infected vaccinated individuals (77). The mechanism of increased susceptibility is unknown, some suggesting that MRKAd5 led to an increased number of $CCR5^{+}CD4^{+}$ T-cells in the mucosa (HIV-1 target cells) of vaccinated individuals, though since mucosal samples were not collected in the trial, this cannot be confirmed. The failure of the STREP trial led many to believe Adenovirus based vaccines, or CTL-based vaccine strategies in general should no longer be pursued, and instead the scientific community should refocus efforts on basic understandings of HIV-1 pathogenesis to better inform future vaccine discovery.

In addition to the STEP trial, the recent HVTN 505 vaccine efficacy trial consisting of a DNA/Ad5 vaccine was halted early in 2013 due to lack of vaccine efficacy in immunized individuals (80). The failure of the HVTN 505 trial furthered the

notion that an alternative strategy for HIV-1 vaccination may need to be pursued, with an increased focus on antibody-based vaccine strategies.

Paradigm 3: The RV144 Trial

The RV144 trial began in September 2003, around a year before the STEP trial, and concluded in October 2009. This trial tested a prime-boost approach in which individuals, primarily high-risk men and women, were immunized first with a canarypox vector ALVAC (vCP1521) expressing HIV-1 clade-B *gag* (HIV_{LAI}) and gp120 (HIV_{CRF01_AE}) linked to the transmembrane anchoring region of gp41 (HIV_{LAI}) on weeks 0, 4, 12, and 24. Individuals were then boosted with AIDSVAX B/E (the same bivalent gp120 mixture from VAX003) on weeks 12 and 24 (45, 81). Initial immunogenicity studies in phase I/II trials showed modest antibody and T-cell responses. Most vaccinated individuals developed neutralizing antibodies, like those in the VAX003 trial, against lab-adaptive strains of HIV-1, but the negative results of the VAX003 trial suggested these responses wouldn't aid in protection (82). The canarypox component of the vaccination was intended to induce CD8 T-cell responses, however initial data suggested that immunization resulted in only 24% of vaccinated individuals developing detectable responses (82).

In 2009 the results of the RV144 trial were published, demonstrating a modest yet significant vaccine efficacy of 31.2%, with almost 60% efficacy within the first year of the trial (83). This result was met with a much skepticism from the scientific community. At face value, the immunogenicity data suggested the RV144 trial would not be any more efficacious than the VAX003 or the STEP trial (84). In 2012, immune-correlates of

protection for the RV144 trial were published, indicating that non-neutralizing antibodies directed towards the V1V2 loop of gp120 are associated with decreased risk of infection, while high levels of gp120-specific serum IgA were associated with increased risk of infection (85). The lack of neutralizing antibodies, which are very difficult to generate against HIV-1 by vaccination, as the correlate of protection suggests that some other antibody effector function such as antibody-dependent cell-mediated cytotoxicity is responsible for protection (86, 87). The modestly positive results of the RV144 trial and the negative results of the STEP trial have reinvigorated the paradigm that antibodies are a crucial component to an effective vaccine. The RV144 trial, being the first trial to show vaccine efficacy, is considered a landmark in HIV-1 vaccine development. While the results are still questioned by some in the scientific field (88), many believe that studying and replicating the results from the RV144 trial will greatly aid in the development of an effective vaccine .

The Role of V1V2-loop directed antibodies in protection from infection.

The results of the RV144 trial suggested that antibodies directed towards the variable loops 1 and 2 (V1V2) on gp120 may be a correlate of protection (85). The V1V2-loop region of gp120 spans an approximately 70 amino acid region in gp120, located on towards the apex of the HIV-1 Env trimer (**Fig. 5a**) (15). This region contains four major beta-strands (A, B, C, D) and two hypervariable loops V1 and V2, as well as several conserved glycans (**Fig. 5b**) (89). The V1 hypervariable loop is formed between the A and B beta strands, and a disulfide bond between the A and B strands stabilizes this loop (**Fig. 5c**). A hairpin loop between strands B and C reaches into the trimer apex and

is recognized by numerous broadly neutralizing antibodies such as PGT145 and PGDM1400. This hairpin is relatively more conserved than the V1 and V2 hypervariable loops (**Fig. 5d**). The second V2 hypervariable loop connects the C and D stands together, a second disulfide bond between strand D and A stabilize the entire region. Within the C strand exists a putative $\alpha_4\beta_7$ binding site, the significance of which is currently being investigated by multiple groups (90).

The targeting of the V1V2 locus in vaccinated individuals was suggested by data showing sera binding to gp120 could be blocked by V1/V2 and V2 specific monoclonal antibodies (91). The major correlate of protection, however, was found by measuring sera reactivity against a gp70-V1V2 scaffold protein, which is composed of the V1V2 region of the Case.A2 (HIV clade-B) attached to the murine leukemia virus gp70 protein, which presents the V1V2 region in a more conformational manner (92). Binding to Case.A2 gp70-V1V23 showed a significant inverse correlation ($p = 0.02$, Multivariate Logistic Regression) with the rate of HIV-1 infection, and stratifying the vaccinated individuals based on their reactivity to gp70-V1V2 showed that the highest responders had the lowest incidence of infection (85).

Further analysis revealed sera binding to a core linear sequence within the V2 loop is also associated with decreased risk of infection in the RV144 trial. This region, called by some as the "V2 Hotspot" spans the hairpin loop between beta strands B and C, as well as beta strand C itself within the V1V2 loop region (residues 166-178 of strain HIV strain HxB2) (**Fig. 6a, b, c**) (93). While several residues within this region are potentially covered by the glycan shield, much of the V2 hotspot protein backbone is exposed at the apex. Interestingly, this sequence immediately precedes the putative $\alpha_4\beta_7$

binding site on gp120 (9, 90). Analysis of the consensus sequences across HIV-1 clades reveal a pattern of conservation of the V2 hotspot with several residues, such as N156 and N160 glycosylation sides and a heavy lysine rich motif between residues 166-172, both having implications in vaccine design (**Fig. 6d**) (94).

Residues within the V2-hotspot region are contact sites for multiple V1V2-apex directed broadly neutralizing antibodies such as CH03 (89) and PG9 (95). Additionally, analysis of the HIV-1 strains found in infected individuals in the RV144 trial showed that strains that matched the vaccine sequence with a lysine at position 169 within the V2 loop region were not found in vaccinated infected individuals, suggesting immune pressure and selection of strains that mismatch the vaccine strain (96). Monoclonal antibodies isolated from vaccinated individuals against the V1V2 loop, CH58 and CH59, overlapped with binding by the bNAbs CH01 and PG9, and while they only had weak neutralization activity, they did mediate ADCC activity, suggesting that non-neutralizing antibody functions may have an important role in protection (87). Additional studies in non-human primate models also demonstrated a correlation between anti-V1V2 antibodies and protection from neutralization resistant SIV (97-99), demonstrating the relevance of studying these antibody responses for vaccine development.

As both the RV144 trial and the VAX033 trial used AIDSVAX B/E as a protein immunization, the discrepancy of vaccine efficacy between the two trials, VAX003 showing no efficacy, raises questions about the differences between the two trials. The RV144 trial consisted of priming with the poxvirus ALVAC before boosting with the gp120 protein AIDSVAX B/E, while the VAX003 trial was repeated immunizations with AIDSVAX B/E alone. One major difference between the trials is that the VAX003 was

mainly given to intravenous drug users, while the RV144 trial was mainly given to high risk men and women, and studies have shown that intravenous transmission differs highly from mucosal transmission (100). Upon closer analysis of the subtypes of IgG responses showed that the RV144 induced statistically higher levels of HIV Env and V2-specific IgG3 than VAX003, and these high IgG3 levels were determined to be a correlate of protection (101). The VAX003 trial, however, induced a larger level of IgG4 subclass antibodies. IgG3 has a high affinity for the Fcγ receptor, suggesting enhanced functionality in Fc-mediated effector functions. The use of the poxvirus ALVAC as a priming agent, which would promote high antiviral activity, may aid in the skewing of IgG subclasses to IgG3 (102), a finding which supports the use of poxvirus vectors in future vaccine trials.

The role of V1V2 and V2-loop directed antibodies in decreasing the risk of infection is still not completely understood. The lack of neutralizing activity in vaccinated individuals suggest that non-neutralizing antibodies are responsible for protection (3). Non-neutralizing antibodies against HIV-1 have been shown to have several antiviral functions, including capturing viral particles and promoting opsonization and sequestration, Fc-mediated effector functions such as antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent cellular cytotoxicity (ADCC) (**Fig. 3c**) (103). Some data suggests that the V2 loop region is important in viral transmission and fitness, as HIV-1 viruses isolated from chronically infected people have highly mutated V1V2 sequences, suggesting viral escape and immune pressure (104). Additionally, the proximity of the $\alpha_4\beta_7$ binding site on gp120 may have relevance, as the initial interactions between HIV-1 virions and host target cells may be aided by gp120 binding

to $\alpha_4\beta_7$, activating other integrins, and stabilizing the viral synapse (**Fig. 3d**) (9, 105). Recent studies in treating SIV-infected rhesus macaques under ART with a $\alpha_4\beta_7$ targeting monoclonal antibody resulted in low or undetectable viral loads after ART treatment was discontinued, indicating some crucial role of $\alpha_4\beta_7$ in viral replication (106).

Current HIV-1 Vaccine Strategies: Development of Novel Immunogens and Prime-Boost Regimens

The failures of the VAX003, VAX004, and the STEP trial, as well as the modest efficacy of the RV144 trial, spurred about a call to renew focus on the basic science of HIV-1, answering fundamental questions surrounding both the virus and its pathogenicity (107). One major area of focus has been in the development of improved HIV-1 Env immunogens that better resemble the native HIV-1 Env trimer or promote potential protective antibody responses. With an increased understanding of the structure of native HIV-1 Env, and the epitopes on Env targeted by broadly neutralizing antibodies, targets on HIV-1 Env for neutralization or other antibody-mediated effector functions have been discovered (8). Many novel immunogens are designed with the intention of presenting neutralizable epitopes while blocking non-neutralizing epitopes. Other immunogens are designed to engage the germline precursors of certain bNAb lineages, such as VRC01. Scaffold-based immunogens aim to prime the antibody response against a specific epitope, which can then be boosted by a full length Env. Many of these novel immunogens will be entering phase-I trials in humans to access their immunogenicity.

Monomeric gp120

Monomeric gp120 has historically been the most evaluated protein immunogen in HIV-1 efficacy trials. Studies characterizing the antigen properties of gp120 have shed light on possible improvements that could be made to the immunogen. Monomeric gp120 readily binds numerous bNAbs, such as VRC01 and b12 (CD4bs), and PGT121 (V3-gylcan), but it does not bind to V1V2-apex binding bNAbs such as PGT145 and PG16, indicating that monomeric gp120 lacks the expression of quaternary epitopes found on native HIV-1 Env (108). Immunization with monomeric gp120 primarily results in antibodies capable of neutralizing Tier-1 (neutralization sensitive) isolates but not Tier-2 or -3 (neutralization resistant) isolates (109). The lack of more effective neutralizing antibody responses against monomeric gp120 may be due to numerous non-neutralizing, yet immunogenic, epitopes found on monomeric gp120, especially within the inner domain of gp120 that is inaccessible on native trimeric HIV-1 Env. The CD4bs on monomeric gp120, while accessible, can be approached by antibodies at angles that are incompatible with native HIV-1 Env, leading to the generation of non-neutralizing CD4bs-directed antibodies (110).

Efforts to improve monomeric gp120 immunogens involve altering the protein to not express certain regions, such as the variable loops, or by attaching *N*-linked glycans to certain sites to hide non-neutralizing antibodies. This strategy has been shown to be effective for reducing non-neutralizing V3-loop directed antibodies via glycosylation of the V3-loop (111). Other modifications of gp120 involve separating the outer domain (OD) from the inner domain. The OD makes up the exposed region of gp120 on trimeric Env, and contains the variable loops, the glycan shield, and the CD4bs (112). OD-based

immunogens have been tested in rabbits in which they generated binding titers against gp120 and neutralizing antibodies against tier-1 and tier-2 viral isolates (113). Additional modifications of OD-based immunogens included engineered OD (eOD) immunogens that bind to the inferred germline precursors of CD4bs class antibodies (114). These immunogens can be used as a priming agent to engage the germ-line precursors, which can then be expanded and matured with other immunogens to induce bNAbs (115).

Native-like Env trimers

Soluble Env immunogens that resemble the Native HIV-1 Env in structure and antigenicity are considered strong candidates for the induction of protective antibody responses. To generate soluble Env trimers, the cytoplasmic and transmembrane tail of gp41 can be removed from the gp160, leaving the complete gp120 and ectodomain of gp41 ($gp41_{ECTO}$). However, cleaved soluble gp140 proteins are very unstable and will readily dissociate into the gp120 and gp41 subunits (116). Early efforts to stabilize gp140 trimers involved removing the native furin cleavage site and adding heterologous trimerization motifs to the C-terminus to generate uncleaved trimeric gp140 ($gp140_{UNC}$) (117). While $gp140_{UNC}$ does form trimers, structurally they do not adequately resemble the native HIV-1 Env spike, as the gp120 subunits do not form the propeller-blade structure and instead suspend from gp41 like "drooping flowers in a vase (118). Furthermore $gp140_{UNC}$ trimers were found to have exposed non-neutralizing epitopes, and the CD4bs on $gp140_{UNC}$ trimers does not accurate recapitulate the CD4bs found on native trimeric HIV-1 Env (110).

The lack of proper folding in $gp140_{UNC}$ suggests that cleavage of gp120 from gp41 is required to have correct folding. One approach to generating cleaved but stabilized trimeric gp140 is to introduce a disulfide bond (SOS) that covalently links gp120 and gp41 together in a proper configuration (116). To improve the folding of this SOS-gp140, stabilizing the pre-fusion state of HIV-1 Env, a mutation was added in a gp41 (I559P) helix region, forming SOSIP-gp140 (119). Further improvements included truncating the gp140 at position 664 to remove a domain of the MPER that caused aggregates to form, generating SOSIP.664-gp140 (20), and these SOSIP.664 based gp140 proteins were found to closely resemble the native HIV-1 Env (120). SOSIP.664 gp140 proteins can be expressed from cell lines but need to be affinity-purified with bNAbs to select properly folded trimers and remove aggregates (121)

Studies with a HIV-1 clade A strain BG505 SOSIP.664 gp140 trimer, using cryo-EM and crystallography, generated the first high resolution images of the HIV-1 Env trimer, a landmark for structure-based HIV-1 vaccine development (14, 15). Furthermore, immunization of rhesus macaques with BG505 SOSIP.664 gp140 induces tier-2 neutralizing antibodies (against the autologous BG505 isolate), a first in non-human primate vaccine studies (122), however no heterologous tier-2 neutralizing activity was generated. Further analysis has attributed autologous tier-2 neutralizing activity to a glycan hole on BG505, located at residue 241, that is present in fewer than 3% of global isolates (123), and restoring this glycan be site-directed mutagenesis ablated the neutralizing activity. The importance of glycan holes for generating neutralizing antibodies via SOSIP.664 gp140 immunization has now been well documented, and

novel immunogens are being designed to direct antibody responses toward specific regions with conserved glycan holes to improve neutralization breadth (21, 94, 124).

One issue with gp140 based HIV-1 Env immunogens is the immunodominance of gp41 over gp120 in antibody responses. During acute infection, humans first make antibodies to gp41, eight days after detectable viremia, but gp120 specific antibodies are delayed by an additional two weeks (125). These gp41 antibodies were shown to not impact the control of viremia, suggesting they have little functionality. Furthermore, early gp41-directed antibodies have been shown to be polyreactive, originating from memory B-cells primed against non-HIV antigens such as host or bacterial antigens, and polyreactive gp41-binding antibodies have been isolated from uninfected individuals (126). The immunodominance of gp41 can also be seen in the vaccine setting. A series of human trials evaluated a DNA prime adenovirus serotype 5 (Ad5) boost regimen in which Ad5, in addition to *gag, pol,* and *nef* genes, expressed a trivalent mixture of clade A, B, and C *env* gp140. Analysis of the Env antibody response revealed that 93% of HIV-1 specific antibodies derived from memory B-cells were specific for gp41, and these antibodies were often polyreactive and reacted to host and intestinal microbiota antigens (127). This data suggests that gp140 immunogens may not be ideal for human immunization, and other immunogens that either remove gp41 or mutate regions associated with polyreactive responses should be investigated.

Trimeric gp120 immunogens via cyclic permutation

An alternative approach to generating trimeric HIV-1 Env immunogens without the incorporation of gp41 is via heterologous trimerization domains. These domains can

be used to covalently link together three gp120 subunits into a trimeric configuration. An initial study designed and characterized gp120s with two different trimerization domains, SUM02a, and human cartilage matrix protein (hCMP) (128). The N- and C-termini of gp120 are positioned in such a way that allows for cyclic permutation in which the native N- and C-termini are joined by an amino acid linker chain, and a de novo N- and C-termini is created at a different position on the protein. The insertion of the hCMP trimer domain into the hypervariable V1 loop of a cyclically permutated gp120 resulted in a gp120 that exists primarily as a trimer, binds CD4, the CD4bs-directed bNAb b12, and V2-apex binding bNAbs PG9 and PG16 (128) (**Fig 7**). An additional studied characterized an HIV-1 clade B strain JRFL gp120 cyclically permutated with the hCMP trimer domain inserted into the V1 loop (JRFL-hCMP-V1cyc) (**Fig. 8**). This protein, here-after named cycP-gp120, exists primarily as a trimer, confirmed by both BN-PAGE western blotting and negative stain electron microscopy, and binds to CD4bs-directed (VRC01, VRC03) and V2-apex trimer specific bNAbs (PGT145, PGDM1400). Additionally, cycP-gp120 contains the E168K mutation to allow for binding by PG9 and PG16 (29). Immunogenicity studies in guinea pigs demonstrated that cycP-gp120 generates anti-gp120 antibodies, and tier-2 neutralizing antibodies against multiple isolates (129). The characterization of cycP-gp120 in rabbits and rhesus macaques, as both a priming and boosting immunogen, is further investigated in this dissertation.

Modified Vaccinia Ankara (MVA) as a priming and boosting vector

The modest protective efficacy of the RV144 trial and the failures of the VAX003 and VAX004 trials suggest that the utilization of a poxvirus prime may be important for

generating protective immune responses. One poxvirus vector, modified vaccinia Ankara (MVA) has been extensively studied as a potential HIV-1 vaccine vector, and is currently being tested in human clinical trials (68, 69, 98). MVA62Bsm is an MVA vector which expresses HIV-1 *gag* (HIV-1_{HXB-2}) *pol* (HIV-1_{BH10}) and clade-B strain ADA gp150, a truncated cleaved gp160 lacking the gp41 cytoplasmic domain (130). Upon immunization, MVA-62BSm (here after referred as MVA-HIV), like other MVA vectors, will infect dendritic cells, monocytes, and B-cells (131). Infected cells will then present HIV-1 on the cell surface, and as secreted virus like particles (VLP). To characterize the ADA gp150 presented by MVA-HIV, we infected DF-1 cells with MVA-HIV and measured the binding of different Env-specific bNAbs and non-neutralizing antibodies by flow. Cell-surface gp150 on infected cells bound preferentially to bNAbs over non-neutralizing antibodies, including quaternary specific bNAbs such as PGT145 and PGT151, indicating that HIV-1 Env displayed by MVA-HIV is trimeric and properly folded (**Fig. 9**). MVA62B is currently being tested in humans as a boosting vector after priming with a DNA vaccine (JS7) which expresses HIV-1 *gag, pol,* and strain ADA gp160. MVA62B is a robust boosting vector, eliciting both durable T-cell and antibody responses, demonstrating it was a safe, effective vector in humans (132). This dissertation will examine MVA-HIV as a priming immunization before boosting with a protein. The use of MVA-HIV may promote beneficial antibody responses, as comparisons between the RV144 trial and the VAX003/VAX004 showed that the highest IgG3 (the subtype of antibody that correlated with decreased risk of infection) responses were directed toward the HIV-1 92TH023 strain Env, which is expressed by the ALVAC

poxvirus, but similar trends weren't seen with the MN and A224 HIV-1 Env, which were derived from the protein boosts (101).

Mucosal Vaccine Development for HIV-1

HIV-1 is primarily transmitted across mucosal surfaces via the genital or gastrointestinal route. Immediately post transmission, in the initial hours and days of infection, HIV-1 is considered to be in a vulnerable state as it replicates within local target cells and establishes a viral reservoir (42). Viral dissemination is marked by CD4+ T-cell depletion in the gastrointestinal tract resulting in loss of important IL-17 and IL-22 producing CD4+ T-cells and a subsequent breakdown of the gut barrier (133). The resulting immune activation leads to disease progression. An effective vaccine capable of preventing the transmission and establishment of infection therefore would most likely require mucosal immunity (134). However, most licensed vaccines are administered systemically by the intramuscular or subcutaneous route, which would result in primarily a systemic immune response. Mucosal vaccination offers multiple advantages over systemic delivery, such as non-invasive administration, the generation of mucosal and systemic immune responses, and practicality for mass vaccination (135). Disadvantages of mucosal vaccine development stem from the difficulty in effectively characterizing immune responses at mucosal sites as well as developing effective vaccine routes that will reliably generate the desired mucosal response. Nevertheless, an effective mucosal vaccine for HIV-1 may provide an easy and practical method of generating protective immune responses at the site of transmission.

Mucosal immunity to HIV-1 infection

Both cellular and humoral immunity at mucosal sites are thought to be important for preventing and/or controlling HIV-1 infection. Numerous studies have implicated mucosal antibodies in protection from SIV or SHIV infection in rhesus macaques (136-139). In addition to local mucosal antibody response, transudated IgG may also play a role in preventing infection. Passive immunization with IgG1 subclass monoclonal bNAbs in rhesus macaques, delivered intravenously, protects from vaginal challenge with a pathogenic SHIV challenge (140). The role of SIV-specific T-cell responses in the mucosal tissue has also been documented, as vaccine induced T-cell responses in colorectal and vaginal tissue have both mediated resistance to SIV infection and viral control (141-143). Additionally, recent studies have emphasized the importance of the inflammatory state within the vaginal tissue in risk of HIV-1 infection. One study monitoring HIV-1 acquisition in South African women found that women mono-colonized with *Lactobacillus crispatus* were at a significantly decreased risk of HIV-1 infection, while women with diverse vaginal microbiome populations were at increased risk (144). This discrepancy was attributed to decreased numbers of HIV-1 target cells and pro-inflammatory cytokines in women mono-colonized with *L. crispatus*. In another study examining the efficacy of Tenovir (an antiretroviral drug) gel microbicide as a pre-exposure prophylactic agent in high-risk women found that Tenovir was significantly more efficacious in women with *Lactobacillus*-dominated vaginal microbiomes (145). The combination of mucosal vaccination and probiotic therapy may thus further enhance protection from infection.

Routes of Mucosal Vaccination for HIV-1

Several routes of mucosal vaccination have been tested in non-human primate models. Most mucosal routes aim to deliver antigens to the mucosal-associated lymphoid tissue (MALT), which includes sites such as the nasal-associated lymphoid tissue (NALT) and the gut-associated lymphoid tissue (GALT). While these sites are anatomically separated, the immune responses primed within them are functionally linked, with adaptive responses primed in the MALT often acquiring mucosal-homing features. For example, vaccination targeting the NALT can result in effectors within the female reproductive tract. Systemic immunization, such as intramuscular and subcutaneous immunization, can result in mucosal immune responses, however the magnitude of the mucosal response is generally not as high as mucosal vaccination (146). Numerous routs of mucosal vaccination have been investigated for HIV-1 vaccines, such as intranasal, intratracheal, intrarectal, intravaginal, and oral.

The intranasal (IN) route is attractive due to the ease and practical administration of the immunization, the low dose of immunogen required to induce immune responses, and the ability to generate both systemic and mucosal responses via IN delivery (135). In a study examining a combination of IN and intramuscular (IM) immunization, rhesus macaques immunized via IN and IM combined with gp41-subunit grafted virosomes were protected from intravaginal challenge with pathogenic $SHIV_{SF162P3}$ (139). Another study in which rhesus macaques were immunized IN with an Ad5 vector and boosted IM with envelope proteins resulted in significant protection from mucosal challenge with SIV or SHIV (147, 148).

DNA/MVA vaccine modalities have also been used in IN immunization studies. DNA encoding HIV/SIV genes along with DNA encoding IL-2, IL-12, or IL-15 delivered nasally before boosting with MVA elicited both systemic and mucosal immune responses (149). Upon intrarectal challenge with $SHIV_{89.6}$ all animals were infected, but animals immunized with DNA + IL-12 were protected from $CD4^+$ T-cell loss and AIDS progression. A similar control of viremia was seen in female rhesus macaques immunized IN with DNA/MVA and challenged intra-vaginally with SIV (143). While IN immunization is effective at generating mucosal responses, there are disadvantages of this route as high inflammatory responses in the nasal tissue can cause neurological side effects such as Bell's palsy (150). The potential for neurological side effects is a major hindrance for IN vaccination, and vaccine candidates must be extensively tested for safety for use in this route.

Intratracheal (IT) immunization delivers vaccines directly to the trachea, which can then be taken up by the bronchoalveolar lymphoid tissue containing large B-cell follicles inducing IgA antibody responses (151). This route has been investigated for HIV/SIV immunization, though not as heavily as the IN route. In one study evaluating immunization with formalin-treated SIV in biodegradable microspheres found that priming intramuscularly and boosting IT induced SIV-specific vaginal IgA responses (152). Another study found that priming rhesus macaques IN with an Ad5 vector expressing HIV-1 Env gp140 and subsequently boosting IT with the same vector resulting in protection of 3 out 4 animals from rectal SHIV challenge (148). While IT immunization may be an effective route to generate mucosal responses, the

administration of immunizations IT is somewhat impractical, which limits its application for human use.

Intrarectal (IR) vaccination, while having practicality limitations, is an effective route for generating local antibody responses in the colorectal tissue (153). Rectal immunization has some advantages over oral delivery as IR delivered vaccines avoid the harsh conditions of the stomach which can easily degrade immunogens not formulated to survive such conditions. In one study rhesus macaques were immunized rectally with a synthetic-HIV/SIV-peptide vaccine along with the mucosal adjuvant LT (R192G), derived from the *E. coli* heat labile-enterotoxin. Following intrarectal SHIV challenge animals immunized IR controlled viremia and protected animals from infection more than effectively than subcutaneously immunized or control animals (154).

Intravaginal (Ivag) vaccination has similar practical limits as IR inoculation, but this route has also been studied extensively for HIV-1 vaccine development. The mucosal epithelium in the female reproductive tract (FRT) is more varied than in the rectum, ranging from stratified squamous epithelia in the ectocervix and vagina and simple columnar epithelium in the endocervix and upper reproductive tract. Intravaginal immunization has some additional limitations over IR immunization. The menstrual cycle induces changes to the epithelial layers of the FRT, which can cause immunogenicity of the vaccines to vary depending on when they are administered. Additionally, the menstrual cycle can have effects on viral challenges and infectivity. The lack of dedicated secondary lymphoid tissue in that takes up antigens in the FRT can also lead to Ivag delivered immunizations to be less immunogenic. However, it is possible to achieve vaginal immune responses via this route. In one study recombinant

trimeric HIV-1CN54 clade C gp140 was administered in Carbogel (an excipient licensed for vaginal use in women) vaginally and boosted IM the same gp140 resulted in both systemic and mucosal antibodies (155) Priming IM and then boosting Ivag also promoted the same result. In a study that compared Ivag, oral, and nasal administration of a DNA/MVA SIV vaccine, all routes generated mucosal T-cell and antibody responses in the vaginal secretions (141). However, protective efficacy was better in the oral and nasally administered groups.

Oral vaccination primarily involves delivery of antigens orally through the stomach to reach the GALT (Intragastric, IG). Oral vacation can generate high levels of mucosal homed antibodies, especially within the gastrointestinal tract. However, antigens must be able to survive degradation by enzymes within the saliva and the stomach, as well as effects of dilution and inadequate uptake in the GALT. Additionally, many antigens administered orally will induce immune tolerance in the GALT, which must be overcome if a strong immune response is desired (156). One approach to overcome the harsh stomach conditions is the use of enteric-coated capsules, which will resist stomach acid and dissolve in the small intestine, allowing for uptake within the GALT. Orally delivery of capsules containing an Ad5 vector expressing HIV-1 *gag* and HIV-1 *env* peptides was shown to prime cellular immune responses in the intestine upon boosting IN with the *env* peptides plus mutant cholera toxin adjuvant (157)

Another strategy to bypass the stomach is to deliver vaccinations directly to the oral tissues. The oral tissue is composed of the sublingual tissue, located below the tongue, as well as the buccal tissue, which is the inner lining of the cheeks. The mucosal epithelium of both the sublingual and buccal tissue consists of squamous epithelium,

thicker than the single columnar epithelium seen in the intestinal tract, but not keratinized like the dermis of the skin (158). The sublingual and buccal tissues contain numerous dendritic cell subtypes, and delivery of ovalbumin along with the cholera toxin adjuvant to the sublingual tissue has shown to induce systemic and mucosal antibodies in mice (159). A study in rhesus macaques comparing different mucosal routes of an Ad5-HIVgag and gp120 protein boost found that sublingual immunization was as effective at generating antibody responses as IN, IR, and IVag immunization, though protective efficacy from rectal SIV challenge was similar between groups (137). This dissertation will further investigate the sublingual and buccal tissue was a route of mucosal HIV-1 vaccination in rhesus macaques.

Double-mutant heat-labile toxin as a mucosal adjuvant

The double-mutant heat-labile enterotoxin (dmLT) is a mutated form of the heat-labile enterotoxin (LT) produced by enterotoxigenic *Escherica coli* (ETEC), and is an experimental mucosal adjuvant and potential vaccine candidate for ETEC (160). This enterotoxin, along with the related cholera toxin, are composed of an enzymatically active A-subunit which is in close association with a pentameric B-subunit (161). Wild-type LT induces diarrhea through the ADP-ribosylation activity of the A-subunit, however two mutations to LT (R192G/L211A) (dmLT) removes enterotoxicity while retaining adjuvant activity (162). While the exact mechanism of adjuvant activity is not completely understood, numerous studies show that dmLT is a potent mucosal adjuvant and promotes both the generation of intestinal antigen-specific IgA responses and TH17-responses (163). Additionally, when compared to other adjuvants, such as the TLR-9

agonist CpG, dmLT has been shown to induce $\alpha_4\beta_7$ on antigen-specific CD4+ cells, and promotes a balance of TH_1 and TH_{17} responses in contrast to CpG inducing primarily a TH_1 responses (164). Clinical trials in humans characterizing dmLT as both a mucosal adjuvant and a vaccine antigen are currently underway, and initial results from one study indicate that oral administration of dmLT is safe and immunogenic in humans, with doses up to 100 ug being well tolerated (165).

Summary

The past ten years of HIV-1 vaccine development has seen major advancements in our understanding of the structure and pathogenicity of HIV-1. The modest success of the RV144 trial and the associated correlates of V1V2-directed antibodies in decreasing risk of infection, as well as the crystal structures of the native HIV-1 Env spike informing the design of novel immunogens to aid in developing broadly neutralizing antibodies have brought about new efforts for a protective HIV-1 vaccine. Additionally, increased knowledge about the needs for mucosal immunity to block HIV-1 from establishing infection has brought about the need for novel approaches of generating mucosal immune responses. This dissertation will investigate two aspects of active HIV-1 research, the characterization of a novel HIV-1 Env immunogen in rhesus macaques, and method of sublingual and buccal vaccination to induce strong mucosal responses.

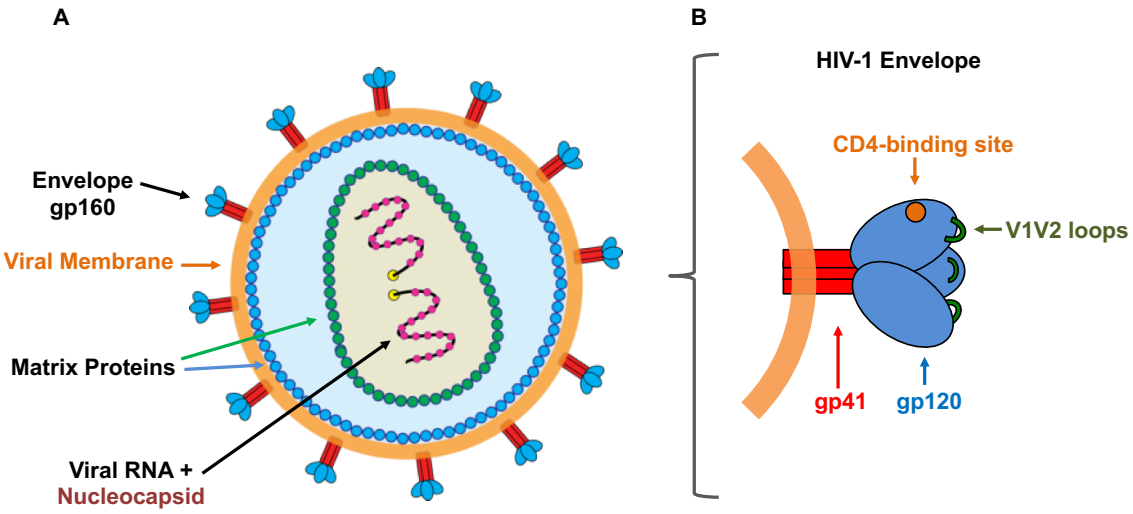

Figure 1: **Structure of HIV-1**. (**A**). HIV-1 viral particle, composed of trimeric HIV-1 Env (gp160) linked to the viral plasma membrane. The viral capsid encloses the viral RNA, which is covered with a nucleoprotein and is packaged with reverse transcriptase (yellow). (**B**). Schematic of HIV-1 Env, consisting of a heterotrimer of gp41 and gp120. The CD4-binding site is located on gp120. In green, the position of the variable loops 1 and 2 (V1V2).

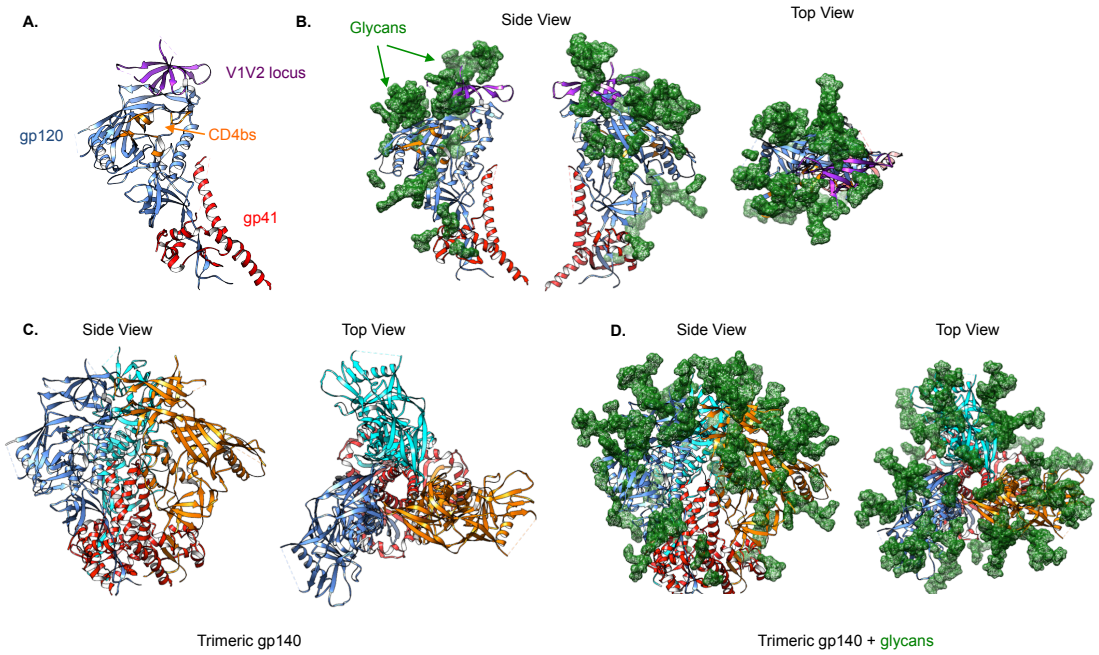

Figure 2: **Structure of HIV-1 Envelope, BG505 SOSIP.664 gp140**. (**A**) gp120 subunit and associated gp41 subunit (red). The conformational CD4 binding site in orange. The V1V2 locus in purple. (**B**) Glycans on gp120:gp41 subunit in green. Side views and top view of gp120:gp41 showing positioning of glycans. (**C**) Trimeric gp140 from side and top. Gp120 subunits in blue, cyan, and orange. Gp41 subunits in red. (**D**) Same structure in (C) with glycans shown in green. Structures derived from PDB 5T3Z.

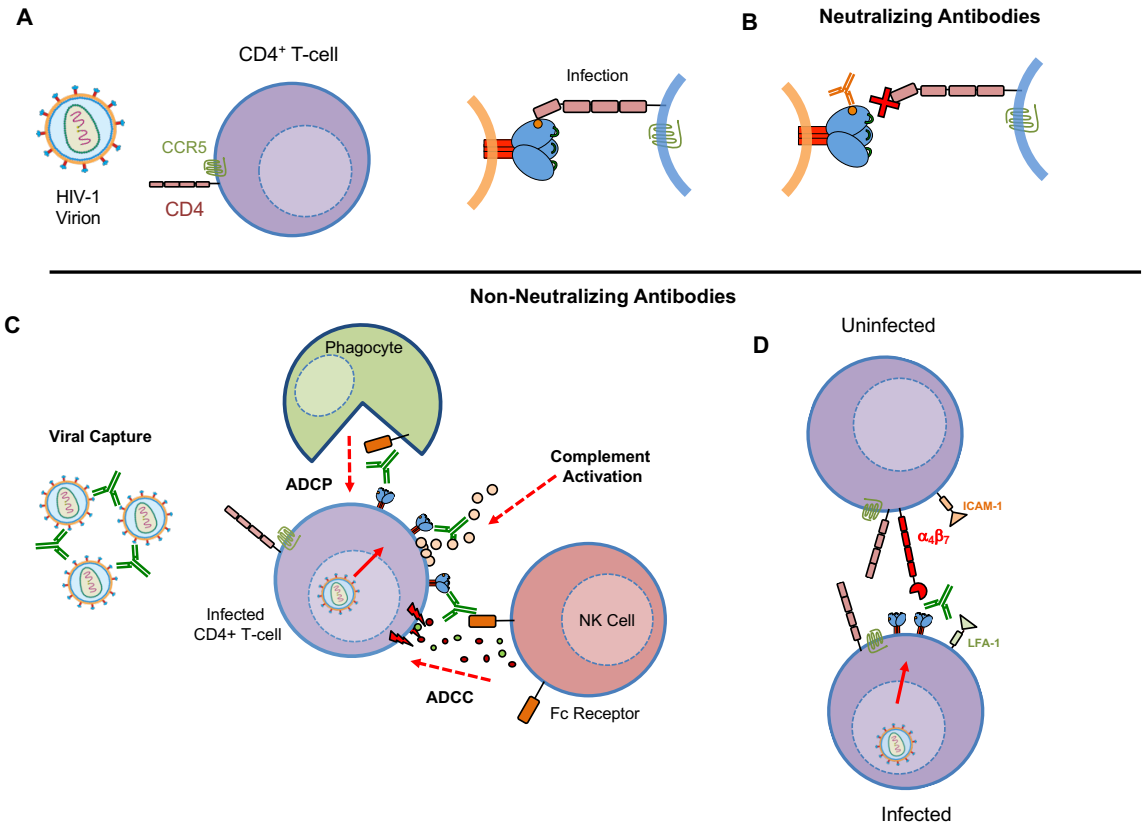

Figure 3: Neutralizing and non-neutralizing antibody effector functions. (A). Schematic of HIV-1 viral particle and a CD4+ T-cell expressing CD4 and CCR5. Binding to CD4 via the CD4bs on gp120 mediates viral entry and infection. (**B**). Neutralizing antibodies which target the CD4bs block binding of gp120 to host CD4, inhibiting infection. (**C**). Non-neutralizing antibody effector functions include viral capture by antibodies, antibody dependent cellular phagocytosis (ADCP) by phagocytic cells, complement activation, and antibody dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells. (**D**). Non-neutralizing antibodies binding to gp120 and blocking a4b7 binding by gp120 inhibits the formation of a viral synapse and cell-to-cell infection.

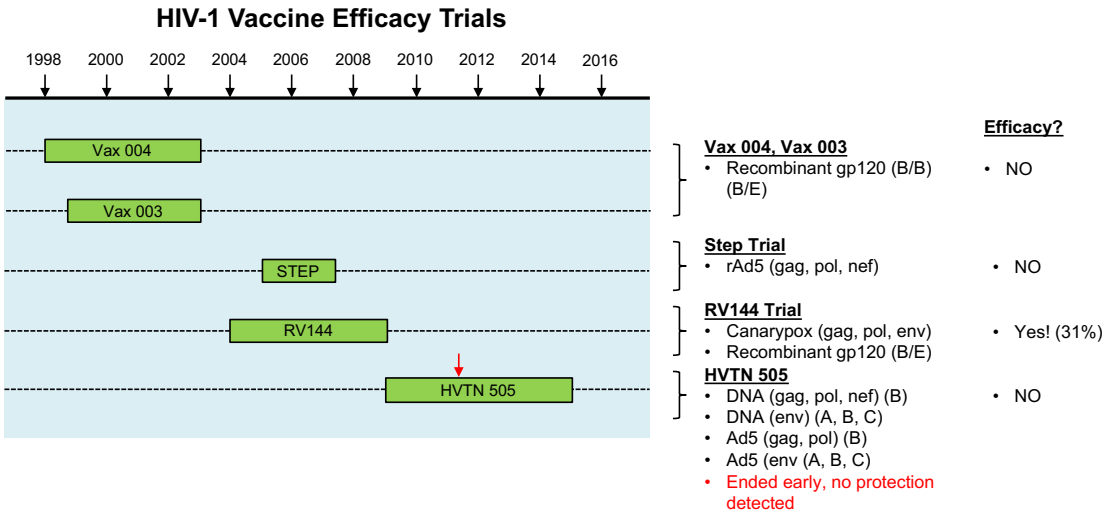

Figure 4: **Timeline of HIV-1 Vaccine efficacy trials (43)**.

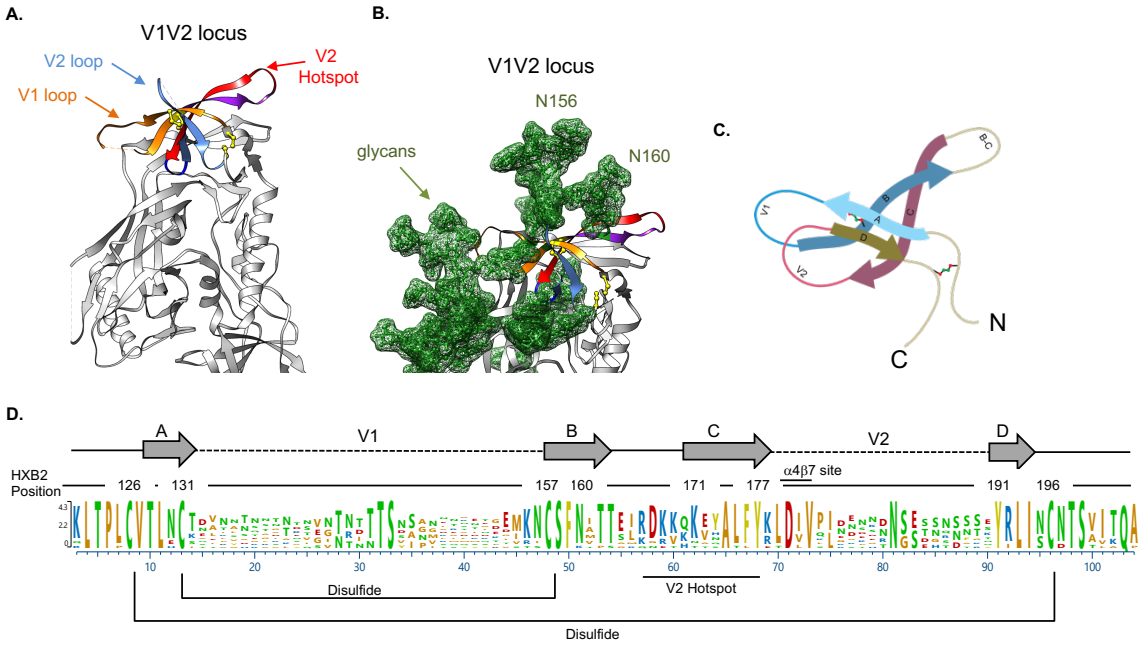

Figure 5: **The V1V2 region of HIV-1 gp120.** (**A**) V1V2 region of BG505 SOSIP.664 gp140. Disulfide bond forming residues in yellow. The hypervariable V1 and V2 loop are unresolved. (**B**) Glycan shield in the V1V2 region. Glycans as N156, and N160, important for binding by multiple V2-directed bNAbs (166). (**C**) Cartoon showing the structure of the V1V2 loop including the A, B, C, D sheets and the V1 and V2 hypervariable loops. (**D**). Protein sequence logo of the V1V2 loop derived from a panel of 15 gp70-V1V2 scaffold proteins, showing conservation and variability of specific residues at different position (167). V2 hotspot and putative $\alpha_4\beta_7$ binding site indicated. HXB2, HIV-1 reference strain.

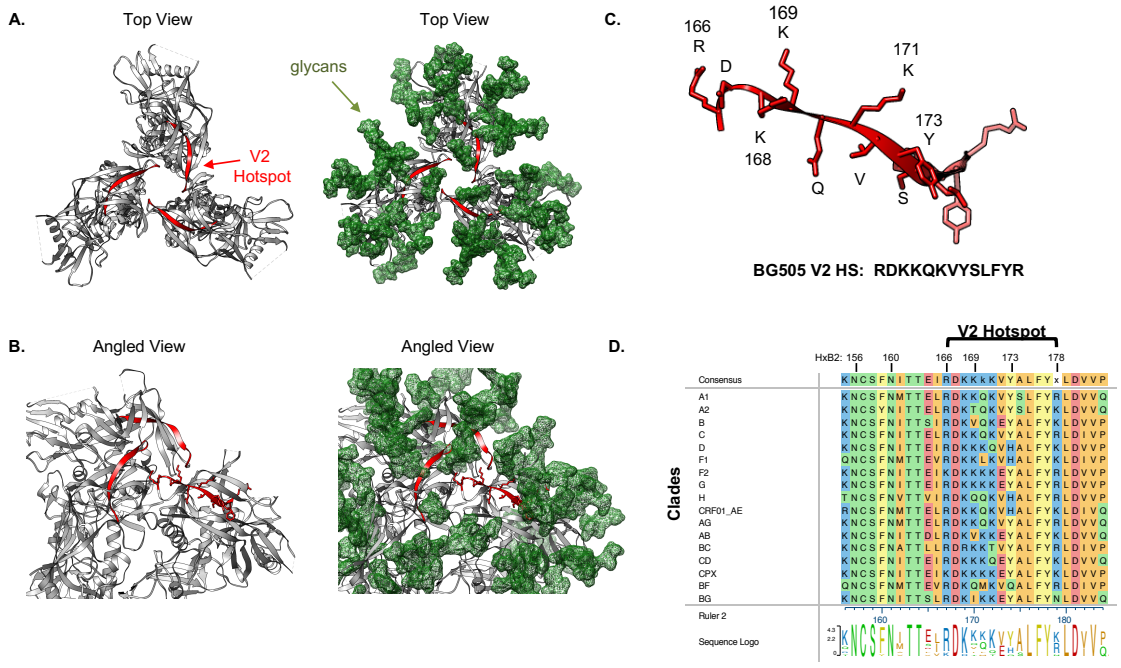

Figure 6: The V2-hotspot region of gp120. (**A**). The V2 hotspot, composed mainly of the C-strand within the V1V2 locus, depicted in red within each gp120 subunit of the BG505 SOSIP.664 gp140 trimer without glycans (left) or with the glycans (right) in green. (**B**). V2-hotspoted visualized from an angle to show accessibility by antibody recognition without (left) and with (right) glycans. (**C**). BG505 V2-hotspot sequence isolated from gp120 subunit, showing orientation of specific amino acid residues. (**D**). Alignment of consensus sequences from major clades of HIV-1, with relevant sites of glycosylation (N156, N160), and the V2-hotspot (position 166-178) indicated. HxB2: HIV-1 reference sequence. Sequences derived from the Los Alamos National Laboratory (Consensus and Ancestral Sequence Alignments, 2004).

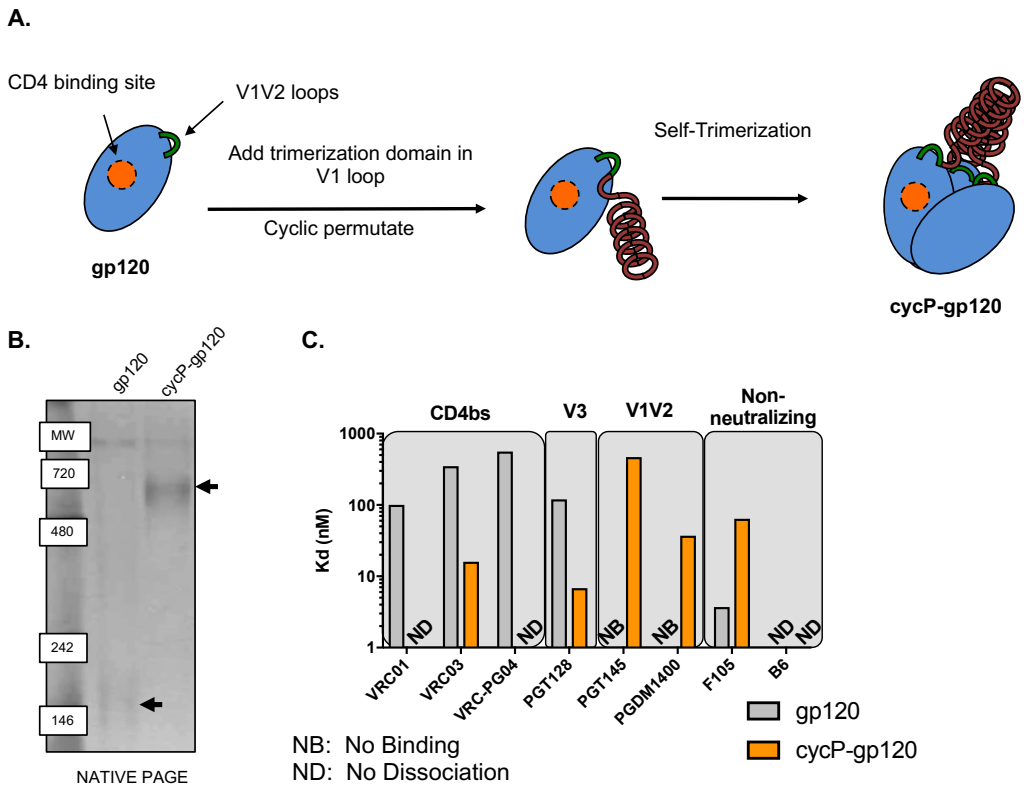

Figure 7: **Generation of trimeric gp120 (cycP-gp120).** (**A**) To generate a trimeric gp120 immunogen, the trimerization domain of human matrix cartilage protein (hCMP) was fused to the de novo N-terminus proximal to the V1 loop of a cyclic permutated gp120. (**B**). Native PAGE gel electrophoresis of monomeric gp120 and cycP-gp120, detected via coomassie staining. cycP-gp120 exists primarily as a trimer. (**C**). Binding affinity for neutralizing (VRC01, VRC03, VRC-PG04, PGT128, PGT145, PGDM1400) and non-neutralizing antibodies (F105, B6) to monomeric gp120 or cycP-gp120, measured by surface plasmon resonance ND (No-Dissociation), NB (No Binding). Adapted from previous studies (129).

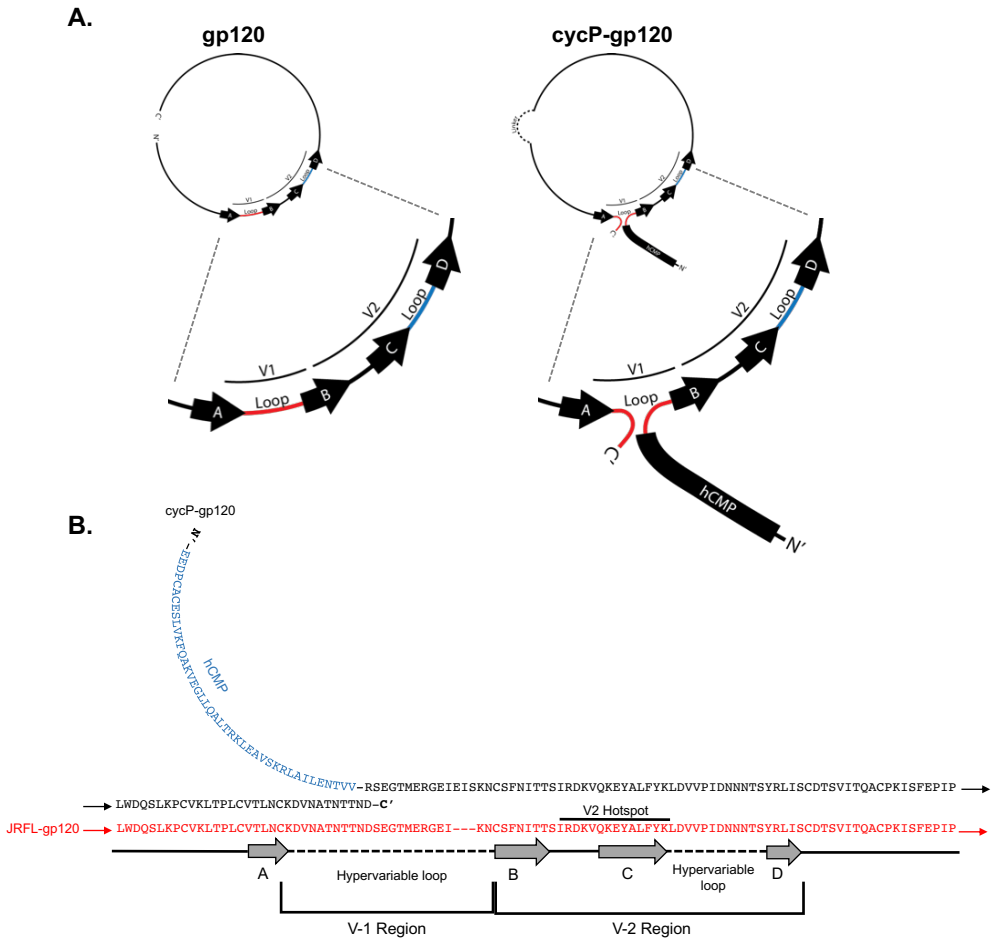

Figure 8: **V1V2 loops in cycP-gp120.** (A) Schematic of gp120 (left) and cycP-gp120 (right) depicting the amino acid linker to the native N- and C- termini. The trimerization domain is then inserted in the de novo N-terminus proximal to the V1-hypervariable loop. (B) Sequences of the V1V2 region of cycP-gp120 (top) overlaid on monomeric gp120 (bottom, red).

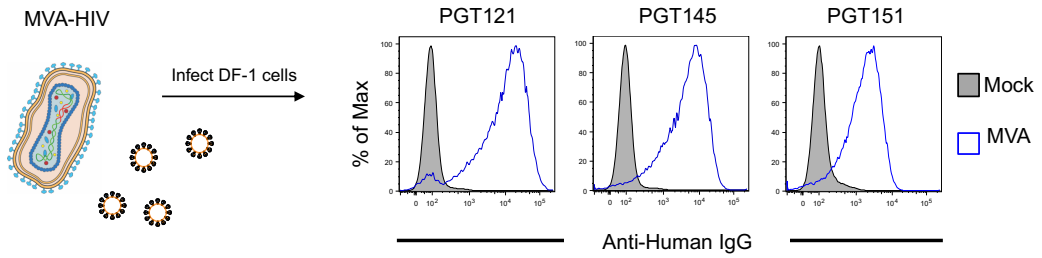

Figure 9: **MVA-HIV expresses trimeric HIV-1 Env**. DF-1 cells were infected with MVA-HIV (MVA-62BSm) expressing HIV-1 gag, pol, and env (strain ADA, cell-surface trimeric gp150). The broadly neutralizing antibodies PGT121, and trimer specific PGT145 and PGT151 were incubated with infected cells. Antibody binding dected by anti-human IgG-Biotin, PE-streptavidin, and analyzed by flow cytometry. Mock, uninfected controls.

Chapter II: A trimeric HIV-1 envelope gp120 immunogen induces potent and broad anti-V1V2 loop antibodies against HIV-1 in rabbits and rhesus macaques

Andrew T. Jones[a,b], Venkateswarlu Chamcha[a,b], Sannula Kesavardhana[c], Xiaoying Shen[f], David Beaumont[f], Raksha Das[c], Linda S. Wyatt[d], Celia C. LaBranche[e], Sherry Stanfield-Oakley[e], Guido Ferrari[e,f], David C. Montefiori[e], Bernard Moss[d], Georgia D. Tomaras[f], Raghavan Varadarajan[c], and Rama Rao Amara[a,b#]

[a]Emory Vaccine Center, Yerkes National Primate Research Center, Emory University, Atlanta, Georgia, USA

[b]Department of Microbiology and Immunology, Emory School of Medicine, Emory University, Atlanta, Georgia, USA

[c]Molecular Biophysics Unit, Indian Institute of Science, India

[d]Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA

[e]Department of Surgery, Duke University Medical Center, Durham, NC, USA

[f]Duke Human Vaccine Institute, Duke University Medical Center, Durham, North Carolina, USA

 # Corresponding Author: Rama Rao Amara, Ph.D.

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Abstract

Trimeric HIV-1 envelope (Env) immunogens are attractive due to their ability to display quaternary epitopes targeted by broadly neutralizing antibodies while obscuring unfavorable epitopes. Results from the RV144 trial highlighted the importance of vaccine induced HIV-1 Env V1V2 directed antibodies, with key regions of the V2 loop as targets for vaccine-mediated protection. We recently reported that a trimeric JRFL-gp120 immunogen, generated by inserting a N-terminal trimerization domain in the V1 loop region of a cyclically permuted gp120 (cycP-gp120), induces neutralizing activity against multiple tier-2 HIV-1 isolates in guinea pigs in a DNA prime/protein boost approach. Here, we tested the immunogenicity of cycP-gp120 in a protein prime/boost approach in rabbits and as a booster immunization to DNA/MVA vaccinated rabbits and rhesus macaques. In rabbits, two cycP-gp120 protein immunizations induced 100-fold higher titers of high avidity gp120-specific IgG compared to two gp120 immunizations, with four total gp120 immunizations being required to induce comparable titers. CycP-gp120 also induced markedly enhanced neutralizing activity against tier-1A and -1B HIV-1 isolates, substantially higher binding and breadth to gp70-V1V2 scaffolds derived from a multi-clade panel of global HIV-1 isolates, and antibodies targeting key regions of the V2-loop region associated with reduced risk of infection in RV144. Similarly, boosting MVA or DNA/MVA primed rabbits or rhesus macaques with cycP-gp120 showed a robust expansion of gp70-V1V2-specific IgG, neutralization breadth to tier-1B HIV-1 isolates and ADCC activity. These results demonstrate that cycP-gp120 serves as a robust HIV Env immunogen that induces broad anti-V1V2 antibodies and promotes neutralization breadth against HIV-1.

Importance

Recent focus in HIV-1 vaccine development has been the design of trimeric HIV-1 envelope (Env) immunogens that closely resemble native HIV-1 Env, with a major goal being the induction of broadly cross-reactive neutralizing antibodies (bNAbs). While the generation of bNAbs are considered a gold-standard in vaccine-induced antibody responses, results from the RV144 trial showed that non-neutralizing antibodies directed towards the V1V2-loop of HIV-1 gp120, specifically the V2 loop region, were associated with decreased risk of infection demonstrating the need for the development of Env immunogens that induce a broad anti-V1V2 antibody response. In this study, we show that a novel trimeric gp120 protein, cycP-gp120, generates high titers of high avidity and broadly cross-reactive anti-V1V2 antibodies, a result not found in animals immunized with monomeric gp120. These results reveal the potential of cycP-gp120 as a vaccine candidate to induce antibodies associated with reduced risk of HIV-1 infection in humans.

Introduction

While great progress has been made in developing anti-retroviral therapies, and preventative measures such as pre-exposure prophylaxis have aided in reducing new infections, an effective vaccine remains crucial to the eradication of Human Immunodeficiency Virus -1 (HIV-1) (168). Continuous efforts to understand HIV-1 pathogenesis and acquisition have aided in combating the HIV/AIDS pandemic, however an effective prophylactic vaccine has yet to be developed. As with most conventional vaccines, a strong humoral response is critical for providing protection from infection. Many successful vaccines provide protection by inducing neutralizing antibodies, which act by binding to and inhibiting infectious virions from entering their target cell. However, the generation of broadly neutralizing antibodies (bNAbs) that recognize diverse HIV-1 isolates has been difficult, with non-neutralizing antibodies being the predominant response in both vaccinations and natural infection (169). While uncommon, the spontaneous generation of bNAbs in naturally infected people has been well documented (17, 170), and passive transfer experiments with isolated bNAbs have provided protection in non-human primates from SIV/HIV (SHIV) chimeric infections (171, 172), demonstrating the importance of bNAbs in preventing HIV-1 infection. However, no current HIV-1 vaccine candidate has been able to induce bNAbs in humans.

The main target of antibodies in an HIV-1 vaccine is the HIV-1 Envelope (Env) glycoprotein, which mediates the initial attachment and fusion to host target cells. HIV-1 Env is first expressed as a precursor peptide, gp160, which forms homotrimers before furin-mediated cleavage separates gp160 into its gp120 and gp41 subunits, resulting in a non-covalently linked heterotrimeric spike (8). Monomeric gp120 has been used in

multiple HIV-1 vaccine efficacy trials, with many failing to induce broadly cross-reactive neutralizing antibodies and confer protection from infection (173). However, in the RV144 vaccine efficacy trial, consisting of an ALVAC-HIV virus vector prime followed by a monomeric gp120 protein boost resulted in a short-term vaccine efficacy of 60% and long-term efficacy of 30% (83). This trial demonstrated for the first time the potential for vaccine-induced protection in humans at risk for HIV-1 infection. Surprisingly, protection was not related to the presence or generation of bNAbs. The major correlate of protection was instead found to be non-neutralizing antibodies specific for the scaffolded V1V2 region of gp120 (85, 174), a finding supported by recent non-human primate (NHP) trials in which protection from neutralization resistant simian immunodeficiency virus (SIV) infection also correlated with anti-V1V2 antibodies (97-99, 101). Further studies have revealed a key role of V2-targed antibodies in RV144 vaccine-mediated protection (96), as vaccine efficacy was higher against viruses matching the vaccine V2 loop at key residues, and antibodies directed towards linear V2-peptides correlated with lower risk of infection (93, 175). Collectively, these results highlight the need for the development of HIV Env immunogens that induce broadly cross-reactive anti-V1V2 antibodies.

We recently described the design and immunogenicity of a novel trimeric gp120 immunogen in guinea pigs (129). This immunogen, hCMP-v1-cyc-gp120 (referred to as cycP-gp120 hereafter), is based on a cyclically permuted gp120 in which a de novo N-terminus is generated within the V1 loop region with the native N- and C- termini joined via an amino acid linker chain (129). To induce a trimeric complex, the human matrix cartilage protein (hCMP) coiled-coil trimerization domain was fused to the de novo N-

termini, resulting in a stabilized, disulfide bond linked trimeric gp120 (128). Binding studies with monoclonal antibodies showed that cycP-gp120 binds to bNAbs with a higher affinity than gp120, similar to gp140 based trimeric immunogens, including quaternary epitope specific bNAbs such as PG9, PG16, and PGT145, demonstrating the existence of well-folded trimers (129). Immunization of guinea pigs with cycP-gp120 using a DNA prime/protein boost modality showed induction of neutralizing antibodies against neutralization resistant (tier-2) HIV-1 isolates. However, the ability of cycP-gp120 immunogen to induce the potentially protective anti-gp70-V1V2 responses is not known. Thus, in this study we tested the immunogenicity of cycP-gp120 protein prime/boost immunizations in rabbits and characterized the neutralizing Ab responses and V1V2 loop-directed responses. In addition, we tested the ability of cycP-gp120 protein to enhance the immunogenicity of our DNA/MVA vaccines in rabbits and rhesus macaques. Our results show that priming and/or boosting with cycP-gp120 induces high levels of high avidity HIV-1 Env specific antibodies, tier-1A and -1B neutralizing antibodies, and the induction of antibody dependent cellular cytotoxicity (ADCC) activity in MVA-primed rabbits. In addition, we show that cycP-gp120 induces a remarkable anti-gp70-V1V2 antibody response, promoting antibodies recognizing V1V2 sequences from a diverse, multi-clade panel of global HIV-1 isolates, as well as antibodies directed towards a core V2-loop region.

Results

Trimeric cyclically permuted gp120 induces high levels of high avidity anti-HIV binding and neutralizing antibodies in rabbits

To characterize the immunogenicity of cycP-gp120 vs. monomeric gp120, we immunized rabbits intramuscularly with 20 ug of either HIV-1 clade B strain JRFL-gp120 or JRFL-cycP-gp120 on weeks 0 and 8 (**Fig. 1a**). The gp120 group received two additional immunizations at weeks 16 and 32. Sera were collected for both groups at weeks 0, 2, 10, and 16. Additional sera collections for gp120 immunized animals were taken at weeks 18, 24, 32, and 34. Antibodies specific for JRFL-gp120 were determined using ELISA in sera taken two weeks post the specified immunization. Rabbits receiving two cycP-gp120 immunizations generated a strong anti-JRFL gp120 serum IgG response (geo*mean titer, 6.1 log*), two logs higher than rabbits receiving two monomeric gp120 immunizations (geo*mean titer, 3.5 log*) (**Fig. 1b,c**). Impressively, two additional gp120 immunizations were required (geo*mean titer, 5.6 log)* to generate equivalent JRFL-gp120 specific antibody responses elicited by the two cycP-gp120 immunizations.

Neutralizing antibody responses (NAb) induced by either gp120 or cycP-gp120 immunization was measured against a multi-clade panel of pseudoviruses that have high (SF162.LS, MW965.26), moderate (BaL.26), or low (ADA, JR-FL, TRO.11) neutralization sensitivity (tier-1A, -1B, -2, respectively). Two cycP-gp120 immunizations generated a strong cross-clade NAb response against tier-1A clade B (SF162.LS) and clade C (MW965.26) viruses (*ID50 range, 1.8-3.2 log)* (**Fig. 1d**). In contrast, two gp120 immunizations did not induce detectable NAbs against either tier-1A isolates. The additional two gp120 boosts did generate NAbs against SF162.LS but only in two of the

four animals, and only one animal generated neutralizing activity against clade C MW965.26. Immunization with cycP-gp120 also induced NAbs against the more resistant tier-1B clade B BaL.26 virus (*ID50 range, 1.5-2.3 log; mean ID50, 1.6 log*). No neutralizing activity against BaL.26 was observed in gp120 immunized rabbits, and no neutralizing antibodies against tier 2 pseudoviruses, including JR-FL, were observed in any of the immunized animals (**Table 1**). However, it is possible that additional immunizations with cycP-gp120 may help to generate these responses as previous studies found that repeated germinal center reactions aid in the generation of autologous tier-2 neutralization activity (122, 176, 177).

Antibody avidity plays an important role in protection from HIV/SIV infection (178-182). To measure antibody avidity against JRFL-gp120, we performed an ELISA in which rabbit sera bound to JRFL-gp120 was incubated with 1.5M sodium thiocyanate (NaSCN), which dissociates weakly bound antibodies, or PBS prior to detection. Rabbits immunized twice with gp120 had low avidity anti-JRFL gp120 antibodies (*mean avidity index of 17.3),* which modestly increased upon subsequent gp120 immunizations (*mean avidity index of 33.4*) (**Fig. 1e**). However, immunization with cycP-gp120 resulted in very high avidity antibodies (*mean avidity index of 78.2*) that were markedly higher than animals immunized with monomeric gp120. As cycP-gp120 is both antigenically distinct from monomeric gp120, as well as a trimeric protein, we also tested antibody avidity against cycP-gp120. As with monomeric gp120, rabbits immunized with cycP-gp120 generated very high avidity antibodies against cycP-gp120 (*mean avidity index of 93)*, significantly higher than gp120 immunized rabbits (*mean avidity index of 73)* (**Fig. 1f**). Taken together, these data suggest that cycP-gp120 is a robust HIV-1 Env immunogen,

capable of generating very high titers of high avidity antibodies, as well as tier-1A and -1B neutralizing antibodies after only two immunizations in rabbits.

CycP-gp120 induces a robust antibody response against V1-V2 variable loops derived from global HIV-1 isolates

Recent vaccine studies in both non-human primates and humans have emphasized the importance of antibodies targeting the V1V2 loop region of gp120 in protecting from HIV-1 infection (85, 97, 98, 101). To test for the presence of binding antibodies to diverse HIV-1 isolates we measured binding to gp120, gp140, and gp70-V1V2 scaffold proteins derived from a global panel of tier-2 HIV-1 isolates from acute/early infection representing multiple clades of HIV. The gp70-V1V2 scaffold proteins included case A2 and C.1086 strains as the presence of binding Abs to these proteins was shown to be associated with reduced risk of infection in RV144 trial (174).

Rabbits immunized twice with cycP-gp120 or four times with gp120 showed comparable binding to gp120/gp140 proteins from multiple isolates including cross-clade reactivity against non-clade B isolates (**Fig. 2a**). The cycP-gp120 immunized animals showed higher binding activity against some isolates and lower binding activity against others compared to animals immunized with four gp120 immunizations. These results demonstrate that both monomeric gp120 and trimeric cycP-gp120 induce comparable titers of binding antibody response against gp120/gp140 proteins from diverse panel of HIV-1 isolates. However, in sharp contrast to binding to gp120/gp140 proteins, rabbits immunized twice with gp120 showed no detectable responses against gp70-V1V2

scaffolds (data not shown), and further gp120 immunizations resulted in only a weak response with 9 out of 16 tested isolates being recognized by all vaccinated animals, suggesting that monomeric JRFL gp120 elicits poor V1V2 scaffold directed antibody responses even after multiple immunizations. Impressively, two cycP-gp120 immunizations resulted in a robust induction of anti-V1V2 antibodies against 15 of the 16 isolates tested (**Fig. 2b**), A strong trend was observed between the overall anti-gp120 response and V1V2-directed antibodies in cycP-gp120 immunized rabbits, a correlation not found in gp120 immunized rabbits (**Fig. 2c**), indicating that cycP-gp120 can preferentially promote V1V2 antibodies as part of the overall antibody response against HIV-1 Env. These results demonstrate that cycP-gp120 serves as an excellent immunogen to induce broadly cross reactive antibody response against V1V2 scaffolds from diverse HIV-1 isolates from multiple clades.

CycP-gp120 induced antibody response is targeted against the V2 hotspot peptide

The generation of cross-clade binding antibodies to gp70-V1V2 scaffolds suggests that these antibodies are targeted against a conserved epitope in the V1V2 region or against a specific conformation found on these scaffolds. Recent studies have identified V2-directed antibody responses as a correlate of protection in the RV144 trial, with antibodies targeting a core "hotspot" region within the V2 loop associating with a decreased risk of infection (93). This region corresponds to residues 166-178 (strain HxB2) of gp120, a region proximal to the putative $\alpha_4\beta_7$ binding site motif (90). To test for the generation of cross-clade reactive V2-targeting antibodies, we measured binding of rabbit sera to five 15-mer peptides overlapping by 12 amino acids corresponding to the

V2 hotspot region of the clade C.1086 HIV-1 gp120 (**Fig. 3a**). The hotspot region of C.1086 shares 61% identity to clade-B JRFL sequence. Surprisingly, despite incomplete homology, sera from rabbits immunized with cycP-gp120 showed strong binding to two of the five peptides tested, with three of the four animals binding to a core peptide encoding the entirety of the hotspot region, and one rabbit responding to the subsequent overlapping peptide (**Fig. 3b**). In contrast, no measurable binding activity was detected in rabbits immunized with monomeric gp120 (**Fig. 3c**). Furthermore, we found a direct association (trend) between binding to V2 peptides (peptide #3 or #4) with the overall binding to C.1086 gp70-V1V2 scaffold (**Fig. 3d**).

To further characterize the V2 hotspot-directed response, we synthesized V2 hotspot peptides from multiple clade-B and clade-C viral strains that represent the immunogen strains (JR-FL, ADA) and potential SHIV strains (clade-B SF162P3, clade-C 1157 and 1157-Y173H that are currently being used in non-human primate vaccine protection studies (183). As with C.1086, cycP-gp120 immunized rabbits developed a broad anti-V2 hotspot antibody response to multiple V2 hotspot peptides (**Fig. 3e**). Very low or no reactivity was found in gp120-immunized rabbits against any of the peptides. The V2 peptide sequences show great diversity and differ from the JR-FL vaccine strain at several residues (**Fig. 3f**), suggesting that V2-hotspot directed antibodies induced by cycP-gp120 are flexible and capable of binding to multiple diverse HIV-1 isolates. As V2 directed antibody responses have been shown to correlate with protection in both human and non-human primate vaccine studies, the ability of cycP-gp120 to induce a strong cross-reactive V2-hotspot response greatly adds to its potential as a vaccine candidate.

CycP-gp120 serves as a strong booster immunization in rabbits primed with MVA-HIV

The results from the VAX003 and RV144 trials suggest that the implementation of a poxvirus vector prime in the RV144 trial may be important for promoting protective immune responses, and recent studies suggest these responses can be attributed to the promotion of distinct IgG subclasses between the two trials (101, 184). There is now a concerted effort in the HIV-1 vaccine field to evaluate the use of protein booster immunizations in viral vector priming immunizations, in particular for protein immunogens that strongly induce gp70-V1V2 responses. In light of this, we evaluated the ability of cycP-gp120 to boost humoral immune responses primed by another recombinant poxvirus, Modified Vaccinia Ankara (MVA), engineered to express trimeric membrane bound gp150 derived from HIV-1 clade B strain ADA (MVA-HIV) (130) on infected cells and virus like particles, a vector currently being evaluated in human trials (69).

To test the use of MVA-HIV and cycP-gp120 in a poxvirus prime – protein boost scheme, we immunized rabbits intramuscularly with MVA-HIV on weeks 0 and 8 (130) followed by cycP-gp120 immunizations on weeks 16 and 32. Two MVA-HIV immunizations induced strong binding antibodies against JRFL-gp120 (*mean titer, 5.1 log)*, and these responses were boosted by approximately one log upon subsequent immunization with cycP-gp120 before contracting over the next 14 weeks before a final cycP-gp120 boost (*mean titer, 5.7 log*) (**Fig. 4a**). Neutralizing antibody responses were measured at week 10, post the second MVA-HIV immunization, week 18, post the first cycP-gp120 boost, and week 34, post the final cycP-gp120 boost. Encouragingly,

boosting once with cycP-gp120 expanded both tier-1A and -1B neutralizing antibodies (**Fig. 4b**). However, the second protein boost did not efficiently recall the antibody response and thus the neutralizing antibody titers were also not boosted following the 2nd protein boost (**Fig. 4b**). Analysis at week 34, two weeks post the second cycP-gp120 immunization, showed an expansion of binding to the global panel of HIV-1 gp120/gp140 proteins, highlighting the ability of cycP-gp120 to promote and expand antibodies with multi-clade breath (**Fig. 4c**). While MVA-HIV immunization induced strong gp120 binding antibodies, responses against gp70-V1V2 scaffolds were much weaker with only 1 out 16 isolates tested, Case.A2, showing 100% positive binding in MVA-HIV immunized animals. However, boosting with two cycP-gp120's greatly expanded in both magnitude and breath of gp70-V1V2 directed response, with 15 out of 16 isolates showing positive binding by all animals (**Fig. 4d**), demonstrating cycP-gp120's propensity for inducing V1V2-directed antibody responses. As with rabbits immunized only with cycP-gp120, boosting MVA-primed rabbits with cycP-gp120 also promoted the generation of V2-hotspot binding antibodies, responses not observed after the initial MVA-HIV immunizations (**Fig. 4e**). These data show that cycP-gp120 acts as a strong boosting immunogen in MVA-HIV primed animals, promoting both V1V2-scaffold and V2-hotspot directed responses.

A major effector function of non-neutralizing antibody responses is antibody dependent cellular cytotoxicity (ADCC), and recent studies examining antibody responses from the RV144 trial suggest that ADCC may be involved with vaccine efficacy (101). To measure ADCC activity in rabbits, we co-cultured HIV-1 JRFL-infected target cells with effector NK cells and serial dilutions of rabbit sera, taken at

different time points. While MVA-HIV immunization lead to a low level of ADCC activity, this activity was boosted after the two cycP-gp120 boosts (**Fig. 5a**), with all four rabbits developing ADCC activity against HIV-1 infected cells. In contrast, immunization with cycP-gp120 alone resulted in only one animal out of four developing ADCC activity (**Fig. 5b**), despite having similar if not greater levels of anti-JRFL gp120 serum IgG titers compared to MVA-HIV primed, cycP-gp120 boosted rabbits (**Fig. 5c,d).** Additionally both groups have strong V1V2-directed breadth and JRFL-gp120 V2 hotspot directed antibodies. While the difference in ADCC titers in animals immunized with cycP-gp120 or MVA-HIV prime cycP-gp120 boost does not reach statistical significance, this data suggests that the development of ADCC activity is not based solely on the magnitude or specificity of the antibody response. Additionally, this data suggests that the utilization of a poxvirus vector prime prior to a protein boost could aid in the development in ADCC functionality.

CycP-gp120 serves as a strong booster immunization to MVA primed antibody responses in rhesus macaques

Our clade B DNA/MVA vaccine has completed phase 2a immunogenicity studies in humans and plans are underway for phase 2b efficacy studies (132). To test the immunogenicity of cycP-gp120 in the non-human primate model, we vaccinated four rhesus macaques intramuscularly with our clade B DNA/MVA HIV vaccine, followed by two subcutaneous boosts with HIV-1 JRCSF cycP-gp120 (**Fig. 6a**). Similar to our results in rabbits, cycP-gp120 served as a potent immunogen in rhesus macaques, boosting both gp140-specific serum IgG as well as neutralizing antibodies against tier-1A and -1B HIV-

1 isolates, with neutralizing antibodies against the moderately resistant HIV-1 BaL.26 being detected only after boosting with cycP-gp120 (**Fig. 6b, c**). Boosting with cycP-gp120 enhanced the magnitude of cross-reactive antibodies specific for gp120 and gp140 antigens, and greatly expanded the global gp70-V1V2 response, with all animals reacting against all scaffolds tested after the second protein boost (**Fig. 5d,e**). Taken together, these data show that cycP-gp120 can serve as a potent vaccine immunogen in rhesus macaques, warranting future studies to further examine its efficacy in HIV/SHIV challenge studies.

Discussion

Recent efforts in HIV-1 vaccine design have focused on the design and implementation of novel trimeric HIV-1 Env immunogens with the goal of generating protective antibody responses, such as bNAbs (185) and V1V2-directed antibodies (186). In this study, we characterized the immunogenicity of a trimeric cycP-gp120 protein in rabbits and rhesus macaques and demonstrate that cycP-gp120 is a robust HIV-1 Env immunogen, capable of generating high titers of high avidity HIV-1 Env specific antibodies, tier-1A and -1B neutralizing antibodies, broadly cross-reactive V1V2 and V2 hotspot directed antibodies, and induces ADCC activity in MVA primed rabbits. These results highlight the potential of cycP-gp120 protein to serve as an optimal protein immunogen for homologous and heterologous prime/boost HIV-1 vaccines.

A striking feature of cycp-gp120 is the ability to induce broadly cross-reactive V1V2-directed antibody responses, a major correlate of decreased HIV-1 risk in the RV144 trial (85, 174, 184), measured against a global panel of multi-clade HIV-1 gp70-V1V2 scaffolds. Furthermore, we show that cycP-gp120 induces heterologous, cross-clade reactive antibodies targeting specific regions of the V2 loop that are associated with decreased risk of infection (96). The mechanisms by which anti-V1V2 antibodies mediate protection from HIV-1 infection are not completely understood, however it was hypothesized that non-neutralizing effector functions such as antibody dependent cellular cytotoxicity (ADCC) or Fc-mediated phagocytosis may be important. The lack of ADCC activity in the cycP-gp120 immunized rabbits despite high V1V2 suggests that V1V2-directed antibody responses alone may not be an indicator of ADCC potential. Both

cycP-gp120 alone immunized and MVA-HIV primed cycP-gp120 boosted rabbits have similar levels of V1V2-directed breath and JRFL-V2 hotspot directed antibody responses, yet MVA-HIV primed cycP-gp120 boosted rabbits generated enhanced ADCC activity against HIV-1 JRFL infected target cells, suggesting some other parameters are necessary for ADCC activity.

While ADCC is thought to be a potential function in which non-neutralizing antibodies aid in protection from infection, there have been other proposed mechanisms by which these antibodies act. It has been reported that the V2 loop of HIV-1 gp120 binds to the integrin $\alpha_4\beta_7$ expressed on activated $CD4^+$ T-cells, mediating viral uptake and infection (9, 90), which could potentially be abrogated by anti-V1V2 antibodies, although this may not be applicable for all HIV-1 strains (187). As discussed above, multiple studies have demonstrated the importance of the V2-region for generating protective antibody responses. Alignment analysis of the gp70-V1V2 scaffolds tested in this study show that the V2 region proximal to the beta-strand C of the V1V2 region is generally more conserved than the hypervariable V1 loop, suggesting that the cross-reactive gp70-V1V2 binding antibodies induced by cycP-gp120 may be focused to this more conserved region in V2 (data not shown). Furthermore, the design of cycP-gp120 involves the introduction of hCMP trimerization domain within the V1 loop, which may result in focusing the antibody response away from the V1 loop to the V2 region (129).

One of the potential issues with gp41 containing HIV-1 Env immunogens in humans is the induction of gp41-biased antibody responses. Thus, a major limitation of gp140-based immunogens, including the SOSIP versions, could be the strong induction of gp41 specific binding antibody response as they may promote cross-reactive

antibodies specific for host proteins as well as microbiota derived antigens (127). A key feature of cycP-gp120 is the lack of the gp41 domain, and we predict that immunization with cycP-gp120 in humans would not induce and potentially diverge the antibody response towards these non-protective epitopes, and instead promote antibody responses to relevant epitopes found on the outer domain of gp120. Additionally, antibody responses primed by viral vectors presenting full-length gp160 would preferentially be boosted towards gp120 localized epitopes rather than gp41 epitopes in a prime/boost vaccination model, which could result in an overall more protective antibody response.

Currently, most soluble trimeric HIV-1 Env protein immunogens are based on HIV-1 Env gp140, a modified version of gp160 lacking the trans-membrane domain of gp41. Most notably, the SOSIP.664 gp140 trimer has been well characterized as a properly formed trimeric gp140 immunogen that displays epitopes for bNAbs, and recent work has shown induction of autologous tier-2 neutralizing antibodies in non-human primates (121, 122, 188). While immunization with cycP-gp120 trimer induced tier-1B neutralizing antibodies, we did not see generation of tier-2 neutralizing antibodies. However, our previous studies with cycP-gp120 in guinea pigs did show induction of tier-2 neutralizing antibodies (129). This discrepancy could be due to differences in immunization regimen, lack of adequate number of immunizations or the species used. In the guinea pig study, we used two DNA primes and two protein boosts with cycP-gp120 whereas in the current study we used only two cycP-gp120 protein boosts. Indeed, recent evidence indicates that repeated germinal center reactions during multiple immunizations help with the generation of neutralizing antibodies (176). In our study neutralizing activity was often developed in conjunction with a strong boost in the overall anti-gp120

binding antibody response, suggesting that the generation of tier-1A and -1B neutralizing antibodies could be a consequence of the overall increase in the magnitude of the response. However, rabbits immunized with monomeric gp120 did not develop tier-1B neutralizing antibody responses, even after repeated immunizations and titers similar to cycP-gp120 immunized rabbits, indicating that the anti-Env titers alone do not predict neutralizing activity.

It is important to note that cycP-gp120 based trimeric immunogens provide multiple advantages compared to SOSIP or NFL gp140 trimeric immunogens. First, since nearly 100% of the expressed protein exists in the trimeric form, purification can be done in a single step using lectin affinity compared to multiple laborious steps required to purify gp140 trimers. As a result, the protein yields will be significantly greater with cycP-gp120 immunogens, which is a critical factor for clinical translation. Second, as described above, cycP-gp120 immunogens lack gp41 region and thus can avoid gp41 dominant antibody response in humans. Third, we do not yet know if gp140 trimeric immunogens can induce a broadly cross-reactive anti-V1V2 antibody response, antibody response against the V2 hotspot region or generate ADCC activity against HIV-infected cells.

The vaccine-mediated protection seen in RV144 trial demonstrated the effectiveness of poxvirus prime/protein boost vaccine strategy over a protein alone vaccine used in Vaxgen trial (101). In this study we show that cycP-gp120 is an effective boosting immunogen in animals rabbits with poxvirus (MVA-HIV) vectors, promoting neutralizing antibodies, broad V1V2 breath, V2-hotspot directed antibodies, and ADCC activity. Similarly, in rhesus macaques cycP-gp120 acts as a potent boosting

immunogen, inducing neutralizing antibodies and desirable V1V2-directed antibody responses. Going forward, it would be important to compare cycP-gp120 to other promising trimeric protein immunogens such as SOSIP and NFL versions that can induce autologous tier 2 neutralizing antibody response. In addition, it would also be important to test the effects of other recently described Env trimer stabilizing mutations in cycP-gp120 background on its immunogenicity. Nevertheless, given our highly encouraging immunogenicity results combined with its relative ease of purification, we believe cycP-gp120 has great potential as a vaccine candidate. Thus, we believe these results warrant further studies to characterize cycP-gp120 in non-human primate challenge models.

Methods:

Ethics Statement

All housing and experiments involving rhesus macaques were conducted at the Yerkes National Primate Research Center, and protocols were approved by the Emory University Institutional Animal Care and Use Committee (IACUC) protocol YER-2002080. Experiments were carried out in accordance to USDA regulations and recommendations derived from the Guide for the Care and Use of Laboratory Animals. Rhesus macaques were housed in pairs in standard non-human primate cages and provided with both standard primate feed (Purina monkey chow) fresh fruit, and enrichment daily, as well as free access to water. Immunizations, blood draws, and other sample collections were performed under anesthesia with ketamine (5-10 mg/kg) or telazol (3-5 mg/kg) performed by trained research staff. Rabbits were housed and immunized by Bioqual Inc. (Rockville, MD) in accordance with IACUC protocol 14-R133.

Animals and Immunizations

Rabbits - Twelve female (4 animals/group) New Zealand White rabbits (age 10-12 weeks) were immunized intramuscularly with 20 ug of recombinant JRFL-gp120 (weeks 0, 8, 16, 32) or JRFL-hCMP-V1cyc-gp120 (cycP-gp120) (weeks 0, 8). Both JRFL-gp120 and cycP-gp120 contain the E168K mutation. An additional group was immunized intramuscularly with $1x10^8$ pfu MVA-62BSm (weeks 0, 8) followed by boosting with cycP-gp120 (weeks 16, 32). JRFL-gp120 and cycP-gp120 immunizations were given with the adjuvant Adjuplex (Sigma). The production of cycP-gp120 was as described

previously (129). JRFL-gp120 was a kind gift from Dr. Richard Wyatt at the Scripps Research Institute.

Rhesus macaques - Four Indian rhesus macaques were immunized intramuscularly with two DNA primes (weeks 0, 12) followed by two MVA-62Bsm boosts (weeks 16, 24), both vaccines encoding HIV-1 clade B HxB2 gag/pol and ADA env. In addition, animals were boosted subcutaneously with 100 ug of JRCSF-cycP-gp120 with Adjuplex at weeks 56, 58.

ELISA and Avidity Assays

Rabbit sera IgG binding to recombinant JRFL-gp120 was measured using JRFL-gp120 (immune-tech) coated ELISA plates and reacted against serial dilutions of sera before detection with anti-rabbit IgG HRP (Southern Biotech). Endpoint titers were measured by calculating the serum dilution required to reach an optical density (O.D) of 0.16, four times the background level of detection. Antibody avidity was calculated by incubating JRFL-gp120 or cycP-gp120 coated ELISA plates with serial dilutions of rabbit sera before incubation for 10 minutes with PBS or 1.5M sodium thiocyanate (NaSCN). Plates were then washed and bound antibodies detected using anti-rabbit IgG HRP. The avidity index was determined by measuring the ratio of the area under curve (AUC) values for PBS or NaSCN treated sera. For rabbit sera binding to V2 peptides, 15-mer peptides corresponding to the HIV-1 C.1086 V2 region were synthesized (Genemed Synthesis Inc.) and diluted in PBS before coating onto ELISA plates (1 ug/ml). Serial dilutions of rabbit sera were reacted against V2 peptides and area under curve analysis (AUC) was used to measure binding. Additional peptides corresponding to the V2-hotspot region of HIV-1 strains JR-FL-E168K (N-RDKVQKEYALFYKLD-C), ADA (N-

RDKVKKDYALFYRLD-C), SF162P3 (N-GNKMQKEYALFYRLD-C), 1157 (N-RDKKQKVYALFYRLD-C), and 1157-Y173H (N-RDKKQKVHALFYRLD-C) (183) were synthesized (Genemed Synthesis Inc.), and binding by rabbit sera diluted 1:100 was measured by OD450 reading. Rhesus macaque serum IgG binding to recombinant HIV-1 SF162 (clade-B) gp140 (immune-tech) was measured by ELISA against serial dilutions of sera and detected with anti-rhesus IgG HRP (Southern Biotech), before quantification against a standard curve against rhesus IgG.

Neutralization Assays

Neutralizing antibody activity was measured in 96-well culture plates by using Tat-regulated luciferase (Luc) reporter gene expression to quantify reductions in virus infection in TZM-bl cells. TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program, as contributed by John Kappes and Xiaoyun Wu. Assays were performed with HIV-1 Env-pseudotyped viruses as described previously (189). Test samples were diluted over a range of 1:20 to 1:43740 in cell culture medium and pre-incubated with virus (~150,000 relative light unit equivalents) for 1 hr at 37 ^{o}C before addition of cells. Following 48 hr incubation, cells were lysed and Luc activity determined using a microtiter plate luminometer and BriteLite Plus Reagent (Perkin Elmer). Neutralization titers are the sample dilution at which relative luminescence units (RLU) were reduced by 50% compared to RLU in virus control wells after subtraction of background RLU in cell control wells. Serum samples were heat-inactivated at 56^{o}C for 1 hr prior to assay. Positive values were reported as being at least 3x baseline values standardized against the negative control virus, SVA-MLV.

Binding Antibody Multiplex Assay (BAMA) measuring gp120, gp140, gp70-V1V2 scaffold binding

BAMA assays were performed as described previously (85, 125). Briefly, serial dilutions (starting at 1:80, six five-fold dilutions) of rabbit or rhesus macaque sera were reacted against beads conjugated to a panel of gp120 (strains 51802, BORI, BJOX002, 254008, CNE20, TT31P.2792, B.6240, A244), uncleaved trimeric gp140 (strains RHPA4259, AE.01.con_env03, 1086.C, C.CH505TF, WITO4160, BF1266, 9004S, SC42261), and gp70-V1V2 scaffold proteins (strains B.CaseA, 7060101641, CM244.ec1, TV1.21, 001428.2.42, CAP210.2.00.E8, C2101.c01, BJOX002000.03.2, BF1266_431a, 96ZM651.02, RHPA4259.7, Ce1086_B2, 62357.14, 700010058, 191084_B7, TT31P.2F10.2792) representing the global panel of HIV-1 Envs. Binding of sera to beads was detected using a secondary biotin-conjugated anti-rabbit IgG and measured via Bio-Plex. Binding is reported as Area Under Curve (AUC) analysis generated from measured MFI's at different serial dilutions. Criteria for positive reactivity was as follows: MFI at 1:80 > 100, MFI at 1:80 > Ag-specific cutoff (95^{th} percentile of all pre-bleed for study for each antigen), MFI > 3-fold that of matched pre-bleed before and after blank bead subtraction.

Antibody Dependent Cellular Cytotoxicity (ADCC) assays

ADCC activity was measured using a luciferase (Luc) based assay against HIV-1 subtype B JR-FL IMC-infected target cells performed as previously described (190). Target and effector cells were co-cultured with serial dilutions of rabbit sera taken from week 0, 10,

18, and 34. Killing activity was detected by reduction in Luciferase activity in each well. Results were considered positive if the % specific killing was >15% after subtraction of activity observed at baseline. ADCC Ab titers were defined as the reciprocal of the highest dilution indicating a positive response.

Statistical analysis.

Statistical analysis was performed using Graphpad Prism v7.0. A two-tailed non-parametric t-test (Mann-Whitney Test) was used for all comparisons unless otherwise noted ($*p < 0.05$). Spearman correlations were used to analyze relationships between multiple variables.

Acknowledgements

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Chapter 2 Figures

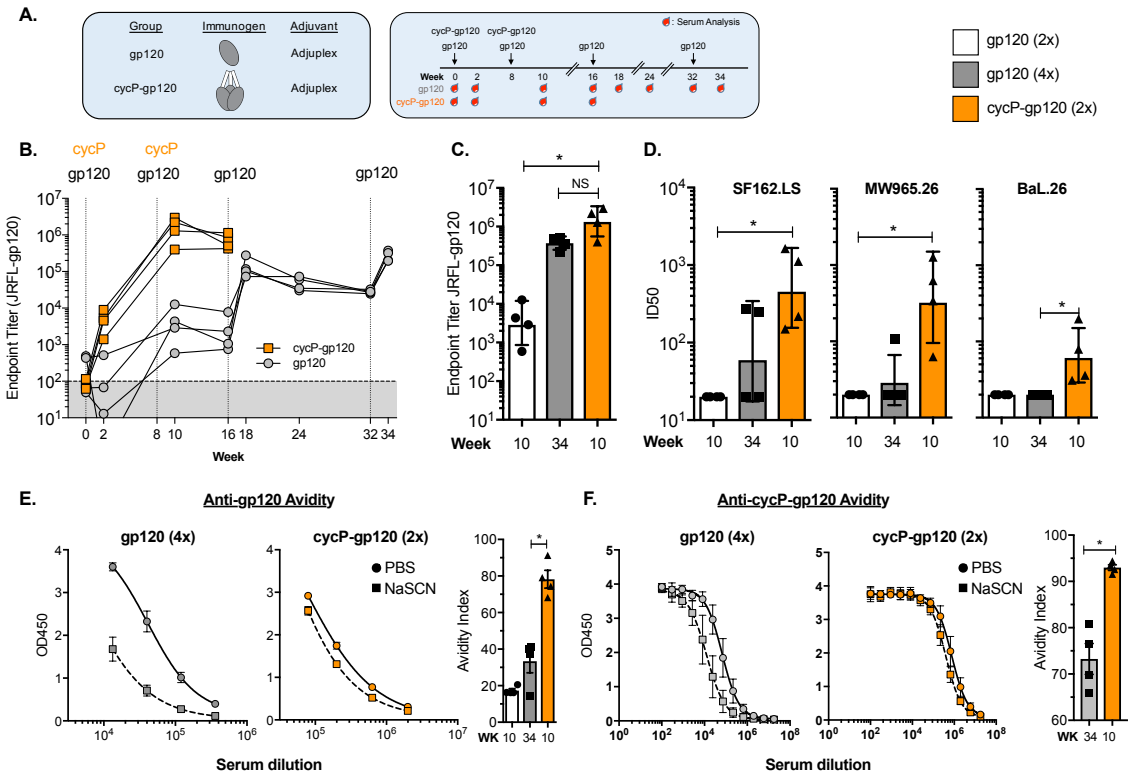


Figure 1: CycP-gp120 induces high titers of high avidity anti-gp120 antibodies and tier-1A and -1B neutralizing antibodies in rabbits. (**A**) Schematic of immunogens and trial design. Rabbits were immunized with cycP-gp120 on weeks 0 and 8, or monomeric gp120 on weeks 0, 8, 16, and 32. Serum was collected on weeks 0, 2, 10, and 16 for both groups, and additionally on weeks 18, 24, 32, and 34 for monomeric gp120 immunized rabbits. (**B**) Kinetics of anti-JRFL gp120 specific IgG titers in serum, measured by endpoint titer. Black dotted lines indicate week of immunization. (**C**) Comparison of JRFL-gp120 specific IgG titers in serum at two weeks post the indicated immunization. (**D**) Neutralizing antibodies measured using TZM-bl cell-based assays against tier-1A (SF162.LS, MW965.26) and -1B (BaL.26) viral isolates. (**E**) Avidity of JRFL-gp120 and (**F**) cycP-gp120 specific IgG measured using 1.5M sodium thiocyanate (NaSCN).

Avidity Index was calculated by measuring the ratio of area under curve (AUC) values of NaSCN to PBS treated samples multiplied by 100. *, $p < 0.05$ (Mann-Whitney Test); ID50, 50% viral infectivity.

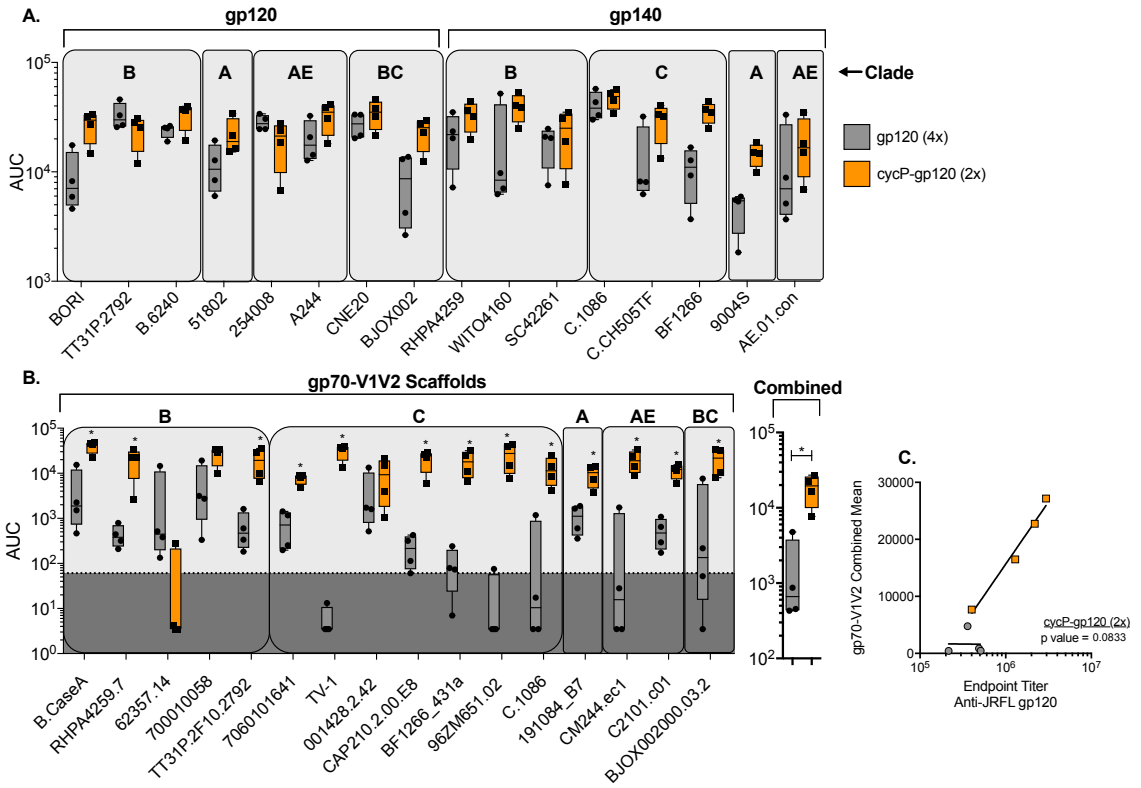


Figure 2: Binding Antibody Multiplex Assay (BAMA) analysis of rabbit immune sera reactivity to a global panel of HIV-1 gp120, gp140, and gp70-V1V2 scaffolds. Serial dilutions of sera from rabbits immunized with gp120 (4x) or cycP-gp120 (2x) were reacted against a multi-clade panel of gp120/gp140 (**A**), and gp70-V1V2 (**B**) scaffold proteins and detected by a binding antibody multiplex assay (BAMA). Data reported as area under curve (AUC) analysis. Individual animal data points are plotted over box-and-whiskers plot. Combined AUC values were measured by calculating average AUC for each animal against all gp70-V1V2 scaffold proteins. Shaded area represents threshold for positive reactivity. (**C**) Correlation analysis of gp70-V1V2 Combined AUC of each immunized animal with their respective anti-JRFL endpoint titers (**Fig. 1c**). *, $p < 0.05$ (Mann-Whitney Test), Spearman correlation test.

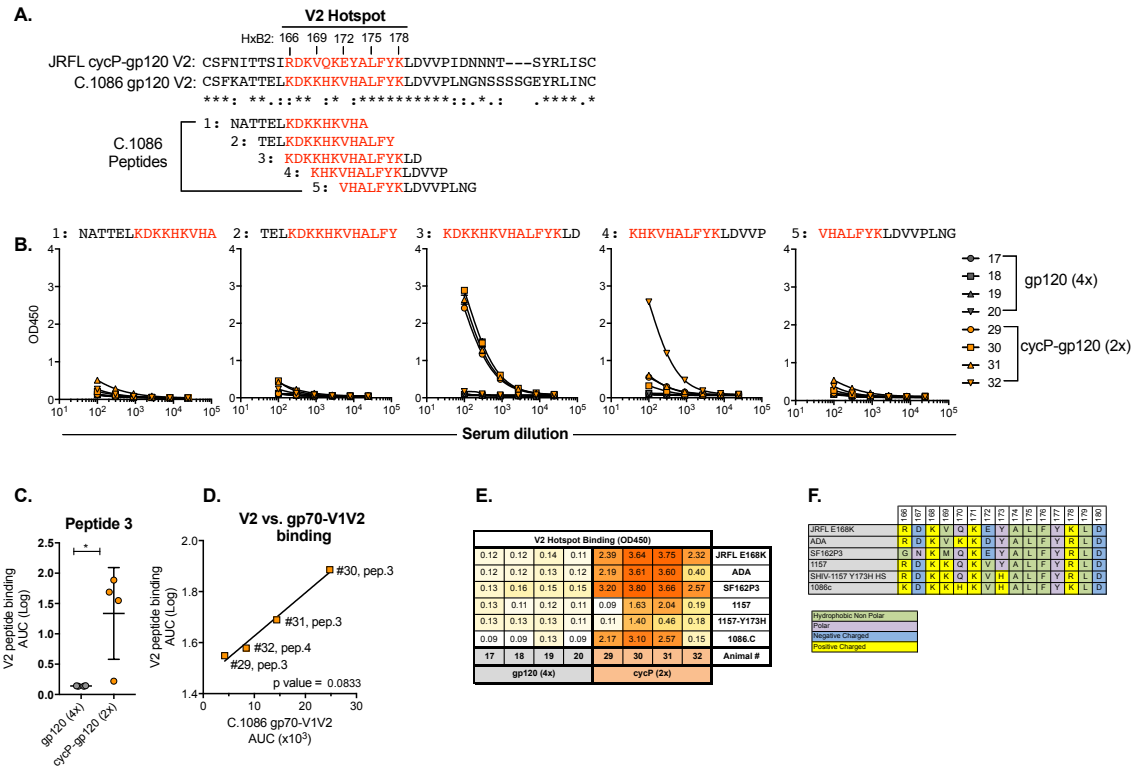
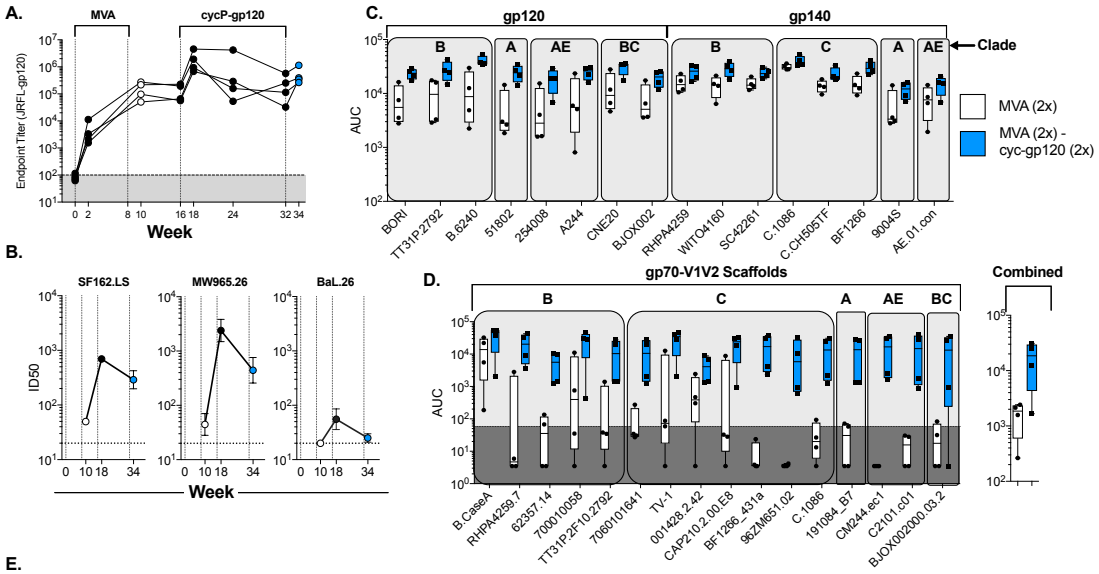


Figure 3: V2-directed antibodies generated by cycP-gp120. (**A**) Alignment of V2 regions of cycP-gp120 and C.1086 gp120. V2 hotspot is indicated in red. (**B**) Overlapping 15-mer peptides derived from C.1086 were reacted against sera from rabbits immunized with gp120 (4x) or cycP (2x) and antibody binding detected by ELISA. Lines represent individual animals, animal number indicated in legend. (**C**) Area under curve analysis of binding to peptide 3. (**D**) Correlation analysis of gp70-V1V2 1086C scaffold binding (**Fig. 2b**) for each cycP-gp120 immunized animal with their respective binding to the indicated V2 peptide. Rabbits #29, 30, and 31 are matched against peptide #3. Rabbit # 32 is matched against peptide #4. (**E**) V2-hotspot binding to peptides derived from different HIV-1 strains, detected by ELISA binding, measured by OD450. (**F**) Sequences of V2-hotspot peptides used in (**E**). *, $p < 0.05$ (Mann-Whitney Test), Spearman correlation test.



E.

V2 Hotspot Binding (OD450)								
0.08	0.08	0.11	0.10	0.21	3.83	3.82	0.72	JRFL E168K
0.08	0.39	0.12	2.36	0.25	3.39	2.34	3.30	ADA
0.09	0.10	0.13	0.10	0.25	3.40	3.66	0.37	SF162P3
0.06	0.06	0.09	0.08	0.21	1.06	0.51	0.24	1157
0.07	0.08	0.11	0.08	0.27	2.38	1.78	0.27	1157-Y173H
0.06	0.06	0.08	0.06	0.17	3.46	3.64	0.30	1086.C
33	34	35	36	33	34	35	36	Animal #
MVA (2x)				MVA (2x) cycP (2x)				

Figure 4: Boosting MVA-primed rabbits with cycP-gp120 promotes neutralizing antibodies, increased gp120/gp140, and gp70-V1V2 directed antibodies. Rabbits were immunized twice with MVA-HIV on weeks 0 and 8, and boosted with cycP-gp120 protein on weeks 16 and 32. (**A**) Kinetic analysis of JRFL-gp120 specific IgG endpoint titers measured via ELISA. (**B**) Neutralizing antibodies measured at weeks 10, 18, and 34 against tier-1A and -1B viruses, reported as ID50 concentrations. (**C-D**) BAMA analysis of sera from week 10 and 34 against gp120/gp140 proteins (**C**) and gp70-V1V2 (**D**) scaffold proteins, reported as area under curve (AUC) analysis. Shaded area represents threshold for positive reactivity. (**E**) V2-hotspot binding, detected by ELISA, from week 10 and week 34 sera.

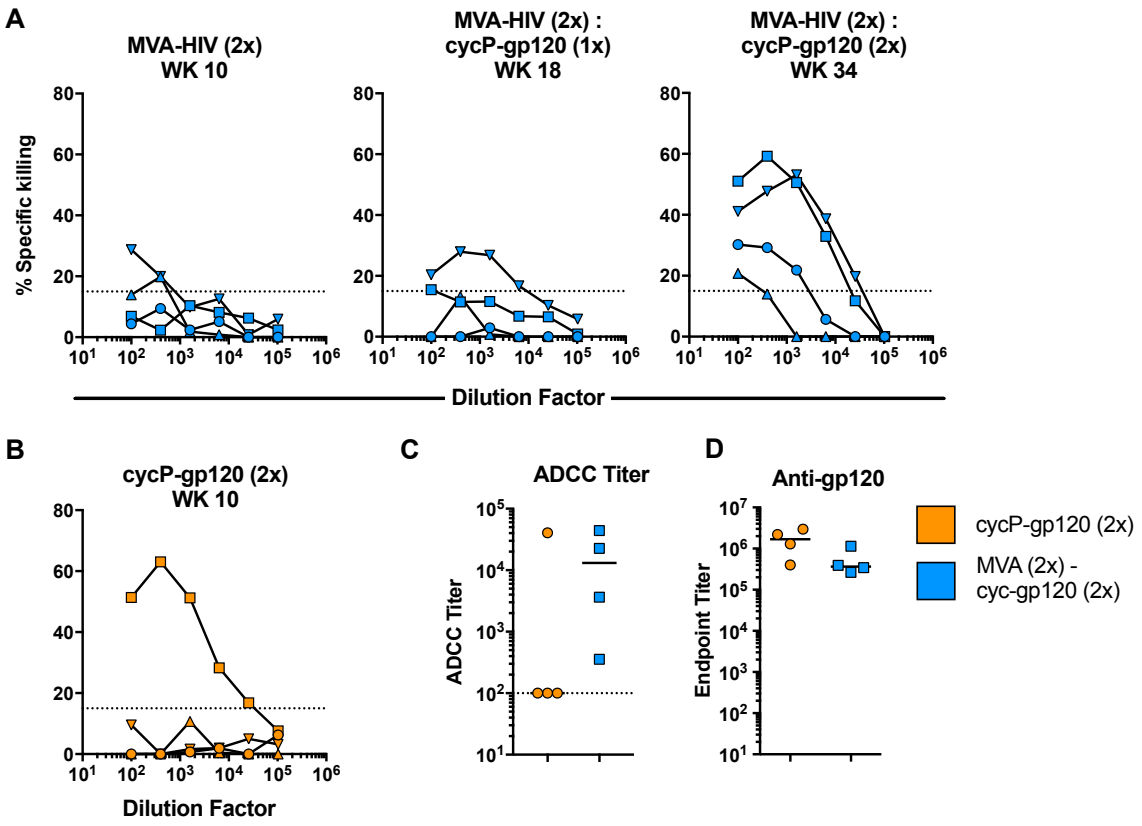


Figure 5: ADCC activity in rabbits immunized with cycP-gp120 or MVA-HIV prime cycP-gp120 boost. ADCC activity against JRFL-infected target cells measured against sera from weeks 10, 18, and 34 in rabbits immunized MVA-HIV followed by boosting with cycP-gp120 (**A**) and week 10 from rabbits immunized with cycP-gp120 alone (**B**). % Specific killing values were subtracted from week 0 sera to remove background ADCC activity. (**C**) ADCC titer and (**D**) anti-gp120 serum IgG titer comparison between cycP-gp120 (2x) and MVA-HIV (2x) – cycP-gp120 (2x) immunized rabbits. ADCC Ab titers were defined as the reciprocal of the highest dilution indicating a positive response.

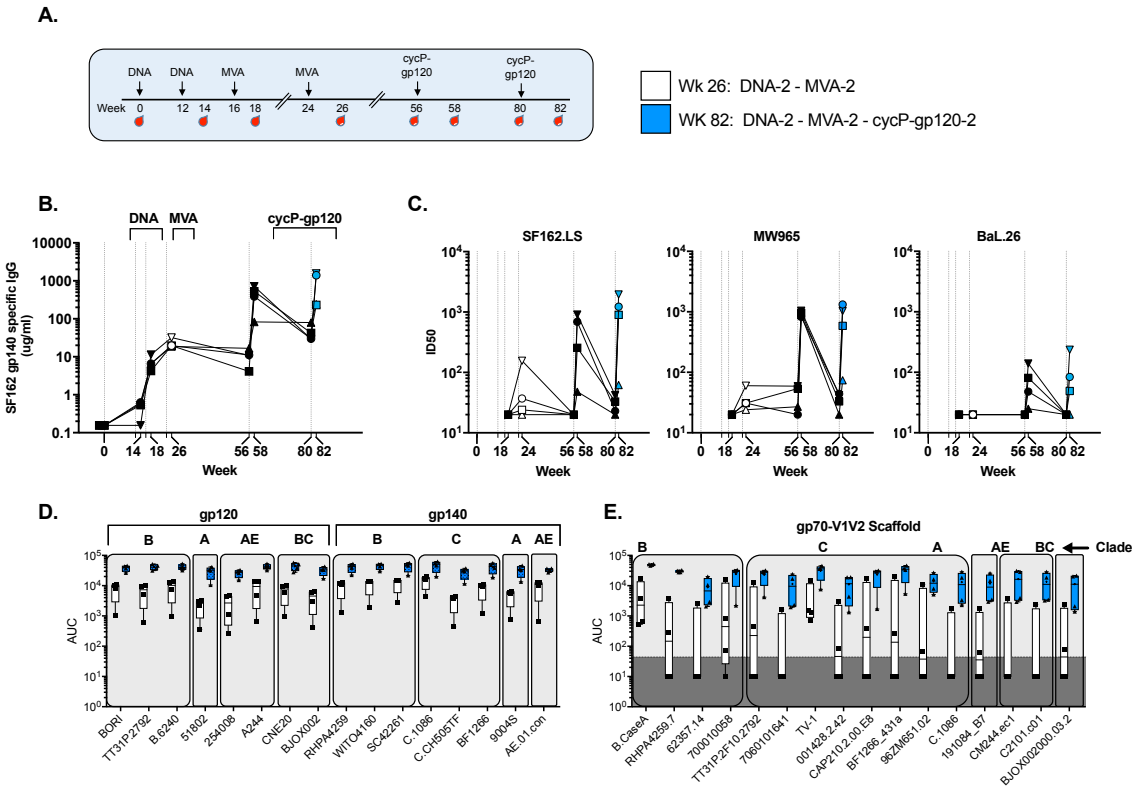


Figure 6: Boosting rhesus macaques primed with a DNA/MVA vaccine with cycP-gp120. (**A**) Study design. Four Rhesus macaques were immunized with a DNA expressing HIV-1 gag/pol/env on weeks 0 and 12 and boosted with MVA-HIV on weeks 16 and 24 before boosting with recombinant JRCSF cycP-gp120 protein on weeks 56 and 80. (**B**) Kinetic analysis of sera IgG specific for HIV-1 SF162 gp140 (ug/ml). (**C**) Neutralizing antibodies against tier-1A (SF162.LS, MW965) and -1B (BaL.26). (**D**) BAMA analysis of week 26 and week 82 sera against a global panel of HIV-1 gp120, gp140 or gp70-V1V2 (**E**) scaffold proteins, reported as area under curve (AUC).

Group	Animal ID	HIV-1 Isolate						
		SVA-MLV	MW965.26	ADA/293T	JR-FL	SF162.LS	BaL.26	TRO.11
		Neg. Ctrl.	Tier 1A	Tier 2	Tier 2	Tier 1A	Tier 1B	Tier 2
			Clade C	Clade B	Clade B	Clade B	Clade B	Clade B
gp120(2)	17	<20	<20	<20	<20	<20	<20	<20
	18	<20	<20	<20	<20	<20	<20	<20
	19	<20	<20	<20	<20	<20	<20	<20
	20	<20	<20	<20	<20	<20	<20	<20
gp120 (4x)	17	<20	**109**	<20	<20	**273**	<20	<20
	18	<20	<20	<20	<20	**250**	<20	<20
	19	<20	**20**	<20	<20	<20	<20	<20
	20	<20	<20	<20	<20	<20	<20	<20
cycP-gp120 (2x)	29	<20	**340**	<20	<20	**218**	**31**	<20
	30	<20	**625**	<20	<20	**1116**	**79**	<20
	31**	<20	**1267**	<20	<20	**1637**	**198**	**41****
	32	<20	**63**	<20	<20	**150**	**36**	<20

Table 1: Neutralizing antibody titers against tier-1A, -1B, and -2 HIV-1 isolates. Neutralizing antibodies generated by gp120 (2x), gp120 (4x), or cycP-gp120 (2x) immunization in rabbits measured by TZM-bl assay against a panel of tier-1A, -1B, and -2 HIV-1 isolates. Data reported as ID50, dilution of sera required to inhibit 50% of viral infectivity. Positive values reported as > 3x the background signal against SVA-MLV. **Cell toxicity noted in first two dilutions of animal # 31 with all viruses, titers of up 1:180 may not reflect true neutralizing activity.

Chapter III: Systemic or Oral MVA/Protein HIV-1 immunization with a needle-free injector induces protective antibody responses in rhesus macaques

Andrew T. Jones[1,2], Korey Walter[3], Xiaoying Shen[4], Celia C. LaBranche[5], Linda S. Wyatt[6], Georgia Tomaras[4], David C. Montefiori[5], Bernard Moss[6], Dan H. Barouch[7], John D. Clements[8], Pamela A. Kozlowski[3], Raghavan Varadarajan[9], and Rama Rao Amara[1,2]

[1]Emory Vaccine Center, Yerkes National Primate Research Center, Emory University, Atlanta Georgia, USA; [2]Department of Microbiology and Immunology, Emory School of Medicine, Emory University, Atlanta, Georgia, USA; [3]Department of Microbiology, Immunology and Parasitology, Louisiana State University Health Sciences Center, New Orleans, LA, USA; [4]Duke Human Vaccine Institute, Duke University Medical Center, Durham, North Carolina, USA; [5]Department of Surgery, Duke University Medical Center; Durham, NC, USA; [6]Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA ; [7]Ragon Institute of MHG, MIT, and Harvard, Boston, MA, USA; [8]Department of Microbiology and Immunology, Tulane University School of Medicine, New Orleans, LA, USA; [9]Molecular Biophysics Unit, Indian Institute of Science, Bangalore, India,

Abstract

In the immediate hours and days post mucosal transmission, HIV-1 is at a vulnerable state due to localized replication and low or unestablished viral reservoirs. Thus, vaccines that induce strong mucosal immune responses are ideal for targeting HIV-1 at this state. Here we evaluate the sublingual and buccal tissues (SL/B) as a route of oral mucosal vaccination in rhesus macaques compared to systemic vaccination. To aid in antigen uptake we utilized a modified needle-free injector to deliver immunizations across the mucosal epithelium into the underlining tissue. Rhesus macaques were immunized twice with a modified vaccinia Ankara (MVA) vaccine expressing HIV-1 Gag, Pol and Envelope antigens, followed by two immunizations with the recombinant trimeric cycP-gp120 immunogen along with the mucosal adjuvant dmLT. Animals were immunized via three different routes, needle-free sublingual and buccal injection (n = 5), topical application to the sublingual and buccal tissue (n = 4), or the conventional intradermal (MVA) and subcutaneous (protein) routes (ID/SC). All animals were challenged intrarectally approximately 5 months after the final immunization with a pathogenic SHIV-SF162P3 for a maximum of 6 challenges. Systemic immunization (ID/SC) induced strong IgG responses in serum and mucosal secretions but failed to induce IgA responses. Impressively, needle-free oral immunization generated a robust HIV Env-specific IgG and IgA antibody response both in blood and mucosal compartments that are at least 10-fold higher compared to responses in ID/SC immunized animals. Following intrarectal challenge, all 5 controls became infected by 3 challenges and we observed a significant delay in acquisition of infection in both vaccinated groups ($p= 0.02$ for oral, $p= 0.007$ for ID/SC and $p= 0.002$ for combined with an estimated

vaccine efficacy of ~70% per exposure) compared to unvaccinated controls. Two of the six ID/SC animals remained uninfected at the end of 6 challenges. Correlates of protection include binding antibodies to a key region of the V2-loop associated with protection in the RV144 trial, as well as antibody-mediated cell-mediated virus inhibition at the pre-challenge timepoint. Our results show that SL/B immunization via a needle-free injector is a practical route to generate both systemic and mucosal antibodies, as well as the protective efficacy of MVA prime followed by gp120 trimer boost.

Introduction

Human Immunodeficiency virus (HIV) -1 is most commonly transmitted across mucosal surfaces via sexual contact. Within in the first days and weeks of infection, HIV-1 is localized to the mucosal tissue, replicating in close-by target cells, before systemic dissemination, seroconversion, and massive CD4+ T-cell depletion (42). During this early phase, however, HIV-1 is in a vulnerable state. The viral reservoir is limited in size or unestablished, and the genetic bottleneck and host factor derived pressure reduces the viral founder population (191). The induction of immune effectors present at mucosal tissues via vaccination may therefore be critical in preventing HIV-1 infection. This hypothesis is supported by numerous studies in non-human primates. The vaccine-mediated protection from simian immunodeficiency virus (SIV) via rhesus cytomegalovirus vectors (RhCMV) expressing SIV genes is attributed to a strong effector memory T-cell response present in both blood and mucosal tissues which can immediately target and kill SIV-infected cells upon mucosal transmission (192). Furthermore, passive transfer studies with broadly neutralizing antibodies (bNAbs) demonstrate that bNAbs transferred to non-human primates before or during the initial acute phase can confer sterilizing immunity and eliminate viral reservoirs, but transferring bNAbs post the acute phase will result in a transient control of viremia and selection for escape viral variants (140, 193). These studies suggest that an effective HIV-1 vaccine should combat and clear the virus before dissemination and viral escape can occur.

Mucosal vaccination is an effective method of generating both mucosal and systemic immune responses. Mucosal vaccines are also attractive as they are relatively

non-invasive and can be administered on a large scale (135). Mucosal vaccines for HIV-1 have been heavily studied in animal models but there have been few human clinical trials evaluating mucosal vaccination and mucosal responses have rarely been characterized in previous clinical trials (194). Multiple routes of mucosal HIV/SIV immunization have been investigated in non-human primates, including intrarectal, intravaginal, intranasal, intratracheal, oral, and sublingual delivery. While these routes have been shown to induce mucosal responses, each have disadvantages such as intrarectal and intravaginal being impractical for mass vaccination and generating primarily local immune responses, neurological risks associated with intranasal immunization, and the degradation of antigens in of oral immunizations, as well as the induction of oral tolerance.

Oral vaccination conventionally involves the ingestion of an antigen which must survive the harsh conditions of the stomach to be taken up in the gut-associated lymphoid tissue (GALT) to prime immune responses. Examples of licensed oral vaccinations for humans include the live-attenuated polio vaccine, Rotarix and RotTeq for rotavirus infections, and the cholera vaccine (195). Current strategies in oral vaccine development involve using enteric-coated capsules that survive through the stomach intact to be dissolved in the small intestine (196) and engineering commensal bacteria to express vaccine antigens on the cell wall (197). An alternative strategy for oral vaccination is to target the sublingual (below the tongue) and buccal (inner cheek) tissue within the oral mucosa for antigen delivery, bypassing the need for the antigen to travel through the stomach and small intestine. While this method does not directly result in antigen uptake in the GALT, studies have shown that sublingual immunization can result in cellular and

humoral immune responses in the female genital tract, demonstrating that immunization of the oral mucosa can induce gut-homing adaptive responses (198, 199).

Unlike the single cell columnar epithelium in small intestine, the sublingual and buccal epithelium consists of a stratified squamous epithelium that may inhibit the natural uptake of vaccine antigens (158). Delivery of antigens into the sublingual and buccal tissue can be aided by needle-free injectors, which drive the immunizations through the epithelium into the underlining tissue. Needle-free injectors have been extensively studied as a tool to deliver drugs and vaccinations through the skin without the use of a needle (48, 200). Most needle-free injectors are designed to deliver injections intramuscularly through the arm, however needle-free injectors for sublingual immunization are also being developed (201). There are multiple advantages of needle-free injectors over needle-based delivery, especially in the oral tissue, as they are non-invasive and allow for dispersal of vaccine antigens into the underlying tissue rather than concentrated at an injection site. In this study, we will evaluate a needle-free injector in the delivery of HIV-1 vaccines to the sublingual and buccal tissue of rhesus macaques and compare this delivery to topical application of immunizations to the oral tissues, as well as systemic immunization via intradermal and subcutaneous injection.

A major focus of HIV-1 vaccine research is the design and characterization of novel immunogens that can promote favorable antibody responses. While the generation of broadly neutralizing antibodies (bNAbs) is a major goal of HIV-1 vaccine research, results from the RV144 trial demonstrated that non-neutralizing antibodies against the V1V2 loop of HIV-1 gp120 correlates with decreased risk of HIV-1 infection (85). The association of V1V2-directed antibodies in decreased risk or delayed acquisition of

HIV/SIV infection has been shown in non-human primate studies (97-99), demonstrating the importance of V1V2-directed antibodies in protection. Mechanisms of protection via V1V2-directed antibodies are hypothesized to be non-neutralizing antibody effector functionality such as antibody dependent cellular cytotoxicity (ADCC), as V1V2-directed monoclonal antibodies isolated from RV144 vaccinated individuals showed ADCC activity (87). Further studies have identified a key region within the V2 loop, the "V2 Hotspot", which is associated with decreased risk of infection in the RV144 trial (93), and it was found that RV144 vaccinated individuals had increased protection against HIV-1 viruses matching the vaccine strain at position 169, located within the hotspot (96). Thus, vaccine immunogens that generate V1V2-directed antibodies, with an emphasis on V2-loop directed antibodies, are of great interest for HIV-1 vaccine development.

Chapter II of this dissertation characterized the immunogenicity *in vivo* of a novel trimeric gp120 immunogen, generated by the fusion of the trimerization domain of the human matrix cartilage protein (hCMP) to the V1 loop of a cyclically permutated gp120. This immunogen, named cycP-gp120, exists as a trimer and binds to trimer specific V2-apex directed bNAbs such as PGT145 and PGDM1400 (129). In rabbits and non-human primates we found that cycP-gp120 is a robust immunogen, generating highly cross-reactive V1V2-directed antibodies and V2 hotspot directed antibodies (167). As a boosting immunogen for rabbits primed with modified vaccinia Ankara (MVA), a poxvirus engineered to express HIV-1 (MVA-HIV), cycP-gp120 promoted V1V2 and V2 hotspot directed antibodies, as well as ADCC activity.

In this study we characterize further the MVA-HIV prime cycP-gp120 boost regimen in rhesus macaques, delivering vaccines mucosally via needle-free injection to the sublingual and buccal tissue, topical application to the sublingual and buccal tissue, or intradermal and subcutaneous (ID/SC) injection. Immunizations with cycP-gp120 will be adjuvanted with the mucosal adjuvant double mutated heat-labile enterotoxin (dmLT), derived from *Escherichia coli*, which has been shown to boost mucosal immune responses (163, 164). We show that needle-free injection to the sublingual and buccal tissue generates strong systemic and mucosal antibody responses within the rectal and vaginal secretions, significantly higher than ID/SC animals. To test the vaccine efficacy of MVA-HIV/cycP-gp120 immunization, we challenged immunized animals intrarectally with a heterologous pathogenic $SHIV_{SF162P3}$. Both needle-free delivery and ID/SC injection resulted in a significant delay of SHIV infection, with correlates of protection consisting of peak serum IgG binding titers to gp120, rectal IgG specific for gp120 at pre-challenge, antibodies directed towards the $SHIV_{SF162P3}$ V2-hotspot, and antibody dependent cell-mediated viral inhibition (ADCVI). These results demonstrate the potential of MVA-HIV/cycP-gp120 as a possible vaccine candidate in future HIV-1 vaccine trials, as well as needle-free injection to the sublingual and buccal tissue as an effective and easy route to generate strong systemic and mucosal antibody responses.

Results

Antigen presenting cells in the sublingual and buccal tissue

While antigen presenting cells in the sublingual and buccal tissue have been described for mice and humans, they have not been extensively characterized in rhesus macaques (202). Langerhans cells (LCs) are a major subset of migratory dendritic cells in the epidermis and have multiple roles in immunity, from maintaining immune homeostasis in the epidermis to activation and presentation of antigens upon inflammation (203, 204). To characterize LCs in the sublingual and buccal tissue in rhesus macaques, we stained paraffin-embedded sections of rhesus macaque sublingual and buccal tissue with hematoxylin and eosin (H&E) stain to image the general architecture of the epidermis and underlying lamina propria (**Fig. 1a**) and immunohistochemistry with an anti-langerin monoclonal antibody (**Fig. 1b**). LC's could be detected in both the sublingual and tissue, primarily residing in the mucosal epithelium. To further characterize dendritic cell subsets in the sublingual and buccal tissue, biopsied tissue samples were homogenized and analyzed via flow cytometry. Antigen presenting cells were gated as HLA-DR^{+}CD45^{+}CD3^{-}CD20^{-} live cells. In both the sublingual and buccal tissue, we detected a population of CD14+DC-Sign+ dendritic or macrophage like cell (203, 205) (**Fig. 1c**). Additionally, we detected conventional myeloid dendritic cells (CD14^{-}CD16^{-}CD123^{-}BDCA1^{+}), but not plasmacytoid dendritic cells (CD14^{-}CD16^{-}CD123^{+}BDCA1^{-}) which may only be present during inflammation (202). These data show there is a diverse population of antigen presenting cells in the sublingual and buccal tissue, ranging from Langerhans cells to conventional myeloid dendritic cell subsets.

The vitamin A metabolite, retinoic acid, is important for the generating of gut-homing adaptive immune responses (206, 207). Dendritic cells process vitamin A to retinoic acid via retinal dehydrogenase (RALDH) enzymes (208, 209), the activity of which can be detected via the ALDEFLUOR^{TM} kit (STEMCELL) (**Fig. 2a**). To characterize the expression of RALDH enzymes in the sublingual and buccal tissues, as well oral mucosa draining submandibular and submental lymph nodes, we homogenized tissue biopsies, incubated with the ALDEFLUOR^{TM} reagent, and stained for flow cytometry analysis. We found that BDCA1^{+} DCs, gated on CD45^{+}CD3^{-}CD20^{-}HLA-DR^{+} live cells in the submandibular, submental, and the inguinal showed RALDH activity (**Fig. 2b**). However, this activity wasn't found in DCs within the sublingual and buccal tissue themselves, suggesting that tissue DCs activate RALDH activity upon activation and migration to the lymph node, or lymph node resident DCs express RALDH at a basal level. Taken together, this data demonstrates that the oral mucosa contains numerous antigen presenting cell subsets, and the draining lymph nodes contain gut-homing features, suggesting the oral mucosa is a viable route for mucosal vaccination.

Needle-Free Injector for sublingual and buccal vaccination

The stratified squamous epithelium of the sublingual and buccal tissue may hinder the efficient uptake of vaccine antigens. To aid in antigen delivery, needle-free injectors can be used to effectively drive immunizations through the epithelium and into the underlying tissue without the use of a needle stick. In this study we used a modified version of the Syrijet Mark-II Needless Injector (Keystone Industries), designed for the use in dentistry to inject local anesthesia to the oral mucosa (210) (**Fig. 3a**). The caps of

sterile water cartridges used to flush the Syrijet were removed, the water extracted and replaced with immunization preps, and finally replaced with a new sealed cap (**Fig. 3b**). To test the efficiency of using the Syrijet to inject into the sublingual and buccal tissue, we injected an uninfected rhesus macaque with 100 ul of sterile PBS and visualized the injection site before and after injection (**Fig. 3c**). The Syrijet injects effectively into both the sublingual and buccal tissue with minimal damage and bleeding, and no additional swelling or damage was reported in the following days post injection.

Immunization of rhesus macaques

Female rhesus macaques (n = 15) were immunized twice with $1x10^8$ pfu of modified vaccinia Ankara (MVA) expressing HIV-1 clade B *gag, pol, env* (strain ADA, trimeric gp150 on MVA-infected cells and virus like particles) (MVA-HIV) (130) and boosted twice with 100 ug of the recombinant trimeric gp20 (strain JRFL, E168K mutation), cycP-gp120, along with the mucosal adjuvant dmLT (130, 164) (**Fig. 4**) Immunizations were separated into three groups: topical application (n = 4) of immunizations to the buccal and sublingual tissue to allow for the natural uptake of antigens, needle-free injection (n = 5) via Syrijet to the buccal and sublingual tissue, and intradermal (MVA-HIV) and subcutaneous (cycP-gp120 + dmLT) (n = 6) injection to the right and left thighs as a systemic control (ID/SC). Doses were split between the sublingual and buccal tissue or the left and right thigh. Immunizations were done on weeks 0 and 15 for MVA-HIV, and 23 and 31 for cycP-gp120 (for some topical and ID/SC animals, immunizations were done on weeks 0, 5, 17, and 25 though this did not impact the immunogenicity outcome). The mucosal adjuvant dose for the two oral

groups was 25 ug per site injection for the first protein boost, and 1 ug per site for the second protein boost in needle-free injected animals. The ID/SC immunized animals received 1 ug per site of dmLT for both protein immunizations. 19 weeks after the second boost, animals were challenged weekly with an intrarectal challenge of a pathogenic clade-B $SHIV_{SF162P3}$ virus (211). Five unimmunized female macaques were used as an unvaccinated control.

Needle-free injection of the sublingual and buccal mucosa induces a strong systemic and mucosal antibody response

To track the antibody responses to the immunizations in the sera, we performed ELISAs against HIV-1 gp120 (strain ADA) on the day of immunization and two weeks post immunization. MVA-HIV immunization induced gp120-specific serum IgG in both needle-free oral and ID immunized macaques, however we did not detect antibody responses in the topically immunized animals, indicating that needle-free oral injection greatly enhance vaccine uptake (**Fig. 5a**). Surprisingly, upon the first cycP-gp120 boost, we saw a massive expansion in gp120-specific serum IgG in the needle-free oral injected animals (*mean titer, 1289 ug/ml*), almost a log higher than ID/SC (*mean titer, 131 ug/ml*). The antibody responses contracted in both groups before being boosted a second time and contracting to the pre-challenge timepoint (*mean titer, 30 ug/ml vs. 10 ug/ml*). Similar to serum IgG, anti-gp120 serum IgA was significantly expanded in needle-free orally immunized animals upon cycP-gp120 boost (**Fig. 5b**). This data shows that needle-free oral immunization is an effective route to generate high titers of vaccine-specific serum IgG and IgA.

Along with the systemic antibody responses, we monitored the induction of mucosally localized IgG and IgA in the rectal, vaginal, and salivary secretions. Due to the variable amount of total IgG and IgA isolated from secretions, we measured HIV-1 Env specific antibodies as specific activity, calculated as the ratio of ng anti-gp120 IgG or IgA to ug total IgG or IgA. As with what was seen in sera, boosting with cycP-gp120 via needle-free oral injection resulted in a large expansion of rectal, vaginal, and salivary IgG, significantly higher than ID/SC immunization (**Fig. 6a**). Impressively, needle-free oral immunization also induced significant levels of rectal, vaginal, and salivary vaccine-specific IgA, with vaginal IgA remaining significantly higher than ID/SC immunized animals until the pre-challenge timepoint (**Fig. 6b, c**). Additionally, we detected very low to no antibody responses in the topically immunized animals, demonstrating the effectiveness of the needle-free injector in delivering the immunizations across the opal epithelium. From these results we have shown that needle-free oral delivery to the sublingual and buccal tissue is an easy and practical method of generating substantial systemic and mucosal antibody responses.

Neutralizing Antibody Responses

The generation of neutralizing antibodies to HIV-1 is a long-sought goal of HIV-1 vaccination. To test the presence of neutralizing antibodies generated by MVA-HIV/cycP-gp120 immunization, we measured the neutralizing activity of sera against a multi-clade panel of pseudoviruses that have high (SF162.LS, MW965.26), moderate (BaL.26), or low (ADA, JR-FL, TRO.11) neutralization sensitivity (tier-1A, -1B, -2, respectively). Needle-free oral immunization induced a significantly higher titer of

neutralizing antibodies against the neutralization sensitive clade-B SF162.LS and the moderately resistant clade-B BaL.26 isolate after the first and second protein boosts (**Fig. 7**) than ID/SC immunization. At the pre-challenge time point, however, both responses were not significantly different. Additionally, we detected no tier-2 (neutralization resistant) neutralizing antibody responses, which is in line with our previous results (167), suggesting that mutations to cycP-gp120, or additional homologous immunizations (129), may be required to generate tier-2 neutralizing activity.

Boosting with cycP-gp120 induces broadly cross-reactive antibodies to gp120 and gp140 antibodies, and strong cross-reactive V1V2- and V2 hotspot- directed antibody responses

To address the global diversity of HIV-1, an effective vaccine should ideally recognize HIV-1 isolates from multiple strains and clades. To characterize the cross-reactivity of cycP-gp120 induced antibodies, we measured antibodies binding to a global panel of gp120, gp140 and gp70-V1V2 scaffold proteins via binding antibody multiplex assay (BAMA) (212). Upon boosting with cycP-gp120 we observed a strong cross-reactive antibody response against multiple clades of gp120 and gp140 antigens, reacted to by all immunized animals (**Fig. 8a**). Responses trended higher for needle-free orally immunized animals, however at the pre-challenge timepoint both groups had similar levels of reactivity (**Fig. 8b**). These results show the high broadly-reactive antibody response generated by cycP-gp120, a crucial component to an HIV-1 vaccine candidate.

As results from the RV144 trial suggest that antibodies directed towards the V1V2 loop of gp120 are associated with protection (85), the generation of these

antibodies, especially broadly reactive V1V2-directed antibodies, is of great interest in HIV-1 vaccine development. We measured antibody response to Case.A2 gp70-V1V2 (clade-B), the antigen reported in the RV144 analysis, in the serum as well as rectal and vaginal secretions. Needle-free oral immunization resulted in significantly higher titers of anti-gp70-V1V2 responses in the sera following the first protein boost, with similar trends observed in the rectal and vaginal secretions (**Fig. 9a**). To further measure the cross-reactivity of V1V2-directed antibodies, sera was screened against beads conjugated to a panel of 16 gp70-V1V2 scaffolds representing the global diversity of HIV-1, with binding quantified via binding antibody multiplex assay (BAMA). Two weeks post the first protein boost, needle-free orally immunized animals generated a substantial broadly cross-reactive gp70-V1V2 response against multiple clades of isolates, significantly higher that ID/SC immunization, demonstrating not only the high immunogenicity of the sublingual and buccal route, but also the maturation of the antibody responses upon needle-free oral delivery (**Fig. 9b**). At the pre-challenge timepoint, responses to gp70-V1V2 scaffolds have contracted in both groups, with no significance between the groups, suggesting that through contraction and further boosting in ID/SC immunized animals the V1V2 response leveled to a setpoint (**Fig. 9c**)

To measure antibody responses to the V2 Hotspot peptide (spanning positions 166-178 of HIV-1 stain HXB2) (93), we synthesized 13 amino-acid peptides corresponding to the V2 hotspot of cycP-gp120 (clade-B JRFL, E168K), MVA-HIV (clade-c ADA), SHIVSF162P3 (clade-B) (211), and $SHIV_{1157(QNE)Y173H}$ (clade-C) (183). Needle-free oral immunization generated a strong cross-reactive V2 hotspot response, recognizing not only the MVA-HIV and cycP-gp120 vaccine strains (ADA, JRFL

E168K), but also the heterologous $SHIV_{SF162P3}$ and to a lesser extent $SHIV_{1157(QNE)Y173H}$ (**Fig. 10a**). ID/SC immunization did result in detectable V2 hotspot binding responses, but at a much lower level than needle-free oral immunization, and responses between the two groups were similar after the second protein boost (**Fig. 10b**). The cross-reactivity of V2 antibody responses to heterologous strains (**Fig. 10c**) demonstrate the remarkable ability of cycP-gp120 to generate V2-hotspot binding antibodies.

Needle-free sublingual and buccal injection and ID/SC immunization generates significant protection from $SHIV_{SF162P3}$ challenge

To test the vaccine efficacy of MVA-HIV/cycP-gp120, we challenged animals 19 weeks after the second protein boost with a weekly intrarectal challenge (1:100 dilution) of SHIVSF162P3 for maximum of six weeks (211). Five unvaccinated female macaques were used as a control group. All unvaccinated animals were infected by the third challenge, however we detected a significant delay in acquisition in infection in both the needle-free orally immunized ($p = 0.019$) and ID/SC ($p = 0.0074$) immunized animals, with two ID/SC immunized animals remaining uninfected after the sixth challenge (**Fig. 11a, b**). Upon combining the two groups together, we detect an even greater significant protection ($p = 0.0022$) (**Fig. 11c**). Tracking viral loads following infection, we detected a trend for lower set-point viral loads in immunized animals, but these did not reach significance (**Fig. 11d**). This data demonstrates the protective efficacy of MVA-HIV/cyc-gp120 immunization against a heterologous pathogenic SHIV challenge, a crucial feature of a protective HIV-1 vaccine candidate, achievable through either

mucosal vaccination of the sublingual and buccal tissue, or through intradermal and subcutaneous injection.

Immune-correlates of protection from $SHIV_{SF162P3}$ infection

Due to the low sample size in the immunization groups, elucidating clear correlates of protection can be challenging. However, several parameters of the immune response correlated or highly trended with protection from infection. Additionally, as the routes and adjuvant doses for the needle-free oral and ID/SC group have varied, the groups have been separated for many analyses due to confounding factors, such as high vaccine-specific serum IgA, unique each group (85). Binding titers against gp120 (strain ADA) after the first protein boost, the peak of the overall antibody response, trended with delayed acquisition in the needle-free orally immunized, and was significantly correlated with protection in the ID/SC immunized animals ($r = 0.8827$, $p = 0.0333$) (**Fig. 12a**). Similarly, we observed a positive trend in the gp120-specific rectal IgG levels taken at pre-challenge, indicating that mucosally localized rectal IgG may be important for protection from infection (**Fig. 12b**).

Binding antibodies to gp70-V1V2 scaffolds did not correlate with protection (data not shown). However, the peak binding to the JRFL-E168K V2 hotspot strongly correlated with protection in needle-free orally immunized animals ($r = 0.9747$, $p = 0.0333$), peak binding in the ID/SC immunized animals showed a high positive trend towards protection ($r = 0.7945$, $p = 0.0667$) (**Fig. 12c**). A similar trend was seen in binding to the $SHIV_{SF162P3}$ challenge virus V2 hotspot, in which both groups showed a strong positive trend toward protection (**Fig. 12d**). This data suggests that V2-hospot

antibodies do play a role in protection from infection, supporting the finding from the RV144 study as well as studies in non-human primates showing the importance of the V2 region in vaccine protection (93, 96, 97, 183).

Interestingly, we found a strong positive trend in protection in binding to a gp120 antigen from the BAMA assay measuring global cross-reactivity (**Fig. 8a**). Post the first cycP-gp120 boost we found a positive correlation ($r = 0.9747$, $p = 0.033$) in needle-free orally immunized and a strong positive trend ($r = 0.7945$, $p = 0.0667$) in ID/SC immunized macaques with reactivity to B.6240 gp120 and vaccine protection (**Fig. 13a**). Upon alignment of protein sequences from the gp120 and gp140 antigens used in the BAMA assay and the SHIV challenge virus sequence, we found that $SHIV_{SF162P3}$ was most closely related to B.6240 (**Fig. 13b**). Furthermore, we found that of all the gp120 and gp140 antigens tested, only B.6240 and $SHIV_{SF162P3}$ shared the same V2 hotspot region (**Fig. 13c**), providing more evidence of the importance of the V2 region in vaccine-mediated protection. None of the gp70-V1V2 scaffolds contained a matching V2 hotspot sequence to the $SHIV_{SF162P3}$, possibly explaining the lack of correlation between gp70-V1V2 binding and protection.

As neutralizing antibodies against tier-1A or -1B did not correlate with protection, and we did not detect detectable neutralization of the $SHIV_{SF162P3}$ challenge virus, non-neutralizing antibody effector functionality may be involved with protection. To access the functionally of vaccine-induced antibodies, we cultured infected target cells with vaccine sera and measured viral output in an antibody dependent cell-mediated viral inhibition (ADCVI) assay. We found a strong inverse correlation with protection, in which sera from protected or late infected animals inhibited viral grown and infection

(**Fig. 14**). The mechanism of inhibition could be multiple antibody-mediated functionalities, including Fc-mediated phagocytosis, opsonization, ADCC, or blocking of the cell-to-cell viral synapse. Taken together, this data shows that MVA-HIV/cycP-gp120 induce strong antibody-mediated protection associated high binding titers to HIV-1 Env in the sera and rectal secretions, V2 hotspot binding antibodies, and ADCVI functionality.

Discussion

This study demonstrates for the first-time protective efficacy of a trimeric gp120 immunogen, cycP-gp120, against a heterologous tier-2 neutralization resistant SHIV intra-rectal challenge (213). Vaccine-mediated protection was found to be associated with non-neutralizing antibodies directed towards the V2 region of the challenge virus, and ADCVI activity. Furthermore, this study describes the use of needle-free injectors targeting the sublingual and buccal tissue for vaccine delivery as a viable and practical approach for generating strong systemic and mucosal antibody responses. These findings suggest that MVA-HV and cycP-gp120 vaccine platforms should be investigated further in clinical trials.

Needle-free sublingual and buccal immunization generated substantial titers of HIV-1 Env binding antibodies, neutralizing antibodies, and V1V2- and V2 hotspot-directed antibodies upon boosting with cycP-gp120, significantly higher than ID/SC immunization. Additionally, needle-free immunization induced high levels of mucosally localized vaccine-specific IgG and IgA. Notably, Env-specific vaginal IgG appeared to be robust and long-lasting, supporting previous studies indicating sublingual immunization promotes vaginal responses (158, 198). The larger magnitude of the antibody response in needle-free immunized animals could be due to having a higher dose of adjuvant in the needle-free oral immunized group compared to ID/SC for the first protein boost, and thus we cannot say that needle-free oral immunization is superior to ID/SC without further studies comparing the rotes. However, the ability of needle-free injection to the sublingual and buccal tissue to generate strong systemic and mucosal antibody responses highlight the attractiveness of this route for mucosal vaccine

development, as this route is as easy and practical as intranasal delivery, yet does not carry any risks of CNS-related side effects (150).

In this study we also tested the double mutated heat-labile enterotoxin (dmLT), derived from *Escherichia coli*, as a mucosal adjuvant. dmLT has been tested extensively as a mucosal adjuvant, promoting IgA and T_H17 responses *in vivo* (163). Adjuvanting cycP-gp120 with dmLT resulted in the generation of rectal and vaginal IgA responses when delivered through needle-free oral injection. Additionally, dmLT is being tested as a vaccine candidate for Enterotoxigenic *Escherichia coli* (ETEC) (165). In our study adjuvanting cycP-gp120 wit dmLT induced anti-dmLT antibodies in both the needle-free oral immunization group and the ID/SC group (**Fig. 15**), suggesting the needle-free sublingual and buccal route may be advantageous in generating anti-dmLT antibody responses by vaccination.

Despite generating significantly higher systemic and mucosal antibodies post cycP-gp120 boost in needle-free immunized animals compared to ID/SC immunized macaques, we did not see significantly enhanced vaccine protection in needle-free orally immunized animals. This could be due to several factors. At the pre-challenge timepoint, 19 weeks after the second protein boost, gp120-specific serum and rectal IgG levels are comparable between both needle-free orally immunized and ID/SC immunized animals. Results from the RV144 trial suggested that Env-specific sera IgA may mitigate the otherwise protective antibody responses (85). Needle-free oral immunization resulted in significantly higher vaccine-specific sera IgA than ID/SC immunization, responses of which did not correlate significantly with protection (**Fig. 16**). Similarly, rectal IgA responses against gp120 also did not correlate with protection, suggesting that these

responses may not be desirable in rectal transmission. Previous studies have demonstrated that HIV-1 Env specific vaginal IgA can inhibit HIV-1 transcytosis across the mucosal epithelium (139, 214). As needle-free oral immunization resulted in a significant vaginal IgA response, this route may provide enhanced protection in an intra-vaginal challenge model. The dichotomy between the requirements to protect from vaginal vs rectal transmission presents a challenge for HIV-1 vaccine research, as an ideal vaccine should be able to protect both males and females from infection. Future studies will evaluate needle-free oral immunization in the context of intra-vaginal challenge to fully characterize this route in vaccine-mediated protection.

In addition to the antibody responses, we tracked the cellular immune responses generated by vaccination over time. While we did not detect strong $CD8^+$ T-cell responses, we did detect $CD4^+$ T-cell responses against HIV-1 antigens, which peaked after the first protein boost (**Fig. 17a, b**). Interestingly, we measured strong IFN-γ, TNF-α, and IL-2 cytokine production by $CD4^+$ T-cells in PBMCs when stimulated with Envelope peptides, indicating that this vaccine regimen can generate multifunctional T-cell responses. Additionally, we did not detect any skewing towards an activated target cell phenotype within the rectal tissue ($CCR5^+HLA\text{-}DR^+$ $CD4^+$ T-cells) at the pre-challenge timepoint, suggesting that needle-free oral immunization, while generating high levels of mucosal immune responses, does not result in the generation of higher levels of HIV-1 target cells (**Fig. 17c**).

A current major focus of HIV-1 vaccine research is the design characterization of novel HIV-1 Env immunogens that generate protective antibody responses with an emphasis on broadly neutralizing antibody responses. Modified gp140 trimers, such as

the BG505 SOSIP.664 gp140 trimer, are of key focus as they resemble native HIV-1 Env, bind to bNAbs, and can induce autologous tier-2 neutralizing antibodies in non-human primates (8, 122). While neutralizing antibodies generated by SOSIP gp140 have generally been restricted to autologous strains, targeting specific glycan holes, improvements to the immunogens show promise in generating heterologous tier-2 neutralization (215). In this study, MVA-HIV/cycP-gp120 immunization did not generate tier-2 neutralizing antibodies, despite being highly immunogenic. This may be due to the vaccine strains being heterologous rather than homologous, and most studies with SOSIP gp140 immunization were based off multiple homologous immunizations to mature the antibody response. Recent studies have shown the requirement of multiple germinal center reactions for the development of tier-2 neutralizing antibodies, supporting the hypothesis that high levels of antibody maturation are required to generate these responses (176). However, the ability of SOSIP gp140-based immunogens to protect against a heterologous pathogenic SHIV challenge has yet to be determined. There are several advantages of the cycP-gp120 to gp140-based trimers. The purification of cycP-gp120 does not require affinity purification to remove improperly folded trimers, which is necessary for gp140 immunogens (121). Additionally, the gp41 region has been reported to be immunodominant, targeted by polyreactive antibody responses in humans that cross-react with microbial antigens (127). This immunodominance may diverge the antibody response away from favorable, relevant epitopes on gp120.

Major correlates of protection in this study involved not only the serum and rectal IgG response, but responses directed towards a key region within the V2-loop of gp120. The importance of the V1V2 region in protection from infection was demonstrated in the

RV144 trial in which non-neutralizing V1V2-directed antibodies correlated with decreased risk of infection (85). Further studies determined that a region within the V2-loop, termed the V2-hotspot, correlated highly with decreased risk of infection and contained sites of vaccine-mediated immune pressure (93, 96). Antibodies against the V1V2 loop have been isolated and characterized as having ADCC activity, which have informed further immunogen design to induce these antibodies (87, 183). The mechanism V2-directed antibodies from protecting from infection is not completely understood. The V2-hotspot peptide backbone is exposed at the apex of the trimer, with certain residues possibly masked by N156 and N160 glycans (216), indicating it may be accessible to antibody binding on the native trimeric Env spike. In addition to V2-hotspot directed antibodies, we show that antibody-dependent cell-mediated viral inhibition (ADCVI) correlates with delayed acquisition of infection. The mechanisms of ADCVI could include several antibody-mediated effector functions such as opsonization, Fc-mediated phagocytosis, and ADCC. Binding to the trimeric spike by V2-directed antibodies could potentially mediate these responses without directly neutralizing the virus. Furthermore, proximal to the V2-hotspot is a putative $\alpha_4\beta_7$ binding site, the importance of which in SIV/HIV infection has been demonstrated by anti-$\alpha_4\beta_7$ monoclonal therapy reducing mucosal transmission or viral rebound of SIV (105, 106, 217). It is hypothesized that gp120 binding to $\alpha_4\beta_7$ may initialize contact with a target cell, promoting the formation of the viral synapse, which is crucial for cell-to-cell transmission (218, 219).

Taken together, this study demonstrates the vaccine-mediated protection of MVA-HIV/cycP-gp120 as well as the viability of the needle-free sublingual and buccal route.

Furthermore, correlates of protection support V1V2-directed antibodies, in particular, V2-directed antibodies, as desirable antibody responses. Future studies will further characterize the MVA-HIV/cycP-gp120 vaccine platform in non-human primates and humans, as well as needle-free oral delivery in generating protective antibody responses against other mucosal routes of transmission.

Methods

Ethics Statement

All housing and experiments involving rhesus macaques were conducted at the Yerkes National Primate Research Center, and protocols were approved by the Emory University Institutional Animal Care and Use Committee (IACUC) protocol YER-2003491. Experiments were carried out in accordance to USDA regulations and recommendations derived from the Guide for the Care and Use of Laboratory Animals. Rhesus macaques were housed in pairs in standard non-human primate cages and provided with both standard primate feed (Purina monkey chow) fresh fruit, and enrichment daily, as well free access to water. Immunizations, blood draws, and other sample collections were performed under anesthesia with ketamine (5-10 mg/kg) or telazol (3-5 mg/kg) performed by trained research staff

Animals and Immunization

Indian rhesus macaques were immunized on weeks 0 and 15 with $1x10^8$ pfu MVA-62Sm expressing HIV-1 gag, pol, and env (strain ADA gp150) (130), and boosted with 100 ug of recombinant JRFL-hCMP-V1cyc (cycP-gp120) (129) in combination with the mucosal adjuvant double mutated heat-labile enterotoxin (dmLT) (162). Animals were separated into three groups based on the route of immunization. Group 1, Topical application: Immunizations were applied topically by pipette to the buccal and sublingual tissue, with the animals remaining in upright position for one minute to allow for natural absorption. dmLT dose was 25 ug per tissue site for each timepoint. The dose of MVA-HIV and cycP-gp120 was split between both tissue sites. Group 2, Needle-free

oral injection: Immunizations were delivered via the Syrijet Mark-II Needless Injector (Keystone Industries) to the sublingual and buccal tissues. MVA-HIV and cycP-gp120 doses were split between both tissue sites. dmLT dose was 25 ug per tissue site for the first cycP-gp120 boost, and 1 ug per tissue site for the second cycP-gp120 boost. Group 3, Intradermal and subcutaneous injection: MVA-HIV was delivered intradermally and cycP-gp120 was delivered subcutaneously to the right and left thigh, the dose being split between each thigh. dmLT dose for each timepoint was 1 ug per site. The production of cycP-gp120 was described previously (129).

Intrarectal SHIV challenge

Rhesus macaques were challenged intra-rectally with HIV-1 clade-B Env expressing neutralization resistant SHIV-SF162P3 (1:100) diluted in sterile RPMI weekly for a maximum of six challenges as previously described (211). Plasma samples taken one week post each challenge were analyzed by PCR for detection of viremia (>60 copies/ml). Viral loads were monitored up to 12 weeks post infection to determine the peak and set-point viral loads.

Antibody Detection Assays

Rhesus macaque sera IgG binding to recombinant ADA-gp120 was measured using ADA-gp120 (immune-tech) coated ELISA plates and reacted against serial dilutions of sera before detection with anti-rhesus IgG HRP (Southern Biotech). Titers of gp120-specific IgG was quantified using an anti-rhesus IgG standard curve. Peptides corresponding to the V2-hotspot region of HIV-1 strains JR-FL-E168K (N-

RDKVQKEYALFYKLD-C), ADA (N-RDKVKKDYALFYRLD-C), SF162P3 (N-GNKMQKEYALFYRLD-C), and 1157-Y173H (N-RDKKQKVHALFYRLD-C) (183) were synthesized (Genemed Synthesis Inc.) and binding of macaque sera (1:100 dilution) was measured by ELISA (OD 450 reading).

For mucosal antibody responses, IgA and IgG were isolated from Weck-Cel swabs as previously described (220). Anti-gp120 (strain ADA) ng IgG and IgG was quantified in rectal, vaginal, and salivary secretions and divided to ug of total IgG or IgA isolated from the secretions to calculate the specific activity. Specific activities are considered significant if values are greater than the mean specific activity in pre-immunization samples plus three standard deviations.

Neutralization Assays

Neutralizing antibody activity was measured in 96-well culture plates by using Tat-regulated luciferase (Luc) reporter gene expression to quantify reductions in virus infection in TZM-bl cells. TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program, as contributed by John Kappes and Xiaoyun Wu. Assays were performed with HIV-1 Env-pseudotyped viruses as described previously (189). Test samples were diluted over a range of 1:20 to 1:43740 in cell culture medium and pre-incubated with virus (~150,000 relative light unit equivalents) for 1 hr at 37 ^{o}C before addition of cells. Following 48 hr incubation, cells were lysed and Luc activity determined using a microtiter plate luminometer and BriteLite Plus Reagent (Perkin Elmer). Neutralization titers are the sample dilution at which relative luminescence units (RLU) were reduced by 50% compared to RLU in virus control wells after subtraction of

background RLU in cell control wells. Serum samples were heat-inactivated at 56^{o}C for 1 hr prior to assay. Positive values were reported as being at least 3x baseline values standardized against the negative control virus, SVA-MLV.

ADCVI Assays

Antibody dependent cell-mediated viral inhibition assays were done as previously described (136, 221). Briefly, PHA and IL-2 stimulated rhesus peripheral blood mononuclear cells (PBMCs) were infected with SHIV-SF162p3 and plated in a 96-well round bottom plate. To these cells rhesus primary NK effectors cells were added with or without immune sera from immunized rhesus macaques. After seven days incubation, supernatants were collected and viral output was measured via p27 capture ELISA.

Binding Antibody Multiplex Assay (BAMA) measuring gp120, gp140, gp70-V1V2 scaffold binding

BAMA assays were performed as described previously (85, 125). Briefly, serial dilutions (starting at 1:80, six five-fold dilutions) of rabbit or rhesus macaque sera were reacted against beads conjugated to a panel of gp120 (strains 51802, BORI, BJOX002, 254008, CNE20, TT31P.2792, B.6240, A244), uncleaved trimeric gp140 (strains RHPA4259, AE.01.con_env03, 1086.C, C.CH505TF, WITO4160, BF1266, 9004S, SC42261), and gp70-V1V2 scaffold proteins (strains B.CaseA, 7060101641, CM244.ec1, TV1.21, 001428.2.42, CAP210.2.00.E8, C2101.c01, BJOX002000.03.2, BF1266_431a, 96ZM651.02, RHPA4259.7, Ce1086_B2, 62357.14, 700010058, 191084_B7, TT31P.2F10.2792) representing the global panel of HIV-1 Envs. Binding of sera to

beads was detected using a secondary biotin-conjugated anti-rabbit IgG and measured via Bio-Plex. Binding is reported as Area Under Curve (AUC) analysis generated from measured MFI's at different serial dilutions. Criteria for positive reactivity was as follows: MFI at 1:80 > 100, MFI at 1:80 > Ag-specific cutoff (95th percentile of all pre-bleed for study for each antigen), MFI > 3-fold that of matched pre-bleed before and after blank bead subtraction.

Flow Cytometr and T-cell responses

PBMCs were stimulated with Gag (clade-B) or Env (clade-B consensus) peptides along with anti-CD28 and anti-CD49d co-stimulatory molecules for two hours before the addition of GolgiStop and GolgiPlug and incubated for an additional four hours. PBMCs were then stained for analysis by flow cytometry with antibodies against CD3 (BD), CD4 (BD), CD8 (BD), IFN-γ (BD), TNF-α (BD), and IL-2 (BD). Live-cells were detected using the LIVE/DEAD (Life) reagent. Rectal Biopsy samples were digested with collagenase and stained for phenotypic markers, CD3, CD4, CD8, CD45RA, CCR7, CCR5, HLA-DR, Ki67, and LIVE/DEAD for flow cytometry analysis

Statistical analysis.

Statistical analysis was performed using Graphpad Prism v7.0. A two-tailed non-parametric t-test (Mann-Whitney Test) was used for all comparisons unless otherwise noted (*$p < 0.05$). Spearman correlations were used to analyze relationships between multiple variables. Alignment of protein sequences done in MegAlign Pro (DNASTAR).

Chapter 3 Figures

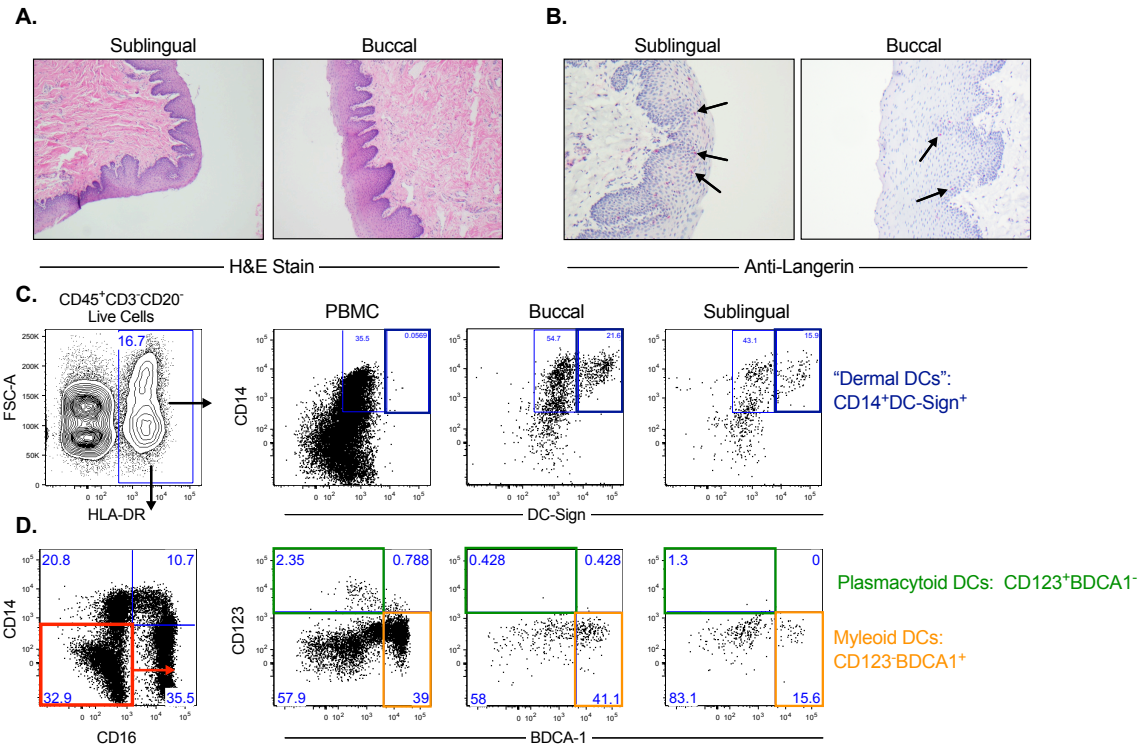


Figure 1: Dendritic Cells in the Sublingual and Buccal Tissue. Paraffin embedded sublingual and buccal tissue sections were stained with Hematoxylin and eosin (**A**) and imaged by light microscopy or anti-Langerin (**B**) and analyzed by immunohistochemistry. Langerin$^+$ cells are stained red. (**C**) Flow cytometry analysis of PBMC, sublingual, and buccal cells stained for dendritic cell markers. (**C**) CD45$^+$CD3$^-$ CD20$^-$HLA-DR$^+$ cells gated for CD14 and DC-Sign (Dermal DCs). (**D**) CD45$^+$CD3$^-$ CD20$^-$HLA-DR$^+$CD14$^-$CD16$^-$ cells gated for CD123 and BDCA-1 (Plasmacytoid DCs, CD123$^+$BDCA-1$^-$) (Myeloid DCs, CD123$^-$BDCA-1$^+$).

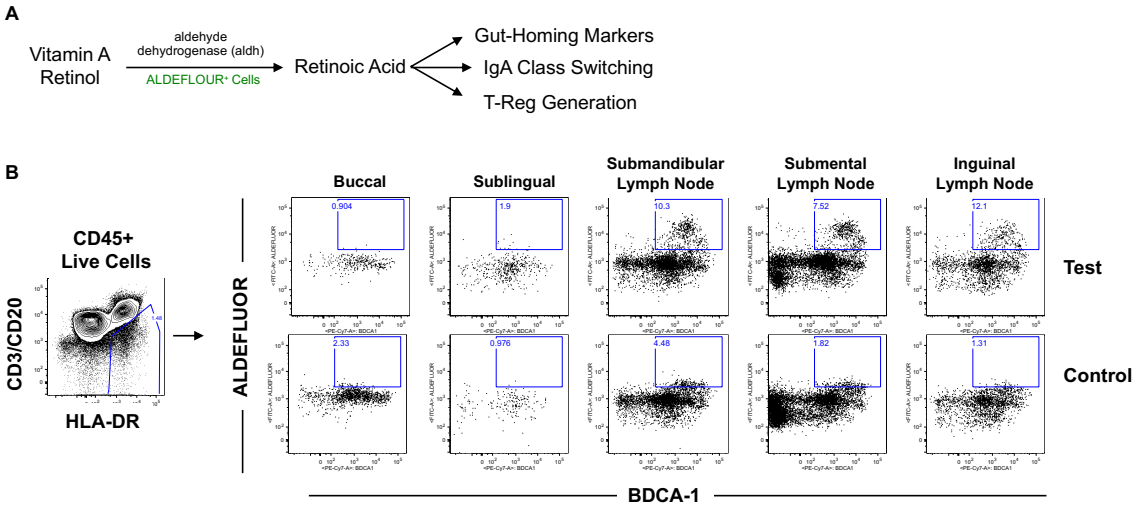


Figure 2: Detection of aldehyde dehydrogenase (aldh) activity in dendritic cells in oral tissue and lymph nodes. (A) Vitamin A is metabolized to Retinoic Acid via aldh activity, which then mediates the generation of gut-homing markers, IgA class switching, and T_{REG} generation. $Aldh^+$ cells can be labeled with the ALDEFLUOR™ kit (STEMCELL). (B) $CD45^+CD3^-CD20^-HLA\text{-}DR^+BDCA\text{-}1^+$ cells stained with the test ALDEFLUOR™ reagent or a control reagent. $ALDEFLUOR^+$ cells contain alhd activity.

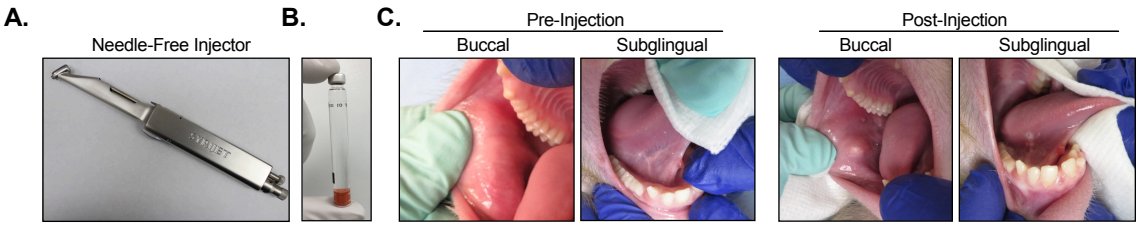


Figure 3: **Sublingual and buccal immunization with a needle-free injector** (**A**) Needle-free injector used to deliver immunizations to the sublingual and buccal tissue. (**B**) Water cartridges were modified to contain immunizations. (**C**) Sublingual and buccal tissue of a rhesus macaque before and five minutes after PBS injection with the needle-free injector.

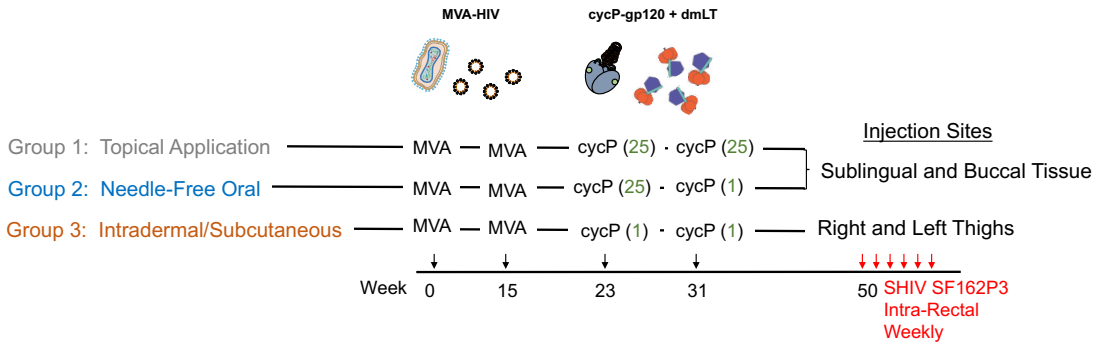


Figure 4: Vaccine scheme. Rhesus macaques (n = 15) were immunized twice with MVA-HIV ($1x10^8$ pfu) and boosted twice with recombinant trimeric gp120 (cycP-gp120) (100 ug) with the mucosal adjuvant dmLT (ug dose in green). 19 weeks after the second boost the animals were challenged intra-rectally with pathogenic SHIV-SF162P3. Animals were immunized via topical application to the buccal and sublingual tissue (n = 4), needle-free injection to the sublingual and buccal tissue (n = 5), or intradermally (MVA-HIV) and subcutaneously (protein) (n = 6). MVA-HIV and cycP-gp120 doses were split between the buccal and sublingual tissue, or the right and left thighs.

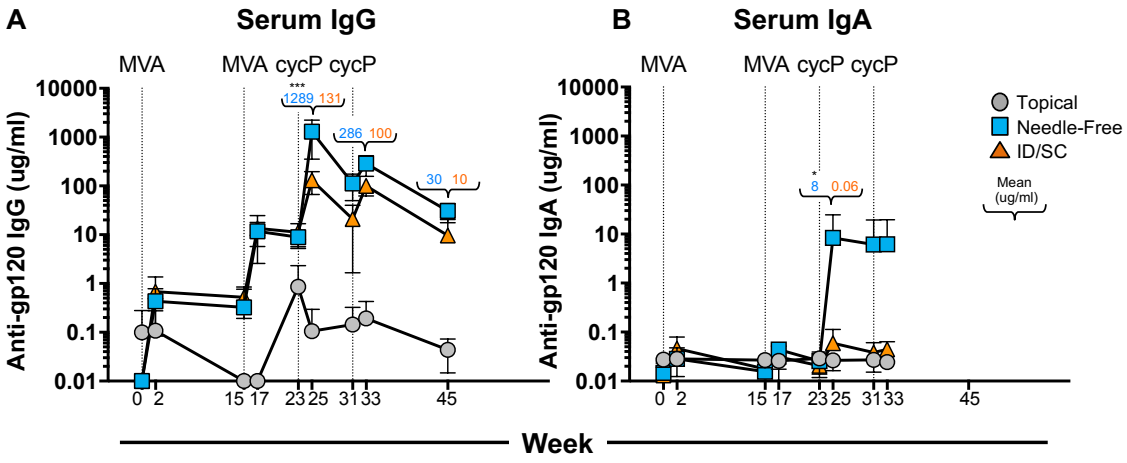


Figure 5: Anti-gp120 antibodies in the sera. Kinetics of the IgG (**A**) and IgA (**B**) anti-gp120 (ADA) antibody response in the sera, measured by ELISA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *(Two-Way ANOVA)*.

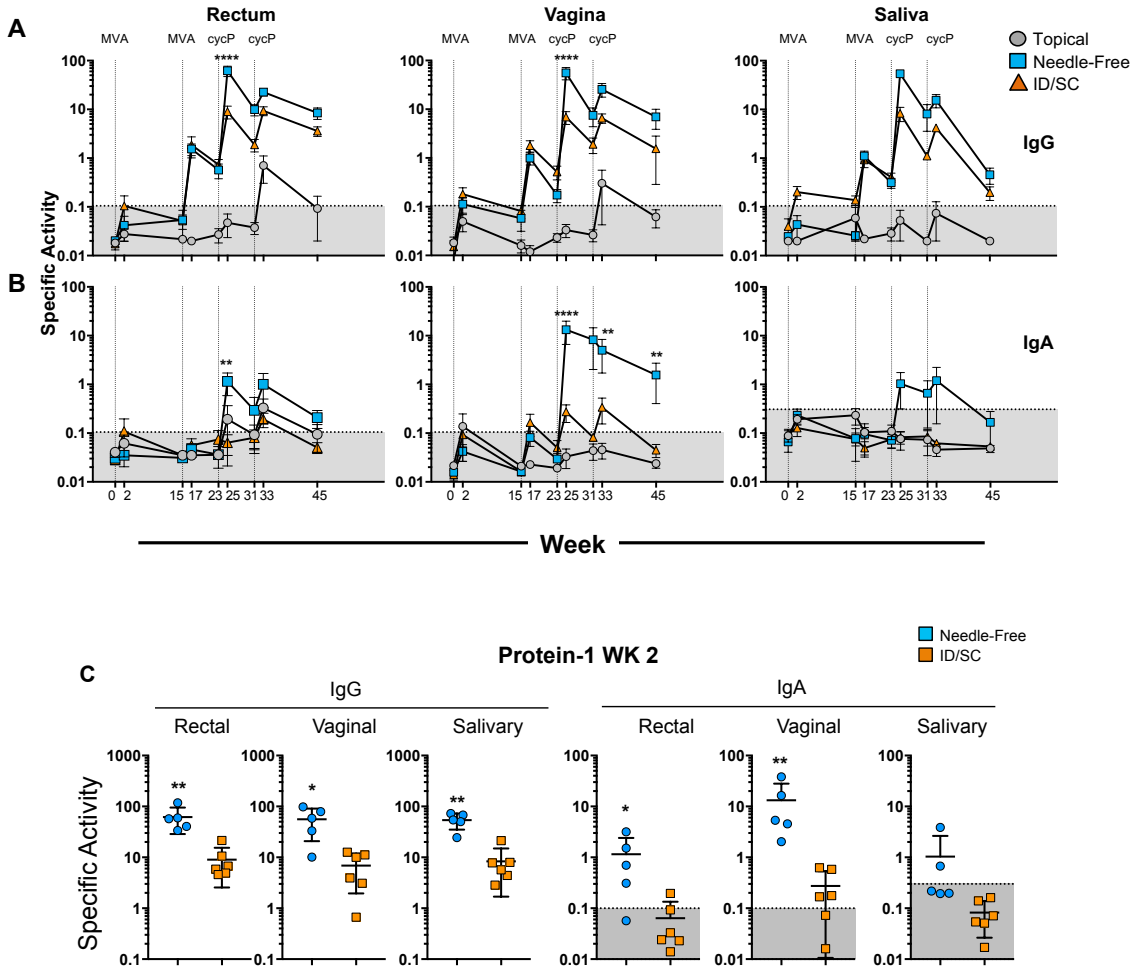


Figure 6: Anti-gp120 antibodies in the mucosal secretions. Kinetics of specific activity of anti-gp120 rectal, vaginal, and salivary IgG (A) or IgA (B) in mucosal secretions. Specific activity calculated as the ratio of ng anti-gp120 IgG or IgA to total IgG or IgA isolated from the secretions. (C) Specific activities of rectal, vaginal, and salivary IgG and IgA two weeks after the first protein boost. ** $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Two-Way ANOVA) (A-B) (Mann-Whitney) (C).*

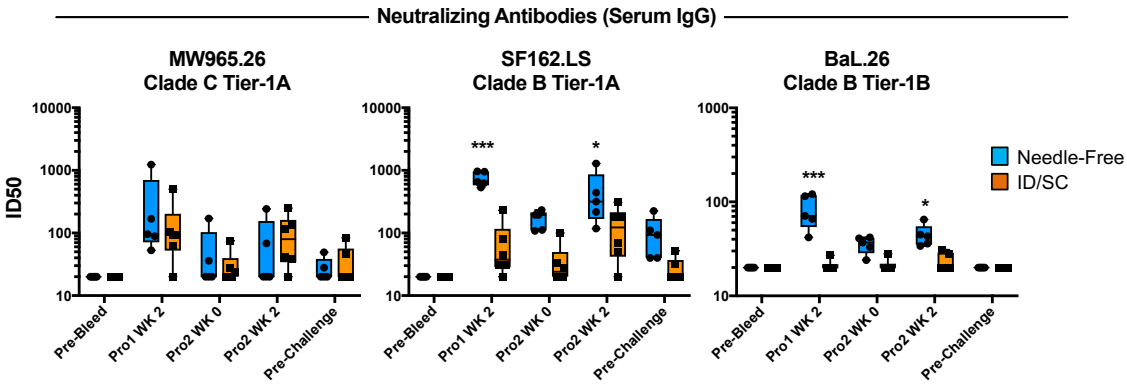


Figure 7: Neutralizing Antibodies: Serum neutralizing activity against neutralization sensitive MW965.26 (clade C) and SF162.LS (clade B) or moderately resistant BaL.26 (clade B) HIV-1 isolates measured by TZM-bl assay. ID50, sera dilution required to reduce virus infectivity by 50%. ** $p < 0.05$, ****$p < 0.0001$ (2-Way ANOVA, multiple comparisons).*

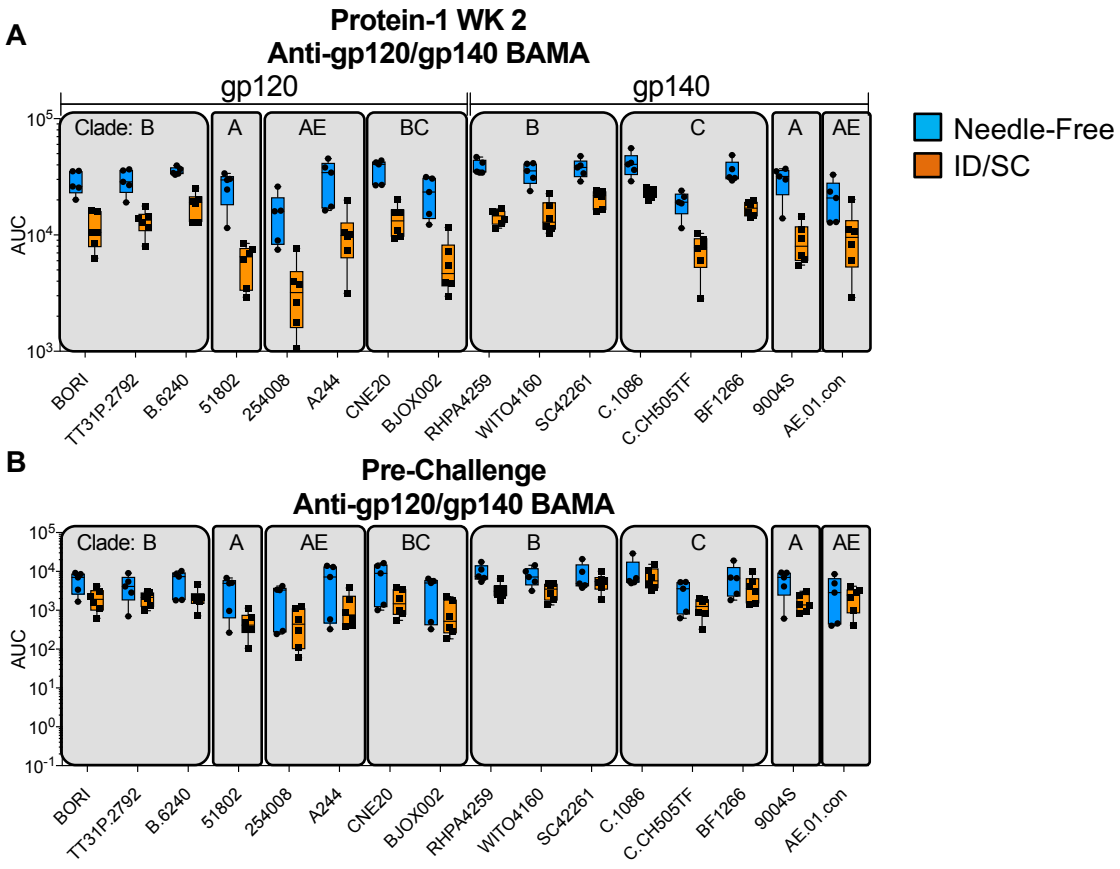


Figure 8: Anti-Env antibody breadth and cross-reactivity. Binding of immune sera to a panel of gp120 and gp140 antigens, measured at the protein-1 week 2 timepoint (**A**) or the pre-challenge timepoint (**B**). Sera reactivity to gp120 and gp140 antigen-conjugated beads measured by Binding Antibody Multiplex Assay (BAMA), and quantified as Area Under Curve (AUC) analysis.

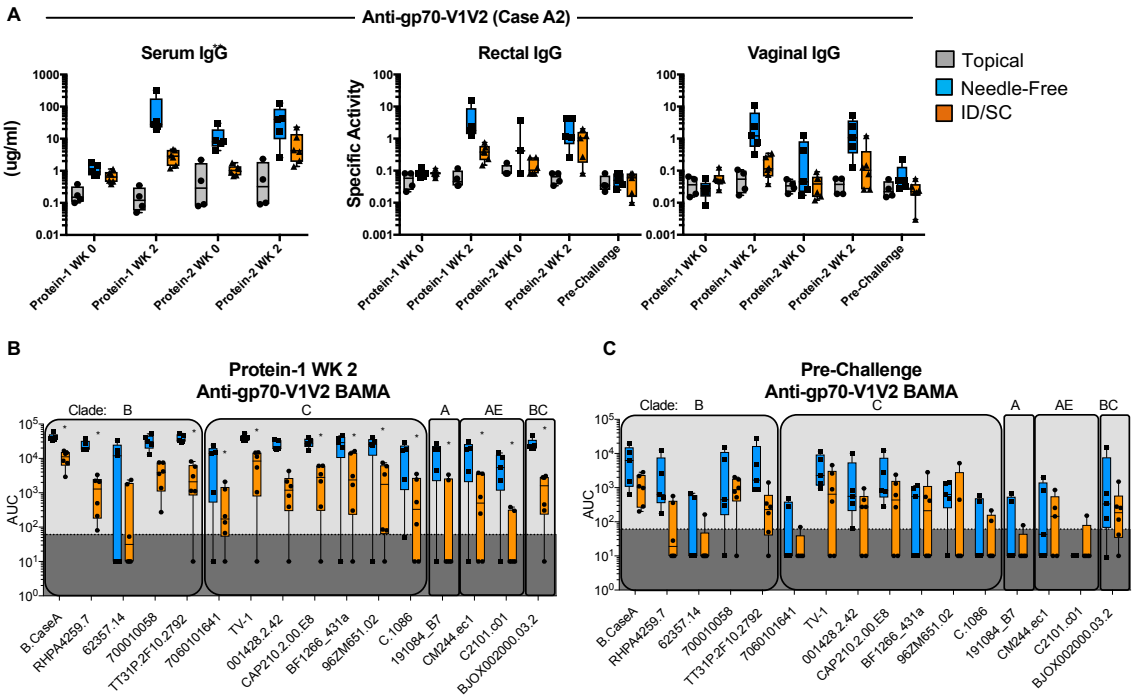


Figure 9: Anti-V1V2 antibody response. (**A**) Anti-gp70-V1V2 (Case.A2) antibody response in serum, rectal, and vaginal IgG in topically immunized, needle-free orally immunized, and ID/SC immunized macaques. Serum responses quantified as ug/ml, rectal and vaginal responses measured as specific activity. Serum IgG binding to a panel of gp70-V1V2 scaffolds representing the global diversity of HIV-1 across multiple clades, measured by BAMA analysis at the protein-1 week 2 (B) and pre-challenge (C) time point. *$p < 0.05$ (*Two-Way ANOVA*, *multiple comparisons*).

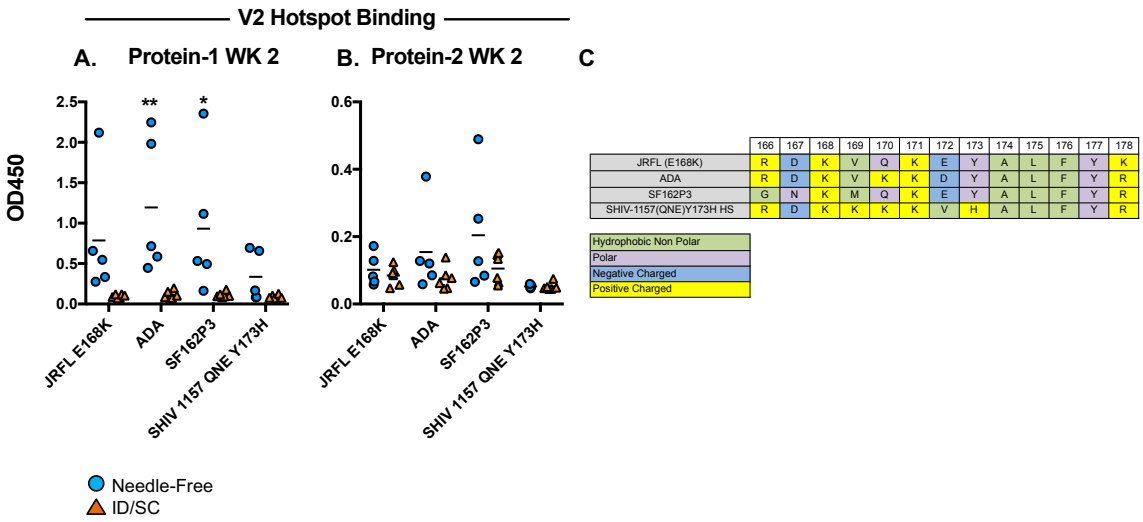


Figure 10: Anti-V2 Hotspot antibodies. Serum IgG binding to peptides corresponding to the V2-hotspot of HIV-1 strain JRFL-E168K (cycP-gp120), ADA (MVA-HIV), SHIVSF162P3, and SHIV-1157-Q(N)E-Y173H (clade-C) measured by ELISA and 1:100 dilution at protein-1 week 2 (**A**) and protein-2 week 2 (**B**) time points. (**C**) Sequences of V2-hotspots used in (**A, B**). (*Two-Way ANOVA, multiple comparisons*)

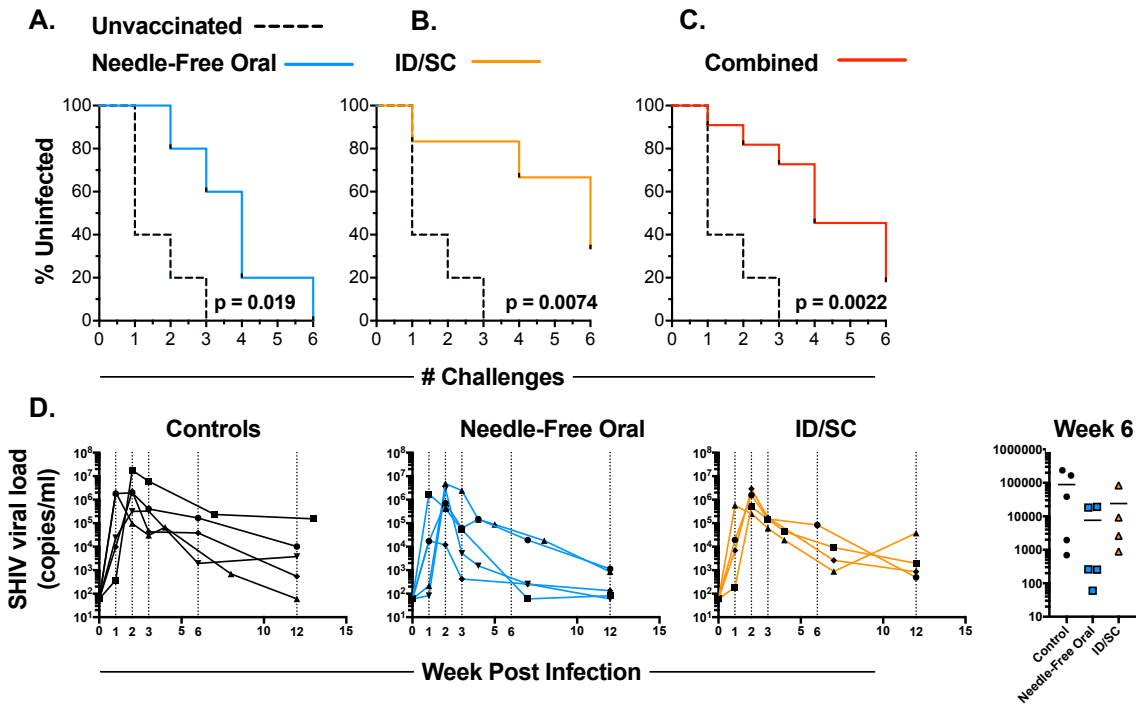


Figure 11: Intra-rectal challenge with pathogenic SHIV-SF162P3. Animals were challenged weekly with an intra-rectal low dose (1:100 dilution) of SHIV-SF162P3. 5 unvaccinated macaques were used as a control. Acquisition of infection in needle-free orally immunized (**A**), ID/SC immunized (**B**), or needle-free oral + ID/SC combined immunized (**C**) macaques. (**D**) Kinetics of viral loads in control, needle-free oral, and ID/SC immunized infected macaques. Right, viral loads at week 6 post infection. Animals were considered infected upon plasma viral load being above background for two consecutive weeks post the indicated challenge. *Log-rank (Mantel-Cox) test.*

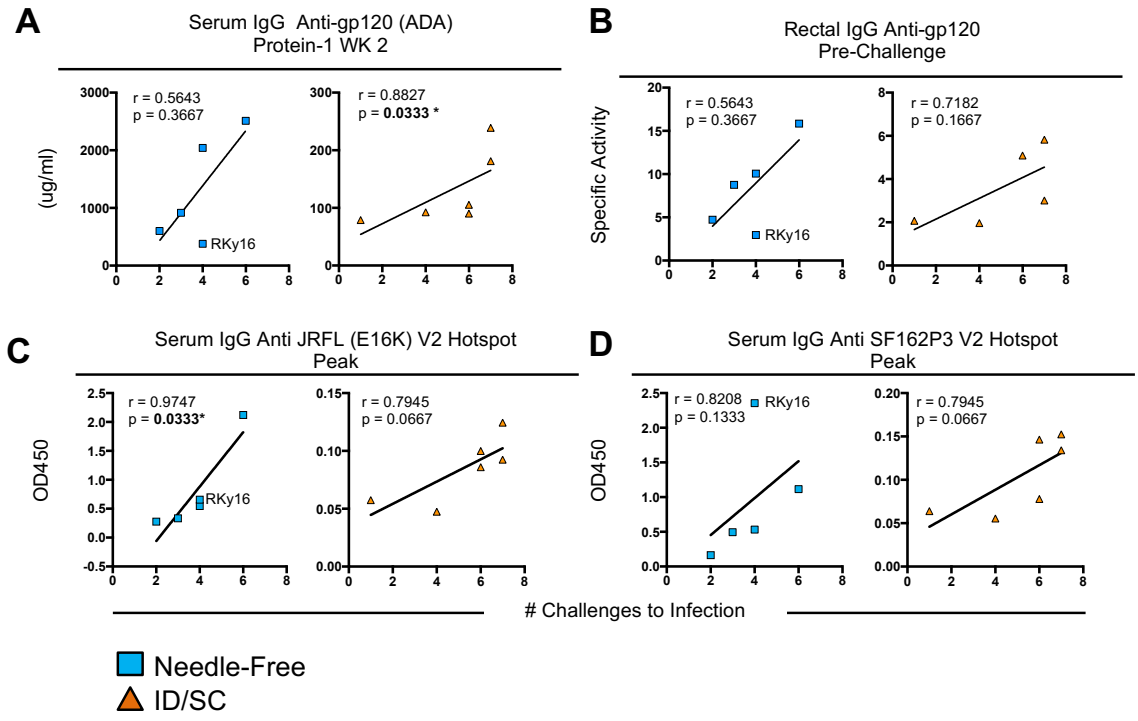


Figure 12: Immune-correlates of protection. Correlations with immune parameters and acquisition of infection. (A) Binding titers against gp120 (ADA) at protein-1 week 2, (B) gp120-specific anti-gp120 IgG at pre-challenge, (**C**) Serum IgG against JRFL-E168K and SHIV-SF162P3 (**D**) hotspot peptides, measured at their peak response, protein-1 WK 2 for needle-free oral immunization, and protein-2 week 2 for ID/SC immunization. Outlier animal in needle-free oral group (RKy16) indicated. Spearman correlations.

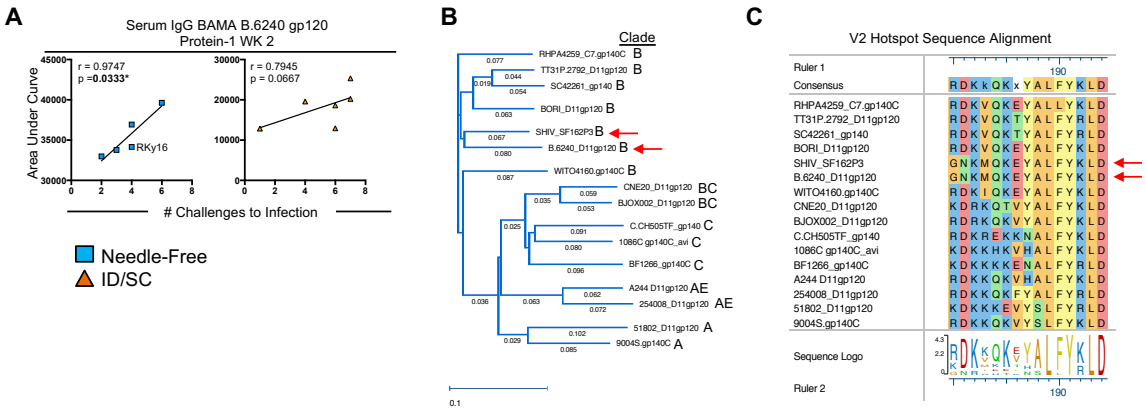


Figure 13: Binding to B.6240 gp120 correlates with protection. (A) Correlation analysis of binding to B.6240 at the protein-1 week 2 timepoint measured by BAMA vs. acquisition of infection. (**B**) Phylogenetic analysis of SHIV.SF162P3 Env and gp120/gp140 antigens used in BAMA anaylsis. (**C**) V2 hotspot alignment of SHIV.SF162P3 gp120 and gp120/gp140 antigens used in BAMA analysis. *Spearman correlation*

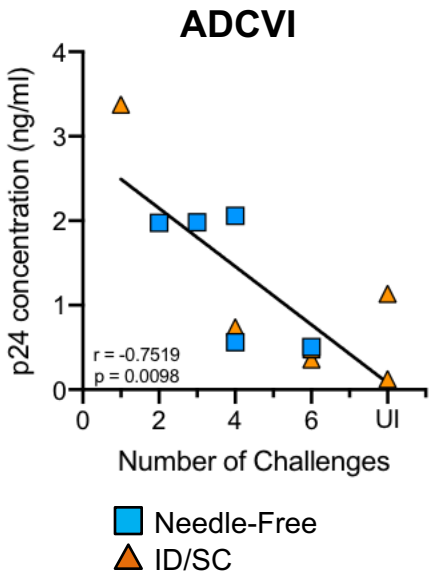


Figure 14. Antibody dependent cell-mediated viral inhibition (ADCVI) correlates with protection. Immune sera from the pre-challenge timepoint was added to activated rhesus PBMCs infected with SHIV-SF16P3 and incubated with effector NK cells, Seven days after incubation viral outgrowth was measured using p24 viral capture assay. Correlation analysis of viral p24 in supernatatnat and number of challenges to infection UI (Uninfected). *Spearman Correlation.*

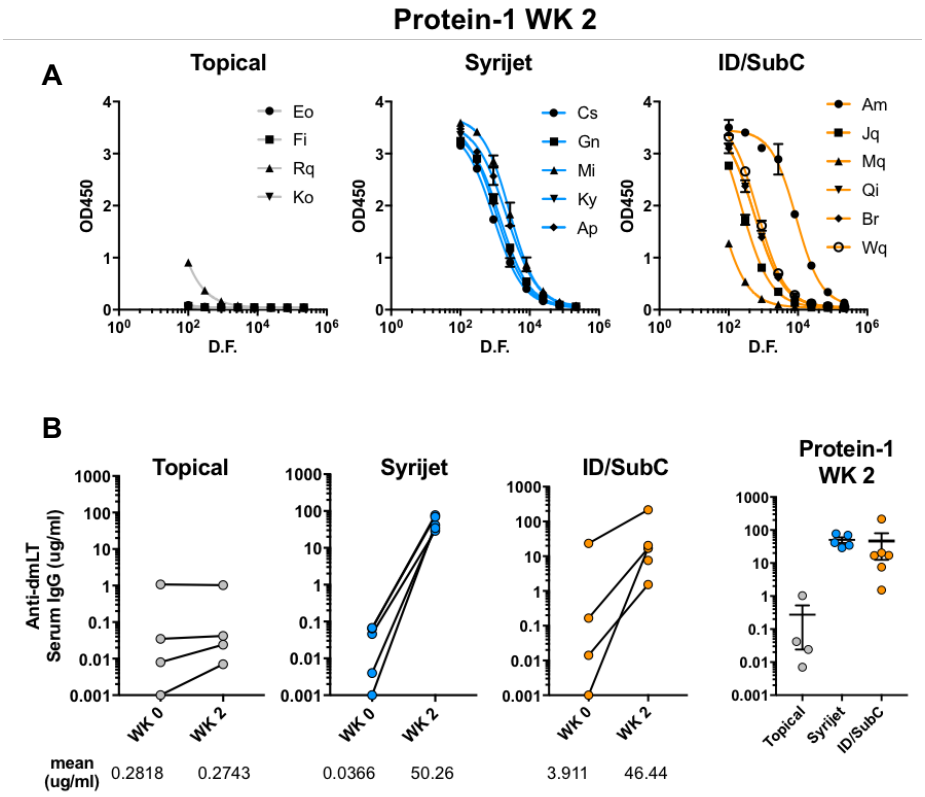


Figure 15: Anti-dmLT antibodies generated by immunization with cycP-gp120 with dmLT. (**A**) Anti-dmLT antibodies in macaques immunized with MVA-HIV and boosted with cycP-gp120 with mucosal adjuvant dmLT. ELISA binding data of sera reacted against dmLT measured two weeks post cycP-gp120 and dmLT immunization. (**B**) Anti-dmLT (ug/ml) in animals before and after immunization with cycP-gp120 and dmLT.

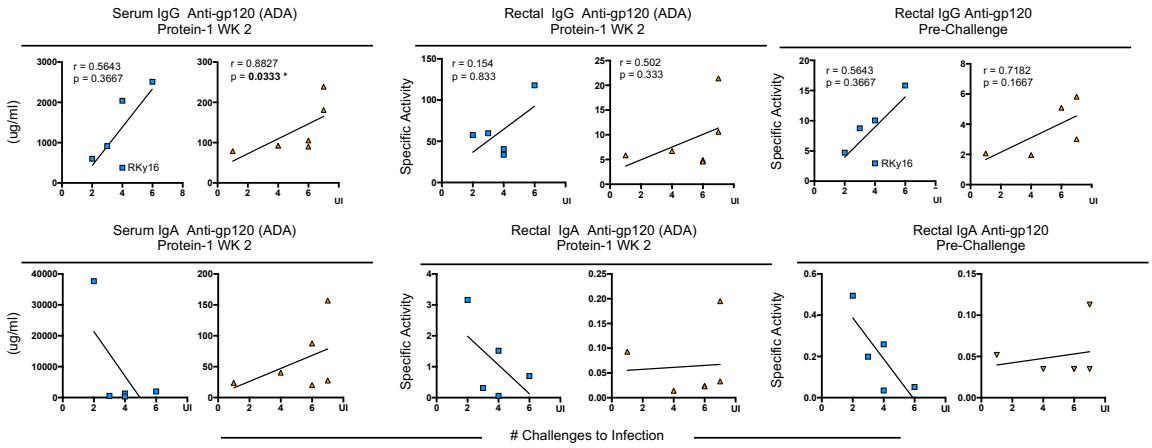


Figure 16: Serum and rectal IgA responses do not correlate with protection.

Correlation analysis of serum and rectal IgG and IgA at protein-1 week 2 and pre-challenge time points with acquisition of infection. *Spearman correlation*.

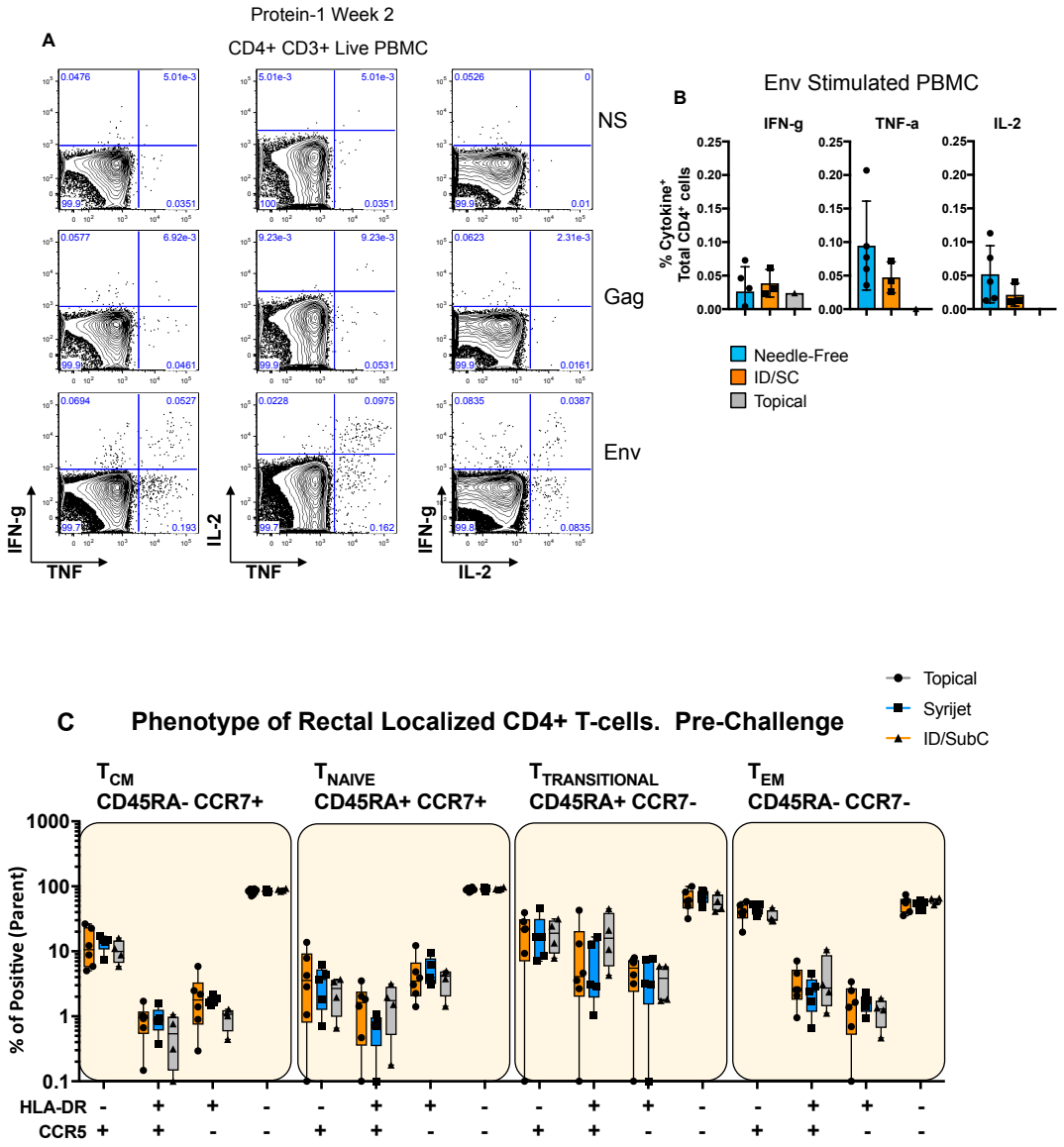


Figure 17: Peak CD4+ T-cell responses and phenotype of rectal CD4+ T-cells at the pre-challenge time-point. (**A**) Flow plots of NS, Gag, and Env peptide stimulated PBMCs, gated on Live $CD4^{+}CD3^{+}$ cells isolated two weeks after the first protein boost. (**B**) Percent cytokine positive CD4+ T-cells reacting to Env peptide stimulation. NS background subtracted from Env responses. (**C**) HLA-DR and CCR5 expression levels of central memory (T_{CM}), naïve (T_{Naive}), transitional memory ($T_{Transitional}$), and effector memory (T_{EM}) rectal CD4+ T-cells taken at the pre-challenge time-point.

Chapter IV: Discussion

The work in this dissertation characterized *in vivo* the immunogenicity of a novel HIV-1 Eng trimer immunogen in rabbits and rhesus macaques. While the design and initial characterization of this immunogen, cycP-gp120, has been previously reported (129), this work is the first to characterize this immunogen in rabbits and rhesus macaques, establish the ability of cycP-gp120 to induce broad cross-reactive V1V2-antibody responses, and induce protection against challenge with a pathogenic SHIV virus. These findings add to the field a new class of Env-immunogens to be studied and improved upon, and further substantiates anti-V2 antibodies as a goal of vaccination.

Additionally, this work established a novel route of mucosal immunization for HIV-1, via needle-free injection to the sublingual and buccal tissue, as a viable method of generating robust systemic and mucosally localized antibody responses. While mucosal vaccination offers many advantages over systemic vaccination, such as ease of use and the efficiency of generating mucosal responses, disadvantages can stem from routes being impractical to vaccinate on a large scale, unwanted inflammation in intranasal immunizations, and immune responses being limited to local sites (135). We show in this work that needle-free oral immunization of the sublingual and buccal tissue offers an easy, practical, and safe method of generating potent systemic and mucosal responses to HIV-1. Furthermore, this work suggests this technique could be expanded to vaccine development for other mucosal pathogens in which strong mucosal immunity is essential for protection.

The RV144 trial and V1V2-directed antibody responses

Results from the RV144 vaccine efficacy trial in Thailand showed for the first-time vaccine-mediated protection from HIV-1 infection (83). While long-term protection was modest at approximately 30% protection (efficacy in the first year was 60%), all other HIV-1 efficacy trials have shown no protection, or in some cases increased risk of infection via vaccination (43). The results of the RV144 trial were met with skepticism, some of which remains to this day (88), however in 2012 a major study was published reporting on the immune correlates of protection (85). This study postulated that non-neutralizing antibody responses to the V1V2 loop region of gp120 is associated with decreased risk of infection, a finding which propelled forward a new paradigm in HIV-1vaccine-development. V1V2-directed antibodies can be generated by immunization and don't require the high levels of somatic hypermutation and/or long HCR3 regions of broadly neutralizing antibodies. Because of these features, V1V2-directed antibodies may be a realistic and practical goal of HIV-1 vaccines.

In the time since results from the RV144 trial were published, numerous studies in non-human primates have again demonstrated the importance of V1V2-directed antibodies in protecting from HIV-1 or SIV infection. One study by Barouch et al. found that antibodies directed toward the V2-loop correlated with protection from SIV infection in immunized rhesus macaques (97). In another study, rhesus macaques generating high titers of rectal anti-V2 antibodies by immunization similar to the RV144 trial were significantly protected from SIV infection when the adjuvant alum was used with the gp120 boosts (99). Additional analysis of the immune responses in the RV144 trial

indicated that antibodies toward the V2-hotspot (residues 166-178) were associated with vaccine-mediated protection (93).

As the V1V2 loops are inherently variable (hence the name variable loops 1 and 2), targeting them by vaccination may not appear to be the optimal strategy. The V1V2 loop region does indeed contain two hypervariable loops. However, there are several regions of general conservation within the V1V2 region, with several residues being highly conserved across multiple strains and clades (**Chapter 1, Fig.5**). Many of this conservation lies around the V2-hotspot, toward the apex of the trimer (**Chapter 1, Fig. 6**). Furthermore, several residues within the V2 hotspot are not buried by the glycan shield, making them a prime target for antibody recognition. The conservation of this region suggests these residues cannot be easily mutated without sacrificing viral fitness.

One issue with antibodies directed toward the V2-hotspot is the specificity of the antibodies toward a specific linear epitope. Initial studies isolating V1V2-directed antibodies, CH58 and CH59, from RV144 vaccinated individuals showed high specificity against K169 and H173 (AE.244 peptide 165-LRDK**K**QKV**H**ALFYKLDIVPIED-186), as alanine substitutes as each of these positions ablated antibody binding (87). Analysis of the V2 region across clades shows that the predominant amino acid residue at position 173 is a tyrosine (Y) instead of a histidine (H), therefor the induction of V2-directed antibodies with high specificity for H173 may be unfavorable for HIV-1 vaccine development. This finding also has implications for human clinical trials. The recently announced HVTN-702 vaccine efficacy trial, taking place in South Africa, will attempt to repeat the results of the RV144 trial. This vaccine will be composed of ALVAC-HIV and bivalent subtype C gp120 (1086.C and TV-1 gp120) with the adjuvant MF59 (222)

(223). Clade-C is predominant in South Africa and the clade-C consensus contains a tyrosine (Y) at position 173 (**Chapter 1, Fig. 6**). However, 1086.C contains a histidine (H) at this position (**Chapter 2, Fig. 3**) (166-KDKKHKV**H**ALFYK-178). Furthermore, 1086.C lacks an asparagine (N) at position 160, which could expose the V2-hotspot loop to B-cells and promote higher binding to the 1086.C hotspot (224). The other clade-C strain in HVTN-702 is TV-1, which does contain a tyrosine (Y) at position 173, may aid in the generation of antibodies recognizing the clade-C consensus sequence. However, this strain does contain the N160 glycosylation site, which may obstruct the V2-hotspot of TV-1 more than 1086.C (166-RDKKHKE**Y**ALFYR-178). Both 1086.C and TV-1 also contain a positively charged histidine (H) at position 170 (H170), while the clade-C consensus contains a polar uncharged glutamine at this position (Q170) (222) (**Chapter 1, Fig. 6**). Unlike the Y173 residue, though, this residue doesn't seem to be as important as Y173 binding by CH58 and CH59 (87), suggesting some flexibility in antibodies recognizing this epitope.

The importance of V2-hotspot matching between vaccine and challenge viruses has been shown in published studies. One study found that by mutating SHIV-1157ipd3N4, which contains a Y173, to SHIV-1157(QNE)Y173H containing an H173 to improve V2 hotspot binding by a pentavalent vaccine containing both Y173 and H173 immunogens resulted in significant vaccine efficacy (183). This suggests the V2-hotspot sequence is an important consideration for vaccine design, however this study did not compare vaccine efficacy between the wild-type SHIV-1157ipd3N4 and the mutated strain. Another study in which rhesus macaques were immunized with 1086.C gp120 showed no protection upon challenge with SHIV-1157ipd3N4, even though the vaccine

was highly immunogenic (225). These studies indicate that the V2-hotspot in vaccine strains, especially at position 173, should be taken into consideration and reflect the regional clade in which they are designed for.

A striking feature of cycP-gp120 is the ability to generate very strong, cross-reactive V1V2-directed antibody responses. Upon immunization with cycP-gp120 (clade-B) in rabbits or rhesus macaques we detect V1V2-binding antibodies against clade-C, A, AE, and BC isolates. Furthermore, cycP-gp120 generates a robust V2-hotspot directed antibody response. In rabbits immunized with cycP-gp120, V2-hotspot directed antibodies recognized heterologous clade-C 1086.C V2 peptides (**Chapter 2, Fig. 3**). Monomeric gp120 generated no binding activity towards these peptides. Interestingly, cycP-gp120 induced antibodies recognized H173 containing 1086.C peptides, even though cycP-gp120 contains a Y173 residue. This indicates that cycP-gp120 can induce antibodies that recognize both Y173 and H173 containing V2-hotspot sequences, and this cross-reactivity was observed in rhesus macaques immunized with MVA-HIV and cycP-gp120 (**Chapter 3, Fig. 10**). Further studies will fully characterize the key regions within the V2 loop that are targeted by cycP-gp120 immunization.

A key correlate of protection from SHIV-SF162P3 challenge in MVA-HIV/cycP-gp120 immunized rhesus macaques was binding to the V2-hotspot of the challenge virus (**Chapter. 3 Fig. 12**). The challenge virus and cycP-gp120 have several common residues within the V2-hotspot. Both contain a Y173, Q170, K168, and a hydrophobic non-polar residue at position 169. Likewise, binding to the B.6240 gp120 antigen in the BAMA analysis also correlated with SHIV protection, and sequence analysis of all the gp120 and gp140 antigens used in the assay revealed that only B.6240 and SHIV-

SF162P3 share the same V2 hotspot sequence. While MVA-HIV/cycP-gp120 immunization generated very strong anti-gp70-V1V2 antibody responses, binding to these scaffolds did not correlate with protection. Interestingly, none of the gp70-V1V2 scaffolds tested by BAMA analysis contained a matching V2-hotspot sequence to SHIV-SF162P3, possibly explaining why binding to these scaffolds did not so correlations with protection. These findings demonstrate a key role of the V2-loop region, focused on the hotspot sequence (positions 166-178) in vaccine-mediated protection.

The mechanism behind cycP-gp120, or any Env immunogen, directing antibody responses toward this region is still unclear. The generation of cycP-gp120 in which the V1 loop has been interrupted and into it fused the hCMP trimer domain, may push antibody responses away from the V1 loop towards the V2 loop (**Chapter 1, Fig. 8**). Furthermore, as the crystal structure of cycP-gp120 has yet to be solved, the structure of the V2-hotspot could potentially be oriented in a way that is highly assessable by B-cells. Addressing these questions, as well as investigating potential mutations to cycP-gp120 to bolster V2-responses, will aid in fully evaluating cycP-gp120 as a vaccine immunogen.

Neutralizing vs. non-neutralizing antibody responses

Results from the RV144 trial, and subsequent studies investigating V1V2-directed antibodies, have rekindled a debate in the HIV-1 vaccine field between non-neutralizing and neutralizing antibodies, and which (or both?) should be a goal of vaccination (226). While historically a gold standard in vaccine development, the generation of broadly neutralizing antibodies (bNAbs) against primary isolates of HIV-1 has long been a challenge in vaccine development (12). bNAbs occur naturally in some infected people,

often the result of heavily somatic hypermutation with many bNAbs featuring long HCDR3 regions capable of penetrating the glycan shield (169). Despite the difficulty in generating bNAbs by vaccination, their ability to prevent infection of SHIV infection via passive transfer into non-human primates demonstrates their appeal in vaccine-elicited antibody responses (171, 227). Neutralization activity against HIV-1 is most commonly characterized against HIV-1 isolates separated into three tiers of neutralization resistance (228). Tier-1A viruses are the most neutralization sensitive, often derived from laboratory-adapted strains, and their Env primarily exists in an open conformation, exposing otherwise buried neutralizable epitopes. Tier-1B viruses are moderately resistant to neutralization and represent a small fraction of circulating viral isolates. Their envelopes are thought to exist primarily in an intermediate stage between open and closed conformation. Tier-2 viruses represent the majority of circulating viral strains and are considered neutralization resistant, with their envelopes existing primarily in the closed conformation, shielding many neutralizable epitopes. Hence, an ultimate goal of HIV-1 vaccine mediated bNAbs would be the induction of broadly cross-reactive antibodies capable of neutralizing diverse tier-2 HIV-1 viral isolates.

One of the most heavily researched immunogens for the generation of tier-2 neutralizing antibodies is the clade-A BG505 SOSIP.664 trimer from which the first native-like crystal structures of trimeric HIV-1 have been solved (8, 14). Immunization with BG505 SOSIP.664 in non-human primates results in autologous tier-2 neutralization of the BG505 viral isolate, one of the first reports to show that vaccine-induced tier-2 neutralizing antibodies is possible in non-human primates (122). However, neutralizing antibody responses induced by SOSIP.664 gp140 immunogens are primarily limited to

the autologous parent strain, often target glycan holes found within the HIV-1 Env specific to the vaccine strain (123). Additionally, the ability of BG505-SOSIP.664 generated antibody responses to prevent a heterologous SHIV challenge in non-human primates has yet to be published. Current efforts in SOSIP-based gp140 immunogen designs are in optimizing the immunogen via stabilizing mutations to eliminate exposure of unwanted epitopes, with the goal of inducing heterologous tier-2 neutralizing antibodies (215).

In our studies with cycP-gp120, we reliably observed the induction of tier-1B neutralizing antibodies after immunization in both rabbits and rhesus macaques. However, we did not generate tier-2 neutralizing antibodies in these experiments. In the original study describing cycP-gp120, homologous immunization with DNA encoding cycP-gp120 followed by cycP-gp120 protein boost did generate tier-2 neutralizing antibodies in guinea pigs (129). One major difference from this study and what is described in this dissertation is that in our studies we utilized a heterologous prime-boost vaccine regimen in which animals were first immunized with MVA-HIV encoding strain ADA gp150 before boosting with strain JRFL-cycP-gp120. While this may aid in the generation of broadly cross-reactive antibodies against HIV-1 Env, a heterologous prime-boost may not be favorable for generating the high antibody maturation needed for tier-2 neutralizing antibodies. Most studies characterizing SOSIP-gp140 induced tier-2 neutralizing antibodies are based on repeated immunizations with the same recombinant protein (122, 177). Repeated germinal center responses have also been shown to be crucial for the generation of tier-2 neutralizing antibodies (176). Hence the ability of cycP-gp120 to generate tier-2 neutralizing antibodies may still be possible by

vaccination, and current efforts in designing MVA vectors which express cycP-gp120 should allow for a homologous MVA/cycP-gp120 immunization regimen to be tested.

Other major efforts in generating neutralizing vaccination involve sequential immunizations with immunogens designed to first engage bNAb precursor antibodies and guide their maturation into bNAb functionality by modified immunogens (114, 229). This strategy has been demonstrated in knock-in mice expressing bNAb-class precursor genes, however the feasibility of this approach in complex animals such as non-human primates and humans has yet to be shown (230-232). A major limitation to this vaccine strategy, and SOSIP-gp140 repeated immunization-based strategies, is the requirement of numerous immunizations in humans to induce the desired result, which has practicality issues when considering the economic and social requirements an effective HIV-1 vaccine would demand. Furthermore, for germline-targeting immunogens, the immunized individual needs to not only contain the correct alleles to express the bNAb-precursor B-cells, but they must be engaged and expanded over other, potentially more prevalent, naïve B-cells which may recognize the immunogen at an unwanted epitope. This is compounded by the observation that gp140 based immunogens tend to highly skew the antibody response towards the gp41 region due to cross-reactivity of polyreactive antibodies also specific for microbial antigens (127). These hurdles cause the generation of bNAbs by vaccination, while likely still possible, a major resource intensive endeavor of HIV-1 vaccine research.

Research of non-neutralizing antibodies against HIV-1 have seen resurgence of interest since the results of the RV144 trials were published. While seemingly counter-intuitive, the role in non-neutralizing antibodies has been shown to be a correlate of

protection from HIV, SIV, and SHIV infection in both human and non-human primate studies (226). An Ad26 prime and MVA boost expressing SIVsme543 Env prime/boost regimen in rhesus macaques resulted in significant vaccine protection from the neutralization-resistant SIVmac251 (97). Protection correlated with V2-binding responses and tier-1 neutralizing antibodies. Another study by the same group demonstrated a mosaic Ad26-MVA vaccine regimen induced protective responses against a pathogenic SHIV-SF16P3 challenge (211). While this study did generate antibody responses that could neutralize the challenge virus, most protective efficacy was observed against binding titers against Env itself, as well as tier-1 neutralizing antibodies against a sensitive SF162 strain. Finally, numerous analysis of the RV144 vaccinated individuals revealed a strong association of non-neutralizing antibody effector functions as correlates of protection (101), and only weak neutralizing responses were detected in vaccinated individuals (233).

There are several proposed mechanisms for non-neutralizing antibody mediated protection from HIV-1 infection (**Chapter 1, Fig. 3**). These include mechanisms to trap HIV-1 virions in the lumen of the genital or gastrointestinal tract by capturing viral particles and inhibiting them from transcytosis. Fc-receptor mediated functions include antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cell-mediated viral inhibition (ADCVI), antibody-dependent cellular phagocytosis (ADCP), and complement activation (226). It is likely that a combination of these mechanisms would be at work in preventing HIV-1 infection, and several of these functions may depend on the compartment in which HIV-1 is transmitted. Inhibition of viral transcytosis was reported a major correlate of protection in a study evaluating mucosal vaccination and

intra-vaginal SHIV challenge (139). Additional studies examine the mucin layers outside the cervio-vaginal epithelium suggest they may hinder HIV-1 movement, and antibodies which can bind to both mucin and HIV-1 may bolster this hindrance (234, 235). ADCC activity is thought to primarily be mediated by NK-cell killing, though monocytes may also be involved (236). Studies in RV144 vaccinated individuals found that higher titers of V1V2-directed IgG3 antibodies correlated with higher ADCC activity and decreased risk of infection (101). ADCP is thought to be mediated by macrophages, immature dendritic cells, and neutrophils and is directed by binding to FcγRII to antibodies bound to viral antigens (237). ADCVI is thought to be a combination of multiple antibody-mediated effector functions, including ADCC, phagocytosis, and innate immune activation (238). ADCVI has been associated with vaccine-mediated protection in numerous vaccine studies, suggesting it may be a crucial metric in the overall assessment of the antibody functionality against HIV-1 (136, 138, 239, 240).

The studies in this dissertation describe cycP-gp120 as a potent immunogen in generating non-neutralizing antibody effector functionality. In rabbits, we found that using a combination of MVA-HIV prime and cycP-gp120 boost generated ADCC functionality, which was not seen in cycP-gp120 alone immunization. This discrepancy could be due to the poxvirus MVA-HIV prime, which is also hypothesized to be an importance distinction between the RV144 trial (ALVAX + AIDSVAX) and the VAX003/VAX004 trials (AIDSVAX alone) (101). Furthermore, while we did not detect neutralization of the challenge virus in the vaccine efficacy trial, we found a strong inverse correlation with ADCVI activity and risk of infection, indicating that non-neutralizing antibody functionality is a principal component of vaccine-mediated

protection. The relationship between cycP-gp120 inducing V1V2, and V2-hotspot binding antibodies and non-neutralizing antibody functionality may be due to multiple mechanisms. Anti-V1V2 antibodies exhibiting ADCC functionality have been extensively described as a leading mediator of anti-viral antibody functionality (87, 101, 174). Additionally, antibodies directed towards the V2-hotspot are in close proximity to the putative $\alpha_4\beta_7$ binding site on gp120, and may block initial interactions between viral particles or virally infected cells and uninfected target cells express $\alpha_4\beta_7$ (105, 217).

One of the key advantages of non-neutralizing antibodies over neutralizing antibodies in the context of HIV-1 vaccine design is that they are easily generated via vaccination. Historically these antibodies have had limited functionality for numerous reasons. Monomeric gp120 immunogens could induce antibodies primarily directed toward the inner domain of gp120, irrelevant epitopes when these regions are shielded in the native HIV-1 Env trimer. Trimeric gp140 immunogens may shield these regions but in return display non-neutralizing epitopes in the gp41 region, highly immunogenic due to polyreactive antibodies which react to both gp41 and microbial productions. CycP-gp120 averts both these issue as it is a trimeric gp120 immunogen that does not express gp41. Because of this, antibody responses are primarily driven toward the outer domain of gp120, residues also observed on native trimeric HIV-1 Env. Additionally, we believe cycP-gp120 has a unique ability in presenting V2 epitopes to B-cells for the development of V2-directed antibodies, a mechanism potentially due to the disruption of the V1 loop in cycP-gp120, which could promote antibody responses towards the V2 loop. As our knowledge of the mechanisms and specificities of non-neutralizing antibodies against HIV-1 Env have grown significantly over the past decade, this knowledge can be used to

inform future designs and optimizations of current immunogens to aid in boosting these highly anti-viral effector functions.

Mucosal vaccination for HIV-1 via sublingual and buccal immunization

A major component of this dissertation was the evaluation of a novel mucosal route of vaccination for HIV-1. As HIV-1 is primarily transmitted across the mucosal epithelium, mucosal immunity to HIV-1 is thought to be crucial in protecting from infection (42). Historically multiple mucosal routes have been tested for HIV-1 vaccination, including intranasal, intrarectal, intravaginal, and oral immunization (194). While sublingual and buccal vaccination has been proposed for a potential route of mucosal HIV-1 vaccination (198), most studies involved applying immunizations topically to the oral tissue and allowing their natural uptake. In this study we used a needle-free injector to aid in antigen transport across the oral epithelium. We show this is an easy and practical route of oral immunization, generated robust titers of systemic and mucosal antibodies with minimal damage to the oral tissue.

Needle-free injectors have been tested extensively for vaccine delivery. Primarily, needle-free injectors have been used in intradermal delivery of vaccines for pathogens such as Influenza (200, 241). Multiple side-effects were reported in intradermal needle-free injections, mostly involving local inflammation and soreness. However, these local injection site reactions occurred more often in people receiving needle-free administration than with a conventional needle and syringe. In our study we did not report any inflammation in rhesus macaques following MVA-HIV immunization

in the sublingual or buccal tissue, even though this was a live vaccina injection. The discrepancy could be due to immunizations been held in the subcutaneous and muscle tissue in needle-free injection into the arm, while sublingual and buccal needle-free injection may result in more rapid drainage to the draining lymph nodes. However, this hypothesis needs to be further investigated.

While this study is the first to report a needle-free oral injection for HIV-1 vaccination in rhesus macaques, other studies have described devices designed for needle-free oral injection (201, 242). The needle-free injection device used in this study, the Syrijet Mark-II, was designed for use in dentistry to deliver anesthetics. Modifications were required to instead deliver immunizations. Additionally, this device is no longer commercially available, and manufacturer support will soon end, indicating that an alternative device will need to be adopted soon for future studies. We believe this study demonstrates the feasibility and practicality of the sublingual and oral tissue as a site for mucosal immunization, and future experiment will build upon this study with new devices and vaccine formulations.

Despite seeing higher immune responses in needle-free orally immunized rhesus macaques, we did not detect higher protection from SHIV infection than macaques immunized via the ID/SC pathway (**Chapter 3, Fig. 11**). A major issue with HIV-1 vaccination is the generation of detrimental vaccine responses in the quest to generate favorable responses. Vaccines which generate high levels of mucosally localized activated $CD4^+CCR5^+$ T-cells are prime targets for HIV-1 infection, especially within the rectal tissue (243). However, we did not detect any differences in $CD4^+CCR5^+$ T-cells in the rectal tissue at the pre-challenge time point between needle-free orally

immunized and ID/SC immunized macaques, indicating that needle-free injection did not result in higher levels of target cells.

Another key finding from the RV144 trial is the negative association of vaccine-induced sera IgA and protection from infection. This study, we found similar results suggesting that the high sera and rectal IgA was either not associated or negatively associated with protection for SHIV infection (**Chapter 3. Fig. 16**). This lack of protection could be due to the majority of antibody-mediated effector functionality being caused by IgG-associated functions. Furthermore, IgA responses may have a larger importance in intravaginal challenge than intrarectal challenge, as IgA-inhibition of viral transcytosis has been a reported correlate of protection from SHIV infection (139). The high vaginal IgA responses observed in needle-free orally immunized rhesus macaques may thus be crucial for blocking intra-vaginal transmission (**Chapter 3. Fig 6**).

In summary, this dissertation has added to the field of HIV-1 vaccine research in the characterization of a novel immunogen which generates high levels of desirable anti-V1V2 and V2 directed antibody responses as well as vaccine-mediated protection from a pathogenic SHIV-SF162P3 challenge, a crucial feature of a potential vaccine candidate. Furthermore, we describe the use of the sublingual and buccal immunization pathway, aided by the use of needle-free injection, as a practical and viable approach to not only HIV-1 vaccine design, but for multiple vaccinations that require a strong systemic and mucosal immune response. We believe these finding will great inform future studies to optimize not only cycP-gp120 as a vaccine candidate, but the oral tissue as an ideal mucosal vaccination route.

References

1. UNAIDS. UNAIDS Fact Sheet. http://www.unaids.org/en/resources/fact-sheet. Accessed
2. Fauci AS, Marston HD. 2014. Ending AIDS--is an HIV vaccine necessary? N Engl J Med 370:495-8.
3. Excler JL, Michael NL. 2016. Lessons from HIV-1 vaccine efficacy trials. Curr Opin HIV AIDS 11:607-613.
4. Watts JM, Dang KK, Gorelick RJ, Leonard CW, Bess JW, Jr., Swanstrom R, Burch CL, Weeks KM. 2009. Architecture and secondary structure of an entire HIV-1 RNA genome. Nature 460:711-6.
5. Levy JA. 1993. HIV pathogenesis and long-term survival. AIDS 7:1401-10.
6. Korber B, Gaschen B, Yusim K, Thakallapally R, Kesmir C, Detours V. 2001. Evolutionary and immunological implications of contemporary HIV-1 variation. Br Med Bull 58:19-42.
7. Haynes BF, Mascola JR. 2017. The quest for an antibody-based HIV vaccine. Immunol Rev 275:5-10.
8. de Taeye SW, Moore JP, Sanders RW. 2016. HIV-1 Envelope Trimer Design and Immunization Strategies To Induce Broadly Neutralizing Antibodies. Trends Immunol 37:221-232.
9. Arthos J, Cicala C, Martinelli E, Macleod K, Van Ryk D, Wei D, Xiao Z, Veenstra TD, Conrad TP, Lempicki RA, McLaughlin S, Pascuccio M, Gopaul R, McNally J, Cruz CC, Censoplano N, Chung E, Reitano KN, Kottilil S, Goode DJ, Fauci AS. 2008. HIV-1 envelope protein binds to and signals through integrin

alpha4beta7, the gut mucosal homing receptor for peripheral T cells. Nat Immunol 9:301-9.

10. Cao L, Diedrich JK, Kulp DW, Pauthner M, He L, Park SR, Sok D, Su CY, Delahunty CM, Menis S, Andrabi R, Guenaga J, Georgeson E, Kubitz M, Adachi Y, Burton DR, Schief WR, Yates Iii JR, Paulson JC. 2017. Global site-specific N-glycosylation analysis of HIV envelope glycoprotein. Nat Commun 8:14954.
11. Doores KJ, Bonomelli C, Harvey DJ, Vasiljevic S, Dwek RA, Burton DR, Crispin M, Scanlan CN. 2010. Envelope glycans of immunodeficiency virions are almost entirely oligomannose antigens. Proc Natl Acad Sci U S A 107:13800-5.
12. Burton DR, Hangartner L. 2016. Broadly Neutralizing Antibodies to HIV and Their Role in Vaccine Design. Annu Rev Immunol 34:635-59.
13. Poignard P, Moulard M, Golez E, Vivona V, Franti M, Venturini S, Wang M, Parren PW, Burton DR. 2003. Heterogeneity of envelope molecules expressed on primary human immunodeficiency virus type 1 particles as probed by the binding of neutralizing and nonneutralizing antibodies. J Virol 77:353-65.
14. Lyumkis D, Julien JP, de Val N, Cupo A, Potter CS, Klasse PJ, Burton DR, Sanders RW, Moore JP, Carragher B, Wilson IA, Ward AB. 2013. Cryo-EM structure of a fully glycosylated soluble cleaved HIV-1 envelope trimer. Science 342:1484-90.
15. Julien JP, Cupo A, Sok D, Stanfield RL, Lyumkis D, Deller MC, Klasse PJ, Burton DR, Sanders RW, Moore JP, Ward AB, Wilson IA. 2013. Crystal structure of a soluble cleaved HIV-1 envelope trimer. Science 342:1477-83.

16. Ndung'u T, Lu Y, Renjifo B, Touzjian N, Kushner N, Pena-Cruz V, Novitsky VA, Lee TH, Essex M. 2001. Infectious simian/human immunodeficiency virus with human immunodeficiency virus type 1 subtype C from an African isolate: rhesus macaque model. J Virol 75:11417-25.

17. Hraber P, Seaman MS, Bailer RT, Mascola JR, Montefiori DC, Korber BT. 2014. Prevalence of broadly neutralizing antibody responses during chronic HIV-1 infection. AIDS 28:163-9.

18. Sather DN, Armann J, Ching LK, Mavrantoni A, Sellhorn G, Caldwell Z, Yu X, Wood B, Self S, Kalams S, Stamatatos L. 2009. Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection. J Virol 83:757-69.

19. Bachmann MF, Rohrer UH, Kundig TM, Burki K, Hengartner H, Zinkernagel RM. 1993. The influence of antigen organization on B cell responsiveness. Science 262:1448-51.

20. Klasse PJ. 2007. Modeling how many envelope glycoprotein trimers per virion participate in human immunodeficiency virus infectivity and its neutralization by antibody. Virology 369:245-62.

21. Crooks ET, Tong T, Chakrabarti B, Narayan K, Georgiev IS, Menis S, Huang X, Kulp D, Osawa K, Muranaka J, Stewart-Jones G, Destefano J, O'Dell S, LaBranche C, Robinson JE, Montefiori DC, McKee K, Du SX, Doria-Rose N, Kwong PD, Mascola JR, Zhu P, Schief WR, Wyatt RT, Whalen RG, Binley JM. 2015. Vaccine-Elicited Tier 2 HIV-1 Neutralizing Antibodies Bind to Quaternary

Epitopes Involving Glycan-Deficient Patches Proximal to the CD4 Binding Site. PLoS Pathog 11:e1004932.

22. Munro JB, Gorman J, Ma X, Zhou Z, Arthos J, Burton DR, Koff WC, Courter JR, Smith AB, 3rd, Kwong PD, Blanchard SC, Mothes W. 2014. Conformational dynamics of single HIV-1 envelope trimers on the surface of native virions. Science 346:759-63.

23. Burton DR, Pyati J, Koduri R, Sharp SJ, Thornton GB, Parren PW, Sawyer LS, Hendry RM, Dunlop N, Nara PL, Lamacchia M, Garratty E, Stiehm ER, Bryson YJ, Cao Y, Moore JP, Ho DD, Barbas III CF. 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science 266:1024-7.

24. Trkola A, Purtscher M, Muster T, Ballaun C, Buchacher A, Sullivan N, Srinivasan K, Sodroski J, Moore JP, Katinger H. 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J Virol 70:1100-8.

25. Zwick MB, Labrijn AF, Wang M, Spenlehauer C, Saphire EO, Binley JM, Moore JP, Stiegler G, Katinger H, Burton DR, Parren PW. 2001. Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. J Virol 75:10892-905.

26. Shibata R, Igarashi T, Haigwood N, Buckler-White A, Ogert R, Ross W, Willey R, Cho MW, Martin MA. 1999. Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. Nat Med 5:204-10.

27. Baba TW, Liska V, Hofmann-Lehmann R, Vlasak J, Xu W, Ayehunie S, Cavacini LA, Posner MR, Katinger H, Stiegler G, Bernacky BJ, Rizvi TA, Schmidt R, Hill LR, Keeling ME, Lu Y, Wright JE, Chou TC, Ruprecht RM. 2000. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. Nat Med 6:200-6.

28. Simek MD, Rida W, Priddy FH, Pung P, Carrow E, Laufer DS, Lehrman JK, Boaz M, Tarragona-Fiol T, Miiro G, Birungi J, Pozniak A, McPhee DA, Manigart O, Karita E, Inwoley A, Jaoko W, Dehovitz J, Bekker LG, Pitisuttithum P, Paris R, Walker LM, Poignard P, Wrin T, Fast PE, Burton DR, Koff WC. 2009. Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high-throughput neutralization assay together with an analytical selection algorithm. J Virol 83:7337-48.

29. Walker LM, Phogat SK, Chan-Hui P-Y, Wagner D, Phung P, Goss JL, Wrin T, Simek MD, Fling S, Mitcham JL, Lehrman JK, Priddy FH, Olsen OA, Frey SM, Hammond PW, Protocol GPI, Kaminsky S, Zamb T, Moyle M, Koff WC, Poignard P, Burton DR. 2009. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. Science 326:285-289.

30. Wu X, Yang ZY, Li Y, Hogerkorp CM, Schief WR, Seaman MS, Zhou T, Schmidt SD, Wu L, Xu L, Longo NS, McKee K, O'Dell S, Louder MK, Wycuff DL, Feng Y, Nason M, Doria-Rose N, Connors M, Kwong PD, Roederer M, Wyatt RT, Nabel GJ, Mascola JR. 2010. Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. Science 329:856-61.

31. Scheid JF, Mouquet H, Ueberheide B, Diskin R, Klein F, Oliveira TY, Pietzsch J, Fenyo D, Abadir A, Velinzon K, Hurley A, Myung S, Boulad F, Poignard P, Burton DR, Pereyra F, Ho DD, Walker BD, Seaman MS, Bjorkman PJ, Chait BT, Nussenzweig MC. 2011. Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding. Science 333:1633-7.

32. Julien JP, Sok D, Khayat R, Lee JH, Doores KJ, Walker LM, Ramos A, Diwanji DC, Pejchal R, Cupo A, Katpally U, Depetris RS, Stanfield RL, McBride R, Marozsan AJ, Paulson JC, Sanders RW, Moore JP, Burton DR, Poignard P, Ward AB, Wilson IA. 2013. Broadly neutralizing antibody PGT121 allosterically modulates CD4 binding via recognition of the HIV-1 gp120 V3 base and multiple surrounding glycans. PLoS Pathog 9:e1003342.

33. Sok D, van Gils MJ, Pauthner M, Julien JP, Saye-Francisco KL, Hsueh J, Briney B, Lee JH, Le KM, Lee PS, Hua Y, Seaman MS, Moore JP, Ward AB, Wilson IA, Sanders RW, Burton DR. 2014. Recombinant HIV envelope trimer selects for quaternary-dependent antibodies targeting the trimer apex. Proc Natl Acad Sci U S A 111:17624-9.

34. Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien JP, Wang SK, Ramos A, Chan-Hui PY, Moyle M, Mitcham JL, Hammond PW, Olsen OA, Phung P, Fling S, Wong CH, Phogat S, Wrin T, Simek MD, Protocol GPI, Koff WC, Wilson IA, Burton DR, Poignard P. 2011. Broad neutralization coverage of HIV by multiple highly potent antibodies. Nature 477:466-70.

35. Falkowska E, Le KM, Ramos A, Doores KJ, Lee JH, Blattner C, Ramirez A, Derking R, van Gils MJ, Liang CH, McBride R, von Bredow B, Shivatare SS, Wu

CY, Chan-Hui PY, Liu Y, Feizi T, Zwick MB, Koff WC, Seaman MS, Swiderek K, Moore JP, Evans D, Paulson JC, Wong CH, Ward AB, Wilson IA, Sanders RW, Poignard P, Burton DR. 2014. Broadly neutralizing HIV antibodies define a glycan-dependent epitope on the prefusion conformation of gp41 on cleaved envelope trimers. Immunity 40:657-68.

36. Moldt B, Rakasz EG, Schultz N, Chan-Hui PY, Swiderek K, Weisgrau KL, Piaskowski SM, Bergman Z, Watkins DI, Poignard P, Burton DR. 2012. Highly potent HIV-specific antibody neutralization in vitro translates into effective protection against mucosal SHIV challenge in vivo. Proc Natl Acad Sci U S A 109:18921-5.

37. Shingai M, Nishimura Y, Klein F, Mouquet H, Donau OK, Plishka R, Buckler-White A, Seaman M, Piatak M, Jr., Lifson JD, Dimitrov DS, Nussenzweig MC, Martin MA. 2013. Antibody-mediated immunotherapy of macaques chronically infected with SHIV suppresses viraemia. Nature 503:277-80.

38. Caskey M, Klein F, Lorenzi JC, Seaman MS, West AP, Jr., Buckley N, Kremer G, Nogueira L, Braunschweig M, Scheid JF, Horwitz JA, Shimeliovich I, Ben-Avraham S, Witmer-Pack M, Platten M, Lehmann C, Burke LA, Hawthorne T, Gorelick RJ, Walker BD, Keler T, Gulick RM, Fatkenheuer G, Schlesinger SJ, Nussenzweig MC. 2015. Viraemia suppressed in HIV-1-infected humans by broadly neutralizing antibody 3BNC117. Nature 522:487-91.

39. Prevention CfDCa. 2016. HIV Surveillance Report. http://www.cdc.gov/hiv/library/reports/hiv-surveillance.html. Accessed

40. Dosekun O, Fox J. 2010. An overview of the relative risks of different sexual behaviours on HIV transmission. Curr Opin HIV AIDS 5:291-7.

41. Boily MC, Baggaley RF, Wang L, Masse B, White RG, Hayes RJ, Alary M. 2009. Heterosexual risk of HIV-1 infection per sexual act: systematic review and meta-analysis of observational studies. Lancet Infect Dis 9:118-29.

42. Haase AT. 2011. Early events in sexual transmission of HIV and SIV and opportunities for interventions. Annu Rev Med 62:127-39.

43. Esparza J. 2013. A brief history of the global effort to develop a preventive HIV vaccine. Vaccine 31:3502-18.

44. Francis DP, Petricciani JC. 1985. The prospects for and pathways toward a vaccine for AIDS. N Engl J Med 313:1586-90.

45. McKinnon LR, Card CM, Diseases CII, Global Health Training P. 2010. HIV vaccine efficacy trials: A brief history, and options for going forward. AIDS Rev 12:209-17.

46. Dowdle W. 1986. The search for an AIDS vaccine. Public Health Rep 101:232-3.

47. Valenzuela P, Medina A, Rutter WJ, Ammerer G, Hall BD. 1982. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. Nature 298:347-50.

48. McAleer WJ, Buynak EB, Maigetter RZ, Wampler DE, Miller WJ, Hilleman MR. 1984. Human hepatitis B vaccine from recombinant yeast. Nature 307:178-80.

49. Mascola JR, McNeil JG, Burke DS. 1994. AIDS vaccines. Are we ready for human efficacy trials? JAMA 272:488-9.

50. Berman PW, Gregory TJ, Riddle L, Nakamura GR, Champe MA, Porter JP, Wurm FM, Hershberg RD, Cobb EK, Eichberg JW. 1990. Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. Nature 345:622-5.

51. Emini EA, Schleif WA, Nunberg JH, Conley AJ, Eda Y, Tokiyoshi S, Putney SD, Matsushita S, Cobb KE, Jett CM, et al. 1992. Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. Nature 355:728-30.

52. Belshe RB, Graham BS, Keefer MC, Gorse GJ, Wright P, Dolin R, Matthews T, Weinhold K, Bolognesi DP, Sposto R, et al. 1994. Neutralizing antibodies to HIV-1 in seronegative volunteers immunized with recombinant gp120 from the MN strain of HIV-1. NIAID AIDS Vaccine Clinical Trials Network. JAMA 272:475-80.

53. Sawyer LS, Wrin MT, Crawford-Miksza L, Potts B, Wu Y, Weber PA, Alfonso RD, Hanson CV. 1994. Neutralization sensitivity of human immunodeficiency virus type 1 is determined in part by the cell in which the virus is propagated. J Virol 68:1342-9.

54. AIDS.gov. "A Timeline of AIDS". Accessed

55. Anonymous. 1994. HIV vaccines get the green light for Third World trials. Nature 371:644.

56. Berman PW. 1998. Development of bivalent rgp120 vaccines to prevent HIV type 1 infection. AIDS Res Hum Retroviruses 14 Suppl 3:S277-89.

57. Flynn NM, Forthal DN, Harro CD, Judson FN, Mayer KH, Para MF, rgp HIVVSG. 2005. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. J Infect Dis 191:654-665.

58. Pitisuttithum P, Gilbert P, Gurwith M, Heyward W, Martin M, van Griensven F, Hu D, Tappero JW, Bangkok Vaccine Evaluation G. 2006. Randomized, Double□Blind, Placebo□Controlled Efficacy Trial of a Bivalent Recombinant Glycoprotein 120 HIV□1 Vaccine among Injection Drug Users in Bangkok, Thailand. J Infect Dis 194:1661-1671.

59. Cohen J. 2003. Public health. AIDS vaccine trial produces disappointment and confusion. Science 299:1290-1.

60. Cohen J. 2003. HIV/AIDS. Vaccine results lose significance under scrutiny. Science 299:1495.

61. Mascola JR, Snyder SW, Weislow OS, Belay SM, Belshe RB, Schwartz DH, Clements ML, Dolin R, Graham BS, Gorse GJ, Keefer MC, McElrath MJ, Walker MC, Wagner KF, McNeil JG, McCutchan FE, Burke DS. 1996. Immunization with envelope subunit vaccine products elicits neutralizing antibodies against laboratory-adapted but not primary isolates of human immunodeficiency virus type 1. The National Institute of Allergy and Infectious Diseases AIDS Vaccine Evaluation Group. J Infect Dis 173:340-8.

62. Walker BD, Plata F. 1990. Cytotoxic T lymphocytes against HIV. AIDS 4:177-84.

63. Johnson RP, Walker BD. 1991. Identification of HIV-1 cytotoxic T-lymphocyte epitopes and implications for vaccine development. Biotechnol Ther 2:137-46.

64. Fynan EF, Webster RG, Fuller DH, Haynes JR, Santoro JC, Robinson HL. 1993. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proc Natl Acad Sci U S A 90:11478-82.

65. Tang DC, DeVit M, Johnston SA. 1992. Genetic immunization is a simple method for eliciting an immune response. Nature 356:152-4.

66. Lu S, Manson K, Wyand M, Robinson HL. 1997. SIV DNA vaccine trial in macaques: post-challenge necropsy in vaccine and control groups. Vaccine 15:920-3.

67. Robinson HL, Montefiori DC, Johnson RP, Manson KH, Kalish ML, Lifson JD, Rizvi TA, Lu S, Hu SL, Mazzara GP, Panicali DL, Herndon JG, Glickman R, Candido MA, Lydy SL, Wyand MS, McClure HM. 1999. Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. Nat Med 5:526-34.

68. Amara RR, Villinger F, Altman JD, Lydy SL, O'Neil SP, Staprans SI, Montefiori DC, Xu Y, Herndon JG, Wyatt LS, Candido MA, Kozyr NL, Earl PL, Smith JM, Ma HL, Grimm BD, Hulsey ML, Miller J, McClure HM, McNicholl JM, Moss B, Robinson HL. 2001. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Science 292:69-74.

69. Goepfert PA, Elizaga ML, Sato A, Qin L, Cardinali M, Hay CM, Hural J, DeRosa SC, DeFawe OD, Tomaras GD, Montefiori DC, Xu Y, Lai L, Kalams SA, Baden LR, Frey SE, Blattner WA, Wyatt LS, Moss B, Robinson HL, National Institute of A, Infectious Diseases HIVVTN. 2011. Phase 1 safety and immunogenicity

testing of DNA and recombinant modified vaccinia Ankara vaccines expressing HIV-1 virus-like particles. J Infect Dis 203:610-9.

70. Kibler KV, Gomez CE, Perdiguero B, Wong S, Huynh T, Holechek S, Arndt W, Jimenez V, Gonzalez-Sanz R, Denzler K, Haddad EK, Wagner R, Sekaly RP, Tartaglia J, Pantaleo G, Jacobs BL, Esteban M. 2011. Improved NYVAC-based vaccine vectors. PLoS One 6:e25674.

71. Tartaglia J, Perkus ME, Taylor J, Norton EK, Audonnet JC, Cox WI, Davis SW, van der Hoeven J, Meignier B, Riviere M, et al. 1992. NYVAC: a highly attenuated strain of vaccinia virus. Virology 188:217-32.

72. Pantaleo G, Esteban M, Jacobs B, Tartaglia J. 2010. Poxvirus vector-based HIV vaccines. Curr Opin HIV AIDS 5:391-6.

73. Gomez-Roman VR, Robert-Guroff M. 2003. Adenoviruses as vectors for HIV vaccines. AIDS Rev 5:178-85.

74. Cohen J. 2001. AIDS research. Merck reemerges with a bold AIDS vaccine effort. Science 292:24-5.

75. Shiver JW, Fu TM, Chen L, Casimiro DR, Davies ME, Evans RK, Zhang ZQ, Simon AJ, Trigona WL, Dubey SA, Huang L, Harris VA, Long RS, Liang X, Handt L, Schleif WA, Zhu L, Freed DC, Persaud NV, Guan L, Punt KS, Tang A, Chen M, Wilson KA, Collins KB, Heidecker GJ, Fernandez VR, Perry HC, Joyce JG, Grimm KM, Cook JC, Keller PM, Kresock DS, Mach H, Troutman RD, Isopi LA, Williams DM, Xu Z, Bohannon KE, Volkin DB, Montefiori DC, Miura A, Krivulka GR, Lifton MA, Kuroda MJ, Schmitz JE, Letvin NL, Caulfield MJ, Bett

AJ, Youil R, et al. 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature 415:331-5.

76. Priddy FH, Brown D, Kublin J, Monahan K, Wright DP, Lalezari J, Santiago S, Marmor M, Lally M, Novak RM, Brown SJ, Kulkarni P, Dubey SA, Kierstead LS, Casimiro DR, Mogg R, DiNubile MJ, Shiver JW, Leavitt RY, Robertson MN, Mehrotra DV, Quirk E, Merck VSG. 2008. Safety and immunogenicity of a replication-incompetent adenovirus type 5 HIV-1 clade B gag/pol/nef vaccine in healthy adults. Clin Infect Dis 46:1769-81.

77. Cohen J. 2007. AIDS research. Promising AIDS vaccine's failure leaves field reeling. Science 318:28-9.

78. Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, Gilbert PB, Lama JR, Marmor M, Del Rio C, McElrath MJ, Casimiro DR, Gottesdiener KM, Chodakewitz JA, Corey L, Robertson MN, Step Study Protocol T. 2008. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. Lancet 372:1881-1893.

79. Duerr A, Huang Y, Buchbinder S, Coombs RW, Sanchez J, del Rio C, Casapia M, Santiago S, Gilbert P, Corey L, Robertson MN, Step HST. 2012. Extended follow-up confirms early vaccine-enhanced risk of HIV acquisition and demonstrates waning effect over time among participants in a randomized trial of recombinant adenovirus HIV vaccine (Step Study). J Infect Dis 206:258-66.

80. Hammer SM, Sobieszczyk ME, Janes H, Karuna ST, Mulligan MJ, Grove D, Koblin BA, Buchbinder SP, Keefer MC, Tomaras GD, Frahm N, Hural J, Anude

C, Graham BS, Enama ME, Adams E, DeJesus E, Novak RM, Frank I, Bentley C, Ramirez S, Fu R, Koup RA, Mascola JR, Nabel GJ, Montefiori DC, Kublin J, McElrath MJ, Corey L, Gilbert PB, Team HS. 2013. Efficacy trial of a DNA/rAd5 HIV-1 preventive vaccine. N Engl J Med 369:2083-92.

81. Thongcharoen P, Suriyanon V, Paris RM, Khamboonruang C, de Souza MS, Ratto-Kim S, Karnasuta C, Polonis VR, Baglyos L, Habib RE, Gurunathan S, Barnett S, Brown AE, Birx DL, McNeil JG, Kim JH, Thai AVEG. 2007. A phase 1/2 comparative vaccine trial of the safety and immunogenicity of a CRF01_AE (subtype E) candidate vaccine: ALVAC-HIV (vCP1521) prime with oligomeric gp160 (92TH023/LAI-DID) or bivalent gp120 (CM235/SF2) boost. J Acquir Immune Defic Syndr 46:48-55.

82. Nitayaphan S, Pitisuttithum P, Karnasuta C, Eamsila C, de Souza M, Morgan P, Polonis V, Benenson M, VanCott T, Ratto-Kim S, Kim J, Thapinta D, Garner R, Bussaratid V, Singharaj P, el-Habib R, Gurunathan S, Heyward W, Birx D, McNeil J, Brown AE, Thai AVEG. 2004. Safety and immunogenicity of an HIV subtype B and E prime-boost vaccine combination in HIV-negative Thai adults. J Infect Dis 190:702-6.

83. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, Premsri N, Namwat C, de Souza M, Adams E, Benenson M, Gurunathan S, Tartaglia J, McNeil JG, Francis DP, Stablein D, Birx DL, Chunsuttiwat S, Khamboonruang C, Thongcharoen P, Robb ML, Michael NL, Kunasol P, Kim JH, Investigators M-T. 2009. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N Engl J Med 361:2209-2220.

84. Cohen J. 2009. HIV/AIDS research. Beyond Thailand: making sense of a qualified AIDS vaccine "success". Science 326:652-3.

85. Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, Evans DT, Montefiori DC, Karnasuta C, Sutthent R, Liao H-X, DeVico AL, Lewis GK, Williams C, Pinter A, Fong Y, Janes H, DeCamp A, Huang Y, Rao M, Billings E, Karasavvas N, Robb ML, Ngauy V, de Souza MS, Paris R, Ferrari G, Bailer RT, Soderberg KA, Andrews C, Berman PW, Frahm N, De Rosa SC, Alpert MD, Yates NL, Shen X, Koup RA, Pitisuttithum P, Kaewkungwal J, Nitayaphan S, Rerks-Ngarm S, Michael NL, Kim JH. 2012. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. N Engl J Med 366:1275-1286.

86. Wren L, Kent SJ. 2011. HIV Vaccine efficacy trial: glimmers of hope and the potential role of antibody-dependent cellular cytotoxicity. Hum Vaccin 7:466-73.

87. Liao HX, Bonsignori M, Alam SM, McLellan JS, Tomaras GD, Moody MA, Kozink DM, Hwang KK, Chen X, Tsao CY, Liu P, Lu X, Parks RJ, Montefiori DC, Ferrari G, Pollara J, Rao M, Peachman KK, Santra S, Letvin NL, Karasavvas N, Yang ZY, Dai K, Pancera M, Gorman J, Wiehe K, Nicely NI, Rerks-Ngarm S, Nitayaphan S, Kaewkungwal J, Pitisuttithum P, Tartaglia J, Sinangil F, Kim JH, Michael NL, Kepler TB, Kwong PD, Mascola JR, Nabel GJ, Pinter A, Zolla-Pazner S, Haynes BF. 2013. Vaccine induction of antibodies against a structurally heterogeneous site of immune pressure within HIV-1 envelope protein variable regions 1 and 2. Immunity 38:176-86.

88. Desrosiers RC. 2017. Protection against HIV Acquisition in the RV144 Trial. J Virol 91.

89. Gorman J, Soto C, Yang MM, Davenport TM, Guttman M, Bailer RT, Chambers M, Chuang GY, DeKosky BJ, Doria-Rose NA, Druz A, Ernandes MJ, Georgiev IS, Jarosinski MC, Joyce MG, Lemmin TM, Leung S, Louder MK, McDaniel JR, Narpala S, Pancera M, Stuckey J, Wu X, Yang Y, Zhang B, Zhou T, Program NCS, Mullikin JC, Baxa U, Georgiou G, McDermott AB, Bonsignori M, Haynes BF, Moore PL, Morris L, Lee KK, Shapiro L, Mascola JR, Kwong PD. 2016. Structures of HIV-1 Env V1V2 with broadly neutralizing antibodies reveal commonalities that enable vaccine design. Nat Struct Mol Biol 23:81-90.

90. Tassaneetrithep B, Tivon D, Swetnam J, Karasavvas N, Michael NL, Kim JH, Marovich M, Cardozo T. 2014. Cryptic determinant of alpha4beta7 binding in the V2 loop of HIV-1 gp120. PLoS One 9:e108446.

91. Alam SM, Liao HX, Tomaras GD, Bonsignori M, Tsao CY, Hwang KK, Chen H, Lloyd KE, Bowman C, Sutherland L, Jeffries TL, Jr., Kozink DM, Stewart S, Anasti K, Jaeger FH, Parks R, Yates NL, Overman RG, Sinangil F, Berman PW, Pitisuttithum P, Kaewkungwal J, Nitayaphan S, Karasavva N, Rerks-Ngarm S, Kim JH, Michael NL, Zolla-Pazner S, Santra S, Letvin NL, Harrison SC, Haynes BF. 2013. Antigenicity and immunogenicity of RV144 vaccine AIDSVAX clade E envelope immunogen is enhanced by a gp120 N-terminal deletion. J Virol 87:1554-68.

92. Pinter A, Honnen WJ, Kayman SC, Trochev O, Wu Z. 1998. Potent neutralization of primary HIV-1 isolates by antibodies directed against epitopes present in the V1/V2 domain of HIV-1 gp120. Vaccine 16:1803-11.

93. Zolla-Pazner S, deCamp AC, Cardozo T, Karasavvas N, Gottardo R, Williams C, Morris DE, Tomaras G, Rao M, Billings E, Berman P, Shen X, Andrews C, O'Connell RJ, Ngauy V, Nitayaphan S, de Souza M, Korber B, Koup R, Bailer RT, Mascola JR, Pinter A, Montefiori D, Haynes BF, Robb ML, Rerks-Ngarm S, Michael NL, Gilbert PB, Kim JH. 2013. Analysis of V2 antibody responses induced in vaccinees in the ALVAC/AIDSVAX HIV-1 vaccine efficacy trial. PLoS One 8:e53629.

94. Voss JE, Andrabi R, McCoy LE, de Val N, Fuller RP, Messmer T, Su CY, Sok D, Khan SN, Garces F, Pritchard LK, Wyatt RT, Ward AB, Crispin M, Wilson IA, Burton DR. 2017. Elicitation of Neutralizing Antibodies Targeting the V2 Apex of the HIV Envelope Trimer in a Wild-Type Animal Model. Cell Rep 21:222-235.

95. McLellan JS, Pancera M, Carrico C, Gorman J, Julien JP, Khayat R, Louder R, Pejchal R, Sastry M, Dai K, O'Dell S, Patel N, Shahzad-ul-Hussan S, Yang Y, Zhang B, Zhou T, Zhu J, Boyington JC, Chuang GY, Diwanji D, Georgiev I, Kwon YD, Lee D, Louder MK, Moquin S, Schmidt SD, Yang ZY, Bonsignori M, Crump JA, Kapiga SH, Sam NE, Haynes BF, Burton DR, Koff WC, Walker LM, Phogat S, Wyatt R, Orwenyo J, Wang LX, Arthos J, Bewley CA, Mascola JR, Nabel GJ, Schief WR, Ward AB, Wilson IA, Kwong PD. 2011. Structure of HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9. Nature 480:336-43.

96. Rolland M, Edlefsen PT, Larsen BB, Tovanabutra S, Sanders-Buell E, Hertz T, deCamp AC, Carrico C, Menis S, Magaret CA, Ahmed H, Juraska M, Chen L, Konopa P, Nariya S, Stoddard JN, Wong K, Zhao H, Deng W, Maust BS, Bose

M, Howell S, Bates A, Lazzaro M, O'Sullivan A, Lei E, Bradfield A, Ibitamuno G, Assawadarachai V, O'Connell RJ, deSouza MS, Nitayaphan S, Rerks-Ngarm S, Robb ML, McLellan JS, Georgiev I, Kwong PD, Carlson JM, Michael NL, Schief WR, Gilbert PB, Mullins JI, Kim JH. 2012. Increased HIV-1 vaccine efficacy against viruses with genetic signatures in Env V2. Nature 490:417-20.

97. Barouch DH, Liu J, Li H, Maxfield LF, Abbink P, Lynch DM, Iampietro MJ, SanMiguel A, Seaman MS, Ferrari G, Forthal DN, Ourmanov I, Hirsch VM, Carville A, Mansfield KG, Stablein D, Pau MG, Schuitemaker H, Sadoff JC, Billings EA, Rao M, Robb ML, Kim JH, Marovich MA, Goudsmit J, Michael NL. 2012. Vaccine protection against acquisition of neutralization-resistant SIV challenges in rhesus monkeys. Nature 482:89-93.

98. Iyer SS, Gangadhara S, Victor B, Shen X, Chen X, Nabi R, Kasturi SP, Sabula MJ, Labranche CC, Reddy PB, Tomaras GD, Montefiori DC, Moss B, Spearman P, Pulendran B, Kozlowski PA, Amara RR. 2016. Virus-Like Particles Displaying Trimeric Simian Immunodeficiency Virus (SIV) Envelope gp160 Enhance the Breadth of DNA/Modified Vaccinia Virus Ankara SIV Vaccine-Induced Antibody Responses in Rhesus Macaques. J Virol 90:8842-54.

99. Vaccari M, Gordon SN, Fourati S, Schifanella L, Liyanage NP, Cameron M, Keele BF, Shen X, Tomaras GD, Billings E, Rao M, Chung AW, Dowell KG, Bailey-Kellogg C, Brown EP, Ackerman ME, Vargas-Inchaustegui DA, Whitney S, Doster MN, Binello N, Pegu P, Montefiori DC, Foulds K, Quinn DS, Donaldson M, Liang F, Lore K, Roederer M, Koup RA, McDermott A, Ma ZM, Miller CJ, Phan TB, Forthal DN, Blackburn M, Caccuri F, Bissa M, Ferrari G,

Kalyanaraman V, Ferrari MG, Thompson D, Robert-Guroff M, Ratto-Kim S, Kim JH, Michael NL, Phogat S, Barnett SW, Tartaglia J, Venzon D, Stablein DM, et al. 2016. Adjuvant-dependent innate and adaptive immune signatures of risk of SIVmac251 acquisition. Nat Med 22:762-70.

100. Bar KJ, Li H, Chamberland A, Tremblay C, Routy JP, Grayson T, Sun C, Wang S, Learn GH, Morgan CJ, Schumacher JE, Haynes BF, Keele BF, Hahn BH, Shaw GM. 2010. Wide variation in the multiplicity of HIV-1 infection among injection drug users. J Virol 84:6241-7.

101. Yates NL, Liao HX, Fong Y, deCamp A, Vandergrift NA, Williams WT, Alam SM, Ferrari G, Yang ZY, Seaton KE, Berman PW, Alpert MD, Evans DT, O'Connell RJ, Francis D, Sinangil F, Lee C, Nitayaphan S, Rerks-Ngarm S, Kaewkungwal J, Pitisuttithum P, Tartaglia J, Pinter A, Zolla-Pazner S, Gilbert PB, Nabel GJ, Michael NL, Kim JH, Montefiori DC, Haynes BF, Tomaras GD. 2014. Vaccine-induced Env V1-V2 IgG3 correlates with lower HIV-1 infection risk and declines soon after vaccination. Sci Transl Med 6:228ra39.

102. Teigler JE, Phogat S, Franchini G, Hirsch VM, Michael NL, Barouch DH. 2014. The canarypox virus vector ALVAC induces distinct cytokine responses compared to the vaccinia virus-based vectors MVA and NYVAC in rhesus monkeys. J Virol 88:1809-14.

103. Mayr LM, Su B, Moog C. 2017. Non-Neutralizing Antibodies Directed against HIV and Their Functions. Front Immunol 8:1590.

104. Sagar M, Wu X, Lee S, Overbaugh J. 2006. Human immunodeficiency virus type 1 V1-V2 envelope loop sequences expand and add glycosylation sites over the

course of infection, and these modifications affect antibody neutralization sensitivity. J Virol 80:9586-98.

105. Arthos J, Cicala C, Nawaz F, Byrareddy SN, Villinger F, Santangelo PJ, Ansari AA, Fauci AS. 2018. The Role of Integrin alpha4beta7 in HIV Pathogenesis and Treatment. Curr HIV/AIDS Rep doi:10.1007/s11904-018-0382-3.

106. Byrareddy SN, Arthos J, Cicala C, Villinger F, Ortiz KT, Little D, Sidell N, Kane MA, Yu J, Jones JW, Santangelo PJ, Zurla C, McKinnon LR, Arnold KB, Woody CE, Walter L, Roos C, Noll A, Van Ryk D, Jelicic K, Cimbro R, Gumber S, Reid MD, Adsay V, Amancha PK, Mayne AE, Parslow TG, Fauci AS, Ansari AA. 2016. Sustained virologic control in SIV+ macaques after antiretroviral and alpha4beta7 antibody therapy. Science 354:197-202.

107. Kaiser J. 2008. AIDS research. Review of vaccine failure prompts a return to basics. Science 320:30-1.

108. Yasmeen A, Ringe R, Derking R, Cupo A, Julien JP, Burton DR, Ward AB, Wilson IA, Sanders RW, Moore JP, Klasse PJ. 2014. Differential binding of neutralizing and non-neutralizing antibodies to native-like soluble HIV-1 Env trimers, uncleaved Env proteins, and monomeric subunits. Retrovirology 11:41.

109. Kim M, Qiao ZS, Montefiori DC, Haynes BF, Reinherz EL, Liao HX. 2005. Comparison of HIV Type 1 ADA gp120 monomers versus gp140 trimers as immunogens for the induction of neutralizing antibodies. AIDS Res Hum Retroviruses 21:58-67.

110. Tran K, Poulsen C, Guenaga J, de Val N, Wilson R, Sundling C, Li Y, Stanfield RL, Wilson IA, Ward AB, Karlsson Hedestam GB, Wyatt RT. 2014. Vaccine-

elicited primate antibodies use a distinct approach to the HIV-1 primary receptor binding site informing vaccine redesign. Proc Natl Acad Sci U S A 111:E738-47.

111. Garrity RR, Rimmelzwaan G, Minassian A, Tsai WP, Lin G, de Jong JJ, Goudsmit J, Nara PL. 1997. Refocusing neutralizing antibody response by targeted dampening of an immunodominant epitope. J Immunol 159:279-89.

112. Yang X, Tomov V, Kurteva S, Wang L, Ren X, Gorny MK, Zolla-Pazner S, Sodroski J. 2004. Characterization of the outer domain of the gp120 glycoprotein from human immunodeficiency virus type 1. J Virol 78:12975-86.

113. Bhattacharyya S, Rajan RE, Swarupa Y, Rathore U, Verma A, Udaykumar R, Varadarajan R. 2010. Design of a non-glycosylated outer domain-derived HIV-1 gp120 immunogen that binds to CD4 and induces neutralizing antibodies. J Biol Chem 285:27100-10.

114. Jardine J, Julien JP, Menis S, Ota T, Kalyuzhniy O, McGuire A, Sok D, Huang PS, MacPherson S, Jones M, Nieusma T, Mathison J, Baker D, Ward AB, Burton DR, Stamatatos L, Nemazee D, Wilson IA, Schief WR. 2013. Rational HIV immunogen design to target specific germline B cell receptors. Science 340:711-6.

115. Dosenovic P, von Boehmer L, Escolano A, Jardine J, Freund NT, Gitlin AD, McGuire AT, Kulp DW, Oliveira T, Scharf L, Pietzsch J, Gray MD, Cupo A, van Gils MJ, Yao KH, Liu C, Gazumyan A, Seaman MS, Bjorkman PJ, Sanders RW, Moore JP, Stamatatos L, Schief WR, Nussenzweig MC. 2015. Immunization for HIV-1 Broadly Neutralizing Antibodies in Human Ig Knockin Mice. Cell 161:1505-15.

116. Binley JM, Sanders RW, Clas B, Schuelke N, Master A, Guo Y, Kajumo F, Anselma DJ, Maddon PJ, Olson WC, Moore JP. 2000. A recombinant human immunodeficiency virus type 1 envelope glycoprotein complex stabilized by an intermolecular disulfide bond between the gp120 and gp41 subunits is an antigenic mimic of the trimeric virion-associated structure. J Virol 74:627-43.

117. Forsell MN, Schief WR, Wyatt RT. 2009. Immunogenicity of HIV-1 envelope glycoprotein oligomers. Curr Opin HIV AIDS 4:380-7.

118. Sliepen K, Sanders RW. 2016. HIV-1 envelope glycoprotein immunogens to induce broadly neutralizing antibodies. Expert Rev Vaccines 15:349-65.

119. Sanders RW, Vesanen M, Schuelke N, Master A, Schiffner L, Kalyanaraman R, Paluch M, Berkhout B, Maddon PJ, Olson WC, Lu M, Moore JP. 2002. Stabilization of the soluble, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. J Virol 76:8875-89.

120. Khayat R, Lee JH, Julien JP, Cupo A, Klasse PJ, Sanders RW, Moore JP, Wilson IA, Ward AB. 2013. Structural characterization of cleaved, soluble HIV-1 envelope glycoprotein trimers. J Virol 87:9865-72.

121. Sanders RW, Derking R, Cupo A, Julien J-P, Yasmeen A, de Val N, Kim HJ, Blattner C, de la Peña AT, Korzun J, Golabek M, de Los Reyes K, Ketas TJ, van Gils MJ, King CR, Wilson IA, Ward AB, Klasse PJ, Moore JP. 2013. A next-generation cleaved, soluble HIV-1 Env trimer, BG505 SOSIP.664 gp140, expresses multiple epitopes for broadly neutralizing but not non-neutralizing antibodies. PLoS Pathog 9:e1003618.

122. Sanders RW, van Gils MJ, Derking R, Sok D, Ketas TJ, Burger JA, Ozorowski G, Cupo A, Simonich C, Goo L, Arendt H, Kim HJ, Lee JH, Pugach P, Williams M, Debnath G, Moldt B, van Breemen MJ, Isik G, Medina-Ramírez M, Back JW, Koff WC, Julien J-P, Rakasz EG, Seaman MS, Guttman M, Lee KK, Klasse PJ, LaBranche C, Schief WR, Wilson IA, Overbaugh J, Burton DR, Ward AB, Montefiori DC, Dean H, Moore JP. 2015. HIV-1 VACCINES. HIV-1 neutralizing antibodies induced by native-like envelope trimers. Science 349:aac4223.

123. McCoy LE, van Gils MJ, Ozorowski G, Messmer T, Briney B, Voss JE, Kulp DW, Macauley MS, Sok D, Pauthner M, Menis S, Cottrell CA, Torres JL, Hsueh J, Schief WR, Wilson IA, Ward AB, Sanders RW, Burton DR. 2016. Holes in the Glycan Shield of the Native HIV Envelope Are a Target of Trimer-Elicited Neutralizing Antibodies. Cell Rep 16:2327-38.

124. Zhou T, Doria-Rose NA, Cheng C, Stewart-Jones GBE, Chuang GY, Chambers M, Druz A, Geng H, McKee K, Kwon YD, O'Dell S, Sastry M, Schmidt SD, Xu K, Chen L, Chen RE, Louder MK, Pancera M, Wanninger TG, Zhang B, Zheng A, Farney SK, Foulds KE, Georgiev IS, Joyce MG, Lemmin T, Narpala S, Rawi R, Soto C, Todd JP, Shen CH, Tsybovsky Y, Yang Y, Zhao P, Haynes BF, Stamatatos L, Tiemeyer M, Wells L, Scorpio DG, Shapiro L, McDermott AB, Mascola JR, Kwong PD. 2017. Quantification of the Impact of the HIV-1-Glycan Shield on Antibody Elicitation. Cell Rep 19:719-732.

125. Tomaras GD, Yates NL, Liu P, Qin L, Fouda GG, Chavez LL, Decamp AC, Parks RJ, Ashley VC, Lucas JT, Cohen M, Eron J, Hicks CB, Liao HX, Self SG, Landucci G, Forthal DN, Weinhold KJ, Keele BF, Hahn BH, Greenberg ML,

Morris L, Karim SS, Blattner WA, Montefiori DC, Shaw GM, Perelson AS, Haynes BF. 2008. Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG antibodies followed by plasma anti-gp41 antibodies with ineffective control of initial viremia. J Virol 82:12449-63.

126. Liao HX, Chen X, Munshaw S, Zhang R, Marshall DJ, Vandergrift N, Whitesides JF, Lu X, Yu JS, Hwang KK, Gao F, Markowitz M, Heath SL, Bar KJ, Goepfert PA, Montefiori DC, Shaw GC, Alam SM, Margolis DM, Denny TN, Boyd SD, Marshal E, Egholm M, Simen BB, Hanczaruk B, Fire AZ, Voss G, Kelsoe G, Tomaras GD, Moody MA, Kepler TB, Haynes BF. 2011. Initial antibodies binding to HIV-1 gp41 in acutely infected subjects are polyreactive and highly mutated. J Exp Med 208:2237-49.

127. Williams WB, Liao HX, Moody MA, Kepler TB, Alam SM, Gao F, Wiehe K, Trama AM, Jones K, Zhang R, Song H, Marshall DJ, Whitesides JF, Sawatzki K, Hua A, Liu P, Tay MZ, Seaton KE, Shen X, Foulger A, Lloyd KE, Parks R, Pollara J, Ferrari G, Yu JS, Vandergrift N, Montefiori DC, Sobieszczyk ME, Hammer S, Karuna S, Gilbert P, Grove D, Grunenberg N, McElrath MJ, Mascola JR, Koup RA, Corey L, Nabel GJ, Morgan C, Churchyard G, Maenza J, Keefer M, Graham BS, Baden LR, Tomaras GD, Haynes BF. 2015. HIV-1 VACCINES. Diversion of HIV-1 vaccine-induced immunity by gp41-microbiota cross-reactive antibodies. Science 349:aab1253.

128. Saha P, Bhattacharyya S, Kesavardhana S, Miranda ER, Ali PS, Sharma D, Varadarajan R. 2012. Designed cyclic permutants of HIV-1 gp120: implications for envelope trimer structure and immunogen design. Biochemistry 51:1836-47.

129. Kesavardhana S, Das R, Citron M, Datta R, Ecto L, Srilatha NS, DiStefano D, Swoyer R, Joyce JG, Dutta S, LaBranche CC, Montefiori DC, Flynn JA, Varadarajan R. 2016. Structure based design of cyclically permuted HIV-1 gp120 trimers that elicit neutralizing antibodies. J Biol Chem doi:10.1074/jbc.M116.725614.

130. Wyatt LS, Earl PL, Vogt J, Eller LA, Chandran D, Liu J, Robinson HL, Moss B. 2008. Correlation of immunogenicities and in vitro expression levels of recombinant modified vaccinia virus Ankara HIV vaccines. Vaccine 26:486-93.

131. Altenburg AF, van de Sandt CE, Li BWS, MacLoughlin RJ, Fouchier RAM, van Amerongen G, Volz A, Hendriks RW, de Swart RL, Sutter G, Rimmelzwaan GF, de Vries RD. 2017. Modified Vaccinia Virus Ankara Preferentially Targets Antigen Presenting Cells In Vitro, Ex Vivo and In Vivo. Sci Rep 7:8580.

132. Goepfert PA, Elizaga ML, Seaton K, Tomaras GD, Montefiori DC, Sato A, Hural J, DeRosa SC, Kalams SA, McElrath MJ, Keefer MC, Baden LR, Lama JR, Sanchez J, Mulligan MJ, Buchbinder SP, Hammer SM, Koblin BA, Pensiero M, Butler C, Moss B, Robinson HL, Group HS, National Institutes of A, Infectious Diseases HIVVTN. 2014. Specificity and 6-month durability of immune responses induced by DNA and recombinant modified vaccinia Ankara vaccines expressing HIV-1 virus-like particles. J Infect Dis 210:99-110.

133. Haase AT. 2005. Perils at mucosal front lines for HIV and SIV and their hosts. Nat Rev Immunol 5:783-92.

134. Brenchley JM. 2013. Mucosal immunity in human and simian immunodeficiency lentivirus infections. Mucosal Immunol 6:657-65.

135. Lycke N. 2012. Recent progress in mucosal vaccine development: potential and limitations. Nat Rev Immunol 12:592-605.

136. Xiao P, Zhao J, Patterson LJ, Brocca-Cofano E, Venzon D, Kozlowski PA, Hidajat R, Demberg T, Robert-Guroff M. 2010. Multiple vaccine-elicited nonneutralizing antienvelope antibody activities contribute to protective efficacy by reducing both acute and chronic viremia following simian/human immunodeficiency virus SHIV89.6P challenge in rhesus macaques. J Virol 84:7161-73.

137. Xiao P, Patterson LJ, Kuate S, Brocca-Cofano E, Thomas MA, Venzon D, Zhao J, DiPasquale J, Fenizia C, Lee EM, Kalisz I, Kalyanaraman VS, Pal R, Montefiori D, Keele BF, Robert-Guroff M. 2012. Replicating adenovirus-simian immunodeficiency virus (SIV) recombinant priming and envelope protein boosting elicits localized, mucosal IgA immunity in rhesus macaques correlated with delayed acquisition following a repeated low-dose rectal SIV(mac251) challenge. J Virol 86:4644-57.

138. Hidajat R, Xiao P, Zhou Q, Venzon D, Summers LE, Kalyanaraman VS, Montefiori DC, Robert-Guroff M. 2009. Correlation of vaccine-elicited systemic and mucosal nonneutralizing antibody activities with reduced acute viremia

following intrarectal simian immunodeficiency virus SIVmac251 challenge of rhesus macaques. J Virol 83:791-801.

139. Bomsel M, Tudor D, Drillet AS, Alfsen A, Ganor Y, Roger MG, Mouz N, Amacker M, Chalifour A, Diomede L, Devillier G, Cong Z, Wei Q, Gao H, Qin C, Yang GB, Zurbriggen R, Lopalco L, Fleury S. 2011. Immunization with HIV-1 gp41 subunit virosomes induces mucosal antibodies protecting nonhuman primates against vaginal SHIV challenges. Immunity 34:269-80.

140. Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, Beary H, Hayes D, Frankel SS, Birx DL, Lewis MG. 2000. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat Med 6:207-10.

141. Manrique M, Kozlowski PA, Cobo-Molinos A, Wang SW, Wilson RL, Martinez-Viedma Mdel P, Montefiori DC, Carville A, Aldovini A. 2014. Resistance to infection, early and persistent suppression of simian immunodeficiency virus SIVmac251 viremia, and significant reduction of tissue viral burden after mucosal vaccination in female rhesus macaques. J Virol 88:212-24.

142. Manrique M, Kozlowski PA, Wang SW, Wilson RL, Micewicz E, Montefiori DC, Mansfield KG, Carville A, Aldovini A. 2009. Nasal DNA-MVA SIV vaccination provides more significant protection from progression to AIDS than a similar intramuscular vaccination. Mucosal Immunol 2:536-50.

143. Manrique M, Kozlowski PA, Cobo-Molinos A, Wang SW, Wilson RL, Montefiori DC, Mansfield KG, Carville A, Aldovini A. 2011. Long-term control of simian immunodeficiency virus mac251 viremia to undetectable levels in half

of infected female rhesus macaques nasally vaccinated with simian immunodeficiency virus DNA/recombinant modified vaccinia virus Ankara. J Immunol 186:3581-93.

144. Gosmann C, Anahtar MN, Handley SA, Farcasanu M, Abu-Ali G, Bowman BA, Padavattan N, Desai C, Droit L, Moodley A, Dong M, Chen Y, Ismail N, Ndung'u T, Ghebremichael MS, Wesemann DR, Mitchell C, Dong KL, Huttenhower C, Walker BD, Virgin HW, Kwon DS. 2017. Lactobacillus-Deficient Cervicovaginal Bacterial Communities Are Associated with Increased HIV Acquisition in Young South African Women. Immunity 46:29-37.

145. Klatt NR, Cheu R, Birse K, Zevin AS, Perner M, Noel-Romas L, Grobler A, Westmacott G, Xie IY, Butler J, Mansoor L, McKinnon LR, Passmore JS, Abdool Karim Q, Abdool Karim SS, Burgener AD. 2017. Vaginal bacteria modify HIV tenofovir microbicide efficacy in African women. Science 356:938-945.

146. Kraehenbuhl JP, Neutra MR. 2013. Mucosal vaccines: where do we stand? Curr Top Med Chem 13:2609-28.

147. Patterson LJ, Malkevitch N, Venzon D, Pinczewski J, Gomez-Roman VR, Wang L, Kalyanaraman VS, Markham PD, Robey FA, Robert-Guroff M. 2004. Protection against mucosal simian immunodeficiency virus SIV(mac251) challenge by using replicating adenovirus-SIV multigene vaccine priming and subunit boosting. J Virol 78:2212-21.

148. Bogers WM, Davis D, Baak I, Kan E, Hofman S, Sun Y, Mortier D, Lian Y, Oostermeijer H, Fagrouch Z, Dubbes R, van der Maas M, Mooij P, Koopman G, Verschoor E, Langedijk JP, Zhao J, Brocca-Cofano E, Robert-Guroff M,

Srivastava I, Barnett S, Heeney JL. 2008. Systemic neutralizing antibodies induced by long interval mucosally primed systemically boosted immunization correlate with protection from mucosal SHIV challenge. Virology 382:217-25.

149. Bertley FM, Kozlowski PA, Wang SW, Chappelle J, Patel J, Sonuyi O, Mazzara G, Montefiori D, Carville A, Mansfield KG, Aldovini A. 2004. Control of simian/human immunodeficiency virus viremia and disease progression after IL-2-augmented DNA-modified vaccinia virus Ankara nasal vaccination in nonhuman primates. J Immunol 172:3745-57.

150. Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, Spyr C, Steffen R. 2004. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med 350:896-903.

151. Randall TD. 2010. Bronchus-associated lymphoid tissue (BALT) structure and function. Adv Immunol 107:187-241.

152. Marx PA, Compans RW, Gettie A, Staas JK, Gilley RM, Mulligan MJ, Yamshchikov GV, Chen D, Eldridge JH. 1993. Protection against vaginal SIV transmission with microencapsulated vaccine. Science 260:1323-7.

153. Kozlowski PA, Williams SB, Lynch RM, Flanigan TP, Patterson RR, Cu-Uvin S, Neutra MR. 2002. Differential induction of mucosal and systemic antibody responses in women after nasal, rectal, or vaginal immunization: influence of the menstrual cycle. J Immunol 169:566-74.

154. Belyakov IM, Hel Z, Kelsall B, Kuznetsov VA, Ahlers JD, Nacsa J, Watkins DI, Allen TM, Sette A, Altman J, Woodward R, Markham PD, Clements JD, Franchini G, Strober W, Berzofsky JA. 2001. Mucosal AIDS vaccine reduces

disease and viral load in gut reservoir and blood after mucosal infection of macaques. Nat Med 7:1320-6.

155. Cranage MP, Fraser CA, Cope A, McKay PF, Seaman MS, Cole T, Mahmoud AN, Hall J, Giles E, Voss G, Page M, Almond N, Shattock RJ. 2011. Antibody responses after intravaginal immunisation with trimeric HIV-1 CN54 clade C gp140 in Carbopol gel are augmented by systemic priming or boosting with an adjuvanted formulation. Vaccine 29:1421-30.

156. Poonam P. 2007. The biology of oral tolerance and issues related to oral vaccine design. Curr Pharm Des 13:2001-7.

157. Mercier GT, Nehete PN, Passeri MF, Nehete BN, Weaver EA, Templeton NS, Schluns K, Buchl SS, Sastry KJ, Barry MA. 2007. Oral immunization of rhesus macaques with adenoviral HIV vaccines using enteric-coated capsules. Vaccine 25:8687-701.

158. Kraan H, Vrieling H, Czerkinsky C, Jiskoot W, Kersten G, Amorij JP. 2014. Buccal and sublingual vaccine delivery. J Control Release 190:580-92.

159. Cuburu N, Kweon MN, Song JH, Hervouet C, Luci C, Sun JB, Hofman P, Holmgren J, Anjuere F, Czerkinsky C. 2007. Sublingual immunization induces broad-based systemic and mucosal immune responses in mice. Vaccine 25:8598-610.

160. Fleckenstein J, Sheikh A, Qadri F. 2014. Novel antigens for enterotoxigenic Escherichia coli vaccines. Expert Rev Vaccines 13:631-9.

161. Freytag LC, Clements JD. 2005. Mucosal adjuvants. Vaccine 23:1804-13.

162. Norton EB, Lawson LB, Freytag LC, Clements JD. 2011. Characterization of a mutant Escherichia coli heat-labile toxin, LT(R192G/L211A), as a safe and effective oral adjuvant. Clin Vaccine Immunol 18:546-51.

163. Norton EB, Lawson LB, Mahdi Z, Freytag LC, Clements JD. 2012. The A subunit of Escherichia coli heat-labile enterotoxin functions as a mucosal adjuvant and promotes IgG2a, IgA, and Th17 responses to vaccine antigens. Infect Immun 80:2426-35.

164. Frederick DR, Goggins JA, Sabbagh LM, Freytag LC, Clements JD, McLachlan JB. 2017. Adjuvant selection regulates gut migration and phenotypic diversity of antigen-specific CD4(+) T cells following parenteral immunization. Mucosal Immunol doi:10.1038/mi.2017.70.

165. El-Kamary SS, Cohen MB, Bourgeois AL, Van De Verg L, Bauers N, Reymann M, Pasetti MF, Chen WH. 2013. Safety and immunogenicity of a single oral dose of recombinant double mutant heat-labile toxin derived from enterotoxigenic Escherichia coli. Clin Vaccine Immunol 20:1764-70.

166. Cale EM, Gorman J, Radakovich NA, Crooks ET, Osawa K, Tong T, Li J, Nagarajan R, Ozorowski G, Ambrozak DR, Asokan M, Bailer RT, Bennici AK, Chen X, Doria-Rose NA, Druz A, Feng Y, Joyce MG, Louder MK, O'Dell S, Oliver C, Pancera M, Connors M, Hope TJ, Kepler TB, Wyatt RT, Ward AB, Georgiev IS, Kwong PD, Mascola JR, Binley JM. 2017. Virus-like Particles Identify an HIV V1V2 Apex-Binding Neutralizing Antibody that Lacks a Protruding Loop. Immunity 46:777-791 e10.

167. Jones AT, Chamcha V, Kesavardhana S, Shen X, Beaumont D, Das R, Wyatt LS, LaBranche CC, Stanfield-Oakley S, Ferrari G, Montefiori DC, Moss B, Tomaras GD, Varadarajan R, Amara RR. 2018. A Trimeric HIV-1 Envelope gp120 Immunogen Induces Potent and Broad Anti-V1V2 Loop Antibodies against HIV-1 in Rabbits and Rhesus Macaques. J Virol 92.

168. Fauci AS, Marston HD. 2014. Ending AIDS--is an HIV vaccine necessary? N Engl J Med 370:495-498.

169. Sadanand S, Suscovich TJ, Alter G. 2016. Broadly Neutralizing Antibodies Against HIV: New Insights to Inform Vaccine Design. Annu Rev Med 67:185-200.

170. Burton DR, Mascola JR. 2015. Antibody responses to envelope glycoproteins in HIV-1 infection. Nat Immunol 16:571-576.

171. Gautam R, Nishimura Y, Pegu A, Nason MC, Klein F, Gazumyan A, Golijanin J, Buckler-White A, Sadjadpour R, Wang K, Mankoff Z, Schmidt SD, Lifson JD, Mascola JR, Nussenzweig MC, Martin MA. 2016. A single injection of anti-HIV-1 antibodies protects against repeated SHIV challenges. Nature 533:105-9.

172. Shingai M, Donau OK, Plishka RJ, Buckler-White A, Mascola JR, Nabel GJ, Nason MC, Montefiori D, Moldt B, Poignard P, Diskin R, Bjorkman PJ, Eckhaus MA, Klein F, Mouquet H, Cetrulo Lorenzi JC, Gazumyan A, Burton DR, Nussenzweig MC, Martin MA, Nishimura Y. 2014. Passive transfer of modest titers of potent and broadly neutralizing anti-HIV monoclonal antibodies block SHIV infection in macaques. J Exp Med 211:2061-74.

173. Tomaras GD, Plotkin SA. 2017. Complex immune correlates of protection in HIV-1 vaccine efficacy trials. Immunol Rev 275:245-261.

174. Zolla-Pazner S, deCamp A, Gilbert PB, Williams C, Yates NL, Williams WT, Howington R, Fong Y, Morris DE, Soderberg KA, Irene C, Reichman C, Pinter A, Parks R, Pitisuttithum P, Kaewkungwal J, Rerks-Ngarm S, Nitayaphan S, Andrews C, O'Connell RJ, Yang Z-Y, Nabel GJ, Kim JH, Michael NL, Montefiori DC, Liao H-X, Haynes BF, Tomaras GD. 2014. Vaccine-induced IgG antibodies to V1V2 regions of multiple HIV-1 subtypes correlate with decreased risk of HIV-1 infection. PLoS One 9:e87572.

175. Gottardo R, Bailer RT, Korber BT, Gnanakaran S, Phillips J, Shen X, Tomaras GD, Turk E, Imholte G, Eckler L, Wenschuh H, Zerweck J, Greene K, Gao H, Berman PW, Francis D, Sinangil F, Lee C, Nitayaphan S, Rerks-Ngarm S, Kaewkungwal J, Pitisuttithum P, Tartaglia J, Robb ML, Michael NL, Kim JH, Zolla-Pazner S, Haynes BF, Mascola JR, Self S, Gilbert P, Montefiori DC. 2013. Plasma IgG to linear epitopes in the V2 and V3 regions of HIV-1 gp120 correlate with a reduced risk of infection in the RV144 vaccine efficacy trial. PLoS One 8:e75665.

176. Havenar-Daughton C, Carnathan DG, Torrents de la Pena A, Pauthner M, Briney B, Reiss SM, Wood JS, Kaushik K, van Gils MJ, Rosales SL, van der Woude P, Locci M, Le KM, de Taeye SW, Sok D, Mohammed AU, Huang J, Gumber S, Garcia A, Kasturi SP, Pulendran B, Moore JP, Ahmed R, Seumois G, Burton DR, Sanders RW, Silvestri G, Crotty S. 2016. Direct Probing of Germinal Center

Responses Reveals Immunological Features and Bottlenecks for Neutralizing Antibody Responses to HIV Env Trimer. Cell Rep 17:2195-2209.

177. Pauthner M, Havenar-Daughton C, Sok D, Nkolola JP, Bastidas R, Boopathy AV, Carnathan DG, Chandrashekar A, Cirelli KM, Cottrell CA, Eroshkin AM, Guenaga J, Kaushik K, Kulp DW, Liu J, McCoy LE, Oom AL, Ozorowski G, Post KW, Sharma SK, Steichen JM, de Taeye SW, Tokatlian T, Torrents de la Pena A, Butera ST, LaBranche CC, Montefiori DC, Silvestri G, Wilson IA, Irvine DJ, Sanders RW, Schief WR, Ward AB, Wyatt RT, Barouch DH, Crotty S, Burton DR. 2017. Elicitation of Robust Tier 2 Neutralizing Antibody Responses in Nonhuman Primates by HIV Envelope Trimer Immunization Using Optimized Approaches. Immunity 46:1073-1088 e6.

178. Lai L, Vodros D, Kozlowski PA, Montefiori DC, Wilson RL, Akerstrom VL, Chennareddi L, Yu T, Kannanganat S, Ofielu L, Villinger F, Wyatt LS, Moss B, Amara RR, Robinson HL. 2007. GM-CSF DNA: an adjuvant for higher avidity IgG, rectal IgA, and increased protection against the acute phase of a SHIV-89.6P challenge by a DNA/MVA immunodeficiency virus vaccine. Virology 369:153-67.

179. Lai L, Kwa S, Kozlowski PA, Montefiori DC, Ferrari G, Johnson WE, Hirsch V, Villinger F, Chennareddi L, Earl PL, Moss B, Amara RR, Robinson HL. 2011. Prevention of infection by a granulocyte-macrophage colony-stimulating factor co-expressing DNA/modified vaccinia Ankara simian immunodeficiency virus vaccine, vol 204, p 164-73, United States.

180. Kwa S, Lai L, Gangadhara S, Siddiqui M, Pillai VB, Labranche C, Yu T, Moss B, Montefiori DC, Robinson HL, Kozlowski PA, Amara RR. 2014. CD40L-adjuvanted DNA/modified vaccinia virus Ankara simian immunodeficiency virus SIV239 vaccine enhances SIV-specific humoral and cellular immunity and improves protection against a heterologous SIVE660 mucosal challenge. J Virol 88:9579-89.

181. Xiao P, Zhao J, Patterson LJ, Brocca-Cofano E, Venzon D, Kozlowski PA, Hidajat R, Demberg T, Robert-Guroff M. 2010. Multiple vaccine-elicited nonneutralizing antienvelope antibody activities contribute to protective efficacy by reducing both acute and chronic viremia following simian/human immunodeficiency virus SHIV89.6P challenge in rhesus macaques. Journal of virology 84:7161-73.

182. Pegu P, Vaccari M, Gordon S, Keele BF, Doster M, Guan Y, Ferrari G, Pal R, Ferrari MG, Whitney S, Hudacik L, Billings E, Rao M, Montefiori D, Tomaras G, Alam SM, Fenizia C, Lifson JD, Stablein D, Tartaglia J, Michael N, Kim J, Venzon D, Franchini G. 2013. Antibodies with High Avidity to the gp120 Envelope Protein in Protection from Simian Immunodeficiency Virus SIVmac251 Acquisition in an Immunization Regimen That Mimics the RV-144 Thai Trial. Journal of Virology 87:1708-1719.

183. Bradley T, Pollara J, Santra S, Vandergrift N, Pittala S, Bailey-Kellogg C, Shen X, Parks R, Goodman D, Eaton A, Balachandran H, Mach LV, Saunders KO, Weiner JA, Scearce R, Sutherland LL, Phogat S, Tartaglia J, Reed SG, Hu SL, Theis JF, Pinter A, Montefiori DC, Kepler TB, Peachman KK, Rao M, Michael

NL, Suscovich TJ, Alter G, Ackerman ME, Moody MA, Liao HX, Tomaras G, Ferrari G, Korber BT, Haynes BF. 2017. Pentavalent HIV-1 vaccine protects against simian-human immunodeficiency virus challenge. Nat Commun 8:15711.

184. Chung AW, Ghebremichael M, Robinson H, Brown E, Choi I, Lane S, Dugast AS, Schoen MK, Rolland M, Suscovich TJ, Mahan AE, Liao L, Streeck H, Andrews C, Rerks-Ngarm S, Nitayaphan S, de Souza MS, Kaewkungwal J, Pitisuttithum P, Francis D, Michael NL, Kim JH, Bailey-Kellogg C, Ackerman ME, Alter G. 2014. Polyfunctional Fc-effector profiles mediated by IgG subclass selection distinguish RV144 and VAX003 vaccines. Sci Transl Med 6:228ra38.

185. Sanders RW, Moore JP. 2017. Native-like Env trimers as a platform for HIV-1 vaccine design. Immunol Rev 275:161-182.

186. Zolla-Pazner S, Powell R, Yahyaei S, Williams C, Jiang X, Li W, Lu S, Wang S, Upadhyay C, Hioe CE, Totrov M, Kong X. 2016. Rationally Designed Vaccines Targeting the V2 Region of HIV-1 gp120 Induce a Focused, Cross-Clade-Reactive, Biologically Functional Antibody Response. J Virol 90:10993-11006.

187. Perez LG, Chen H, Liao HX, Montefiori DC. 2014. Envelope glycoprotein binding to the integrin alpha4beta7 is not a general property of most HIV-1 strains. J Virol 88:10767-77.

188. Pugach P, Ozorowski G, Cupo A, Ringe R, Yasmeen A, de Val N, Derking R, Kim HJ, Korzun J, Golabek M, de Los Reyes K, Ketas TJ, Julien JP, Burton DR, Wilson IA, Sanders RW, Klasse PJ, Ward AB, Moore JP. 2015. A native-like SOSIP.664 trimer based on an HIV-1 subtype B env gene. J Virol 89:3380-95.

189. Li M, Gao F, Mascola JR, Stamatatos L, Polonis VR, Koutsoukos M, Voss G, Goepfert P, Gilbert P, Greene KM, Bilska M, Kothe DL, Salazar-Gonzalez JF, Wei X, Decker JM, Hahn BH, Montefiori DC. 2005. Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J Virol 79:10108-25.

190. Pollara J, Bonsignori M, Moody MA, Liu P, Alam SM, Hwang KK, Gurley TC, Kozink DM, Armand LC, Marshall DJ, Whitesides JF, Kaewkungwal J, Nitayaphan S, Pitisuttithum P, Rerks-Ngarm S, Robb ML, O'Connell RJ, Kim JH, Michael NL, Montefiori DC, Tomaras GD, Liao HX, Haynes BF, Ferrari G. 2014. HIV-1 vaccine-induced C1 and V2 Env-specific antibodies synergize for increased antiviral activities. J Virol 88:7715-26.

191. Whitney JB, Hill AL, Sanisetty S, Penaloza-MacMaster P, Liu J, Shetty M, Parenteau L, Cabral C, Shields J, Blackmore S, Smith JY, Brinkman AL, Peter LE, Mathew SI, Smith KM, Borducchi EN, Rosenbloom DI, Lewis MG, Hattersley J, Li B, Hesselgesser J, Geleziunas R, Robb ML, Kim JH, Michael NL, Barouch DH. 2014. Rapid seeding of the viral reservoir prior to SIV viraemia in rhesus monkeys. Nature 512:74-7.

192. Hansen SG, Ford JC, Lewis MS, Ventura AB, Hughes CM, Coyne-Johnson L, Whizin N, Oswald K, Shoemaker R, Swanson T, Legasse AW, Chiuchiolo MJ, Parks CL, Axthelm MK, Nelson JA, Jarvis MA, Piatak M, Jr., Lifson JD, Picker LJ. 2011. Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. Nature 473:523-7.

193. Yamamoto H, Matano T. 2016. Patterns of HIV/SIV Prevention and Control by Passive Antibody Immunization. Front Microbiol 7:1739.

194. Tuero I, Robert-Guroff M. 2014. Challenges in mucosal HIV vaccine development: lessons from non-human primate models. Viruses 6:3129-58.

195. Zhu Q, Berzofsky JA. 2013. Oral vaccines: directed safe passage to the front line of defense. Gut Microbes 4:246-52.

196. Davitt CJ, Lavelle EC. 2015. Delivery strategies to enhance oral vaccination against enteric infections. Adv Drug Deliv Rev 91:52-69.

197. Chamcha V, Jones A, Quigley BR, Scott JR, Amara RR. 2015. Oral Immunization with a Recombinant Lactococcus lactis-Expressing HIV-1 Antigen on Group A Streptococcus Pilus Induces Strong Mucosal Immunity in the Gut. J Immunol 195:5025-34.

198. Hervouet C, Luci C, Cuburu N, Cremel M, Bekri S, Vimeux L, Maranon C, Czerkinsky C, Hosmalin A, Anjuere F. 2010. Sublingual immunization with an HIV subunit vaccine induces antibodies and cytotoxic T cells in the mouse female genital tract. Vaccine 28:5582-90.

199. Cuburu N, Kweon MN, Hervouet C, Cha HR, Pang YY, Holmgren J, Stadler K, Schiller JT, Anjuere F, Czerkinsky C. 2009. Sublingual immunization with nonreplicating antigens induces antibody-forming cells and cytotoxic T cells in the female genital tract mucosa and protects against genital papillomavirus infection. J Immunol 183:7851-9.

200. Ravi AD, Sadhna D, Nagpaal D, Chawla L. 2015. Needle free injection technology: A complete insight. Int J Pharm Investig 5:192-9.

201. Miller CS, Greenberg RN. 2017. MucoJet: A novel oral microjet vaccination system. Oral Dis doi:10.1111/odi.12697.

202. Hovav AH. 2014. Dendritic cells of the oral mucosa. Mucosal Immunol 7:27-37.

203. Collin M, McGovern N, Haniffa M. 2013. Human dendritic cell subsets. Immunology 140:22-30.

204. Seneschal J, Clark RA, Gehad A, Baecher-Allan CM, Kupper TS. 2012. Human epidermal Langerhans cells maintain immune homeostasis in skin by activating skin resident regulatory T cells. Immunity 36:873-84.

205. Ochoa MT, Loncaric A, Krutzik SR, Becker TC, Modlin RL. 2008. "Dermal dendritic cells" comprise two distinct populations: CD1+ dendritic cells and CD209+ macrophages. J Invest Dermatol 128:2225-31.

206. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. 2004. Retinoic acid imprints gut-homing specificity on T cells. Immunity 21:527-38.

207. Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, Otipoby KL, Yokota A, Takeuchi H, Ricciardi-Castagnoli P, Rajewsky K, Adams DH, von Andrian UH. 2006. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. Science 314:1157-60.

208. Ganguly S, Manicassamy S, Blackwell J, Pulendran B, Amara RR. 2011. Adenovirus type 5 induces vitamin A-metabolizing enzymes in dendritic cells and enhances priming of gut-homing CD8 T cells. Mucosal Immunol 4:528-38.

209. Duester G. 1998. Alcohol dehydrogenase as a critical mediator of retinoic acid synthesis from vitamin A in the mouse embryo. J Nutr 128:459S-462S.

210. Ram D, Peretz B. 2002. Administering local anaesthesia to paediatric dental patients -- current status and prospects for the future. Int J Paediatr Dent 12:80-9.

211. Barouch DH, Stephenson KE, Borducchi EN, Smith K, Stanley K, McNally AG, Liu J, Abbink P, Maxfield LF, Seaman MS, Dugast AS, Alter G, Ferguson M, Li W, Earl PL, Moss B, Giorgi EE, Szinger JJ, Eller LA, Billings EA, Rao M, Tovanabutra S, Sanders-Buell E, Weijtens M, Pau MG, Schuitemaker H, Robb ML, Kim JH, Korber BT, Michael NL. 2013. Protective efficacy of a global HIV-1 mosaic vaccine against heterologous SHIV challenges in rhesus monkeys. Cell 155:531-9.

212. Yates NL, deCamp AC, Korber BT, Liao HX, Irene C, Pinter A, Peacock J, Harris LJ, Sawant S, Hraber P, Shen X, Rerks-Ngarm S, Pitisuttithum P, Nitayapan S, Berman PW, Robb ML, Pantaleo G, Zolla-Pazner S, Haynes BF, Alam SM, Montefiori DC, Tomaras GD. 2018. HIV-1 Envelope Glycoproteins from Diverse Clades Differentiate Antibody Responses and Durability among Vaccinees. J Virol doi:10.1128/JVI.01843-17.

213. Seaman MS, Janes H, Hawkins N, Grandpre LE, Devoy C, Giri A, Coffey RT, Harris L, Wood B, Daniels MG, Bhattacharya T, Lapedes A, Polonis VR, McCutchan FE, Gilbert PB, Self SG, Korber BT, Montefiori DC, Mascola JR. 2010. Tiered categorization of a diverse panel of HIV-1 Env pseudoviruses for assessment of neutralizing antibodies. J Virol 84:1439-52.

214. Zhou M, Ruprecht RM. 2014. Are anti-HIV IgAs good guys or bad guys? Retrovirology 11:109.

215. Saunders KO, Verkoczy LK, Jiang C, Zhang J, Parks R, Chen H, Housman M, Bouton-Verville H, Shen X, Trama AM, Scearce R, Sutherland L, Santra S, Newman A, Eaton A, Xu K, Georgiev IS, Joyce MG, Tomaras GD, Bonsignori M, Reed SG, Salazar A, Mascola JR, Moody MA, Cain DW, Centlivre M, Zurawski S, Zurawski G, Erickson HP, Kwong PD, Alam SM, Levy Y, Montefiori DC, Haynes BF. 2017. Vaccine Induction of Heterologous Tier 2 HIV-1 Neutralizing Antibodies in Animal Models. Cell Rep 21:3681-3690.

216. Gristick HB, von Boehmer L, West AP, Jr., Schamber M, Gazumyan A, Golijanin J, Seaman MS, Fatkenheuer G, Klein F, Nussenzweig MC, Bjorkman PJ. 2016. Natively glycosylated HIV-1 Env structure reveals new mode for antibody recognition of the CD4-binding site. Nat Struct Mol Biol 23:906-915.

217. Byrareddy SN, Kallam B, Arthos J, Cicala C, Nawaz F, Hiatt J, Kersh EN, McNicholl JM, Hanson D, Reimann KA, Brameier M, Walter L, Rogers K, Mayne AE, Dunbar P, Villinger T, Little D, Parslow TG, Santangelo PJ, Villinger F, Fauci AS, Ansari AA. 2014. Targeting alpha4beta7 integrin reduces mucosal transmission of simian immunodeficiency virus and protects gut-associated lymphoid tissue from infection. Nat Med 20:1397-400.

218. Cicala C, Arthos J, Fauci AS. 2011. HIV-1 envelope, integrins and co-receptor use in mucosal transmission of HIV. J Transl Med 9 Suppl 1:S2.

219. Agosto LM, Uchil PD, Mothes W. 2015. HIV cell-to-cell transmission: effects on pathogenesis and antiretroviral therapy. Trends Microbiol 23:289-95.

220. Kozlowski PA, Lynch RM, Patterson RR, Cu-Uvin S, Flanigan TP, Neutra MR. 2000. Modified wick method using Weck-Cel sponges for collection of human

rectal secretions and analysis of mucosal HIV antibody. J Acquir Immune Defic Syndr 24:297-309.

221. Forthal DN, Landucci G, Cole KS, Marthas M, Becerra JC, Van Rompay K. 2006. Rhesus macaque polyclonal and monoclonal antibodies inhibit simian immunodeficiency virus in the presence of human or autologous rhesus effector cells. J Virol 80:9217-25.

222. Wang Z, Lorin C, Koutsoukos M, Franco D, Bayat B, Zhang Y, Carfi A, Barnett SW, Porter F. 2016. Comprehensive Characterization of Reference Standard Lots of HIV-1 Subtype C Gp120 Proteins for Clinical Trials in Southern African Regions. Vaccines (Basel) 4.

223. Hsu DC, O'Connell RJ. 2017. Progress in HIV vaccine development. Hum Vaccin Immunother 13:1018-1030.

224. Zambonelli C, Dey AK, Hilt S, Stephenson S, Go EP, Clark DF, Wininger M, Labranche C, Montefiori D, Liao HX, Swanstrom RI, Desaire H, Haynes BF, Carfi A, Barnett SW. 2016. Generation and Characterization of a Bivalent HIV-1 Subtype C gp120 Protein Boost for Proof-of-Concept HIV Vaccine Efficacy Trials in Southern Africa. PLoS One 11:e0157391.

225. Eudailey JA, Dennis ML, Parker ME, Phillips BL, Huffman TN, Bay CP, Hudgens MG, Wiseman RW, Pollara JJ, Fouda GG, Ferrari G, Pickup DJ, Kozlowski PA, Van Rompay KKA, De Paris K, Permar SR. 2018. Maternal HIV-1 Env Vaccination for Systemic and Breast Milk Immunity To Prevent Oral SHIV Acquisition in Infant Macaques. mSphere 3.

226. Excler JL, Ake J, Robb ML, Kim JH, Plotkin SA. 2014. Nonneutralizing functional antibodies: a new "old" paradigm for HIV vaccines. Clin Vaccine Immunol 21:1023-36.

227. Shingai M, Donau OK, Plishka RJ, Buckler-White A, Mascola JR, Nabel GJ, Nason MC, Montefiori D, Moldt B, Poignard P, Diskin R, Bjorkman PJ, Eckhaus MA, Klein F, Mouquet H, Cetrulo Lorenzi JC, Gazumyan A, Burton DR, Nussenzweig MC, Martin MA, Nishimura Y. 2014. Passive transfer of modest titers of potent and broadly neutralizing anti-HIV monoclonal antibodies block SHIV infection in macaques. J Exp Med 211:2061-2074.

228. Montefiori DC, Roederer M, Morris L, Seaman MS. 2018. Neutralization tiers of HIV-1. Curr Opin HIV AIDS 13:128-136.

229. Jardine JG, Kulp DW, Havenar-Daughton C, Sarkar A, Briney B, Sok D, Sesterhenn F, Ereno-Orbea J, Kalyuzhniy O, Deresa I, Hu X, Spencer S, Jones M, Georgeson E, Adachi Y, Kubitz M, deCamp AC, Julien JP, Wilson IA, Burton DR, Crotty S, Schief WR. 2016. HIV-1 broadly neutralizing antibody precursor B cells revealed by germline-targeting immunogen. Science 351:1458-63.

230. Sok D, Briney B, Jardine JG, Kulp DW, Menis S, Pauthner M, Wood A, Lee EC, Le KM, Jones M, Ramos A, Kalyuzhniy O, Adachi Y, Kubitz M, MacPherson S, Bradley A, Friedrich GA, Schief WR, Burton DR. 2016. Priming HIV-1 broadly neutralizing antibody precursors in human Ig loci transgenic mice. Science 353:1557-1560.

231. Steichen JM, Kulp DW, Tokatlian T, Escolano A, Dosenovic P, Stanfield RL, McCoy LE, Ozorowski G, Hu X, Kalyuzhniy O, Briney B, Schiffner T, Garces F,

Freund NT, Gitlin AD, Menis S, Georgeson E, Kubitz M, Adachi Y, Jones M, Mutafyan AA, Yun DS, Mayer CT, Ward AB, Burton DR, Wilson IA, Irvine DJ, Nussenzweig MC, Schief WR. 2016. HIV Vaccine Design to Target Germline Precursors of Glycan-Dependent Broadly Neutralizing Antibodies. Immunity 45:483-496.

232. Escolano A, Steichen JM, Dosenovic P, Kulp DW, Golijanin J, Sok D, Freund NT, Gitlin AD, Oliveira T, Araki T, Lowe S, Chen ST, Heinemann J, Yao KH, Georgeson E, Saye-Francisco KL, Gazumyan A, Adachi Y, Kubitz M, Burton DR, Schief WR, Nussenzweig MC. 2016. Sequential Immunization Elicits Broadly Neutralizing Anti-HIV-1 Antibodies in Ig Knockin Mice. Cell 166:1445-1458 e12.

233. Montefiori DC, Karnasuta C, Huang Y, Ahmed H, Gilbert P, de Souza MS, McLinden R, Tovanabutra S, Laurence-Chenine A, Sanders-Buell E, Moody MA, Bonsignori M, Ochsenbauer C, Kappes J, Tang H, Greene K, Gao H, LaBranche CC, Andrews C, Polonis VR, Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Self SG, Berman PW, Francis D, Sinangil F, Lee C, Tartaglia J, Robb ML, Haynes BF, Michael NL, Kim JH. 2012. Magnitude and breadth of the neutralizing antibody response in the RV144 and Vax003 HIV-1 vaccine efficacy trials. J Infect Dis 206:431-41.

234. Shukair SA, Allen SA, Cianci GC, Stieh DJ, Anderson MR, Baig SM, Gioia CJ, Spongberg EJ, Kauffman SM, McRaven MD, Lakougna HY, Hammond C, Kiser PF, Hope TJ. 2013. Human cervicovaginal mucus contains an activity that hinders HIV-1 movement. Mucosal Immunol 6:427-34.

235. Fahrbach KM, Malykhina O, Stieh DJ, Hope TJ. 2013. Differential binding of IgG and IgA to mucus of the female reproductive tract. PLoS One 8:e76176.

236. Kramski M, Parsons MS, Stratov I, Kent SJ. 2013. HIV-specific antibody immunity mediated through NK cells and monocytes. Curr HIV Res 11:388-406.

237. Holl V, Peressin M, Moog C. 2009. Antibody-Mediated Fcgamma Receptor-Based Mechanisms of HIV Inhibition: Recent Findings and New Vaccination Strategies. Viruses 1:1265-94.

238. Asmal M, Sun Y, Lane S, Yeh W, Schmidt SD, Mascola JR, Letvin NL. 2011. Antibody-dependent cell-mediated viral inhibition emerges after simian immunodeficiency virus SIVmac251 infection of rhesus monkeys coincident with gp140-binding antibodies and is effective against neutralization-resistant viruses. J Virol 85:5465-75.

239. Hessell AJ, Hangartner L, Hunter M, Havenith CE, Beurskens FJ, Bakker JM, Lanigan CM, Landucci G, Forthal DN, Parren PW, Marx PA, Burton DR. 2007. Fc receptor but not complement binding is important in antibody protection against HIV. Nature 449:101-4.

240. Florese RH, Demberg T, Xiao P, Kuller L, Larsen K, Summers LE, Venzon D, Cafaro A, Ensoli B, Robert-Guroff M. 2009. Contribution of nonneutralizing vaccine-elicited antibody activities to improved protective efficacy in rhesus macaques immunized with Tat/Env compared with multigenic vaccines. J Immunol 182:3718-27.

241. McAllister L, Anderson J, Werth K, Cho I, Copeland K, Le Cam Bouveret N, Plant D, Mendelman PM, Cobb DK. 2014. Needle-free jet injection for

administration of influenza vaccine: a randomised non-inferiority trial. Lancet 384:674-81.

242. Aran K, Chooljian M, Paredes J, Rafi M, Lee K, Kim AY, An J, Yau JF, Chum H, Conboy I, Murthy N, Liepmann D. 2017. An oral microjet vaccination system elicits antibody production in rabbits. Sci Transl Med 9.

243. Okoye AA, Picker LJ. 2013. CD4(+) T-cell depletion in HIV infection: mechanisms of immunological failure. Immunol Rev 254:54-64.