Antibodies in Vaccine Protection against SIV and HIV-1 Infection

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Abstract

The properties of human immunodeficiency virus type 1 (HIV-1) and its simian counterpart SIV that enable persistent replication in the face of robust cellular, antibody, and innate immune responses have complicated efforts to develop a safe and effective vaccine. Vaccine protection against HIV-1 infection may require a combination of immune mechanisms. However, the types of immune responses that can be induced by vaccination to prevent HIV-1 infection remain unclear.

The features of the viral envelope glycoprotein (Env) that confer inherent resistance to neutralization by antibodies also interfere with the development of antibody responses. We therefore vaccinated rhesus macaques with single-cycle SIV (scSIV) strains expressing Env proteins mutated to remove features that interfere with the induction of antibody responses. Antibodies capable of neutralizing Env-modified but not wild-type SIV were selectively enhanced.

Identifying the immune responses underlying complete protection by live-attenuated SIV against pathogenic SIV challenge may provide guidance for HIV-1 vaccine design. To test the hypothesis that antibodies not measurable by assays for virus neutralization correlate with protection by live-attenuated SIV, we developed a novel assay for antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC activity
increased progressively over time after inoculation, and was measurable against viruses expressing heterologous Env proteins from independent SIV isolates when neutralization was undetectable. Two separate pathogenic SIV<sub>mac</sub>251 challenge experiments took advantage of either the strain specificity or the time-dependent development of immunity to overcome complete protection by live-attenuated SIV. In both experiments, macaques inoculated with live-attenuated SIV that remained uninfected by SIV<sub>mac</sub>251 had significantly higher ADCC activity than those that became infected.

We also measured ADCC for the primary immune correlates analysis of a recent HIV-1 vaccine clinical trial in Thailand (RV144) that reported modest vaccine protection (31%). There was a nonsignificant trend towards lower risk of infection among vaccinees with high versus low relative ADCC activity. However, Env-specific IgA correlated with risk, prompting an analysis stratified by IgA levels. Among vaccinees with low Env-specific IgA, there was lower risk of infection among those with higher ADCC activity.

These observations suggest that antibodies that direct ADCC may contribute to vaccine protection against SIV and HIV-1 infection.
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CHAPTER 1: INTRODUCTION

A primer on HIV-1 vaccine science
1. A. THE GLOBAL HIV-1 PANDEMIC

Scale of the HIV-1 pandemic

The pandemic caused by human immunodeficiency virus type 1 (HIV-1) is the great catastrophe of our time. Approximately 30 million people have died of acquired immunodeficiency virus syndrome (AIDS)\(^1\), which is caused by infection with HIV-1\(^2-6\). Globally, 33 million people are currently infected by HIV-1\(^1,7\) (Fig. 1.1). Prevalence rates exceed 10% in many sub-Saharan African countries, and are highest in Swaziland, at 26%\(^1\). South Africa, Nigeria, India, Kenya, Mozambique, Tanzania, Uganda, the United States, and Zimbabwe each have over 1 million people living with HIV-1 infection\(^7\). HIV-1 has already caused and continues to cause vast suffering.

**Figure 1.1. Global distribution of HIV-1 infections**\(^1,7\). HIV-1 is a pandemic disease, with the majority of infections and highest infection rates occurring in Africa. However, the United States is among the 10 countries with the largest total number of people living with HIV-1.
Emergence and features of the HIV-1 pandemic

HIV-1 was first identified as a lentivirus associated with the syndrome now known as AIDS in homosexual men in the United States 1981. High transmission rates among homosexual men and intravenous (i.v.) drug users enabled the virus to spread rapidly among these high-risk populations. However, HIV-1 predominantly affects the general population in the countries with the highest prevalence rates. In sub-Saharan Africa today, women are disproportionately infected by HIV-1, comprising 76% of HIV-positive people. Among teenage girls aged 15-19 in Lesotho, nearly 8% are HIV-positive. A prominent theme of the HIV-1 pandemic is that infection rates tend to be highest among otherwise vulnerable populations.

Consequences of the HIV-1 pandemic

Consequences of the HIV-1 pandemic cause additional hardship. Shortened life expectancies, high morbidity, and the cost of caring for the sick handicaps economic development, perpetuating poverty. The 16 million children orphaned by AIDS are an appalling but prominent component of this vicious cycle. These forces have the potential to fuel regional instability. HIV-1 has the potential to spawn additional public health crises, seen in a resurgence of tuberculosis (TB). The effects of HIV-1 have ramifications beyond those who are infected.
Need for a safe and effective HIV-1 vaccine

A safe and effective vaccine for HIV-1 is urgently needed. Widespread awareness of the risk of HIV-1 infection through unprotected sex or the sharing of needles has not led to an adequate reduction in the spread of HIV-1 infection. Approximately 2.6 million new infections continue to occur per year\(^1\). An international public health program to test all potentially at-risk individuals for HIV-1 infection, and to treat with highly active antiretroviral therapy (HAART) all persons identified as infected, could theoretically reduce new infections by curtailing viral replication in the would-be transmitter\(^{14-16}\). However, a vast and expensive “test and treat” program has yet to materialize. Only a safe and effective vaccine for HIV-1 can end the pandemic.

1. B. RESISTANCE TO CELLULAR AND INNATE IMMUNITY

Viral resistance to host immune responses complicates vaccine development

The difficulty of vaccinating against HIV-1 stems from features that enable it to replicate persistently for years\(^{17-20}\) in the face of vigorous antibody, T-cell, and innate immune responses. In particular, the inherent resistance of primate lentiviruses to neutralization of viral infectivity by antibodies has impeded efforts to develop a vaccine that prevents HIV-1 infection. Once infection has occurred, HIV-1 and SIV circumvent immunity by evolving to escape virus-specific immune responses, and by inducing immunodeficiency. Thus, the resistance of primate lentiviruses to host immunity has prevented the development of a safe and effective vaccine.
Resistance to innate antiviral restriction factors

A vaccine against HIV-1 might have been superfluous, if not for viral resistance to proteins that mediate innate immunity. The cytidine deaminase APOBEC3G is an innate antiviral restriction factor that can induce lethal hypermutation of nascent viral cDNA\textsuperscript{8,21-24} and interfere with reverse transcription\textsuperscript{25}. However, the Vif protein of HIV-1 binds APOBEC3G and the E3 ubiquitin ligase complex Cul5/ElonginB/ElonginC/Rbx1 to promote its ubiquitylation and proteosomal degradation, thereby freeing the virus from restriction\textsuperscript{26-29}. BST-2 is an interferon-inducible restriction factor, which prevents virus release by tethering virions to the producer cell, as well as promoting the internalization and degradation of virus particles\textsuperscript{30-33}. However, the Vpu protein of HIV-1 and the Nef protein of SIV have evolved to oppose restriction by BST-2 through mechanisms that inhibit its surface expression\textsuperscript{30,31,34,35}. In an example of genetic malleability and rapid evolution to oppose innate immunity, nef-deleted SIV acquired the ability to downmodulate BST-2 through mutations in the cytoplasmic tail of the SIV envelope glycoprotein\textsuperscript{36}. Rhesus macaque TRIM5\textalpha{} and owl monkey TRIM-Cyp can block infection by HIV-1 but not SIV\textsubscript{mac}239\textsuperscript{37,38}. The current level of resistance of SIV\textsubscript{mac}239 to rhesus TRIM5\textalpha{} probably evolved after its cross-species transmission from sooty mangabeys, since common rhesus TRIM5 alleles partially restrict the sooty mangabey viruses SIV\textsubscript{sm}E660 and SIV\textsubscript{sm}E543-3 but not SIV\textsubscript{mac}239\textsuperscript{39-41}. Clearly, HIV-1 can replicate in the presence of human TRIM5\textalpha{}\textsuperscript{37}. Thus, countermeasures against innate antiviral restriction factors have enabled the primate lentiviruses to replicate in their hosts.
Resistance to cellular immune responses

Downregulation of MHC class I. The primate lentiviruses interfere with effective cellular immunity though multiple mechanisms. CD8\(^+\) T-cells can inhibit viral replication and also kill virus-infected cells\(^{42-55}\). However, recognition of viral infection by CD8\(^+\) T-cells depends upon presentation of viral peptides by MHC class I molecules\(^{56}\). The Nef proteins of HIV-1 and SIV are capable of downregulating MHC class I molecules, thereby interfering with CD8\(^+\) T-cell recognition of viral peptides presented by MHC class I\(^{57}\). The absence of this immune-evasion function probably contributes to the lower replication and pathogenicity of nef-deleted strains of SIV or HIV-1, relative to wild-type viruses\(^{58,59}\). Although MHC class I downregulation impairs recognition of virus-infected cells by CD8\(^+\) T-cells, natural killer (NK) cells respond to the absence of MHC class I by killing cells missing these surface molecules\(^{60}\). SIV and HIV-1 solve this dilemma with characteristic elegance by selectively downregulating a subset of MHC class I molecules\(^{61-63}\). HIV-1 Nef reduces recognition by the majority of virus-specific CD8\(^+\) T-cells by selectively downregulating the MHC class I molecules HLA-A and HLA-B, while leaving HLA-C and HLA-E expressed on the surface of the virus-infected cell\(^{61}\). While HLA-C represents the product of a unique duplication of the HLA-B locus that occurred in apes, rhesus macaques possess different duplications within the MHC locus\(^{64,65}\). Using a similar strategy, SIV Nef proteins contribute to immune evasion by selectively downregulating specific macaque and sooty mangabey MHC alleles\(^{62}\). Selective downregulation of MHC class I interferes with CD8\(^+\) T-cell-mediated immunity.
**CD8⁺ T-cell escape.** The primate lentiviruses evolve to escape CD8⁺ T-cell responses during ongoing infection *in vivo*. Novel mutations arise during HIV-1 infection, which abolish MHC-restricted lysis by CD8⁺ T-cells of targets pulsed with the mutant peptide. Subsequent demonstrations of escape included studies showing that CD8⁺ T-cell escape is not only a hallmark of chronic infection, but emerges early during acute infection. A loss of controlled viral replication could be temporally associated with the emergence of CD8⁺ T-cell escape variants, and the number of mutations in known CD8⁺ T-cell epitopes correlated with viral load. Furthermore, CD8⁺ T-cell escape can be achieved through mutations in flanking amino acids that affect the processing of peptides for presentation by MHC class I, without affecting epitope itself. Nevertheless, a loss of controlled viral replication and progression to AIDS can occur without CD8⁺ T-cell escape. The genetic plasticity of primate lentiviruses routinely permits the escape of CD8⁺ T-cell recognition through ongoing evolution *in vivo*, and represents a prominent immune evasion mechanism.

**Tropism for CD4⁺ T-cells.** Induction of immunodeficiency by the primate lentiviruses is itself an immune evasion mechanism. Direct infection of CD4⁺ T-cells and depletion of this lymphocyte population impairs immune responses. The extent of CD4⁺ T-cell depletion is predictive of progression to immunodeficiency and the onset of AIDS-defining illness. This association is probably due in part to the loss of CD4⁺ T-cell functions critical to the orchestration of immune responses. CD4⁺ T-cells produce cytokines and support lymphoid architecture critical to promoting antibody and cell-mediated immune responses. Thus, a biological property of immunodeficiency
viruses as basic as their tropism for CD4\(^+\) T-cells is part of a multifaceted immune evasion strategy.

**Chronic immune activation and negative regulation.** Viral persistence may contribute to immune evasion by inducing negative regulatory pathways that dampen the efficacy of cellular immune responses. Tolerance to self versus responsiveness to pathogens is perhaps the central concept in understanding the regulation of immunity\(^8^4\). To prevent harm to healthy cells, and to prevent an uncontrolled proliferation of lymphocytes that would characterize leukemia or lymphoma, immune functions are negatively regulated by multiple mechanisms. Self-reactive T-cells normally undergo programmed cell death (apoptosis), and thus do not undergo clonal expansion. However, as redundant mechanisms to eliminate self-reactive T-cells and B-cells, and to prevent uncontrolled proliferation, these cells are also deleted by apoptosis in the periphery, or enter an unresponsive state known as anergy\(^8^4\). Consistent with a role for chronic antigenic stimulation in immunodeficiency, T-cell activation predicted shorter survival better than viral load measurements in HIV-1 patients\(^8^5\). In the context of chronic viral infection, the reduced capacity of T-cells to respond to antigen has been called “dysfunction” or “exhaustion”\(^8^6-9^2\). However, since this state bears similarities to anergy and includes programmed cell death, the term “negative regulation” may be more accurate. The negative regulation of immune responses in the context of chronic infection with HIV-1 or SIV may undermine cellular immunity and contribute to the development of AIDS-defining immunodeficiency.
**Chronic immune activation due to increased microbial translocation.**

Microbial translocation across the intestinal epithelium contributes to the chronic immune activation induced by HIV-1 or SIV infection\(^93,^94\). The gastrointestinal tract is colonized by a high density of commensal bacteria (\(10^9-10^{12}\) bacterial cells per gram in the colon), which exist in mutually-beneficial homeostasis with their host\(^95\). However, the integrity of gut mucosal immunity is compromised during HIV-1 or SIV infection, due at least in part to the depletion of gut CD4\(^+\) T-cells early after infection\(^96-100\). In people infected with HIV-1 and in rhesus macaques infected with SIV, lipopolysaccharide (LPS), a major component of the cell wall of gram-negative bacteria, is detectable in plasma at elevated levels\(^93,^94\). Elevated LPS in plasma indicates that bacteria are exiting the lumen of the intestine and transiting across a breached mucosal barrier. LPS is one of several molecules recognized by receptors for pathogen-associated molecular patterns (PAMPs). Binding of PAMPs such as LPS to their receptors activates the secretion of interferon and proinflammatory cytokines. Consequently, higher plasma LPS is correlated with T-cell activation and interferon concentration in plasma. Thus, microbial products that translocate across a compromised mucosal barrier may contribute to chronic immune activation in HIV-1 and SIV infection. Chronic immune activation, as both a direct and an indirect consequence of HIV-1 or SIV infection, may facilitate viral replication by contributing to immunodeficiency.

**Negative regulation of immunity through Fas.** One pathway HIV-1 and SIV exploit within the normal regulatory framework of the immune system is the induction of CD95/Fas on T-cells responding to viral infection. CD95/Fas is used as a marker for
activated T-cells\textsuperscript{101}. Interaction between CD95/Fas and its ligand (FasL) initiates apoptosis\textsuperscript{102}. HIV-1-specific CD8\textsuperscript{+} T-cells express CD95/Fas, and hence are subject to depletion by this mechanism\textsuperscript{103}. The Nef proteins of HIV-1 and SIV induce FasL expression, conveying a pro-apoptotic signal to virus-specific CD8\textsuperscript{+} T-cells\textsuperscript{104,105}. This pro-apoptotic signal is also received by uninfected CD4\textsuperscript{+} T-cells, and thus serves as an additional mechanism promoting CD4\textsuperscript{+} T-cell depletion\textsuperscript{106,107}. Thus, the CD95/Fas pro-apoptotic pathway is exploited by HIV-1 and SIV.

**Negative regulation of immunity through PD-1.** Chronic viral infection can induce a state of unresponsiveness in virus-specific T-cells, without deletion of these cells from the circulating lymphocyte population\textsuperscript{108}. This may be due to a combination of negative regulatory mechanisms that include expression of PD-1 (programmed death 1)\textsuperscript{86}. The virus-specific T-cells from HIV-1-infected people and SIV-infected macaques express PD-1, and blocking the PD-1 ligand promotes the effector functions of these cells, suggesting that chronic infection by the primate lentiviruses promotes T-cell unresponsiveness associated with PD-1 expression\textsuperscript{86}. The PD-1 pathway is one mechanism through which cellular immune responses can be functionally inactivated by chronic viral infection.

**Negative regulation of NK cells.** NK cells are capable of killing virus-infected cells, but are functionally impaired in chronic HIV-1 infection. NK cells can be induced to kill targets by several triggers, including the absence of MHC class I, mentioned above\textsuperscript{60}. Antibody-dependent cell-mediated cytotoxicity (ADCC) is another mechanism
by which NK cells can kill virus-infected targets\textsuperscript{109,110}. NK cells will kill virus-infected cells by ADCC when a lattice of antibody-antigen interactions crosslinks CD16, a receptor for IgG isotype antibodies that is expressed on the surface of NK cells. CD16 crosslinking induces the NK cell to degranulate, releasing perforin and granzyme, which kill the virus-infected cell. However, much of the cytolytic CD56\textsuperscript{dim} CD16\textsuperscript{pos} population of NK cells becomes replaced by a population of unresponsive CD56\textsuperscript{neg} CD16\textsuperscript{pos} NK cells in the context of chronic HIV-1 infection\textsuperscript{111-113}. The functional impairment of the ability of CD56\textsuperscript{neg} CD16\textsuperscript{pos} cells to secrete cytokines, degranulate, and lyse target cells appears to be due to a regulatory mechanism that includes a shift in the equilibrium of phosphoinositide metabolism and the downregulation of perforin expression\textsuperscript{112-116}.

Furthermore, HIV-1 patients have lower levels of CD16 expression on NK cells, perhaps due to the sloughing off of CD16 by matrix metalloproteinases following stimulation in the context of chronic viral infection\textsuperscript{117-120}. Chronic infection appears to compromise NK function, and thereby promote viral persistence. Thus, viral persistence itself is an immune evasion mechanism that may partially explain AIDS-defining immunodeficiency.

1. C. RESISTANCE TO ANTIBODY RESPONSES

\textit{Basic context for understanding neutralization resistance}

\textbf{An antibody-resistant fusion machine.} To infect a cell, enveloped viruses must fuse the viral and cellular membranes, which delivers the viral genome into the cell. For retroviruses, this membrane fusion is mediated by the viral envelope glycoprotein (Env)\textsuperscript{121}. Antibodies can block the entry of viruses into cells, and thereby neutralize viral infectivity\textsuperscript{122-124}. However, Env is able to mediate fusion of the viral and cellular
membranes in the presence of antibodies against it. This capability is necessary for the persistent replication of these viruses in the face of vigorous Env-specific antibody responses. The features that enable Env to resist the antibody responses mounted during ongoing infection render Env resistant to vaccine-elicited antibody responses. Thus, the difficulty of vaccinating against HIV-1 and SIV is due to features of Env that have evolved to enable membrane fusion and persistent viral replication in the presence of Env-specific antibody responses. The same features that minimize the binding of antibodies to Env also interfere with the ability of B-cells to interact with Env. Therefore, the features that confer resistance to existing antibody responses interfere with the development of effective antibody responses in the context of natural infection, and thereby also interfere with attempts to elicit antibody responses by vaccination. In addition, the Env protein has enormous sequence plasticity, which enables variants to arise that escape antibody responses. Continual antigenic escape since the beginning of the HIV-1 pandemic has generated considerable sequence diversity. The inherent resistance of Env to antibodies and the enormous sequence diversity of circulating HIV-1 isolates pose significant obstacles to the development of an effective vaccine against HIV-1.

**Gross anatomy of Env.** Env is a class I viral fusion protein, the prototypical example of which is the hemagglutinin (HA) protein of influenza. Like all class I fusion proteins, Env is trimeric. Although synthesized as a 160 kilodalton (kDa) glycoprotein (gp160) precursor, it is cleaved by the protease furin into 2 polypeptides, gp120 and gp41 (Fig. 1.2a). The names SU and TM refer to the N-terminal surface...
and C-terminal transmembrane domains of retroviral Env proteins. Using retrovirus terminology, SU is gp120, and TM is gp41. Env is heavily glycosylated, with approximately 24 N-linked glycans in gp120 that account for half of its molecular weight, and 3-4 N-linked glycans in gp41 (Fig. 1.2a). Gp120 also possesses 5 loops created by disulfide linkages, the sequences of which are highly variable, and are thus designated as variable loops 1-5 (V1, V2, V3, V4, and V5) (Fig. 1.2a). The binding sites for the viral receptor (CD4) and the coreceptor are on gp120. Primate lentiviruses can utilize one or more type of chemokine receptor as a coreceptor. These chemokine receptors, which belong to the broader family of G protein-coupled receptors (GPCRs), include CCR2, CCR3, CCR5, CXCR4, GPR1, GPR15/BOB, and STRL33/BONZO. Interactions between gp120 and CD4 plus the coreceptor induce conformational changes in gp120 and gp41 that drive membrane fusion (Fig. 1.2c-g). However, antibodies can bind to Env such that they prevent the interactions necessary for membrane fusion, and thus neutralize virus infectivity. Similarly, recombinant soluble CD4 (sCD4), expressed as a secreted protein that is truncated before the transmembrane domain, can also inhibit entry of HIV-1 and SIV. An exception to direct interaction with Env as a mechanism of neutralization is that antibodies can also inhibit fusion by binding directly to CD4 or the coreceptor. Env mediates fusion of the viral and cellular membranes, unless antibodies or sCD4 bind to Env and neutralize virus infectivity.
**Figure 1.2. Major features and conformational rearrangements of Env.** The SIV Env protein is depicted schematically, indicating N-linked glycans, variable loops, and the cleavage site between gp120 and gp41 (a). Env trimer reconstructed from cry-electron tomography (b). Landmarks of the gp120 crystal structure, including the CD4-binding loop (yellow), bridging sheet (dashed box), inner domain 7-stranded β-sandwich (blue), “topological layers” (green, brown, and magenta), and outer domain (red) (c). NMR structure of the post-fusion 6-helix bundle of gp41 (d). Cartoon diagrams of the native trimer (e), gp120 bound to CD4 and CCR5 plus the extended PHI conformation of gp41 (f), and the post-fusion 6-helix bundle (g).

**T-cell line-adapted viruses are not resistant to neutralization.** The extent to which HIV-1 Env proteins are inherently resistant to neutralizing antibodies, and the problem this poses to HIV-1 vaccine development, was not initially appreciated and remains controversial. HIV-1 strains isolated earlier in the disease course typically use
CCR5 but not CXCR4 as a coreceptor, whereas strains isolated from AIDS patients late in the disease course frequently use CXCR4 instead of CCR5, or are capable of using both (*i.e.* are dual-tropic)\(^{154,155}\). Although there are neutralization-sensitive variants of HIV-1 and SIV that utilize CCR5, no highly neutralization-resistant CXCR4-tropic or dual-tropic strains have been characterized to date, suggesting that CXCR4 usage may not be compatible with a high degree of resistance to neutralization by antibodies and sCD4\(^{156-160}\). Nonetheless, the immortalized CD4\(^+\) T-cell lines utilized for growing HIV-1 and also for various assays express CXCR4 but not CCR5\(^{161}\). The sensitivity of HIV-1 or SIV to neutralization can be increased by passaging these viruses in CD4\(^+\) T-cell lines, or in stimulated lymphocytes\(^{162-170}\). This loss of resistance to neutralization may be due to adaptation to CXCR4 and to prolonged replication in the absence of host antibody responses. Commonly used lab strains such as HIV-1\(\text{HxB2}\) and HIV-1\(\text{MN}\) were highly passaged, first in primary cells, and then in an immortalized CD4\(^+\) T-cell line, after being obtained from AIDS patients\(^{2,171-174}\). Consequently, these lab strains are easily neutralized by antisera elicited by vaccination with recombinant gp120\(^{175-177}\) (Fig. 1.3), by sCD4\(^{162}\), and also by monoclonal antibodies with various specificities\(^{178}\). After the experimental infection of chimpanzees and the accidental infection of a laboratory worker with HIV-1\(\text{HxB2}\), this T-cell line-adapted strain acquired a neutralization-resistant phenotype, suggesting that its previous neutralization

**Figure 1.3. Neutralization of T-cell line-adapted strains but not primary isolates of HIV-1\(^{177}\).**
sensitivity was an adaptation to growth in vitro\textsuperscript{179,180}. The testing of several vaccine products developed by companies in the early 1990’s was discontinued when antibody responses elicited by these vaccines were found to have no effect on the infectivity of HIV-1 strains that were minimally passaged or cloned directly without growth in vitro, known as primary isolates, despite their ability to neutralize T-cell line-adapted lab strains\textsuperscript{166,176,177}.

**Many antibodies fail to neutralize T-cell line adapted viruses.** Despite the sensitivity of T-cell line-adapted strains such as HIV-1\textsubscript{HxB2} to neutralization, many gp120-specific monoclonal antibodies still fail to neutralize these viruses\textsuperscript{178,181}. This failure to neutralize T-cell line-adapted strains can be understood in terms of the ability of antibodies specific for gp120 to bind monomeric versus trimeric forms of the protein. Secreted, soluble trimeric forms of Env protein can be produced by mutating the furin cleavage site at the gp120/gp41 junction, plus adding a stop codon at the beginning of the membrane-spanning portion of gp41\textsuperscript{182,183}. These soluble trimers are generally designated “gp140,” since they have a lower molecular weight than gp160, due to the absence of the cytoplasmic tail of gp41. The affinity of antibodies for gp120 or gp140 is typically assessed by enzyme-linked immunosorbent assay (ELISA). A subset of gp120-specific antibodies have significantly higher affinities by ELISA for gp120 than for preparations containing trimeric gp140\textsuperscript{182,184}. Although trimerization may decrease binding through conformational effects, this reduced binding to preparations containing gp140 appears to often be due to the occlusion of antibody epitopes by trimerization\textsuperscript{182,184}. Preparations of trimeric gp140 often appear to contain a fraction of dissociated
monomeric protein, which may account for the incomplete elimination of signal against epitopes occluded specifically in the context of trimeric gp140\textsuperscript{185}. Since inter-subunit interfaces are not surface-exposed, they have probably not evolved to be poor targets for antibodies. Due to occlusion in the trimer, antibodies that recognize the interfaces between gp120 subunits and between gp120 and gp41 cannot neutralize even T-cell line-adapted viruses.

**Neutralizing, non-neutralizing, and silent faces of gp120.** Neutralizing and non-neutralizing gp120-specific antibodies can be assigned to competition groups, based upon reciprocal binding inhibition experiments\textsuperscript{181,186}. For example, preincubation with a saturating concentration of the CD4-binding site antibody b12 inhibits the binding of a sCD4-IgG fusion construct, and preincubation with a saturating concentration of sCD4-IgG inhibits binding of b12\textsuperscript{181}. Thus, sCD4-IgG and b12 belong to the same competition group. Antibodies that bind gp120 but fail to neutralize even T-cell line-adapted viruses map to competition groups that are distinct from those that can neutralize T-cell line-adapted viruses\textsuperscript{181,184}. Although a crystal structure for gp120 was not yet available, the authors of this early work were able to assign the distinct competition groups to separate neutralizing and non-neutralizing faces of gp120 (Fig. 1.4). The spatial locations of the neutralizing and non-neutralizing faces of gp120 were identified when the amino acids necessary for the binding of various monoclonal antibodies were mapped onto the first HIV-1 gp120 crystal structure (Fig. 1.5)\textsuperscript{187,188}. The localization of neutralization epitopes to one surface, the neutralizing face, suggests that the other faces of gp120 are not exposed in the trimer\textsuperscript{187,188}. This crystal structure also identified a third face of gp120 as
an immunogenically silent face, due to the absence of monoclonal antibodies that mapped
to the underlying surfaces\textsuperscript{187,188}. Thus, a subset of gp120-specific antibodies competes
for binding to epitopes at interfaces between Env subunits that are occluded in the trimer.
Consequently, these antibodies cannot neutralize even T-cell line-adapted viruses.

\textbf{Figure 1.4. Some gp120-specific monoclonal antibodies map to surfaces that are
occluded in the trimer.} This drawing combines the cross-competition analysis of
monoclonal antibody specificities conducted by Moore \textit{et al.}\textsuperscript{181} with differences in the
relative ability to bind gp120 versus gp140 conducted by Wyatt \textit{et al.}\textsuperscript{184} Competition
groups and monoclonal antibodies were drawn on the neutralizing face, which contains
the CD4-binding site (a), and the non-neutralizing face (b). The intensity of red shading
is proportional to the reduction in binding for gp140 relative to gp120.
Figure 1.5. Crystal structure of HIV-1 gp120 in complex with sCD4 and monoclonal antibody 17b. A ribbon structure for gp120 in the CD4-bound conformation is oriented with the side proximal to the virus membrane at top (a). A similarly-oriented space-filling structure is colored to indicate the silent, non-neutralizing, and neutralizing faces of gp120 (b). The CD4 and 17b polypeptide chains are not shown.

Antibodies that bind monomeric gp120 but not Env expressed on cells. As observed for neutralization of T-cell line-adapted viruses, a subset of gp120-specific antibodies cannot bind Env that is expressed on the cell surface. Among monoclonal antibodies that bound gp120, only the subset that were capable of binding to cells expressing Env were also capable of neutralizing virus infectivity. Despite having a similar ability to bind gp120 as sera from HIV-1 patients, sera from rabbits immunized with recombinant gp120 protein poorly bound to cells transfected with DNA encoding an Env derived from HIV-1 HxB2. The inability of these gp120-specific antibodies to bind Env expressed on the cell surface suggests that these antibodies recognized surfaces that reside at interfaces between subunits. Therefore, antibodies that recognize the interfaces between gp120 subunits and between gp120 and gp41 are occluded within Env trimers on virions and cells.
Antibodies that neutralize but do not bind monomeric gp120. The converse to the inability of many gp120-specific antibodies to bind Env trimers is the class of antibodies that bind trimeric Env but not monomeric gp120. For example, although most of the Env-specific monoclonal antibodies isolated by Walker et al. from an HIV-1 patient with a potent neutralizing antibody response were non-neutralizing, most of those capable of neutralizing virus infectivity did not bind monomeric gp120 and gp41 (Fig. 1.6)\textsuperscript{191}. Neutralizing antibodies that bind Env on virus particles and cells but do not bind monomeric gp120 or gp41 subunits are quaternary antibodies specific for conformational structures that depend upon interactions with other subunits of the Env trimer\textsuperscript{192-195}. Thus, most antibodies that bind monomeric Env subunits cannot neutralize, and many neutralizing antibodies cannot bind monomeric Env subunits.

Figure 1.6. Neutralization versus gp120 and gp41 binding\textsuperscript{191}. Most gp120- or gp41-specific monoclonal antibodies failed to neutralize infectivity, and most neutralizing antibodies failed to bind recombinant gp120 or gp41 in an ELISA.
Primary isolates of HIV-1 and SIV are resistant to neutralization. Unlike T-cell line-adapted viruses, primary isolates of HIV-1 are highly resistant to neutralization by antibodies and sCD4\textsuperscript{162,170,196-204}. For example, a panel of reagents including sCD4-IgG, monoclonal antibodies, and patient sera failed to neutralize a panel of primary HIV-1 isolates, despite having similar abilities to bind recombinant gp120 proteins from these viruses\textsuperscript{200}. The neutralization resistance of HIV-1 strains has recently been organized into a 3-tier system (Fig. 1.7)\textsuperscript{202}. In this tiered system, T-cell line-adapted viruses and some primary isolates comprised tier 1, the most neutralization-sensitive group. However, the majority of primary isolates fell into tier 2. On average, pooled HIV-1 patient plasma had 50% neutralization titers against tier 2 viruses of approximately 80 to 300. Those with average 50% neutralization titers by pooled plasma of less than 80 comprised tier 3. The primary isolates of SIV used in challenge studies, SIV\textsubscript{mac}239, SIV\textsubscript{mac}251, and SIV\textsubscript{sm}E543-3, were judiciously selected as models for HIV-1 infection and pathogenesis, since they are also resistant to neutralization by antibodies and sCD4\textsuperscript{158,164,205,206}. Whereas neutralization of SIV\textsubscript{mac}251 is undetectable for sera from most infected animals, the 50% neutralization titer against T-cell line-adapted SIV\textsubscript{mac}251\textsubscript{TCLA} is in excess of a 5120-fold dilution of serum\textsuperscript{164}. Therefore, neutralization resistance is a phenotype that can be lost, and the highly dilute concentrations of antibody that neutralize T-cell line-adapted viruses demonstrate that primary viruses resist neutralization by high concentrations of these antibodies\textsuperscript{156,166}. The high level of resistance of primary isolates of HIV-1 and SIV to neutralization is a consequence of having evolved to replicate persistently for years in the face of robust host antibody responses.
Figure 1.7. Tiered ranking system for neutralization sensitivity. The sensitivity of 109 primary HIV-1 isolates to neutralization by plasma pools representing different clades of HIV-1 was compared. The top and bottom of each vertical bar represent the highest and lowest neutralizing antibody titer against each virus listed below, and the means are indicated by black dots.

Difference between transmitted versus chronic variants? Characterizing the HIV-1 strains that establish infection in new hosts is a critical element of HIV-1 vaccine research. It is essential that vaccine candidates are evaluated in non-human primate challenge studies and in vitro assays using viruses that are similar to transmitted HIV-1 isolates, since these are the viruses a vaccine will confront. Initial attempts to deduce the characteristics of transmitted HIV-1 strains from viruses isolated during acute infection concluded that transmitted strains were comparatively sensitive to neutralization, had shorter variable loop sequences, and fewer N-linked glycans than viruses isolated during chronic infection. However, since this particular study, Derdeyn et al. shaped the conventional wisdom on the characteristics of transmitted HIV-1 variants, it may be worthwhile to present the caveats of the paper. Only 8 transmission pairs were studied,
and the viruses from only 5 of these couples were compared for differences in neutralization sensitivity. Although it would be difficult to make statistically meaningful conclusions based on the study of only 5 viruses, significant differences were observed by considering multiple isolates from the new host in aggregate. However, since these viruses were likely to share a single recent common ancestor and are therefore not independent, it would be more realistic to only consider the characteristics of the most recent common ancestor. Also, the significance of differences in the length of variable loop sequences was evaluated using a 1-sided t-test, although 2-sided statistical tests are standard for this type of comparison. Fewer N-linked glycans was a function of shorter variable loops, which are rich in potential sites for glycosylation. Furthermore, the viruses characterized were isolated during acute infection, during which antibody responses are initially absent. It is theoretically possible that mutations that reduce resistance to neutralization may be tolerated prior to the emergence of effective antibody responses, and viruses harboring such mutations may increase the average neutralization sensitivity of acute-phase variants. Another report showing shorter V1V2 sequences and fewer N-linked glycans was also limited to the study of acute-phase variants, rather than their most recent common ancestor. Therefore, although greater neutralization sensitivity for transmitted HIV-1 strains reigned as the conventional wisdom, caveats complicate this conclusion.

**Transmitted viruses are resistant to neutralization.** Newer analyses suggest that the transmitted viruses that establish new HIV-1 infections are at least as resistant to neutralization as viruses isolated during chronic infection. The genetic sequence of
the virus or viruses responsible for establishing infection in a new host can frequently be deduced using a phylogenetic analysis of sequences obtained by single genome amplification (SGA) during acute infection (Fig. 1.8)\textsuperscript{209,210}. Using SGA, a single variant can be identified as a recent common ancestor in the majority of infections, although the frequency of multiple variants establishing infection is higher among homosexual men than among heterosexual men and women\textsuperscript{209-214}. Since it is conceivable that only the descendants of a single variant are detected due to the outgrowth of other transmitted variants by one virus, the most recent common ancestor in the acutely infected host is known as the transmitted/founder (t/f) virus. Comparison of the neutralization resistance of 55 American HIV-1 clade B t/f viruses to 29 viruses obtained during chronic infection revealed that the t/f viruses were at least as resistant to neutralization by a panel of reagents as the chronic viruses\textsuperscript{209}. Among these, the t/f viruses were significantly more resistant to the monoclonal antibodies b12, 2F5, and 4E10 (Fig. 1.9). In addition to neutralization, additional features of t/f variants have been characterized. Among the 55 t/f variants discussed above, 54 used CCR5 but not CXCR4 as a coreceptor, while one was dual-tropic. Another study reported that t/f viruses were somewhat more sensitive to neutralization than chronic isolates, but 3 of the 24 t/f viruses tested in this study were dual-tropic\textsuperscript{215}. In contrast to early associations between the CCR5 usage of viruses present early in infection and macrophage tropism, more recent analyses indicate that t/f viruses replicate poorly in macrophages\textsuperscript{214}. Thus, the t/f viruses found by phylogenetic analysis to initiate infection in new hosts generally utilize CCR5 but not CXCR4, replicate in T-cells but not macrophage, and are resistant to neutralization.
Figure 1.8. Deduction of the t/f virus sequence by phylogenetic analysis\(^{214}\). The HIV-1 sequences used in this analysis were derived from a man with acute HIV-1 infection from heterosexual contact, who had detectable viral RNA and viral p24 antigen in plasma, but had not yet developed detectable antibody responses. Of 18 genomes obtained by SGA, 3 were identical to the deduced t/f sequence. Brackets indicate nucleotide substitutions that occur in more than one variant.

Figure 1.9: Neutralization of t/f and chronic-phase isolates of HIV-1\(^{209}\). The t/f viruses were significantly more resistant to neutralization by b12, 2F5, and 4E10 than the chronic viruses. HIVIG is pooled IgG purified from HIV-1 patients. Both HIVIG and 2F5 neutralized all the chronic viruses, whereas a subset of t/f viruses were not neutralized by the highest concentrations of these reagents tested. The t/f and chronic viruses were comparably resistant to sCD4, although 6 of 55 (11%) were not neutralized by the highest sCD4 concentration tested.
Fusion

Env undergoes global rearrangements to fuse the viral and cellular membranes. To comprehend the basis of lentiviral antibody resistance, it is necessary to understand how the Env fuses the viral and cellular membranes. Binding to CD4 and CCR5 is associated with extensive conformational changes in gp120. Biophysical studies suggest that binding of gp120 to its receptor and coreceptor incurs a high, thermodynamically unfavorable entropy cost\textsuperscript{216,217}. Isothermal titration calorimetry shows that binding of gp120 to CD4 incurs an entropy (-\(T\Delta S\)) cost of approximately 50 kcal/mol, which is a relatively large value for a protein-protein interaction\textsuperscript{216,217}. The binding of CD4 alone incurs approximately 90\% of the total entropy cost of binding both CD4 and a monoclonal antibody used as a surrogate for CCR5 (monoclonal antibodies 17b or 48d)\textsuperscript{217}. However, binding of 17b or 48d first, before CD4, incurs approximately 60\% of the total entropy cost\textsuperscript{217}. Therefore, binding of either CD4 or CCR5 facilitates the binding of the other. This suggests the binding of CD4 and CCR5 may be more cooperative and simultaneous than sequential, as it is typically imagined. These high entropy costs indicate that disordered regions of gp120 become ordered upon binding CD4 and CCR5\textsuperscript{216,217}.

General agreement between crystallographic and biophysical data. A crystal structure of the unliganded conformation of gp120\textsuperscript{218} (\textit{i.e.} not bound to CD4 or a monoclonal antibody) exhibits extensive differences when compared with any of the liganded gp120 co-crystals, and in this respect is consistent with the biophysical data\textsuperscript{216,217}. The liganded co-crystal structures include gp120 bound to both CD4 and the
coreceptor binding site-specific monoclonal antibodies 17b\textsuperscript{187}, 48d\textsuperscript{152}, 412d\textsuperscript{219}, or X5\textsuperscript{219}, or alternatively, gp120 bound to the CD4-binding site-specific monoclonal antibodies b12\textsuperscript{220}, b13\textsuperscript{221}, or F105\textsuperscript{221}. Consistent with the entropic costs associated with binding these ligands, the unliganded SIV gp120 crystal structure generally has less secondary structure than the liganded crystal structures. However, this unliganded SIV gp120 structure was shown to be inconsistent with cryo-electron tomography reconstructions of Env as it exists on virions, suggesting that some of the differences between the crystal structures may be artifacts of the conditions or truncations necessary for crystallization, of the absence of gp41 or trimerization, or of differences between SIV and HIV-1, rather than changes induced by binding CD4 and CCR5\textsuperscript{150}. Nevertheless, the general trends indicated by the unliganded SIV gp120 crystal structure are consistent biophysical data indicating that extensive conformational changes, including an ordering of disordered amino acids, occur upon binding CD4 and CCR5\textsuperscript{216-218}. Therefore, some but perhaps not all of the differences between the unliganded and liganded gp120 structures probably represent conformational changes induced by binding CD4 and CCR5.

**Conformational changes at the CD4 binding loop.** Adopting the CD4-bound conformation creates novel secondary structure in the CD4 binding site. Indeed, the ordering of interactions in secondary structure is consistent with the high entropy cost incurred by binding CD4. The CD4 binding loop of HIV-1 and SIV gp120 has the conserved sequence motif GGDPE. The DPE and the following 2 or 3 amino acids form an $\alpha$-helix in all of the liganded gp120 structures, which does not exist in the unliganded gp120 structure, suggesting a critical secondary structure for interaction with CD4 does
not exist in the unliganded conformation\textsuperscript{152,187,218-221} (Fig. 1.2c). The DPE residues make multiple contacts with residue F43 of CD4, which is critical for the interaction\textsuperscript{187,222}. The GG of GGDPE extends a \(\beta\)-sheet that is part of CD4 in the gp120 co-crystal structure with CD4 and 17b, and this structural element could not exist in the absence of CD4\textsuperscript{187} (Fig. 1.2c). Thus, novel secondary structures form within the CD4 binding site upon interaction with CD4.

**Formation of the bridging sheet.** Binding of CD4 to gp120 creates a 4-stranded \(\beta\)-sheet, designated the “bridging sheet”\textsuperscript{152,187} (Fig. 1.2c). This bridging sheet is absent from the unliganded SIV gp120 structure\textsuperscript{218}. The stem of the V1V2 loop, which is part of the gp120 outer domain, contributes 2 \(\beta\)-strands to the bridging sheet, but the other 2 \(\beta\)-strands of the bridging sheet are part of the gp120 inner domain. Thus, as with the CD4 binding site, novel secondary structures are induced to form the coreceptor binding site. The backbone of the bridging sheet is hydrogen bonded with the E of the conserved GGDPE motif in the CD4 binding site, and therefore may contribute to stabilization of the CD4-bound conformation of the CD4 binding site\textsuperscript{152,187}. The bridging sheet also contains residues that interact with the coreceptor. Formation of the bridging sheet may change the orientation of the inner and outer domains of gp120\textsuperscript{223}.

**Conformational changes in the inner domain of gp120.** Binding of CD4 and CCR5 induces additional conformational changes away from the CD4 binding site, in the inner domain of gp120. A 7-stranded \(\beta\)-sandwich within the inner domain is thought to directly contact gp41, due to its probable location in cryo-electron tomographic images\textsuperscript{150},
and due to the locations of mutations that affect association of gp120 with gp41\textsuperscript{152,224-229} (Fig. 1.2c). Three loops extend from this 7-stranded β-sandwich, each of which contains an α-helix, at least in the CD4-bound conformation\textsuperscript{152} (Fig. 1.2c). These 3 loops have been designated as topological layers 1, 2, and 3\textsuperscript{151,152}. It is layer 2 that contributes 2 β-strands from the inner domain to form the 4-stranded bridging sheet, together with the stem of the V1V2 loop\textsuperscript{152}. The affinity for CD4 and CCR5 can be reduced by mutations at the interface between layers of the inner domain, but restored by compensatory mutations that stabilize the CD4-bound conformation, suggesting contacts between the layers of the inner domain of gp120 also affect the formation of the CD4-bound conformation\textsuperscript{151,230}. Therefore, transition to the conformation induced by binding CD4 and CCR5 reshuffles the inner domain of gp120\textsuperscript{151}. The induction of novel secondary structure and other rearrangements in the CD4 binding loop, in the bridging sheet, and in the inner domain of gp120 would be expected to incur entropy costs, as indicated by the biophysical data.

**Extension of gp41.** Adopting the CD4-bound conformation causes gp120 to disassociate from gp41, and promotes structural rearrangements in gp41 that initiate membrane fusion\textsuperscript{223,231}. The 7-stranded β-sandwich of the inner domain of gp120 is thought to transmit these conformational adjustments to gp41, due to its probable direct interaction with gp41\textsuperscript{151}. Cleavage of gp160 to create gp120 and gp41 during its initial processing endows gp41 with a hydrophobic N-terminal peptide, which remains sequestered in the unliganded Env trimer\textsuperscript{152}. Upon interaction with CD4 and CCR5, and the concomitant conformational rearrangements in gp120, the N-terminal region of the
gp41 ectodomain forms a coiled coil of α-helices\textsuperscript{223}. Formation of this extended coiled coil translocates the fusion peptide away from the base of gp41, and immerses it in the membrane of the target cell (Fig. 1.2f)\textsuperscript{223}. This extended gp41 conformation is designated as the “pre-hairpin intermediate,” or “PHI.” The PHI represents a meta-stable state, which precedes the lower-energy, highly stable 6-helix bundle that takes the shape of trimer of hairpins (Fig. 1.2d and g)\textsuperscript{153,223,232-234}.

**6-helix bundle formation.** Fusion of the viral and cellular membranes occurs when the extended conformation of the PHI collapses to form the 6-helix bundle (Fig. 1.2d and g)\textsuperscript{223}. The C-terminal region of gp41, which is connected to the transmembrane domain in the viral membrane, collapses onto the N-terminal alpha helices. After fusion, the resulting 6-helix bundle structure has the N-terminal fusion peptide and the C-terminal transmembrane region adjacent to one another in the fused membrane\textsuperscript{153,223,232-234}. The most complete 6-helix bundle structure is a solution structure obtained by NMR\textsuperscript{153}, and is in agreement with crystal structures obtained from truncated forms of gp41\textsuperscript{232-234}. Thus, extensive structural rearrangements in gp120 trigger gp41 to enter a meta-stable state, which subsequently attains the lowest-energy state through a global rearrangement that fuses the viral and cellular membranes.

*Mechanisms of antibody resistance*

**Occlusion by glycosylation.** The central concept in the inherent resistance of primate lentiviruses to antibodies is the masking of Env surfaces. The carbohydrate structures attached to the HIV-1 and SIV Env proteins are usually not immunogenic
themselves, since they are recognized as ‘self,’ and thus camouflage Env from antibody responses. Although the broadly neutralizing monoclonal antibody 2G12 targets an epitope that includes carbohydrate\textsuperscript{235-237}, 2G12 has a highly unusual structure\textsuperscript{238}. Several antibodies specific for a quaternary gp120 epitope that appears to include an N-linked glycan have recently been isolated, but these “PGT” antibodies may be unusual since they are the first reported to belong to the 2G12 competition group\textsuperscript{239}. The occlusion of potential antibody epitopes by glycosylation is responsible for the existence of the “silent face” on gp120\textsuperscript{188}. N-linked glycans are added to the asparagine residues of N-X-S/T motifs, where X is any amino acid except for proline, and the third residue is either serine or threonine. Among more than 10,000 proteins in the SWISS-PROT library having at least 1 such predicted N-linked glycosylation site, only 3 present in the library at the time had a higher density of predicted N-linked glycosylation sites than HIV-1 gp120\textsuperscript{240}. The extraordinary high density of predicted N-linked glycosylation sites is thought to comprise a “glycan shield,” which occludes the access of antibodies to surfaces of Env\textsuperscript{241}. The density of N-linked glycans on the surface of gp120 is great enough that hydrogen bonded glycan clusters create a seamless surface of carbohydrate that covers parts of gp120\textsuperscript{218}. The C-terminal helices of gp41 are also modified by N-linked glycosylation, with 4 commonly predicted sites for N-linked glycosylation in HIV-1 gp41 and 3 predicted sites in SIV gp41. Although numerous monoclonal antibodies could be mapped to linear epitopes on HIV-1 gp41, none of these mapped to epitopes within the region between the 4 N-linked glycans\textsuperscript{242}. Likewise, the same region of SIV\textsubscript{mac239} gp41 did not contain any linear determinants that reacted with pooled serum from SIV\textsubscript{mac239}-
infected macaques$^{132}$. Thus, part of the Env structure is immunologically silent, due to occlusion by carbohydrates.

**Neutralization sensitivity of glycan-deficient mutants.** Mutational analyses support the model that glycosylation of Env masks potential neutralization epitopes. Mutations that remove potential N-linked glycosylation sites from gp120 that are not necessary for the proper folding or function of Env generally lead to a loss of resistance to neutralization$^{156,243-246}$. Although this loss of resistance is probably due in part to antibodies that target epitopes that are sterically occluded by carbohydrates in the native trimer, many of these mutations also cause conformational changes that lead to a global loss of resistance to neutralization, which complicates the interpretation of this phenotype$^{156,244-246}$. Macaques infected with strains lacking pairs of N-linked glycans in SIV$_{mac239}$ gp120, or lacking a group of 5 N-linked glycans, developed antibody responses that neutralized the mutant strains at high titers, suggesting these N-linked glycans occlude potential neutralization epitopes$^{244}$. Removal of the 3$^{rd}$ N-linked glycosylation site on SIV$_{mac239}$ gp120 by mutagenesis selectively increases its susceptibility to neutralization by sCD4 without leading to a general loss of resistance to neutralization by pooled plasma from SIV-infected macaques or selected monoclonal antibodies$^{247}$. Mapping of the 3$^{rd}$ N-linked glycan onto crystal structures for HIV-1 and SIV gp120 suggests it is positioned to sterically hinder the access of CD4 or antibodies to the CD4 binding site$^{187,218,247}$. Although removal of 2 or 3 of the 3 N-linked glycans in SIV$_{mac239}$ gp41 did not appear to render this virus more globally sensitive to neutralization by plasma from wild-type SIV$_{mac239}$-infected macaques, the animals
infected with these strains produced antibodies that neutralized the glycan-deficient strains at high titers$^{132}$. The specificity of these neutralizing antibodies for the glycan-deficient strains, coupled with data showing linear epitopes at the N-linked glycosylation sites were recognized by antibodies, suggests that these N-linked glycans occlude potential neutralization epitopes in gp41. Therefore, the sensitivity of viral mutants lacking specific N-linked glycosylation sites in gp120 or gp41 to neutralization by sCD4 and antibodies indicates that N-linked glycans contribute to neutralization resistance.

**Evidence supporting the importance of glycosylation in vivo.** Experimental infection of macaques with mutant SIV strains lacking specific N-linked glycans suggests that glycosylation is important for immune evasion in vivo. Macaques infected with derivatives of SIV$_{mac}$239 containing mutations that remove specific N-linked glycans in gp120 tend to have lower viral loads than animals infected with wild-type SIV$_{mac}$239$^{244,248}$. Macaques infected with derivatives of SIV$_{mac}$239 lacking 2 or 3 of the 3 N-linked glycans in gp41 developed escape mutations in residues adjacent to the N-X-S/T sites that conferred resistance to the neutralizing antibody responses$^{132}$. These included mutations that created novel N-X-S/T sites. The lower viral loads in animals infected with strains lacking N-linked glycans in gp120$^{244,248}$, and the selection for neutralization escape mutations in viruses lacking N-linked glycans in gp41$^{132}$, suggest that N-linked glycosylation is important for immune evasion in vivo.

**Glycans interfere with the development of antibody responses.** Glycosylation may interfere with the induction of antibody responses capable of binding wild-type Env
proteins. As discussed above, macaques infected with SIV strains lacking specific N-linked glycans produce antibodies that neutralize the glycan deficient strains at high titers. However, in comparison to animals infected with wild-type SIV$_{mac239}$, the animals infected with derivatives of SIV$_{mac239}$ lacking specific N-linked glycans developed antibodies capable of neutralizing wild-type SIV$_{mac239}$ at unusually high titers. Specifically, serum from macaques infected with strains lacking pairs of the 4$^{th}$ and 5$^{th}$, 5$^{th}$ and 6$^{th}$, or 4$^{th}$ and 6$^{th}$ N-linked glycosylation sites on gp120 developed 50% neutralizing antibody titers against wild-type SIV$_{mac239}$ between 200 and 16,000 by 64 weeks post-infection. Similarly, an animal infected with a strain derived from SIV$_{mac239}$ lacking all 3 N-linked glycans in gp41 developed 50% neutralizing antibody titers against SIV$_{mac239}$ of approximately 100 by 24 weeks post-infection, and an animal infected with a strain lacking 2 of 3 N-linked glycans in gp41 also developed relatively high neutralizing antibody titers against wild-type SIV$_{mac239}$ at later time points (Desrosiers, et al., unpublished observations). Animals infected with SIV$_{mac239}$ or SIV$_{mac251}$ often do not produce detectable antibodies capable of neutralizing these viruses, or neutralize them at titers that occasionally reach 100 but are typically lower. Removal of an N-linked glycan also increased the stimulation of antibodies capable of neutralizing SHIV$_{89.6P}$ and SHIV$_{SF162}$ (chimeras containing HIV-1 env genes in a genomic backbone mostly consisting of SIV)$. These higher neutralizing antibody titers suggest that N-linked glycans interfere with the induction of antibody responses capable of neutralizing wild-type viruses. Therefore, in addition to conferring resistance to existing antibody responses, glycosylation also interferes with the development of antibody responses.
**The CD4 binding site is in a recessed cavity.** The architecture of the CD4 binding site renders it poorly accessible to antibodies. In addition to its probable occlusion by the V1V2 loop structure\textsuperscript{150,188} and N-linked glycans\textsuperscript{247}, the residues that interact directly with CD4 lie within a recess between the inner and outer domains of gp120\textsuperscript{187}. This surface cavity extends deep into the gp120 structure, and is partially filled by the phenyl ring belonging to F43 of CD4. The distance of the F43 cavity from the surface of the trimer suggests that the generation of antibodies with the long complementarity determining region (CDR) excursions necessary to reach this surface may be inefficient\textsuperscript{188}. Thus, the recessed nature of the CD4 binding site contributes to the antibody resistance of lentiviral Env proteins.

**Thermodynamics of CD4 binding as an antibody resistance mechanism.** The high entropy cost incurred when gp120 adopts the CD4-bound conformation may be a defense mechanism against antibodies. Induction of the CD4-bound conformation of gp120 incurs an unusually high entropy cost of approximately 50 kcal/mol\textsuperscript{217}. With the notable exception of b12, binding of individual monoclonal antibodies to the CD4 binding site incurs an entropy cost of 19-28 kcal/mol\textsuperscript{217}. These high entropy costs suggest that the binding of antibodies to the CD4 binding site induces many of the energetically unfavorable conformational changes in gp120 that are induced by binding CD4. As described above, the basis for this entropy cost appears to be the induction of ordered secondary structure interactions in the CD4 binding site, the bridging sheet, and the topological layers of the inner domain of gp120\textsuperscript{151,152,187,217}. However, the high
entropy cost of adopting the CD4-bound conformation seems paradoxical, since it would reduce the ability of gp120 to interact with CD4, and thereby interfere with viral entry\textsuperscript{217}. Kwong et al. appear to have resolved this paradox by measuring IC\textsubscript{90} concentrations for monomeric sCD4 and a dodecameric CD4 construct against a panel of 6 primary isolates of HIV-1. These primary isolates were neutralized at concentrations of 0.1 to 2.7 nM by the dodecameric construct, whereas monomeric sCD4 neutralized one strain at 280 nM, and did not reach a measurable IC\textsubscript{90} against the other 5 viruses, even at the highest concentration measured, 1200 nM. Thus, dodecameric CD4 neutralizes primary isolates of HIV-1 at a concentration at least 2 orders of magnitude lower than monomeric sCD4. Therefore, Kwong et al. conclude that the energetic barrier to adopting the CD4-bound conformation can be overcome through high-avidity interactions. By analogy to dodecameric CD4, they propose that membrane-bound CD4, mobile only within a 2-dimensional plane on the surface of a CD4\textsuperscript{+} T-cell, is restricted in movement sufficiently to achieve the avidity necessary to overcome the cost of inducing thermodynamically unfavorable conformational transitions gp120. In contrast, soluble CD4 or free-floating antibodies to the CD4 binding site diffuse away from gp120 more readily, and consequently are unable to neutralize these primary isolates. Thus, the primate lentiviruses are able to discriminate between CD4\textsuperscript{+} T-cells and free-floating antibodies to the CD4 binding site. Therefore, the thermodynamic properties of lentiviral Env proteins render them inherently resistant to antibody responses.

**Thermodynamics of coreceptor binding as an antibody defense mechanism.**

The coreceptor binding site of gp120 is protected from antibodies by properties that are
the same or similar to those protecting the CD4 binding site. The binding of individual coreceptor binding site antibodies to gp120, in the absence of CD4, incurs an entropy cost of 29-32 kcal/mol, which is similar to energetic barrier facing interactions between antibodies and the CD4 binding site\textsuperscript{217}. Therefore, although the experimental evidence supporting the conclusion that high-avidity interactions can overcome this energetic barrier was obtained using interactions between CD4 and the CD4 binding site of gp120, the same properties may be expected to apply to the coreceptor binding site. The binding of either CD4 to the CD4 binding site, or of antibodies to the coreceptor binding site, lowers the energetic barrier for the other\textsuperscript{217}. Thus, as discussed above, induction of the CD4 and coreceptor-bound conformation of gp120 may be cooperative. Therefore, the primate lentiviruses may have evolved to distinguish between antibodies in solution that recognize the CD4 or coreceptor binding sites versus CD4 and CCR5 expressed on the surface of a T-cell by requiring avidity and cooperativity to stabilize these interactions.

Spatial dispersion of the coreceptor binding site. The conformational changes required for gp120 to bind its coreceptor are a defense mechanism against antibody responses. The binding of the CCR5 ligands macrophage inflammatory protein 1α (MIP-1α) and MIP-1β to CCR5\textsuperscript{+} cells is inhibited more completely by complexes of gp120 plus sCD4 than by gp120 alone\textsuperscript{251,252}. Therefore, the binding of gp120 to CCR5 is significantly enhanced by the interaction with CD4. Comparisons between liganded and unliganded gp120 structures appear to show that the amino acids that comprise the coreceptor binding site are spatially-disparate in the unliganded structure, but are assembled in the CD4-bound conformation\textsuperscript{218}. The antibodies whose affinity for Env is
enhanced by the gp120-CD4 interaction are known as CD4-inducible (CD4i) antibodies. Thus, whereas the CD4 binding site and the coreceptor binding site both undergo conformational changes to adopt the CD4-bound conformation, the coreceptor binding site is further masked from antibodies in the unliganded conformation by spatial separation.

**Coreceptor binding site assembly in a sterically constrained space.** Antibodies to the coreceptor binding site are poorly neutralizing due partly to the constrained geometry of CD4 and coreceptor engagement. The natural, bivalent form of the monoclonal antibody b12 neutralizes HIV-1 entry more potently than monovalent Fab fragments, which lack the constant Fc region of the antibody$^{253}$. Presumably, the greater neutralization potency of bivalent antibodies owes to an avidity advantage over the monovalent Fab fragments of the same antibody. However, the monovalent Fab fragments of the coreceptor binding site-specific monoclonal antibodies 17b, 48d, and X5 unexpectedly neutralize HIV-1 entry more potently than the divalent forms of these antibodies that possess the full Fc region$^{254}$. Therefore, the close proximity of the target cell membrane to gp120 when it has adopted the CD4-bound conformation may restrict the access of antibodies with Fc domains, but not Fab fragments. Thus, the coreceptor binding site is protected against neutralizing antibodies by steric constraints that are likely imposed by the proximity of the target cell membrane during receptor engagement.

**Tyrosine sulfation.** Usage of a tyrosine-sulfated coreceptor contributes to the ability of primate lentiviruses to resist antibodies. Inhibition of cellular sulfotransferases
or substitution of phenylalanine or aspartate for the N-terminal tyrosine residues of CCR5 inhibits HIV-1 entry, indicating that fusion requires interaction with sulfo tyrosine residues in the N-terminus of CCR5. Sulfo tyrosine has properties that appear to be ideal for facilitating entry and continuous viral replication despite vigorous Env-specific antibody responses. Sulfo tyrosine residues engage in relatively high-affinity interactions due to the electronegative sulfate group bonded to the phenyl ring of tyrosine, but dock within relatively small recessed surfaces on gp120. Therefore, the primate lentiviruses may have minimized the area of the surface required to interact with CCR5, and thereby minimized the area of conserved surfaces available as potential antibody epitopes. Although people infected by HIV-1 can generate tyrosine-sulfated antibodies against the coreceptor binding site of gp120, such as the monoclonal antibodies 412d and E51, the generation of antibodies bearing this post-translational modification may be an additional obstacle to the development of high-affinity antibodies to the coreceptor binding site. Thus, the properties of sulfo tyrosine make CCR5 particularly well-suited as a coreceptor for viruses that have evolved to persistently replicate in the presence of Env-specific antibodies.

**Protection of gp41 during fusion.** The access of antibodies to fusion intermediate forms of gp41 is limited by steric and kinetic constraints. As described above, receptor and coreceptor engagement permits gp41 to adopt an extended conformation known as the pre-hairpin intermediate (PHI). Collapse of the C-terminal helices of the PHI around the N-terminal helices to form the 6-helix bundle can be blocked by peptide inhibitors, or by the neutralizing antibodies 2F5 and 4E10. These
peptide inhibitors are derived from the N-terminal or C-terminal helices of gp41\textsuperscript{258,259}. A C-terminal peptide, T20, is marketed as a therapeutic under the names Enfuvirtide or Fuzeon. However, by conjugating N-terminal and C-terminal peptides to proteins of increasing size, Hamburger \textit{et al.} demonstrated that the PHI exists in a sterically-constrained space, in which the access of larger proteins (\textit{i.e.} antibodies) is reduced\textsuperscript{260}. N-linked glycans on the C-terminal helices of gp41 and the positioning of CD4-gp120-CCR5 complexes may contribute to this steric constraint. The broadly neutralizing monoclonal antibodies 2F5 and 4E10 have epitopes at the C-terminal membrane-proximal external region (MPER) of the PHI\textsuperscript{261-263}. However, antibodies that recognize fusion intermediates are constrained kinetically, since they bind a transient structure. Transient intermediates may also be poorly immunogenic due to this kinetic constraint. Furthermore, 2F5 and 4E10 bind to lipid membranes\textsuperscript{264,265}, which may be interpreted as autoreactivity during B-cell development, potentially promoting the clonal deletion of B-cells that produce antibodies similar to 2F5 and 4E10. Therefore, gp41 intermediate structures that exist during fusion are protected from antibodies by steric and kinetic constraints.

\textbf{Cell-to-cell transfer of virus as a neutralization resistance mechanism.} It is unclear whether assays for measuring virus neutralization accurately reflect the efficacy of virus neutralization \textit{in vivo}. Assays for measuring neutralizing antibodies typically include a step in which virus and antibody are pre-incubated together for 1 hour, prior to the addition of CD4\textsuperscript{+} target cells\textsuperscript{164,241}. However, the half-life of cell-free virus in plasma is on the order of minutes\textsuperscript{18,266}. The 1-hour pre-incubation step may therefore cause virus
neutralization assays to overestimate neutralization in vivo, where more rapid neutralization kinetics may be required. Furthermore, HIV-1 induces the formation of virological synapses between infected and uninfected T-cells, which facilitate the transfer of virus to the uninfected cell, enhancing the efficiency of infection by 2-4 orders of magnitude \(^{267-270}\). This degree of infectivity enhancement suggests that direct cell-to-cell transfer of virus may be responsible for the majority of ongoing virus replication in vivo. However, antibodies capable of neutralizing cell-free virus have little or no effect on infection mediated by cell-to-cell transfer of virus \(^{271,272}\). Thus, direct transfer of virus through neutralization-resistant virological synapses may render antibodies that neutralize virus in vitro less efficacious in vivo. On the other hand, there are considerations that may cause virus neutralization assays to underestimate virus neutralization in vivo. The highest concentration of serum or plasma tested in virus neutralization assays is typically a 1:8 dilution, whereas plasma in vivo is not diluted. Also, the local concentration of neutralizing antibodies in microenvironments that contain B-cells secreting these antibodies may be higher than is observed in plasma. Therefore, it is unclear how measurements of virus neutralization in vitro translate to virus neutralization in vivo.

D. THE PROBLEM OF SEQUENCE DIVERSITY

*Structural basis for antigenic plasticity in Env*

**Variable loops.** Lentiviral Env proteins are plastic in sequence, which facilitates escape from effective antibody responses. However, this variation is concentrated in specific regions of gp120. The 5 variable loops may present a moving target to host antibody responses. The V1V2 loop of HIV-1 is approximately 70 amino acids long,
whereas the analogous region in SIV_{mac}239 is 100 amino acids\textsuperscript{273}. The large size of this structure, plus its probable position at the top of the trimer in cryo-electron tomographic images of unliganded Env on viruses\textsuperscript{150}, and the location of its stem as part of the bridging sheet between the CD4 and CCR5 binding sites\textsuperscript{187,188}, suggest that V1V2 occludes the access of antibodies to the CD4 and CCR5 binding sites. The extreme neutralization sensitivity of a mutant strain deleted in V1V2 (SIV_{mac}239\Delta V1V2) is consistent with V1V2 serving as a steric block to the access of antibodies against internal regions of the Env protein\textsuperscript{156,273}. Whereas V1V2 is probably at the top of the trimer, V4 and V5 appear to be located at the sides\textsuperscript{188}. Thus, the variable loops may serve as an antigenic decoy, directing antibody responses to easily changeable surfaces, and hindering the access of antibodies to conserved regions of the Env protein.

**Plasticity of the “glycan shield”**. The primate lentiviruses escape antibody responses that arise during ongoing infection through the addition and removal of N-linked glycosylation sites in Env\textsuperscript{241,274}. Sequencing Env variants from an HIV-1 patient from early after infection through over more than 2 years later revealed a shuffling of predicted N-linked glycosylation sites, in which some N-X-S/T motifs were lost while new ones appeared. The Env variants arising later in infection were resistant to autologous serum (\textit{i.e.} from the same person) that neutralized the earlier Env variants, and this resistance to neutralization could be mapped to the N-linked glycosylation sites. Mutations in a putative O-linked glycosylation site in the SIV V1V2 region also emerge during ongoing infection and are associated with neutralization escape\textsuperscript{274,275}. A shifting “glycan shield” is one manifestation of the plasticity of lentiviral Env proteins.
An island of variation in the conserved CD4 binding site. Although the CD4-binding site is relatively conserved, retaining prominent sequence features such as the GGDPE motif across HIV-1 and SIV, the surrounding amino acids nevertheless have a degree of inherent freedom to vary in sequence and escape antibody responses. Despite the conservation of elements of the CD4 binding site, an island of sequence variation lies at its center. A water-filled cavity lies at the interface between CD4 and gp120\textsuperscript{187}. The cavity is lined by residues A281, S364, S365, T455, and R469, using numbering for the gp120 of HIV-1\textsubscript{HXB2}, which do not participate in contacts with CD4 and are variable among HIV-1 isolates. This cavity contrasts with the nearby residues that directly interact with CD4, which exhibit greater sequence conservation. Thus, antibodies to the CD4 binding site that rely at least partly upon interactions with this variable, water-filled cavity may be easily escaped through sequence changes. Therefore, a “variational island” at the center of the CD4-binding site serves a similar function to the variable loops, tolerating amino acid substitutions that permit antibody escape.

Main chain interactions. Despite greater relative sequence conservation, the surfaces of gp120 that interact directly with CD4 also retain a degree of sequence plasticity. Remarkably, 60% of the contacts between gp120 and CD4 are made by main chain atoms of the gp120 polypeptide backbone, rather than side chain atoms\textsuperscript{187}. The dependence on main chain contacts may lessen the requirement for particular amino acid identities, thus increasing the sequence plasticity that permits antibody escape.
**Conformational diversity.** Escape from neutralization can occur through amino acid changes that are not part of the neutralization epitope. Individual amino acid changes at disparate sites in the SIV Env trimer independently conferred resistance to neutralizing sera, indicating that escape occurred through by global effects on the Env protein rather than changing an antibody binding site\textsuperscript{275}. Sequence variation in the inner domain of gp120 may permit antibody escape\textsuperscript{151,152}. Finzi et al. suggest that if movement between the topological layers of the inner domain modulates the transition between the unliganded and CD4-bound conformation, sequence variation in this region might permit conformational diversity. Thus, sequence changes within the inner domain of gp120 could permit antibody escape by altering the conformation of gp120 without requiring sequence changes in the CD4-binding site. Therefore, despite its relative conservation, the CD4 binding site may escape antibodies through sequence plasticity elsewhere in the protein. Through conformational adjustments, the primate lentiviruses are capable of escaping antibody responses without directly changing the amino acid sequences of neutralization epitopes.

*Origins of sequence diversity*

**Mutation rate × time.** The sequence diversity of circulating HIV-1 strains presents a significant obstacle to vaccine development, which is superimposed over the inherent resistance of primary viruses to neutralization. This diversity is a consequence of the high error rate of reverse transcriptase (RT). With every round of replication, RT introduces approximately one new mutation into the ~10,000 base HIV-1 genome per newly infected cell\textsuperscript{276}. The diversity of contemporary HIV-1 strains reflects continuous
virus replication with a turnover rate for infected cells of 1-4 days\textsuperscript{17} ever since crossing the species barrier from chimpanzees some time in between 1884 and 1924\textsuperscript{277-279}.

Insertions, deletions, and recombination provide an additional source of diversity. The sequence diversity generated by continuous replication and mutation over the century-long history of HIV-1 in humans is one of the foremost obstacles confronting vaccine design.

\textbf{Selection for antigenic variation.} Selection for immune escape variants and genetic drift drive the diversification of circulating HIV-1 strains. As mentioned above, the primate lentiviruses adopt sequence changes that escape CD\textsuperscript{8}\textsuperscript{+} T-cell responses\textsuperscript{66-74}, and confer resistance to neutralizing antibodies\textsuperscript{180,241,274,275,280-285}. NK cells can also select for sequence changes in HIV-1\textsuperscript{286}. The most thorough description of neutralizing antibody escape was conducted by Richman \textit{et al.}, who analyzed neutralizing antibody titers for plasma and virus captured at longitudinal time points in a matrix format (\textbf{Table 1.1})\textsuperscript{285}. Most of the 14 treatment-naïve subjects who were identified during acute HIV-1 infection developed antibodies capable of neutralizing autologous virus cloned from plasma collected on their first visit. However, a pattern of sequential neutralization and escape emerged, in which significant neutralizing antibody titers were detected against virus isolated at previous but not contemporaneous time points. The observation that emergent isolates appeared to preemptively escape variant-specific neutralizing antibodies is notable in two respects. First, it illustrates the deftness with which HIV-1 evades immunity. Second, it suggests that nascent, variant-specific antibody responses exert selective pressure, despite poorly neutralizing the contemporaneous virus. More
generally, Richman et al. demonstrate that the continuous selection of Env sequences that escape selective pressure from neutralizing antibodies is a hallmark of HIV-1 infection. Thus, the continuous selection of neutralization escape variants in most individuals infected with HIV-1 over the history of the pandemic has shaped the sequence divergence of modern Env sequences. Furthermore, in parallel to classical Darwinian evolution by natural selection, neutral changes that do not affect viral fitness would also be expected to accrue over time and contribute to antigenic diversity\textsuperscript{287-289}. Therefore, in addition to the inherent resistance of Env to neutralizing antibodies, host immune responses have also promoted antigenic diversity that complicates vaccine development.

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<td>AMPHO</td>
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Table 1.1. Relatively poor neutralization of contemporaneous variants within a single representative donor\textsuperscript{285}.

**HIV-1 group M clades.** The viral variants responsible for the HIV-1 pandemic can mostly be assigned to phylogenetically-distinct lineages that originated in the middle of 20\textsuperscript{th} century\textsuperscript{290}. The pandemic HIV-1 strains all belong to the “major group” (group M), which descended from an ancestral virus that was more closely related to several chimpanzee SIV strains than to the non-pandemic group O and group N strains of HIV-1.
(Fig. 1.10a). Group M can further be divided into clades, lettered A through K (Fig. 1.10b). These clades are thought to represent founder events that occurred in the 1940’s, based on “molecular clock” analyses of the accumulation of mutations over time, which include HIV-1 sequences isolated from samples collected in 1959 and 1960.277,291. Certain clades and founder events are linked with regional epidemics.292 The origin of clade B, which is responsible for the HIV-1 epidemic in the United States and Europe, was a founder event in Haiti estimated to have occurred between 1962 and 1970.293 Likewise, the clade B epidemic in the United States represents a subsequent founder event.293 The HIV-1 strain circulating in India descends from a distinct founder event within clade C.125 Certain strains belong to recognized circulating recombinant forms (CRFs), which are chimeras that resulted from recombination between viruses from two different clades. For example, the founder virus responsible for the epidemic in Thailand is CRF01_AE, which has a clade E gp120 sequence in a clade A backbone.294,295 Clade B circulates as a minor variant in Thailand. Founder viruses responsible for approximately 95% of the epidemic in China are traceable to CRF01_AE from Thailand or to CRF07_BC, which contains a clade C env gene of Indian origin and clade B sequences of Thai origin.292,296,297 Circulating HIV-1 strains belong to distinct phylogenetic lineages linked to founder events, which in some cases have a geographic basis.
Figure 1.10. Phylogenetic relationships among primate lentiviruses\textsuperscript{290,298}. The HIV-1 group M viruses, which are responsible for the global HIV-1 pandemic, fall within a family of related lentiviruses isolated from other primate species (a). HIV-1 group M env gene sequences can be grouped into clades A through K (b). The scale bars represent a nucleotide substitution rate of 10%.

Cross-reactivity of immune responses

Impact of intra- and inter-clade diversity. Sequence diversity limits the cross-reactivity of immune responses. Gaschen \textit{et al.} presented an analysis of inter-clade and intra-clade amino acid similarity across the HIV-1 proteome (Fig. 1.11)\textsuperscript{125}. Amino acid
sequences including all HIV-1 proteins typically diverge by 10-30% between clades, and by 5-15% within a clade. The highest frequency of differences exists in gp120. The median amino acid sequence homology in Env among a group of 23 clade C viruses was 87.5% (range 83.5-90.6%). Since the epitopes recognized by CD8\(^+\) T-cells are typically 8 to 11 amino acids in length, an amino acid substitution rate of 5-15% would imply that CD8\(^+\) T-cell epitopes differ by one amino acid, on average, between any two viruses in a clade. However, since some positions in CD8\(^+\) T-cell epitopes are tolerant to substitutions, the existence of a substitution does not necessarily imply a lack of cross-reactivity\(^{299}\). In addition to documenting serial escape from neutralizing antibodies within an infected individual, as mentioned above, Richman \textit{et al.} also compared the ability of autologous neutralizing sera to cross-neutralize viruses from other individuals (\textit{Table 1.2})\(^{285}\). Significant neutralization was only observed against autologous viruses, and not against heterologous viruses from the other patients in the study, even though all were clade B. These observations show that neutralization-resistant primary isolates of HIV-1 generally lack sensitivity to neutralizing antibodies produced by other HIV-1 patients. Thus, sequence differences limit the efficacy of T-cell and antibody responses.
Figure 1.11. The sequence diversity facing a vaccine against HIV-1\textsuperscript{125}. The amino acid substitution rate per position for 23 full-length HIV-1 clade C isolates compared with an individual clade B isolate (red), 2 individual clade C isolates (blue and purple), and the clade C consensus sequence (green) is indicated. Although the region with the maximum intra-clade diversity exists in gp120, the median level of homology in Env among 23 individual clade C isolates and the clade C consensus sequence was 87.5% (range 83.5-90.6%).

Table 1.2. Development of neutralizing antibody titers against autologous and heterologous HIV-1 strains\textsuperscript{285}.

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Strategies to reduce the sequence diversity facing a vaccine. The extent of antigenic diversity facing a vaccine for HIV-1 may appear daunting, but a vaccine does not need to protect against viruses differing from the immunogen by the typical distance between clades, or even the typical distance among isolates within a clade\textsuperscript{125}. Due to the lower antigenic variation within a clade than between clades, clade-specific vaccines would be expected to have greater efficacy than a pan group M vaccine. In addition to comparing the substitution rate for 23 clade C viruses versus 2 additional clade C isolates
and a clade B isolate, Gaschen et al. also depict the distance to a clade C consensus sequence (Fig. 1.11). This comparison shows that a clade-specific consensus or ancestral sequence in a vaccine immunogen typically halves the distance in sequence between the immunogen and individual circulating variants within a clade. The advantage of clade-specific vaccines is relatively straightforward in countries where one or a small number of clades or circulating recombinant forms predominate (i.e. in Haiti, Thailand, and China). Where epidemics are due to geographically linked founder events within a clade, (i.e. in the United States, Europe, India, and China), a region-specific consensus or ancestral sequence would be even closer to circulating variants than a similar immunogen designed to cover the whole clade. A basket of clade-specific vaccines, used in combination, may be necessary to address the diversity of HIV-1 variants circulating in central Africa. As an alternative or complementary approach to consensus or ancestral vaccines, the use of 3 or more mosaic sequences has been proposed as a strategy to maximize the preservation of T-cell epitopes that include more than one polymorphic amino acid. Although several strategies may help to lower the hurdle of sequence diversity, antigenic variation remains a significant challenge for HIV-1 vaccine design.

**Prevalence of broadly neutralizing antibody responses.** Despite the antigenic diversity of HIV-1 and its inherent resistance to neutralization, some HIV-1 patients develop broadly neutralizing antibody responses after years of infection. The proportion of HIV-1 patients found to develop broadly neutralizing antibody responses depends upon the neutralization sensitivity of the viruses assayed. For example, ability
of patient sera to neutralize different HIV-1 isolates is more closely related to the overall neutralization sensitivity of the viral isolate than to its genetic relatedness with the patient’s virus\(^{307,308}\). Using different definitions of broad neutralization against different panels of test viruses, which include moderately sensitive isolates, 1-25% of HIV-1 patients have been reported to develop moderate to broadly reactive neutralizing antibodies after years of persistent infection\(^{305,306,309-311}\). Although quantifying the breadth of neutralization is complicated by the inclusion of viruses that are relatively sensitive to neutralization, some people infected with HIV-1 for several years develop broadly neutralizing antibodies.

E. GENERATION OF ENV-SPECIFIC ANTIBODY RESPONSES

*Generation of rearranged antibody genes*

The generation of antibodies that circumvent the inherent neutralization resistance of primate lentivirus Env proteins is not trivial. The variable region of an antibody is the domain interacts with the antigen, whereas the constant region interacts with Fc receptors and complement\(^{109,312}\). Variable regions consist of 3 complementarity-determining regions (CDRs) and framework regions. For the antibody heavy chain, CDR1 and CDR2 are derived from one of 40 different functional germline variable (V) segments, and CDR3 is derived through recombination that links the V segment with a diversity (D) segment and a joining (J) segment. Human heavy chain genes have 6 J gene segments and 27 D segments. Nucleotides at the junctions between the V, D, and J segments are usually deleted, but form short palindrome sequences separated by a spacer when they are not. The spacer arises during V-D-J recombination, when terminal
deoxynucleotidyltransferase (TdT) adds random nucleotides to the 3’ end of the V segment, both ends of the D segment, and the 5’ end of the J segment. The rearrangement of light chain genes is similar, except that light chains lack D segments. These DNA recombination events occur independently of antigen.

**Affinity maturation**

**Somatic hypermutation and selection.** The affinity of antibody responses for antigen can increase over time through Darwinian natural selection *in vivo*. B-cells that bind antigen with higher affinity are at a selective advantage, since the primary signal for B-cell proliferation is crosslinking of the B-cell receptor, which is a transmembrane form of antibody produced by alternative splicing. In addition, higher affinity for antigen may facilitate antigen uptake and processing for presentation by MHC class II to CD4+ T-cells, which promote B-cell survival and proliferation. Although V(D)J recombination generates considerable diversity, variable regions among B-cells that encounter antigen are further diversified by somatic hypermutation. The rate of somatic hypermutation is approximately $10^{-3}$ per base pair per round of cell division. However, the frequency of mutations decreases exponentially with distance from the promoter. Thus, more somatic mutations occur in CDR1 than CDR3. The framework regions are also subject to somatic hypermutation, although mutations here are underrepresented, probably due to the higher likelihood of a deleterious effect. Activation-induced cytidine deaminase (AID), which is a paralog of the APOBEC proteins, is essential for somatic hypermutation. Tight regulation of AID at the levels of transcription, phosphorylation by protein kinase A (PKA), and a mechanistic link with
chromosome duplication\textsuperscript{321}, restrict somatic hypermutation to proliferating germinal center B-cells\textsuperscript{322}. Although deamination of cytidines would itself be mutagenic, this activity may promote mutagenesis by recruiting error-prone DNA polymerases\textsuperscript{109,313,323}. Resolution of AID-induced lesions appears to involve the generation of double-stranded breaks, the 3’ ends of which can serve as a substrate for nucleotide addition by TdT\textsuperscript{324}. The activity of TdT may contribute to the observation that insertions or deletions are found in approximately 4% of in-frame variable regions in germinal center B-cells\textsuperscript{325}. These mutations diversify the variable regions of B-cells responding to antigen, and the selective advantage conferred to B-cells with improved affinity for antigen drives an increase in the affinity of the antibody response over time.

**Somatic mutation in Env-specific antibodies.** Env-specific antibodies, and the subset of these antibodies that are capable of neutralizing HIV-1 entry, are highly mutated. Scheid \textit{et al.} compared the number of mutations in 502 Env-specific monoclonal antibodies from the memory B-cells of HIV-1 patients against the number of mutations in non-Env specific memory B-cells\textsuperscript{326}. Env-specific light and heavy chains from memory B-cells had a sum of approximately 40 mutations on average, whereas the corresponding memory B-cell population negative for Env binding had approximately 26 mutations. Larger numbers of mutations were observed in HIV-1 Env-specific antibodies using primers outside of the mutated regions\textsuperscript{327}. Therefore, the Env-specific antibody clones selected during HIV-1 infection are more highly mutated than the rest of the memory B-cell repertoire. Scheid \textit{et al.} found that the CDR3 regions of Env-specific antibodies were significantly longer than the CDR3 regions of antibodies from memory
B-cells that did not bind Env\textsuperscript{326}. Likewise, Huang \textit{et al.} reported that most CD4i antibodies have unusually long CDR3 regions\textsuperscript{328}. The unusually long CDR3 regions of Env-specific antibodies suggest that these antibodies recognize poorly accessible cavities rather than surface epitopes. These properties of Env-specific antibodies may help to counteract features of Env that have evolved to interfere with effective antibody responses.

**Somatic mutation in broadly neutralizing antibodies.** Broadly neutralizing monoclonal antibodies exhibit exceptionally high levels of somatic hypermutation. The heavy and light chains of b12 have 45 amino acid substitutions compared with germ line-encoded sequences\textsuperscript{328}. PG16, a recently isolated broadly neutralizing monoclonal antibody\textsuperscript{191}, has 48 amino acid substitutions relative to the germ line-encoded sequence, plus one of the longest CDR3 regions reported for a human monoclonal antibody to date\textsuperscript{329}. Incredibly, the heavy and light chains of another recently-isolated broadly neutralizing monoclonal antibody, VRC01, have approximately 70 amino acids that differ from the germ line-encoded sequences from which they were derived\textsuperscript{330}. VRC01 also possesses unusual features that are rarely observed in antibodies, including an extra disulfide bond, an N-linked glycan, and a deletion of 2 codons in the light chain\textsuperscript{330}. To elucidate the development of antibodies like VRC01, Wu \textit{et al.} employed deep sequencing (\textit{i.e.} 454-pyrosequencing) to sequence the B-cell repertoire from individuals who made VRC01 and VRC01-like antibodies\textsuperscript{331}. Over $7 \times 10^5$ heavy-chain sequences were obtained from one individual, enabling divergence from the germline sequence to be plotted against similarity to a given antibody sequence (Fig. 1.12)\textsuperscript{331}. These plots
show that VRC01 and VRC01-like antibodies, as a population, diverge from the germline-encoded sequence by approximately 30%. Although it is conceivable that the large number of mutations observed in anti-Env antibodies are a consequence of the persistent nature of HIV-1 infection, rather than a requirement for Env binding, Zhou \textit{et al.} demonstrated that reversion to the genomic sequence of residues involved in contacts between VRC01 and gp120 reduced binding and neutralization\textsuperscript{330}. The number of non-germline residues in VRC01 was significantly correlated with binding and neutralization. In an another remarkable example illustrative of the extent of divergence from germ line-encoded sequences that may be required to neutralize HIV-1, 2G12, which has 51 amino acid substitutions relative to the germ line-encoded sequence\textsuperscript{329}, has a highly unusual tetravalent structure in which the heavy and light chains are swapped between divalent IgG molecules\textsuperscript{238}. Artificial, non-mutated precursor antibodies containing heavy and light chain sequences that correspond to the recombined germ line sequences that gave rise to several broadly neutralizing antibodies lacked any detectable ability to bind Env, indicating that somatic mutation was essential for their capacity to neutralize\textsuperscript{327,330,332}. These data indicate that the generation of neutralizing antibodies requires extensive somatic hypermutation.

\textbf{Figure 1.12. Deep sequencing of mutated heavy chain genes}\textsuperscript{331}. The percent divergence from the germline \textit{IGHV1-2*02} sequence is compared with similarity to an \textit{IGHV1-2*02}-derived neutralizing monoclonal antibody, VRC-PG04. The heavy chain sequences at the top right have diverged away from the \textit{IGHV1-2*02} germline towards VRC-PG04-like sequences.
F. EXPERIMENTAL VACCINES AGAINST SIV AND HIV-1

*Failure of classical vaccine approaches*

**Historical success of classical vaccine approaches.** Eradication of a virus previously responsible for pandemic disease has been achieved by vaccination for smallpox, and nearly for polio\(^333,334\). Successful viral vaccines have been live-attenuated viruses, inactivated (i.e. killed) viruses, and viral proteins. Once HIV-1 could be grown in cell culture conditions in the laboratory in 1984, Robert Gallo famously speculated that a vaccine for HIV-1 could be developed within 2-3 years\(^1\). By analogy to the seasonal influenza vaccine, which consists of inactivated virus and induces neutralizing antibodies\(^335\), a similar approach might reasonably have been expected to be effective against HIV-1 before the problems of inherent neutralization resistance and sequence diversity were appreciated.

**Inactivated virus vaccines tested in macaques.** Early successes attempting to protect rhesus macaques from infection with SIV suggested that an inactivated virus vaccine could prevent acquisition of infection\(^336-339\). However, these vaccines were grown in human cells, and similar vaccines produced in macaque peripheral blood mononuclear cells (PBMC) were not protective\(^340-342\). Furthermore, macaques vaccinated with uninfected human cells were protected against virus grown in human cells, and antibody titer to human antigens correlated with protection\(^343\). The protective effect of inactivated SIV vaccines produced in human cells was traced to antibody responses against human antigens present on the human T-cell lines used to prepare the vaccine.
virus. Thus, studies in the macaque model suggested that inactivated vaccines would fail to protect people from HIV-1 infection.

**Live-attenuated vaccines tested in macaques.** Live-attenuated SIV achieved remarkable initial success, providing complete or apparent sterilizing immunity against i.v. challenge with neutralization-resistant pathogenic strains of SIV, thus demonstrating that vaccine protection against HIV-1 is theoretically possible. The first live-attenuated SIV strain tested as a vaccine concept, SIV\textsubscript{mac}239\textDelta nef, was constructed by deleting 182 nucleotides in the middle of the nef open reading frame. Complete or apparent sterilizing protection is therefore defined as the absence of detectable challenge virus RNA at any time point using RT-PCR primers within the deletion in nef. However, live-attenuated SIV has not been under consideration for use in humans since the late 1990’s due to well-justified safety concerns. SIV\textsubscript{mac}239\textDelta nef establishes a persistent infection in macaques, but has less pathogenic potential than wild-type SIV\textsubscript{mac}239. Peak SIV\textsubscript{mac}239\textDelta nef viral loads are approximately 2 orders of magnitude lower than peak SIV\textsubscript{mac}239 viral loads. Some but not all animals inoculated with SIV\textsubscript{mac}239\textDelta nef control SIV\textsubscript{mac}239\textDelta nef replication to undetectable levels, whereas relatively rare animals with highly protective MHC class I alleles naturally control wild-type SIV\textsubscript{mac}239 infection to undetectable levels. Despite the lower relative pathogenicity of SIV\textsubscript{mac}239\textDelta nef, a subset of adult animals infected with SIV\textsubscript{mac}239\textDelta nef eventually develops AIDS. Alexander et al. demonstrated that SIV\textsubscript{mac}239\textDelta nef evolves in vivo to increase its replicative capacity and pathogenicity. A more attenuated vaccine strain, SIV\textsubscript{mac}239\textDelta3, was therefore engineered by making additional deletions in vpr and the
long terminal repeat (LTR) promoter region\textsuperscript{347}. However, SIV\textsubscript{mac}239\textdelta3 also caused disease in adult macaques after prolonged observation\textsuperscript{348}. Moreover, SIV\textsubscript{mac}239\textdelta3 was pathogenic in neonatal macaques, which raised the specter of mother-to-child transmission of the vaccine strain causing AIDS in babies born to vaccinated mothers\textsuperscript{349,350}. To address these safety concerns, increasingly attenuated SIV strains were engineered (SIV\textsubscript{mac}239\textdeltaX and SIV\textsubscript{mac}239\textdelta4)\textsuperscript{351,352}. However, these strains afforded less consistent protection than SIV\textsubscript{mac}239\textdelta3 against challenge with SIV\textsubscript{mac}251. Therefore, the least attenuated strain capable of consistently providing complete protection against pathogenic SIV challenge lacked an acceptable safety profile.

**Recombinant protein vaccines tested in chimpanzees.** Vaccines based on recombinant protein have been tested for efficacy in humans and non-human primates. As mentioned above, recombinant Env protein vaccines elicited antibodies capable of neutralizing T-cell line-adapted HIV-1 strains, but not primary isolates\textsuperscript{166,176,177}. In challenge studies using chimpanzees, recombinant gp120 protein formulated with an adjuvant elicited gp120 ELISA titers, and in some cases but not others may have protected these animals from infection with neutralization sensitive challenge viruses\textsuperscript{353-356}. While these studies could be interpreted as supporting the capacity of antibody responses to prevent HIV-1 infection, they provide no information on the ability of these vaccines to protect against circulating HIV-1 strains due to their usage of neutralization-sensitive challenge viruses.
Recombinant protein vaccine phase III human clinical trial VAX003.

Repeated inoculations of gp120 formulated with alum adjuvant was tested as a vaccine for HIV-1 in two placebo-controlled, phase III efficacy trials, VAX003 in Thailand and VAX004 in the United States, Canada, and The Netherlands. These recombinant protein vaccines were produced by VaxGen under the brand name AIDSVAX. AIDSVAX B/E, which was tested in Thailand, was derived from the clade B virus HIV-1MN, and from the CRF01_AE isolate HIV-1A244. The 2546 i.v. drug users in Bangkok who participated in VAX003 received 7 inoculations with AIDSVAX B/E. During the course of the trial, 106 vaccine recipients and 105 placebo recipients became infected with HIV-1. Therefore, there was no protection from infection. When infections due to clade B versus CRF01_AE viruses were considered separately, there was also no difference in risk of infection among vaccine versus placebo recipients. No differences were observed in viral RNA loads or CD4+ T-cell counts among vaccine versus placebo recipients either. An attempt was made to correlate risk of infection with different measures of antibody responses. Plasma from the 106 vaccine recipients who became infected with HIV-1 was compared to plasma from 115 randomly selected vaccine recipients that remained uninfected for the ability of antibodies to bind recombinant gp120, a V2 peptide, a V3 peptide, to block an interaction between recombinant gp120 and CD4, and to neutralize HIV-1MN infectivity. The infected versus uninfected vaccine recipients were perfectly matched for activity in these assays. Therefore, multiple immunizations with recombinant gp120 failed to prevent infection or to improve clinical outcomes of infection, and measures of antibody responses provided no evidence of immune protection.
Recombinant protein vaccine phase III human clinical trial VAX004. In VAX004, 5095 homosexual men and 308 women who belonged to high-risk groups for HIV-1 infection were inoculated 7 times with clade B gp120 plus adjuvant immunogens, or just with the adjuvant as a placebo\textsuperscript{358}. Among the VAX004 trial participants, 362 men and 6 women became infected with HIV-1. Kaplan-Meier plots of the time to acquisition appeared identical for vaccine and placebo recipients who were white or reported low or medium behavioral risk, whereas small, non-significant trends were in the direction of vaccine efficacy were reported for non-whites ($P=0.13$), and for people who reported the highest level of behavioral risk ($P=0.29$). Furthermore, as observed in the VAX003 trial, there were no differences in viral RNA loads or CD4\textsuperscript{+} T-cell counts among vaccine versus placebo recipients among participants who became infected during the VAX004 trial. Therefore, immunization with recombinant gp120 provided no protection from HIV-1 infection or disease among high-risk groups for sexual transmission in the VAX004 trial.

Antibody and risk of infection in VAX004. There were small but statistically significant differences between the antibody responses of 239 of the infected vaccine recipients and 163 randomly selected vaccines who remained uninfected\textsuperscript{359}. These differences were observed in the ability of plasma samples to block the binding of recombinant gp120 protein to CD4 (Cox proportional hazards, $P=0.006$, 0.006, and 0.003 for the 2\textsuperscript{nd}, 3\textsuperscript{rd}, and 4\textsuperscript{th} quartiles of CD4 blocking versus the 1\textsuperscript{st} quartile). It is not intuitive that measures of a vaccine-elicited immune response would correlate with a lower
infection rate for a vaccine that provided no protection relative to a placebo. This observation was possible because vaccinees with lower measures of these antibody responses were at an elevated risk of HIV-1 infection, relative to the placebo recipients. One interpretation of this outcome is that VAX004 elicited antibodies that conferred some protection among the higher responders, but also elicited another type of immune response that enhanced the risk of HIV-1 acquisition. Although the authors acknowledged this interpretation, they favored the alternative explanation that antibody responses were a non-causal correlate of lower risk. A follow-up study by Forthal et al. also reported a lower risk of infection for greater antibody-dependent cell-mediated virus inhibition (ADCVI) against the dual-tropic primary isolate HIV-1\textsubscript{92US657} (P=0.019)\textsuperscript{361}. However, the authors present the caveat that the plasma samples for the vaccinees that became infected were, in general, collected at earlier time points after fewer immunizations than the plasma samples for the vaccines that remained uninfected. The authors state that this may have biased the analyses by creating lower ADCVI activity among the infected versus uninfected vaccinees. Therefore, higher antibody responses may have correlated with reduced risk of infection in VAX004, but the absence of vaccine protection complicates the interpretation of this relationship.

\textit{Vaccines based on recombinant viral vectors}

\textbf{Poxvirus vector vaccines tested in macaques.} Vectors based on poxviruses met with early success against neutralization-sensitive challenge viruses, but mostly failed to provide similar protection against neutralization-resistant challenge viruses in non-human primate studies. Hu et al. reported that pig-tailed macaques primed with a modified
vaccinia Ankara (MVA) vector expressing Env, and boosted with recombinant gp160 protein, produced antibodies that neutralized the SIV\textsubscript{mne}CLE11S challenge virus at 50\% titers >100 and were completely protected\textsuperscript{362}. Amara \textit{et al.}, Barouch \textit{et al.}, Robinson \textit{et al.}, and Shiver \textit{et al.} showed that prime-boost vaccine strategies using MVA vectors could reduce viral loads and prevent the rapid disease progression observed among naïve control animals challenged with SHIV\textsubscript{89.6P}\textsuperscript{363-366}, which is a dual-tropic, neutralization-sensitive challenge virus\textsuperscript{367-369}. However, Pal \textit{et al.} reported that among macaques immunized with the canarypox-derived vector ALVAC and boosted with recombinant gp120 protein, the only significant differences in viral loads after challenge with SIV\textsubscript{mac}251 were observed for the comparison between vaccinated macaques that possessed the protective MHC class I allele \textit{Mamu-A*01} versus unvaccinated, \textit{Mamu-A*01}-negative animals\textsuperscript{370}. Also disappointingly, Horton \textit{et al.} and Vogel \textit{et al.} saw only modest differences in viral loads after challenge with SIV\textsubscript{mac}239, and no differences in disease progression for animals immunized with a DNA prime, MVA boost vaccine regimen\textsuperscript{42,48}. However, in a study intended to model breast milk transmission, Van Rompay \textit{et al.} reported that 14 of 16 naïve control animals became infected after oral exposure of neonatal macaques to SIV\textsubscript{mac}251, whereas 11 of 16 MVA-vaccinated and 6 of 16 ALVAC-vaccinated animals were infected\textsuperscript{371}. Therefore, with the exception of the oral transmission study in neonates, poxvirus-based vaccines afforded protection against the neutralization-sensitive challenge strains SIV\textsubscript{mac}CLE11S and SHIV\textsubscript{89.6P}, but not neutralization-resistant viruses SIV\textsubscript{mac}239 and SIV\textsubscript{mac}251.
Poxvirus vector vaccines tested for safety and immunogenicity in humans.

Poxvirus-based vaccines have been evaluated for safety and immunogenicity in several phase I and II human clinical trials. Immunization with ALVAC-vectored gp120 or gp160 alone or in combination with recombinant gp120 protein elicited detectable virus-specific CD8⁺ T-cell responses in comparable minorities of immunized human volunteers³⁷²-³⁷⁴. However, whereas the ALVAC immunogen alone elicited low titer antibodies capable of neutralizing HIV-1MN in a subset of these trial participants, boosting with recombinant gp120 increased the proportion who had detectable neutralizing antibody titers against HIV-1MN to >90%, and also increased the magnitude of these titers. Likewise, some studies evaluating the combination of ALVAC and recombinant gp120 also observed virus-specific CD8⁺ T-cell responses in a minority of participants, and HIV-1MN neutralizing antibody titers in many or all³⁷⁵-³⁷⁷. However, other studies testing immunization with ALVAC in combination with recombinant gp120 were unable to detect virus-specific CD8⁺ T-cell responses, although antibodies capable of neutralizing HIV-1MN were elicited³⁷⁸,³⁷⁹. A recent phase I test of an MVA-based vaccine elicited low-frequency CD8⁺ T-cell responses in less than half the vaccinees, plus antibodies capable of neutralizing HIV-1MN and other tier 1 viruses³⁸⁰. None of the trials reported neutralizing antibodies against neutralization-resistant primary isolates. Therefore, poxvirus vectors sometimes elicit CD8⁺ T-cell responses and antibodies capable of neutralizing T-cell-line adapted viruses, and these antibody titers can be boosted with recombinant protein.
Human clinical trial testing ALVAC/AIDSVAX efficacy in Thailand (RV144).

Due to the limited protection afforded by similar vaccine regimens against neutralization-resistant challenge viruses in the macaque model, the absence of detectable CD8$^+$ T-cell responses in the majority of human volunteers immunized with poxvirus-based vectors, the induction of antibody only capable of neutralizing T-cell line-adapted viruses, and the lack of protection in VAX003/004, the borderline significant protection reported in September of 2009 for recipients of ALVAC and AIDSVAX was unexpected$^{379,381,382}$. The aforementioned observations had previously led leaders of the field to express concern over the futility of proceeding with this trial$^{383}$. Participants in the RV144 trial were immunized with ALVAC-vCP1521 on weeks 0, 4, 12, and 24, and with AIDSVAX B/E (formulated as in VAX003) on weeks 12 and 24$^{379}$. In total, 8197 volunteers received at least one dose of the vaccine, and 8198 received at least one dose of the saline placebo. Among these, 2021 vaccinees and 1832 placebo recipients were excluded from the “per protocol” group, mostly due to having not received all 4 inoculations. CD4$^+$ T-cell responses against Env were detected in approximately one third of vaccine recipients, but no differences in CD8$^+$ T-cell responses were measured among vaccine versus placebo recipients. Nearly all vaccinees made antibodies capable of binding recombinant gp120 in an ELISA. One source of controversy surrounding the RV144 trial was that the “per protocol” analysis did not indicate a statistically significant protective effect (P=0.16). Rather, a statistically significant outcome was only achieved in a “modified intention to treat” (MITT) analysis, which excluded only those participants found to be infected at baseline (P=0.04). In the MITT analysis, 51 vaccinees and 74 placebo recipients became infected with HIV-1, which is a 31% reduction in infections over the
42-month follow-up period (95% CI=1.7 to 51.8%). These P-values and confidence intervals relate to the size of the vaccine effect. However, a Bayesian analysis (i.e. testing the hypothesis that there is no effect) performed later by Gilbert et al. suggested there was a 22% chance that the vaccine had no protective effect. A second controversy stems from the lack of any difference in viral RNA loads or CD4\(^+\) T-cell preservation among vaccine versus placebo recipients who became infected. The expectation that a vaccine capable of preventing HIV-1 infection would also provide some control over viral replication among people who became infected is reasonable. Given the available information, vaccination with a canarypox-based vector and recombinant gp120 probably provided modest protection against HIV-1 infection.

**Adenoviral vector vaccines tested in macaques.** Vaccines based on adenoviral vectors elicit higher magnitude CD8\(^+\) T-cell responses and afford better control of post-challenge viral loads in nonhuman primates than vaccines based on poxviruses. Albeit among small numbers of animals, Shriver et al. presented evidence that prime-boost regimens using vectors derived from adenovirus serotype 5 (Ad5) elicited higher frequencies of virus-specific CD8\(^+\) T-cells and provided better control of SHIV\(_{89.6p}\) than regimens using MVA. Likewise, Wilson et al. reported better protection against SIV\(_{sm}\)E660 challenge by an Ad5-based vaccine among Mamu-A*01-positive macaques than Seth et al. reported for an MVA-based vaccine among Mamu-A*01-positive animals. Nevertheless, the significant protective effect of an Ad5-based vaccine regimen was lost when the Mamu-A*01-positive animals were excluded. Since pre-existing immunity to Ad5-based vectors may decrease their immunogenicity, higher
CD8+ T-cell responses and better protection were attained by priming and boosting with vectors derived from different adenovirus serotypes\textsuperscript{47,387,388}. Although adenoviral vectors improved upon the protection provided by poxvirus-based vectors, and newer strategies may augment their immunogenicity, the protection reported in earlier studies was observed in the contexts of protective MHC class I alleles or challenge with a neutralization-sensitive virus.

\textbf{T-cell-based vaccines and protection against SHIV\textsubscript{89.6P}.} Vaccine control of SHIV\textsubscript{89.6P} replication has historically been ascribed to CD8+ T-cell responses, but evidence suggests that antibody responses contribute significantly. SHIV\textsubscript{89.6P} is highly pathogenic, rapidly causing a nearly complete loss of CD4+ T-cells and germinal centers, progression to AIDS, and death, often within just weeks of infection\textsuperscript{366,369,389-391}. Unvaccinated animals therefore stand little chance of developing an effective antibody response. In fact, vaccinated animals make neutralizing antibody responses after infection with SHIV\textsubscript{89.6P}, whereas naïve control animals do not. For example, the number of animals mounting detectable neutralizing antibody responses against SHIV\textsubscript{89.6P} after infection was 0/4 naïve controls versus 16/16 vaccinees in Amara \textit{et al}\textsuperscript{363}, 2/8 controls versus 12/12 vaccinees in Barouch \textit{et al}\textsuperscript{392}, and 1/6 controls versus 17/18 vaccinees in Letvin \textit{et al}\textsuperscript{393}. Since the later two studies included immunogens that lacked Env, the neutralizing antibodies are not due to the priming of Env-specific antibody responses by vaccination. Therefore, vaccine-elicited T-cell responses mitigated the pathogenicity of SHIV\textsubscript{89.6P} sufficiently to allow for neutralizing antibody responses to develop. These antibodies may be important for the long-term control of SHIV\textsubscript{89.6P} replication.
Human clinical trials testing adenoviral vector vaccine efficacy (STEP/Phambili). Based on various lines of evidence indicating that CD8+ T-cell responses control viral replication, including the success of Ad5 vectors against SHIV89.6P, Merck initiated the STEP trial North America, the Caribbean, South America and Australia, and the Phambili trial in South Africa366,394-396. In the STEP trial, 1494 volunteers were immunized with $3 \times 10^{10}$ Ad5 particles containing HIV-1 clade B gag, pol, and nef genes on weeks 0, 4, and 26, and 1506 volunteers received placebos395. No attempt was made to elicit Env-specific antibodies. The study population consisted of women in high risk groups, homosexual men who reported multiple sex partners or unprotected sex, and in the Caribbean, heterosexual men who reported a combination of other risk factors. However, the STEP trial was aborted early, due to futility and risk of harm. There were 49 HIV-1 infections among 914 male vaccinees, and 33 among 922 male placebo recipients. Women who participated in the trial were excluded from these analyses, since only one became infected. The risk of HIV-1 infection was significantly higher among vaccine versus placebo recipients who had pre-existing antibodies that neutralized Ad5 or were uncircumcised. Among those who became infected with HIV-1, the vaccine had no effect on post-infection viral loads or disease progression394,397. The same Ad5 clade B vaccine was tested in South Africa, which has a clade C epidemic, in the Phambili trial398. Phambili was terminated early in response to the STEP trial. Unlike in STEP, the majority of the infections in the Phambili trial occurred in women, and the overall infection rate was much higher (8% of participants seroconverted). Also as observed in STEP, there were more infections among the vaccine than placebo.
recipients, although the difference was not significant. Thus, repeated immunization of human volunteers with an Ad5-based vaccine lacking an Env component did not confer protection against infection, viral replication, or disease progression, and may have increased the likelihood of HIV-1 acquisition among certain groups.

*Mechanisms of protection by live-attenuated SIV*

**Back to basics.** The limited success of empirical vaccine discovery continues to renew interest in a basic science approach to HIV-1 vaccine development. Identifying immune responses capable of preventing infection by SIV and HIV-1, and understanding the stimuli required to elicit them, may be necessary to develop a vaccine against HIV-1. Live-attenuated SIV remains the only experimental vaccine approach that consistently prevents SIV infection in the most rigorous challenge models. The mechanisms responsible for protection by live-attenuated SIV could therefore serve as a model for the rational design of an HIV-1 vaccine, but they remain enigmatic. Although from an intuitive point of view, one might expect neutralizing antibodies to be associated with complete protection, most animals completely protected from infection with SIVmac251 lack detectable antibodies capable of neutralizing this virus. Therefore, we were interested in identifying an immune response associated with protection by live-attenuated SIV and understanding its development.

**Treatment of complete protection as potentially distinct from control.** Early infection may present unique opportunities to contain viral replication and block systemic dissemination. These opportunities may help to explain why macaques
paradoxically often exhibit complete or apparent sterilizing protection against challenge with SIV\textsubscript{mac239} or SIV\textsubscript{mac251} while SIV\textsubscript{mac239Δnef} viral loads reflect ongoing vaccine strain replication (Reeves \textit{et al.}, manuscripts in preparation). The primary difference between the early events in virus acquisition versus ongoing infection relate to differences between focal virus replication in the mucosa versus systemic infection. An \textit{in situ} hybridization experiment showed that following vaginal challenge of macaques with SIV\textsubscript{mac251}, virus replication is highly focal\textsuperscript{403}. The cells containing viral RNA were adjacent to one another, and only found in a single tissue section. Consistent with the \textit{in situ} data, the vast majority of tissue sections were negative for viral RNA by RT-PCR. In general, viral RNA was restricted to the genital tract until 6 to 10 days after challenge, when it became detectable in the gut, lymph nodes, spleen, and plasma. The authors propose that acquisition typically begins with one infected cell at the portal of entry, which infects its neighbors, and these cells infect other nearby cells, until the number of infected cells becomes sufficient to efficiently seed virus to gut and lymphoid tissue. A single t/f virus being responsible for most sexually transmitted HIV-1 infections also appears consistent with early events being focal in nature\textsuperscript{209-214}. It is conceivable that homing signals (\textit{i.e.} chemokines) produced by virus-specific T-cells, by cells with crosslinked Fc receptors, and by fixed complement could promote the recruitment of effector cells to focal sites of early virus replication. Also, soluble molecules produced locally may be present at significantly higher concentrations at a focal site of virus replication than at a distant site. More generally, there may be features unique to mucosal immunity, or that differ between healthy adults versus hosts with varying degrees of chronic immune activation and dysfunction. Therefore, we treated the
categorical phenomenon of complete or apparent sterilizing protection as distinct from quantitative differences in post-infection viral loads.

**Time-dependence of protection by live-attenuated SIV.** Complete protection by live-attenuated SIV may require a combination of T-cell, antibody, and innate immunity. However, the efficacy of at least one of the immune responses necessary for complete protection increases over time, since animals challenged with pathogenic SIV<sub>mac</sub>251 months after inoculation with live-attenuated SIV are protected from infection, whereas those challenged at early time points become infected. Wyand et al. reported that 0/4 animals challenged with SIV<sub>mac</sub>251 8 weeks after inoculation with SIV<sub>mac</sub>239Δ3 were completely protected, whereas 1/4 challenged at week 20, and 4/6 challenged at week 79 were completely protected<sup>347</sup>. Similarly, Connor et al. reported that 0/4 animals challenged with SIV<sub>mac</sub>251 5 weeks after inoculation with SIV<sub>mac</sub>239Δ<em>nef</em> were completely protected, whereas 2/4 challenged at week 10, 4/4 challenged at week 15, and 3/4 challenged on week 25 were completely protected<sup>345</sup>. The shorter time period required for the maturation of protection by SIV<sub>mac</sub>239Δ<em>nef</em> versus SIV<sub>mac</sub>239Δ3 probably relates to the better protection provided by less attenuated vaccine strains<sup>347,352</sup>. The results of experiments with other challenge strains are also consistent with an increase in protection against superinfection over time<sup>404,405</sup>. Therefore, an immune response that is essential for complete protection by live-attenuated SIV increases in efficacy over time. Cole et al. showed that antibody responses elicited by live-attenuated SIV increase over time in titer and in their ability to resist being stripped from an ELISA plate by a high concentration of urea<sup>406</sup>. Weeks to months are required for neutralizing
antibodies to become detectable in the context of wild-type SIV and HIV-1 infection\textsuperscript{275,285}. Antibodies that have undergone affinity maturation may be responsible for the time-dependent development of protection by live-attenuated SIV, but may be considerably more difficult to elicit by vaccination than T-cell responses.

**Lessons from adoptive and passive transfer studies.** Although the present dissertation specifically addresses a role for antibodies, it is unlikely that antibodies are the only immune response necessary for complete protection by live-attenuated SIV. The immune system has evolved to respond with multiple effector cell populations, which may work cooperatively. This concept was demonstrated in mice by Dittmer et al., who showed that the adoptive transfer of CD4\textsuperscript{+} T-cells, CD8\textsuperscript{+} T-cells, and B-cells in combination reconstituted complete protection, whereas any 2 of these 3 populations failed to confer complete protection\textsuperscript{407}. This adoptive transfer experiment supports the intuitively logical proposition that different arms of adaptive immunity have evolved to operate synergistically as an integrated system.

Passive transfer experiments in the macaque model may offer clues on the relative efficacy of antibodies alone, in the absence of virus-specific T-cells. However, passive transfer of plasma from animals inoculated with live-attenuated SIV strains has yielded mixed results. In one study using adult cynomolgus macaques, passive sera conferred no protection\textsuperscript{408}. Likewise, Desrosiers et al. have observed no protection by passive transfer of IgG purified from animals infected with SIV\textsubscript{mac}239 or SIV\textsubscript{mac}239\Delta3 in unpublished experiments. However, passive transfer completely protected neonatal rhesus macaques from oral challenge with SIV\textsubscript{mac}251\textsuperscript{409}. Experiments using passive transfer of
neutralizing monoclonal antibodies to protect against SHIV challenge suggest that a 50% neutralizing antibody titer in serum >100, or that neutralizes 100% of virus infectivity at low dilutions, is the minimum necessary to achieve complete protection. These concentrations contrast with the low or undetectable antibodies capable of neutralizing the challenge virus in animals completely protected by live-attenuated SIV. Therefore, if antibodies are important for complete protection by live-attenuated SIV, another adaptive immune response (i.e. T-cells) is probably necessary in combination.

**T-cells in protection by live-attenuated SIV.** It is possible that the time dependence of protection by live-attenuated SIV against SIVmac251 challenge relates to the maturation of T-cells, not antibodies. However, in contrast to antibody titers, virus-specific CD8+ T-cell responses decline in frequency after the acute peak of live-attenuated SIV replication (Reeves et al., manuscript in preparation). Also, since adenovirus-based vaccines that do not confer complete protection can elicit higher magnitude T-cell responses than live-attenuated SIV, it is unlikely that the degree of protection afforded by live-attenuated SIV is a function of the overall frequency of virus-specific CD8+ T-cells. For T-cells to account for the time-dependent maturation of protection, one must invoke an increase in effector function over time, which has not yet been shown. Rollman et al. have shown that the CD8+ T-cells of 2 animals inoculated with a live-attenuated SIV strain degranulated with more rapid kinetics than those of 2 animals inoculated with a poxvirus-based vaccine. However, these experiments were conducted with just 2 pairs of animals at a single time point, and it is unclear whether degranulation kinetics are generally more rapid for CD8+ T-cells elicited by live-
attenuated SIV versus prime-boost regimens. In addition to promoting other immune responses, CD4+ T-cells themselves can possess direct effector function against virus-infected cells. Live-attenuated SIV elicits relatively high-magnitude CD4+ T-cell responses, and CD4+ T-cells can recognize and suppress viral replication in macrophages. Although the time-dependence of protection by live-attenuated SIV does not appear to be related to the magnitude of virus-specific CD8+ T-cell responses, other changes may occur over time.

**Protection against heterologous SIV challenge strains.** Live-attenuated SIV affords greater protection against challenge viruses that are more closely related in sequence. SIV_{mac}239 and its derivatives are closely related to SIV_{mac}251, differing in Env by approximately 5% on the amino acid level\(^{417}\). SIV_{sm}E660 is an independent SIV isolate, which differs in Env by 15% from SIV_{mac}239 at the amino acid level. Since this distance is comparable to the median level of intra-clade variation, researchers interested in the cross-reactivity of virus-specific immune responses have tested the degree of protection afforded by vaccines derived from SIV_{mac}239 against challenge with SIV_{sm}E660\(^{384,418-420}\). In contrast to challenge with SIV_{mac}251 at late time points after inoculation with SIV_{mac}239\(\Delta A3\)^{347}, 0/6 animals challenged with SIV_{sm}E660 >2 years after inoculation were completely protected\(^{420}\). Likewise, just 2/10 animals were completely protected against challenge with SIV_{sm}E660 6 months after inoculation with SIV_{mac}239\(\Delta nef\)^{418}. The more limited protection provided by live-attenuated SIV strains derived from SIV_{mac}239 against challenge with SIV_{sm}E660 versus SIV_{mac}251 suggests that protection is mediated by adaptive immune responses susceptible to antigenic
variation. Although there were sequence differences between SIV\textsubscript{mac}239Δnef and SIV\textsubscript{sm}E660 in some of the CD8\textsuperscript{+} T-cell epitopes recognized by these animals, many cross-reacted and expanded in anamnestic responses after superinfection with SIV\textsubscript{sm}E660. However, consistent with the poor neutralization of heterologous HIV-1 isolates\textsuperscript{285}, antibodies capable of neutralizing SIV\textsubscript{sm}E660 were undetectable\textsuperscript{418}. Although weaker than the time-dependence argument, the better protection against SIV\textsubscript{mac}251 than SIV\textsubscript{sm}E660 despite the presence of cross-reactive CD8\textsuperscript{+} T-cells also appears consistent with a role for Env-specific antibodies in protection by live-attenuated SIV.

**Functions of antibodies other than neutralization.** Despite the absence of detectable antibodies capable of neutralizing SIV\textsubscript{mac}251 in most animals completely protected against this challenge strain, the time-dependence of protection, and perhaps its strain specificity, remain consistent with a role for Env-specific antibodies in protection by live-attenuated SIV. The antibodies that bind Env as it exists on virions and virus-infected cells would be expected to have numerous effector functions. As mentioned above, NK cells kill virus-infected cells by ADCC in response to the crosslinking of CD16 by antibodies bound to Env\textsuperscript{109,110}. Effector cells stimulated by the crosslinking of Fc receptors also release soluble factors that interfere with viral replication\textsuperscript{421}. ADCVI assays presumably measure the combined effects of ADCC and noncytolytic inhibition of viral replication\textsuperscript{422,423}. Noncytolytic mechanisms of viral inhibition include the release of molecules that induce an antiviral state and that, perhaps fortuitously, interfere with access to the viral coreceptor. The β-chemokines MIP-1α, MIP-1β, and RANTES directly compete with gp120 for access to CCR5, inhibit HIV-1 and SIV replication, and
contribute to ADCVI\textsuperscript{135,139,251,360,422,424}. The binding of CCR5 to its ligands, and the binding of other chemokine receptors to their respective ligands, which include products of complement fixation, induces the downregulation of CCR5 as part of the signal desensitization built into GPCR signaling cascades\textsuperscript{425-427}. The cytokines and chemokines released subsequent to crosslinking of Fc receptors also promote the homing of immune effector cells\textsuperscript{428,429}. Complement fixation can lyse virions and virus-infected cells\textsuperscript{430,431}, and likewise, produces molecules that promote the homing of immune effector cells\textsuperscript{432-436}. Clearance of virions by phagocytosis may also interfere with viral replication\textsuperscript{437}. HIV-1 replication can be inhibited by \(\alpha\)-defensins\textsuperscript{438}, which are produced by neutrophils\textsuperscript{439}. Thus, the potential antiviral functions of antibodies are numerous and varied, and include neutralization, ADCC, complement fixation, homing, activation, induction of an antiviral state, and interference with access to viral coreceptors. Antiviral functions of antibodies mediated by cells of the innate immune system, such as NK cells, dendritic cells, neutrophils, monocytes and macrophages, or by complement, are examples of the integration of antibody and innate immunity.

**Additional observations supporting a role for antibodies other than neutralization.** Observations outside of live-attenuated SIV also support a role for antibodies other than neutralization. As mentioned above, SIV and HIV-1 appear to preemptively escape neutralizing antibody responses, since plasma poorly neutralizes contemporaneous viral variants, and mutations accumulate in Env in the absence of significant neutralization\textsuperscript{285,440}. Therefore, antibody responses exert selective pressure in the absence of significant neutralization. Although higher Env expression and virion
incorporation increases viral infectivity and resistance to neutralization, the cytoplasmic domains of Env proteins possess internalization signals that limit their surface expression and virion incorporation\textsuperscript{249,441-443}. The limited surface expression of Env may reflect a trade-off that favors resistance to non-neutralizing mechanisms through lower Env expression versus greater infectivity and neutralization resistance through higher Env expression. Early studies testing killed virus vaccines suggest that mechanisms other than neutralization and ADCC can prevent SIV infection\textsuperscript{336-343}. Antibodies to human cellular antigens were responsible for protection of macaques from infection by virus grown in human cells. However, antibodies to human cellular antigens do not neutralize the infectivity of viruses produced in human cells\textsuperscript{159,334}. Although it is possible that the amount human cellular antigen delivered onto a macaque CD4\textsuperscript{+} T cell after fusion of the viral and cellular membranes can serve as a target for ADCC or complement, the amount of protein carried by the virus is relatively small compared with a cell, and may diffuse laterally. Therefore, direct effects of antibody on the virion other than neutralization may prevent infection. These observations bolster the case for the importance of antibody functions other than neutralization.

**Summary.** Development of a vaccine capable of preventing HIV-1 infection is a difficult problem due to properties of the primate lentiviruses that have evolved to enable persistent replication and transmission in the face of host immune responses. Inherent features of the Env protein interfere with the induction and the efficacy of antibody responses, and provide the sequence plasticity that facilitates antigenic diversification\textsuperscript{187,188,217,241,244,251,252,254,274}. No vaccines under consideration for clinical
evaluation are capable of eliciting antibodies that neutralize primary isolates of HIV-1 and SIV representative of CCR5-tropic neutralization-resistant t/f viruses. Although antibodies that neutralize autologous HIV-1 isolates are frequently elicited during natural HIV-1 infection, these typically lack significant ability to neutralize heterologous HIV-1 isolates\textsuperscript{285}, suggesting that antigenic diversity is a significant problem. The level of somatic hypermutation among HIV-1-specific antibodies is unusually high\textsuperscript{326,328}, and is exceptionally high among antibodies that neutralize a broad range of viruses\textsuperscript{191,329-332}. However, the rate of somatic hypermutation of approximately $10^3$ nucleotide substitutions per cell division imposes a speed limit on affinity maturation\textsuperscript{313}. The failure of efforts to elicit antibodies that neutralize primary HIV-1 isolates, and of an empirical approach to HIV-1 vaccine discovery more generally, has renewed interest in basic research to understand mechanisms of vaccine protection. Protection by live-attenuated SIV increases over a time scale on the order of months\textsuperscript{345,347}, consistent with an essential role for the affinity maturation of antibody responses\textsuperscript{322,406}. Although neutralizing antibodies are typically undetectable among most animals completely protected from wild-type SIV infection by vaccination live-attenuated SIV\textsuperscript{345,352}, antibodies may contribute to protection through mechanisms other than neutralization\textsuperscript{110,135,139,360,421-424,428,429,431,436-438,444}. It is therefore our priority to evaluate the possible contribution of antibodies to protection by live-attenuated SIV, and also to understand the induction of antibody responses by live-attenuated SIV. Although the induction of effective antibody responses may represent the most significant obstacle to the development of a safe and effective HIV-1 vaccine, other immune responses will probably be required in
combination, since antibody, T-cell, and innate immunity have evolved to operate synergistically as an integrated system\textsuperscript{407}. 
CHAPTER 2

Envelope-modified scSIV selectively enhances antibody responses and partially protects against repeated, low-dose vaginal challenge
Acknowledgements

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2. A. ABSTRACT

Immunization of rhesus macaques with strains of SIV that are limited to a single cycle of infection elicits T cell responses to multiple viral gene products and antibodies capable of neutralizing lab-adapted SIV, but not neutralization-resistant primary isolates of SIV. In an effort to improve upon the antibody responses, we immunized rhesus macaques with three strains of single-cycle SIV (scSIV) that express envelope glycoproteins modified to lack structural features thought to interfere with the development of neutralizing antibodies. These envelope-modified strains of scSIV lacked either five potential N-linked glycosylation sites in gp120, three potential N-linked glycosylation sites in gp41, or 100 amino acids in the V1V2 region of gp120. Three doses consisting of a mixture of the three envelope-modified strains of scSIV were administered on weeks 0, 6, and 12, followed by two booster inoculations with VSV G trans-complemented scSIV on weeks 18 and 24. Although this immunization regimen did not elicit antibodies capable of detectably neutralizing SIVmac239 or SIVmac251UCD, neutralizing antibody titers to the envelope-modified strains were selectively enhanced. Virus-specific antibodies and T cells were observed in the vaginal mucosa. After twenty weeks of repeated, low-dose vaginal challenge with SIVmac251UCD, six of eight immunized animals versus six of six naïve controls became infected. Although immunization did not significantly reduce the likelihood of acquiring immunodeficiency virus infection, statistically significant reductions in peak and set-point viral loads were observed in the immunized animals relative to the naïve control animals.
2. B. INTRODUCTION

Development of a safe and effective vaccine for HIV-1 is an urgent public health priority, but remains a formidable scientific challenge. Passive transfer experiments in macaques demonstrate neutralizing antibodies can prevent infection by laboratory-engineered SHIV strains. However, no current vaccine approach is capable of eliciting antibodies that neutralize primary isolates with neutralization-resistant Env proteins. Virus-specific T cell responses can be elicited by prime-boost strategies utilizing recombinant DNA and/or viral vectors, which confer containment of viral loads following challenge with SHIV. Unfortunately, similar vaccine regimens are much less effective against SIVmac239 and SIVmac251, which bear closer resemblance to most transmitted HIV-1 isolates in their inability to utilize CXCR4 as a coreceptor and inherent high degree of resistance to neutralization by antibodies or sCD4. Live-attenuated SIV can provide complete or apparent sterile protection against challenge with SIVmac239 and SIVmac251, or at least contain viral replication below the limit of detection. Due to the potential of the attenuated viruses themselves to cause disease in neonatal rhesus macaques, and to revert to a pathogenic phenotype through the accumulation of mutations over prolonged periods of replication in adult animals, attenuated HIV-1 is not under consideration for use in humans.

As an experimental vaccine approach designed to retain many of the features of live-attenuated SIV, without the risk of reversion to a pathogenic phenotype, we and others devised genetic approaches for producing strains of SIV that are limited to a single
cycle of infection\textsuperscript{159,455-459}. In a previous study, immunization of rhesus macaques with single-cycle SIV (scSIV) trans-complemented with VSV G elicited potent virus-specific T-cell responses\textsuperscript{159}, which were comparable in magnitude to T-cell responses elicited by optimized prime-boost regimens based on recombinant DNA and viral vectors\textsuperscript{42,48,363,366,386,448}. Antibodies were elicited that neutralized T-cell line-adapted SIV\textsubscript{mac251TCLA}\textsuperscript{159}. However, despite the presentation of native, trimeric SIV Env on the surface of infected cells and virions, none of the scSIV-immunized macaques developed antibody responses that neutralized SIV\textsubscript{mac239}\textsuperscript{159}. Therefore, we have now introduced Env modifications into scSIV to facilitate the development of neutralizing antibodies.

Most primate lentiviral envelope glycoproteins are inherently resistant to neutralizing antibodies due to structural and thermodynamic properties that have evolved to enable persistent replication in the face of vigorous antibody responses\textsuperscript{132,217,241,244,251,252,254,274,460}. Among these, extensive N-linked glycosylation renders much of the Env surface inaccessible to antibodies\textsuperscript{130,241,243,247,274}. Removal of N-linked glycans from gp120 or gp41 by mutagenesis facilitates the induction of antibodies to epitopes that are occluded by these carbohydrates in the wild-type virus\textsuperscript{132,244}. Consequently, antibodies from animals infected with glycan-deficient strains neutralize these strains better than antibodies from animals infected with the fully-glycosylated SIV\textsubscript{mac239} parental strain\textsuperscript{132,244}. Most importantly with regard to immunogen design, animals infected with the glycan-deficient strains developed higher neutralizing antibody titers against wild-type SIV\textsubscript{mac239}\textsuperscript{132,244}. Additionally, the removal of a single N-linked glycan in gp120 enhanced the induction of neutralizing antibodies against SHIV\textsubscript{89.6P} and SHIV\textsubscript{SF162} in a prime-boost strategy by 20-fold\textsuperscript{250}. These observations suggest that
potential neutralization determinants accessible in the wild-type Env are poorly immunogenic unless specific N-linked glycans in gp120 and gp41 are eliminated by mutagenesis.

The variable loop regions 1 and 2 (V1V2) of HIV-1 and SIV gp120 may also interfere with the development of neutralizing antibodies. Deletion of V1V2 from HIV-1 gp120 permitted neutralizing monoclonal antibodies to CD4-inducible epitopes to bind to gp120 in the absence of CD4, suggesting that V1V2 occludes potential neutralization determinants prior to the engagement of CD4. A deletion in V2 of HIV-1 Env exposed epitopes conserved between clades, improved the ability of a secreted Env trimer to elicit neutralizing antibodies in rabbits, and was present in a vaccine that conferred complete protection against SHIVSF162P4. A deletion of 100 amino acids in V1V2 of SIVmac239 rendered the virus sensitive to monoclonal antibodies with various specificities. Furthermore, 3/5 macaques experimentally infected with V1V2-deleted SIVmac239 resisted superinfection with wild-type SIVmac239. Thus, occlusion of potential neutralization determinants by the V1V2 loop structure may contribute to the poor immunogenicity of the wild-type Env protein.

Here we tested the hypothesis that antibody responses to scSIV could be improved by immunizing macaques with strains of scSIV engineered to eliminate structural features that interfere with the development of neutralizing antibodies. Antibodies to Env-modified strains were selectively enhanced, but these did not neutralize the wild-type SIV strains. We then tested the hypothesis that immunization might prevent infection in a repeated, low-dose vaginal challenge model of heterosexual HIV-1 transmission. Indeed, while all six naïve control animals became infected, two of
eight immunized animals remained uninfected after twenty weeks of repeated vaginal challenge. Relative to the naïve control group, reductions in peak and set-point viral loads were statistically significant in the immunized animals that became infected.
2. MATERIALS AND METHODS

Animals. The animals included in this study were all female Indian-origin rhesus macaques (Macaca mulatta). They were housed in a biosafety level 3 containment facility at the New England Primate Research Center (NEPRC), and given care in accordance with standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the Harvard Medical School Animal Care and Use Committee. These experiments and procedures were approved by the Harvard Medical Area Standing Committee on Animals, and were conducted in accordance to the *Guide for the Care and Use of Laboratory Animals*. The animals in this study were typed for the MHC class I alleles Mamu-A*01, -A*02, -A*08, -A*11, -B*01, -B*03, -B*04, -B*08, -B*17, and -B*29, as well as the MHC class II alleles DRB1*w201, DRB1*0401/06, and DPB1*06. The MHC typing results are summarized in Table 2.1. Typing was performed in Dr. David Watkins’ laboratory at the Wisconsin National Primate Research Center (WNPRC), as previously described by Kaizu *et al*.

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Table 2.1. MHC typing of immunized and naïve macaques.
**Env-modified strains of scSIV.** Mutations were introduced into the 3’ half of the SIV<sub>mac239</sub> genome<sup>132,244,273</sup>. A fragment containing a stop codon followed by two single-nucleotide deletions in nef<sup>456</sup> plus a stop codon at position E767 of Env was cloned into the NheI and SphI sites of the 3’ halves of SIV genomes containing the M5, g123, and ΔV1V2 Envs. Sequence tags gsa, cao, and ggr were previously introduced into scSIV constructs containing the mutated frameshift region, and deletions in pol<sup>468</sup>. SphI-SphI fragments of the 5’ halves containing the three sequence tags were cloned into the SphI sites of the 3’ halves containing the M5, g123, and ΔV1V2 Envs, and stop codons in Env and Nef.

**Preparation of scSIV.** Virus stocks of scSIV were produced by co-transfection of 293T cells with the Gag-Pol expression product pGPfusion, and the proviral DNA for each strain of scSIV<sup>455</sup>. 293T cells were seeded on day 0 at a density of 3x10<sup>6</sup> cells per 100 mm dish in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin. Cells were transfected on day 1 with 5 μg of each plasmid, using the GenJetTM transfection reagent according to the manufacturer’s instructions (SignaGen Laboratories, Gaithersburg, MD). Media was removed on day 2, and cells were washed twice with serum-free DMEM, which was replaced with DMEM supplemented with 10% rhesus serum (Equitech-Bio, Kerrville, TX). Cell culture supernatant was collected on day 3. Cellular debris was removed by centrifugation at 2095 x g, and supernatants were concentrated by repeated, low-speed centrifugation in YM-50 ultrafiltration units (Millipore, Bedford, MA), according to the manufacturer’s instructions. Aliquots of scSIV were cryopreserved at -
80°C until use. The virus concentration was determined by SIV p27 antigen capture ELISA (Advanced BioScience Laboratories, Kensington, MD).

**Immunization of macaques with scSIV.** Eight animals were inoculated intravenously with identical doses containing 5 μg p27 of scSIVmac239M5, scSIVmac239g123, and scSIVmac239ΔV1V2 on weeks 0, 6, and 12. The animals were boosted with 15 μg p27 scSIVmac239 trans-complemented with VSV GNJ (New Jersey serotype) on week 18, and 15 μg p27 scSIVmac239 trans-complemented with VSV G1 (Indiana serotype) on week 24. Animals were anesthetized by 15 mg/kg intramuscular injection of ketamine-HCl, and the concentrated scSIV stock was injected through a 22-gauge catheter aseptically placed into the saphenous vein.

**Neutralizing antibodies.** Neutralizing antibodies were titered by measuring the inhibition over serial, two-fold plasma dilutions of the activity of a secreted alkaline phosphatase (SEAP) reporter gene under transcriptional control of the SIV LTR promoter in a C8166-derived T-cell line. SEAP activity was measured using the Phospha-Light SEAP detection kit (Applied Biosystems, Foster City, CA). Cells incubated without virus or without plasma were used to determine the minimum and maximum SEAP activities, respectively. The nearest values above and below 50% SEAP activity were used to calculate what plasma dilution would intercept the 50% inhibition line.

Virus was incubated with plasma dilutions in 100 μl volumes for 1 hour at 37°C before adding a 100 μl volume containing the C8166-SEAP reporter cells. Due to differences in infectivities between Env mutants, different amounts of input virus and
C8166-SEAP cells were used per well for each strain. Minimizing the amount of input virus and time to reading SEAP activity maximizes the apparent 50% neutralization titer (data not shown). These amounts and incubation times were as follows: 12.5 ng p27 \( \text{SIV}_{\text{mac239M5}} \) and 50,000 cells, read on day 5; 10 ng p27 \( \text{SIV}_{\text{mac239g123}} \) and 50,000 cells, read on day 4; 2.5 ng p27 \( \text{SIV}_{\text{mac239AVV1V2}} \) and 40,000 cells, read on day 3; 2 ng p27 \( \text{SIV}_{\text{mac251TCLA}} \) and 15,000 cells, read on day 3; 0.5 ng p27 \( \text{SIV}_{\text{mac239}} \) and 15,000 cells, read on day 3; 0.5 ng p27 \( \text{SIV}_{\text{mac251UCD}} \) and 15,000 cells, read on day 3.

\( \text{SIV}_{\text{mac239M5}}, \text{SIV}_{\text{mac239g123}}, \) and \( \text{SIV}_{\text{mac239}} \) stocks were produced by transfection of \( 293T \) cells. An uncloned \( \text{SIV}_{\text{mac239AVV1V2}} \) stock that was passed in \( H. \text{saimiri} \)-transformed macaque 221 cells\textsuperscript{273} and expanded in CD8-depleted, PHA-activated rhesus PBMC was selected for the neutralizing antibody assays due to its higher infectivity. \( \text{SIV}_{\text{mac251TCLA}} \) was grown in MT4 cells. The \( \text{SIV}_{\text{mac251UCD}} \) used for neutralization assays was an uncloned stock, expanded from the challenge stock for five days in CD8-depleted, PHA-activated rhesus PBMC.

**Collection and processing of mucosal specimens.** Cervicovaginal and rectal secretions were collected atraumatically with pre-moistened Weck-Cel sponges (Medtronics, Minneapolis, MN), and extracted by centrifugation as previously described\textsuperscript{469}. Vaginal biopsies containing both epithelium and underlying tissue were collected using sterile pinch biopsy forceps. Lymphocytes were isolated from biopsy specimens as previously described\textsuperscript{470,471}. Biopsies were incubated in 1 mM EDTA for 30 minutes at 37°C, then twice incubated for an hour in fresh media containing collagenase Type IV while shaking vigorously. A cell suspension was obtained by mechanical
dispersion of collagenase-treated samples with a blunt-ended 18 G needle, followed by filtration using a 70 µm nylon cell strainer. Cells were layered over a 35%/60% discontinuous Percoll gradient and centrifuged for 20 minutes at 1000 x g to enrich for lymphocytes.

**Mucosal antibodies.** Concentrations of total IgG, total IgA, and antibodies to gp120 and viral lysates were measured by chromagenic ELISA as previously described using microtiter plates coated with goat anti-monkey IgG (MP BioMedicals, Solon, OH), goat anti-monkey IgA (Rockland, Gilbertsville, PA), SIV\textsubscript{mac251} rgp120 (ImmunoDiagnostics, Woburn, MA), or a 500-fold dilution of SIV\textsubscript{mac251} viral lysate (Advanced Biotechnologies Inc, Columbia, MD). Preparations of rhesus macaque serum containing known quantities of each immunoglobulin or gp120-specific antibody were used as standards. Prior to analyses for IgA antibodies, specimens were depleted of IgG using Protein G Sepharose as described previously Plates were developed with biotinylated goat anti-monkey IgA (Alpha Diagnostics, San Antonio, TX) or anti-human IgG (SouthernBiotech, Birmingham, AL) polyclonal antibodies. The concentration of SIV-specific IgG or IgA in secretions was normalized relative to the total IgG or IgA concentration by calculating the specific activity (ng gp120-specific antibody per µg total IgG or IgA). Specific activity (SA) values were considered to be significant if greater than the mean plus three standard deviations of samples from naïve macaques.

**IFNγ ELISPOT assays.** Longitudinal T-cell responses to Gag, Tat, Rev, Vif, Vpr, Vpx, Env, and Nef were measured using pools of 15-mer peptides overlapping by eleven
residues at 2.5 µg/ml. PBMC were plated at 3x10^5 and 1x10^5 cells per well in duplicate wells at each density on Multiscreen 96-well plates (Millipore, Bedford, MA), incubated overnight, and IFNγ was detected using the Mabtech ELISPOT kit for monkey/human IFNγ (Mabtech, Mariemont, OH). Spots were enumerated by an automated ELISPOT reader (Zellnet Consulting, New York, NY). The number of spot forming cells (SFC) per million PBMC was calculated by subtracting the number of background spots in wells that received cells but not peptide.

Full-proteome epitope mapping was expedited through the use of a deconvolution matrix. Each animal was mapped using deconvolution matrices consisting of one 96-well ELISPOT plate containing 92 matrix wells, 3 DMSO-only negative control wells, and one concanavalin A (ConA) positive control well. Peptides covering Gag, Pol, Tat, Rev, Vif, Vpr, Vpx, Env, and Nef were each present in 2 of the 92 matrix wells. The matrix was designed to minimize the number of potentially positive peptides that would require individual testing. Mapping of CD4+ T cell epitopes was performed using PBMC depleted of CD8+ cells by Dynal anti-CD8 magnetic beads (Invitrogen, Carlsbad, CA). CD8+ T cell epitopes were mapped using PBMC depleted of CD4+ cells. Depletions were conducted at a 3:1 bead-to-cell ratio for 45 minutes on a rotator at 4°C, and confirmed by flow cytometry to have reduced the target population to a maximum of 0.1% of lymphocytes. Cells were seeded to the matrix plate at 1x10^5 cells per well. Surplus CD4- or CD8-depleted cells were rested at 37°C overnight. After processing and enumeration of spots, peptides present in two wells deemed positive, defined as greater than 50 SFC per million PBMC and 3 standard deviations above background, were selected for individual testing on the rested CD4- or CD8-depleted PBMC.
**MHC class I tetramer staining.** MHC class I tetramer staining of virus-specific CD8+ T cells was conducted for the *Mamu-A*01- and *A*02-positive macaques. A 200 µl volume of whole blood was incubated for 30 minutes at 37°C with one of the APC-conjugated tetramers, Mamu-A*01 Gag$_{181-189}$ (CM9), Mamu-A*01 Tat$_{28-35}$ (SL8), Mamu-A*02 Nef$_{159-167}$ (YY9), or Mamu-A*02 Gag$_{71-79}$ (GY9). These tetramers were provided by Dr. David Watkins’ laboratory (WNPRC, Madison, WI). Samples were then stained for 30 minutes at room temperature with FITC-conjugated anti-CD3 (clone SP34, BD Biosciences, San Jose, CA), and PerCP-conjugated anti-CD8 (clone SK1, BD Biosciences). Erythrocytes were then eliminated by treatment with FACS Lysing solution (BD Biosciences, San Jose, CA). Samples were washed and fixed in 2% formaldehyde in PBS. A FACSCalibur flow cytometer was used for data collection (BD Biosciences, San Jose, CA). Data was analyzed using the FlowJo version 8.7.1 software package (Tree Star, San Carlos, CA).

**Repeated, low-dose vaginal challenge with SIV$_{mac251 UCD}$.** The SIV$_{mac251 UCD}$ virus stock used for challenges was prepared at the California National Primate Research Center in June, 2004. We have adopted the UCD designation to indicate that this virus stock has its own passage history, in PBMC and in monkeys, although it was derived from a SIV$_{mac251}$ stock originally provided by Dr. Ronald Desrosiers.

Fresh vials of virus were thawed at 37°C, transferred immediately to ice, and diluted 1 to 100 in RPMI tissue culture medium (Invitrogen, Carlsbad, CA) containing no additional additives. Each 1 ml dose contained 1 ng SIV p27 equivalents or 1000 TCID$_{50}$. 


Doses were stored on ice until inoculation. Animals were anesthetized by 15 mg/kg intramuscular injection of ketamine-HCl, and positioned with their hindquarters raised for vaginal inoculation and the following 30 minutes. Four hours later, the above challenge procedure was repeated. Challenge days were spaced one week apart, and were discontinued once viral RNA was detectable in plasma for 2 consecutive weeks.

**Plasma viral RNA loads.** Plasma samples were collected in 0.5 to 1.5 ml volumes of sodium citrate anticoagulant and ultracentrifuged at 20,000 x g for 1 hour to pellet virus. RNA was extracted, reverse transcribed into cDNA, and quantified by real-time PCR as previously described\textsuperscript{473}. The limit of detection for this assay is 30 copies of RNA per ml. The primer/probe sets for the quantitative, multiplex real-time RT-PCR assay for the unique sequence tags ggr, cao and gsa have previously been described by DeGottardi et al\textsuperscript{468}.

**CD4\textsuperscript{+} T cell subsets.** The maintenance or destruction of naïve, central memory, effector memory, CCR5\textsuperscript{+} memory, and total CD4\textsuperscript{+} T cell populations was monitored by flow cytometry. Total CD4\textsuperscript{+} T cell counts and the concentrations of each cell population per µl whole blood were calculated by enumerating lymphocytes per µl of whole blood by complete blood count (CBC), and multiplying by the percentage belonging to each CD4\textsuperscript{+} T cell subpopulation as determined by flow cytometry. Whole blood was stained with FITC-conjugated anti-CD3 (clone SP34, BD Biosciences, San Jose, CA), PerCP-conjugated anti-CD4 (clone L200, BD Biosciences, San Jose, CA), APC-conjugated anti-CD95 (clone DX2, BD Biosciences, San Jose, CA), and PE-conjugated anti-CD28 (clone
CD28.2, BD Biosciences, San Jose, CA) or PE-conjugated anti-CCR5 (clone 3A9, BD Biosciences, San Jose, CA). Erythrocytes were eliminated with FACS Lysing solution (BD Biosciences, San Jose, CA), and cells were washed and fixed in 2% formaldehyde in PBS. Flow cytometry data for CD4$^+$ T cells was collected and analyzed as described for tetramer data.

**Statistical methods.** All statistical evaluations were conducted using SPSS 15.0 (SPSS Inc., Chicago, IL), and Strata MP 10.0 (Strata Corp., College Station, TX). The differences in the ratio of anti-gp120 IgG SA in CVS to plasma was compared between the uninfected immunized animals (n=2) and those immunized and infected (n=6) using a generalized estimation equation (GEE) model. Correlations between repeated measurements within the same subject were accounted for by use of unstructured covariance as well as first order autocorrelation. Both approaches lead to a similar result. In total, 31 measurements from 8 animals were included.

A 2-tailed Mann-Whitney U test on the highest measured log-transformed values between weeks 1 and 5 post-infection was used to determine the significance of differences in peak viral loads. A linear mixed model analysis was used to determine the significance of differences in CD4$^+$ T cell counts and log-transformed set-point viral loads between the immunized versus naïve groups$^{474}$. Set-point viral loads were evaluated for the period between weeks 5 and 67 post-infection, centered on week 28.
2. RESULTS

**Immunization of macaques with Env-modified strains of scSIV.** Rhesus macaques were immunized with three strains of scSIV, each of which had different mutations that deprive the SIV envelope glycoprotein of specific countermeasures against host antibody responses. The wild-type SIV$_{mac239}$ Env has 23 potential N-linked glycosylation sites in gp120, and three potential N-linked glycosylation sites in gp41 (Fig. 2.1a). The 5th, 6th, 8th, 12th and 13th potential N-linked glycosylation sites in gp120 were eliminated by mutagenesis in the Env-modified scSIV strain scSIV$_{mac239M5}$ (Fig. 2.1b)$^{244}$. All three potential N-linked glycosylation sites in gp41 were eliminated in the strain scSIV$_{mac239g123}$ (Fig. 2.1c)$^{132}$. The third Env-modified scSIV strain, scSIV$_{mac239ΔV1V2}$, had 100 amino acids in the V1V2 loop structure deleted (Fig. 2.1d)$^{273}$. These three strains of Env-modified scSIV also had a glutamate to stop codon change to truncate the cytoplasmic tail of gp41, which maximizes Env incorporation onto virions, viral infectivity, and the Env-specific antibody response (Fig. 2.1b-d)$^{249,468}$. In order to maximize CD8$^+$ T-cell responses, each scSIV strain contained a premature stop codon in nef that eliminates 26 amino acids from the C-terminus of the protein required for MHC class I downregulation (Fig. 2.1b-d)$^{475}$. 


Figure 2.1. Animals were immunized with Env-modified single-cycle SIV, and challenged by repeated, low-dose vaginal inoculation. A schematic representation of the wild-type SIV<sub>mac239</sub> Env appears at the top (a). Positions of potential sites of N-linked glycosylation (N-X-S/T motifs) are indicated by tree-like symbols. Features removed from each Env-modified strain are indicated (red). The M5 Env has asparagine to glutamine substitutions that eliminate the 5<sup>th</sup>, 6<sup>th</sup>, 8<sup>th</sup>, 12<sup>th</sup> and 13<sup>th</sup> potential N-linked glycosylation sites in gp120 (b). The g123 Env has all three potential N-linked glycosylation sites in gp41 similarly eliminated (c). Variable loops 1 and 2 of the ΔV1V2 envelope are deleted (d). All of the modified envelopes have a glutamate-to-stop codon change at position 767 (E767*). Eight macaques received an intravenous injection consisting of a mixture of 5 µg p27 equivalents of scSIV<sub>mac239M5</sub>, scSIV<sub>mac239g123</sub>, and scSIV<sub>mac239ΔV1V2</sub> on weeks 0, 6, and 12 (e). All 8 animals also received 15 µg p27 equivalents of VSV G trans-complemented scSIV<sub>mac239</sub> intravenously on weeks 18 and 24. Beginning on week 32, the animals were challenged vaginally with 1000 TCID<sub>50</sub> (1 ng p27) of SIV<sub>mac251_UCD</sub> twice per day of the same day each week for 20 weeks, or until viral RNA was detected in plasma on 2 consecutive weeks.

Eight female rhesus macaques received 3 intravenous inoculations consisting of a mixture of 5 µg p27 equivalents of each of the 3 Env-modified scSIV strains on weeks 0,
6, and 12 to prime antibody responses to Env surfaces with limited accessibility in the native trimer (Fig. 2.1e). These immunogens would be expected to also prime antibody responses to novel surfaces unique to the mutant Envs. Truncation of the Env cytoplasmic tail can also alter the conformation of the ectodomain$^{443,476-478}$. Therefore, to focus the antibody response on surfaces exposed in the native trimer, the animals were boosted on weeks 18 and 24 with 15 μg p27 equivalents of scSIV$_{mac}$239, which expresses the wild-type SIV$_{mac}$239 Env with a full-length cytoplasmic tail (Fig. 1e). These 2 booster doses were trans-complemented with VSV G to maximize infectivity. To prevent neutralization of the second boost by VSV G-specific antibodies$^{159,479}$, scSIV was trans-complemented with the New Jersey serotype (VSV G$_{NJ}$) on week 18, and the Indiana serotype (VSV G$_{I}$) on week 24. This immunization regimen was designed to maximize the stimulation of both Env-specific antibody responses and the magnitude of virus-specific T cell responses.

To determine whether this immunization regimen might reduce the likelihood of viral transmission by the vaginal route, the 8 immunized animals and 6 naïve controls were repeatedly challenged for 20 weeks by low-dose vaginal inoculation of SIV$_{mac}$251$_{UCD}$, beginning on week 32 (Fig. 2.1e). This challenge regimen was expected to be sufficient to infect all the naïve control animals$^{480}$. Thus, significant differences in the number of doses required to establish infection, or in the final number of infections for the immunized versus naïve groups, might indicate that immunization reduced vaginal transmission.
Transient viremia following each dose of Env-modified and VSV G trans-complemented scSIV. Transient viral loads in plasma representing the progeny virions released by cells infected with each Env-modified strain of scSIV were measured independently using a quantitative, multiplex real-time RT-PCR assay specific for unique sequence tags cloned into the pol gene of each strain\(^{468}\). Since cell-free virus is cleared from plasma with a half-life on the order of minutes\(^{18,266}\), these viral RNA load measurements reflect the lifespan of cells productively infected with scSIV in vivo, and thus the duration of antigenic stimulation. The peaks of viremia for scSIV\(_{mac239M5}\) and scSIV\(_{mac239g123}\) were nearly identical, with geometric mean peak viral loads after the first inoculation of $6.8 \times 10^3$ and $6.1 \times 10^3$ RNA copies per ml (Fig. 2.2). However, peak viral loads for scSIV\(_{mac239\Delta V1V2}\) were considerably lower than the other Env-modified strains, resulting in detectable viral loads in only 4 of the 8 immunized animals, the highest of which peaked at 170 copies of viral RNA per ml in Mm 158-02. Except for the first dose of scSIV\(_{mac239M5}\) in Mm 316-98, all single-cycle viral loads for each strain were cleared to below the limit of detection by 3 weeks after inoculation. Relative to the first dose, peak viral loads were lower after the second inoculation for both scSIV\(_{mac239M5}\) (2-tailed Mann-Whitney U test, $P=0.0002$) and scSIV\(_{mac239g123}\) (2-tailed Mann-Whitney U test, $P=0.0002$). Following the booster inoculations with VSV G\(_{NJ}\) and VSV G\(_{I}\) trans-complemented scSIV on weeks 18 and 24, peak geometric mean viral loads were $1.8 \times 10^4$ and $7.8 \times 10^3$ copies of viral RNA per ml, consistent with the infectivity enhancement afforded by VSV G\(^{159}\). These viral load measurements confirm the productive infection of cells in vivo by the Env-modified and VSV G trans-complemented strains of scSIV.
Figure 2.2. Plasma viral RNA following each inoculation with single-cycle SIV. The level of viral RNA in plasma was measured independently for each of these strains using a quantitative, multiplex real-time RT-PCR assay for the unique sequence tags ggr, cao and gsa cloned into each of the three scSIV strains. This assay has a threshold of detection of 30 copies of viral RNA per ml plasma (dashed line).

Neutralization of Env-modified and T-cell line-adapted SIV, but not wild-type SIV_{mac239} or SIV_{mac251}_{UCD}. To determine whether immunization with the Env-modified strains of scSIV significantly enhanced antibody responses to surfaces occluded by the N-linked glycans and V1V2 loops, neutralization of the Env-modified viruses by
plasma samples from this study was compared to neutralization by plasma samples from a previous study, in which animals were immunized with strains of scSIV expressing wild-type envelope glycoproteins from SIV<sub>mac239</sub>, SIV<sub>mac316</sub>, and SIV<sub>mac155T3</sub> (Fig. 2.3a-h). The plasma samples utilized for this comparison were collected 2 weeks after

![Diagram](image)

**Figure 2.3.** Plasmas neutralized Env-modified and T-cell line-adapted SIV, but not wild-type SIV. Neutralizing antibody titers were compared between the animals immunized with Env-modified scSIV that subsequently became infected (blue) or remained uninfected (green), and historical control plasmas from animals immunized with scSIV expressing wild-type Envs (black). Pooled pre-immune plasma served as a negative control (gray). The dashed line indicates 50% inhibition of SEAP activity measured in relative light units (RLU). Plasma samples collected 2 weeks after the third dose of scSIV were tested for neutralization of SIV<sub>mac251_TCLA</sub> (a), SIV<sub>mac239M5</sub> (b), SIV<sub>mac239g123</sub> (c), SIV<sub>mac239ΔV1V2</sub> (d), and SIV<sub>mac239</sub> (i). Fifty-percent neutralization titers are shown for SIV<sub>mac251_TCLA</sub> (e), SIV<sub>mac239M5</sub> (f), SIV<sub>mac239g123</sub> (g), and SIV<sub>mac239ΔV1V2</sub> (h). P-values were determined by 2-tailed Mann-Whitney U tests. Week 26 plasmas were tested for neutralization of SIV<sub>mac239</sub> (j). Plasma samples drawn on week 32 were tested for neutralization of SIV<sub>mac251_UCD</sub> (k).
the third intravenous inoculation with scSIV in both studies. Neutralization assays using plasma samples from both studies were performed in parallel.

No significant difference was detected for neutralization of SIV\textsubscript{mac251\_TCLA} by plasmas from the 2 groups (2-tailed Mann-Whitney U test, P=0.30) (Fig. 2.3a and e). This suggests that any differences in neutralizing antibody titers against the Env-modified strains do not reflect non-specific differences in overall antibody titers between the 2 studies. All of the animals immunized with Env-modified or wild-type strains of scSIV neutralized SIV\textsubscript{mac239M5}, consistent with previous observations that elimination of these carbohydrate attachment sites increases the susceptibility of this virus to neutralization\textsuperscript{156,244}. However, neutralizing antibody titers to SIV\textsubscript{mac239M5} were not significantly different for animals immunized with Env-modified versus wild-type strains of scSIV (2-tailed Mann-Whitney U test, P=0.37) (Fig. 2.3b and f), indicating that scSIV\textsubscript{mac239M5} did not specifically facilitate the induction of antibodies to epitopes occluded by the 5\textsuperscript{th}, 6\textsuperscript{th}, 8\textsuperscript{th}, 12\textsuperscript{th} and 13\textsuperscript{th} N-linked glycans in gp120. In contrast, SIV\textsubscript{mac239g123} was detectably neutralized at low titers by only 2 of the animals immunized with wild-type scSIV, but was neutralized by all of the animals immunized with the Env-modified strains of scSIV, and at titers 5 times higher (2-tailed Mann-Whitney U test, P=0.01) (Fig. 2.3c and g). The difference in 50% neutralization titers between the groups remains statistically significant even if the outlier, Mm 284-99, with a titer of 5763, nearly 200-fold higher than the average for the rest of the group, is excluded from the analysis (2-tailed Mann-Whitney U test, P=0.02). These results suggest that the N-linked glycans in gp41 interfere with the induction of Env-specific
antibody responses, and that the elimination of these glycans by mutagenesis can enhance
the induction of antibodies to surfaces accessible in this N-linked glycan-deficient strain.

Immunization with Env-modified strains also elicited higher neutralizing antibody
titers to the V1V2-deleted virus than did scSIV expressing wild-type Envs. While plasma
from animals immunized with either the Env-modified or wild-type strains of scSIV both
neutralized SIV$_{mac}239\Delta V1V2$, titers for the animals immunized with Env-modified
strains were 7 times higher (2-tailed Mann-Whitney U test, $P=0.01$) (Fig. 2.3d and h).
Indeed, the animals immunized with Env-modified scSIV were all able to neutralize
SIV$_{mac}239\Delta V1V2$ infectivity to $<2\%$, and had 50% neutralization titers that ranged from
3.1x$10^3$ to 8.4x$10^4$. Thus, in spite of the comparably poor take of infection by
scSIV$_{mac}239\Delta V1V2$ (Fig. 2.2), immunization with Env-modified scSIV enhanced the
induction of antibodies to determinants revealed by deletion of the V1V2 loops.

Although immunization with Env-modified strains of scSIV enhanced the
induction of antibodies that neutralize SIV$_{mac}239g123$ and SIV$_{mac}239\Delta V1V2$, plasma
samples collected 2 weeks after the third dose of Env-modified scSIV (week 14) and 2
weeks after the second VSV G scSIV boost (week 26) failed to detectably neutralize
SIV$_{mac}239$ (Fig. 2.3i and j). Likewise, plasma collected on the first day of challenge
(week 32) did not detectably neutralize the SIV$_{mac}251_{UCD}$ challenge strain (Fig. 2.3k).

**SIV-specific antibodies in mucosal secretions.** Mucosal secretions were tested
for SIV-specific antibodies before and after challenge (Fig. 2.4). Vaginal and rectal
secretions were collected using pre-moistened Weck-Cel sponges to avoid abrasion of the
mucosal epithelium$^{469}$. This approach permits the nearly exclusive collection of
secretions, without drawing fluid from the underlying tissue. To control for significant variability in the concentrations of total IgG and IgA in mucosal secretions over time and over the menstrual cycle, the specific activity (SA) of antibody titers to gp120 or viral lysates was calculated by dividing the μg amount of SIV-specific antibody by the mg amount of total IgG or IgA in the sample (Fig. 2.4).

Gp120-specific IgG was detectable in the cervicovaginal secretions (CVS) of all the immunized animals prior to challenge (Fig. 2.4a). Comparison of the specific activity of IgG in CVS versus plasma revealed a positive correlation (Fig. 2.4b) (Spearman $R_s=0.91$, $P<0.0001$). This correlation demonstrates that the proportion of total IgG specific for gp120 in the vaginal mucosa closely mirrors the proportion found in plasma. Indeed, the median ratio of the specific activities in CVS versus plasma was 1:1 (Fig. 2.4c). However, the 2 immunized animals that remained SIV-negative after the 20-week challenge period had significantly higher pre-challenge ratios of specific activity in CVS versus plasma than the 6 animals that subsequently became infected (Fig. 2.4c) (ratio (95% CI): 3.6 (3.1, 4.0), $P<0.001$).

Gp120-specific IgA was measured in CVS, rectal secretions, and plasma (Fig. 2.4d and e). Prior to challenge, none of the animals had detectable gp120-specific IgA titers in plasma (data not shown) or CVS (Fig. 2.4d). Likewise, with the exception of Mm 305-99, none of the animals had detectable Env-specific IgA titers in rectal secretions (Fig. 2.4e). High-titer gp120-specific IgA responses were observed post-infection in both CVS and rectal secretions for all 6 of the scSIV-immunized animals that became infected (Fig. 2.4d and e).
Figure 2.4. Virus-specific antibody responses were detected in mucosal secretions.
Anti-gp120 IgG specific activity (SA) was monitored longitudinally in CVS of immunized animals prior to infection (black), from the week each became infected (blue), and for the 2 that did not become infected (green) (a). Mucosal antibody responses in the naïve animals were measured prior to infection (gray) and post-infection (red). The 20-week challenge phase is shaded. The dashed line indicates the limit of detection, defined as the mean plus three standard deviations of negative samples. Anti-gp120 IgG SA in CVS and plasma are correlated (Spearman $R_s=0.91$, $P<0.0001$) (b). The ratios of anti-gp120 SA in CVS versus plasma were significantly higher at the 5 pre-challenge time points in the 2 animals that remained SIV-negative (c) (ratio (95% CI): 3.6 (3.1, 4.0), $P<0.001$). IgG samples containing readings below the limit of detection, defined as 3 standard deviations above the average SA for naïve controls, were excluded from correlative and ratio analyses (b and c). IgA SA was monitored against gp120 in CVS (d) and rectal secretions (e), and against viral lysate in CVS (f) and rectal secretions (g).
IgA in CVS, rectal secretions, and plasma was tested for reactivity against a SIV\textsubscript{mac}251 viral lysate preparation (Fig. 2.4f and g). No IgA specific for viral lysates was detectable in plasma prior to challenge (data not shown), but low levels were detectable in CVS from Mm 234-99 (Fig. 2.4f), and in rectal secretions from Mm 259-03 and Mm 261-00 (Fig. 2.4g). High levels of viral lysate-specific IgA were detectable in CVS and rectal secretions after infection of all of the immunized animals (Fig. 4f and g). Thus, the virus-specific IgA detected in some animals prior to challenge, and the high-titer IgA responses that developed in immunized animals that became infected, may have contributed to the control of viral replication.

**Breadth of virus-specific T cell responses elicited by immunization with scSIV.** T-cell responses to the 8 viral antigens expressed by scSIV were measured longitudinally during the immunization phase of this experiment by IFN\textgamma ELISPOT assays (Fig. 2.5). Each animal responded to multiple viral antigens. These responses were boosted by inoculation with VSV G scSIV on weeks 18 and 24.

The breadth of the CD4$^+$ and CD8$^+$ T-cell responses elicited by scSIV and SIV\textsubscript{mac}239\textDelta nef was assessed by whole-proteome, deconvolution epitope mapping. CD4$^+$ T-cell epitopes were defined on week 25 (Table 2.2), and CD8$^+$ T-cell epitopes were defined on week 26 (Table 2.3). CD4$^+$ T-cell epitopes were analyzed using PBMC depleted of CD8$^+$ cells by anti-CD8 antibody-coated magnetic beads, and CD8$^+$ T-cell epitopes were analyzed using PBMC depleted of CD4$^+$ cells by anti-CD4 antibody-coated magnetic beads. In cases where consecutive 15-mer peptides both scored positive, the overlapping eleven amino acid sequence is shown due to the high probability that a
**Figure 2.5.** T-cell responses were detectable against all proteins expressed by scSIV. IFNγ ELISPOT responses against peptide pools of overlapping 15-mers representing the Gag, Tat, Rev, Vif, Vpr, Vpx, Env, and Nef proteins were measured over the immunization period (weeks 0-32). The limit of detection was 50 spot forming cells (SFC) per $10^6$ PBMC.

A single epitope exists within this region (**Tables 2.2 and 2.3**). Due to routinely low PBMC yields from Mm 241-01, a comprehensive analysis of T-cell epitopes was not possible for this animal. The 7 immunized animals that were mapped recognized an average of 5.4 CD4$^+$ and 4.3 CD8$^+$ T-cell epitopes. The same mapping protocol was used
to determine that animals chronically infected with live-attenuated SIV<sub>mac239Δnef</sub> recognized an average of 5.2 CD4<sup>+</sup> T-cell epitopes (n=5 animals) (Table 2.4), and 5.2 CD8<sup>+</sup> T-cell epitopes (n=17 animals) (Table 2.5). The T-cell responses measured 1-2 weeks after boosting with scSIV versus during chronic infection with SIV<sub>mac239Δnef</sub> were generally similar (Fig. 2.6). However, CD8<sup>+</sup> T-cell responses were higher in magnitude after boosting with scSIV than during chronic SIV<sub>mac239Δnef</sub> infection (2-tailed Mann-Whitney U test, P=0.0263). Thus, the breadth of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses elicited by scSIV were comparable to the magnitude and breadth of those observed during persistent infection with live-attenuated SIV.

### Table 2.2. CD4<sup>+</sup> T-cell responses elicited by scSIV in 7 macaques.

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### Table 2.3. CD8⁺ T-cell responses elicited by scSIV in 7 macaques.

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### Table 283-03

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### Table 290-03

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### Table 325-06

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Table 2.5. CD8$^+$ T-cell epitopes elicited by SIVmac239Δnef in 17 macaques.

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**Figure 2.6. Magnitude and breadth of T-cell responses elicited by scSIV and SIV\textsubscript{mac239Δnef}.** Deconvolution epitope mapping was performed on CD4\(^+\) T-cell responses 1 week after boosting with VSV G\(_1\) scSIV, and on CD8\(^+\) T-cell responses 2 weeks after boosting with VSV G\(_1\) scSIV. The magnitude (a) and breadth (b) of the T-cell responses were compared to those assessed by deconvolution epitope mapping during chronic infection with SIV\textsubscript{mac239Δnef}. The higher magnitude CD8\(^+\) T-cell responses in scSIV versus SIV\textsubscript{mac239Δnef} was significant (2-tailed Mann-Whitney U test, \(P=0.0263\)).

**Virus-specific CD8\(^+\) T-cells in peripheral blood and vaginal mucosa.**

Immunization with scSIV elicited virus-specific CD8\(^+\) T-cells that were detectable in peripheral blood and the vaginal mucosa by MHC class I tetramer staining (Fig. 2.7). Tetramer\(^+\) CD8\(^+\) T-cells were detected in peripheral blood following the first inoculation in each of the Mamu-A*01 and -A*02-positive animals, except Mm 305-99. These responses were boosted by immunization with VSV G scSIV on weeks 18 and 24. Virus-specific CD8\(^+\) T-cell responses identified by tetramer staining mirror the longitudinal IFN\(\gamma\) ELISPOT results (Fig. 2.5).

The magnitude of MHC class I tetramer\(^+\) CD8\(^+\) T-cell responses elicited by immunization with scSIV varied from animal to animal. Mm 284-99 had the highest pre-challenge peak responses, with 6.7% of CD8\(^+\) T-cells recognizing Mamu-A*01 Gag\(_{181-189}\) (CM9), and 2% of CD8\(^+\) T-cells recognizing each Mamu-A*01 Tat\(_{28-35}\) (SL8) and Mamu-
A*02 Nef_{159-167} (YY9) (Fig. 2.7d). Mm 305-99 had the lowest magnitude response to Mamu-A*01 Gag_{181-189} (CM9), which became detectable only after boosting with VSV G scSIV, and reached a maximal frequency of 0.5% of CD8^{+} T-cells (Fig. 2.7c). The CD8^{+} T-cell response to Mamu-A*01 Tat_{28-35} (SL8) never exceeded the limit of detection in this animal. These observations are consistent with the absence of T-cell responses to peptides containing this epitope for both the longitudinal analysis of IFNγ ELISPOT responses to the Tat peptide pool (Fig. 2.5), and to the individual 15-mer peptides containing this epitope (Table 2.3). Thus, even among Mamu-A*01-positive macaques, there was considerable variation in the magnitude of CD8^{+} T-cell responses elicited by immunization with scSIV.
Figure 2.7. Virus-specific CD8+ T-cells were detected in peripheral blood and the vaginal mucosa. CD8+ T-cells binding the MHC class I tetramers Mamu-A*01 Gag181-189 (CM9), Mamu-A*01 Tat28-35 (SL8), Mamu-A*02 Nef159-167 (YY9), and Mamu A*02 Gag71-79 (GY9) were detected in peripheral blood (a-c). The limit of detection indicated by the dotted line is 0.05% of CD8+ T-cells. The 20-week challenge period is shaded. Tetramer+ CD8+ T-cell frequencies are shown prior to infection (black), and from the week each animal became infected (blue) (a, b and e). Frequencies of tetramer+ CD8+ T-cells are shown for the 2 immunized animals that remained uninfected (green) (c and d). Similar frequencies of CD8+ T-cells were tetramer+ among lymphocytes isolated from vaginal biopsies versus peripheral blood (f).
SIV-specific CD8+ T-cell frequencies were also measured in lymphocytes isolated from biopsies of the vaginal mucosa on week 26, 2 weeks after boosting with VSV G scSIV. Tetramer+ cell frequencies among lymphocytes isolated from these biopsies were similar to the frequencies observed in peripheral blood samples processed in parallel (Fig. 2.7f). Frequencies of Mamu-A*01 Gag181-189 (CM9)-specific CD8+ T-cells in the biopsies ranged from 0.8% in Mm 305-99 to 3.1% in Mm 284-99. These biopsy results confirm that intravenous immunization with scSIV elicited SIV-specific CD8+ T-cells that trafficked to the vaginal mucosa, and were therefore present at the site of challenge.

Extraordinarily high anamnestic CD8+ T-cell responses ensued following infection of immunized animals. Cumulative Mamu-A*01 Gag181-189 (CM9) plus Mamu-A*01 Tat28-35 (SL8) responses peaked at 22% and 28% of CD8+ T-cells in peripheral blood from Mm 259-03 and Mm 261-00, respectively (Fig. 2.7a and b). Likewise, an astonishing 44% of CD8+ T-cells recognized the Mamu-A*02 Nef159-167 (YY9) epitope in Mm 158-02 (Fig. 2.7e). In contrast, the percentage of Mamu-A*01 Gag181-189 (CM9)-specific CD8+ T-cells declined steadily in Mm 284-99 from 1.7% to 0.13% of CD8+ T-cells during the 20-week challenge period (Fig. 2.7d). In the case of Mm 305-99, Mamu-A*01 Gag181-189 (CM9)-specific CD8+ T-cells increased in frequency on weeks 40-41 and 46-47, perhaps reflecting the simulation of recall responses by the challenge virus (Fig. 2.7e). Robust anamnestic T-cell responses were observed in the 6 immunized animals that became infected, but not in the 2 that remained uninfected.

**Outcome of repeated, low-dose vaginal challenge.** Repeated, low-dose vaginal inoculation with SIVmac251UCD was conducted to model heterosexual transmission of
HIV-1. Beginning on week 32, each of the 8 immunized animals and 6 naïve control animals were challenged vaginally with 1000 TCID\textsubscript{50} (1 ng p27) of SIV\textsubscript{mac251UCD}.

Inoculations were administered in the morning and again in the afternoon of the same day each week, for 20 consecutive weeks. Challenge doses were discontinued when viral RNA was detected in plasma for 2 consecutive weeks for any given animal. All naïve control animals were expected to become infected\textsuperscript{480}, and a significant difference in the number of doses required to establish infection, or in the final number of animals becoming infected in the immunized group versus the naïve controls would indicate that immunization prevented transmission. All 6 naïve control animals and 6 of the 8 immunized animals became infected (Fig. 2.8). Four of the 6 naïve animals were infected by a single clone, as determined by single genome amplification (SGA), and analysis of sequences encoding Env at the first viral RNA-positive time point (Eric Hunter, Emory University, personal communication). A Weibull regression analysis was performed to determine whether the immunized animals required significantly more doses than the naïve controls to establish infection. The number of doses required to establish infection in each group computed a hazard (risk) ratio of 0.38 (95% CI=0.12, 1.21), where a ratio of 1 would mean the groups are at equal risk of becoming infected. Although there was a trend toward greater resistance to infection for the scSIV-immunized animals, this analysis did not reveal a statistically significant difference in the risk of infection relative to the control animals (P=0.10).
The repeated, low-dose challenge regimen resulted in animals becoming infected on different weeks (Fig. 2.9a). Viral load measurements were therefore synchronized to the last week in which viral RNA was undetectable in each animal to compare differences in peak and set-point viral loads (Fig. 2.9b). All viral load analyses excluded the two immunized animals that remained uninfected. Peak viral loads were reduced 0.72 log for the immunized group relative to the control group (Fig. 8C). The blunting of peak viral loads in the immunized group was statistically significant (P=0.0038, two-tailed Mann-Whitney U test). Geometric mean viral loads were 1.1, 1.2, 1.1, and 1.0 log lower on weeks 4, 6, 8, and 12 post-infection in the immunized group relative to the naïve controls. A linear mixed model analysis of chronic phase log viral loads also revealed a statistically significant difference in set-point viral loads for the period between 5 and 67 weeks post-infection, centered on week 28 (P=0.004) (Fig. 8C). Despite the convergence of viral loads after week 23 due to the loss of two rapidly progressing naïve control
animals on weeks 16 and 23, viral loads for the immunized group were 0.96-log lower for this period (95% CI=0.31, 1.6). Thus, immunization of macaques according to a prime-boost regimen with Env-modified and VSV G trans-complemented scSIV resulted in statistically significant containment of viral replication.

Figure 2.9. Post-challenge plasma viral RNA loads. Viral loads were measured at weekly intervals beginning on week 32 using a real time RT-PCR assay with a limit of detection of 30 copies of RNA per ml of plasma for the immunized animals that became infected (blue) or remained uninfected (green), and for the naïve controls (red) (a)\cite{473}. Viral load measurements were synchronized to the last week in which viral RNA was undetectable in each animal that became infected (b). Comparison of geometric mean viral loads for the infected animals revealed acute peak viremia was reduced by 0.72 log (2-tailed Mann-Whitney U test, P=0.0038), and was lower by 1.1, 1.2, 1.1, and 1.0 log on weeks 4, 6, 8, and 12 in the immunized group (c). Set-point viral loads were significantly reduced for the period between weeks 5 and 67 post-infection, centered on week 28, as determined by a mixed linear model analysis (P=0.004; 0.96-log lower, 95% CI=0.31, 1.6). Due to progression to AIDS, naïve control animals Mm 230-04, Mm 215-98, Mm 358-01, Mm 128-01 were euthanized weeks 16, 23, 43, and 51 post-infection, and immunized animals Mm 316-98 and Mm 158-02 were euthanized 38 and 44 weeks post-infection.
Despite statistically significant differences in viral loads, immunization did not result in significant preservation of CD4+ T-cells. CD4+ T-cell counts in peripheral blood were monitored beginning on the first day of challenge to determine whether the targets of SIV infection were better protected in the scSIV-immunized animals (Fig. 2.10a). Frequencies of naïve, central memory, and effector memory subsets were defined by staining for CD28 and CD95 (Fig. 2.10b-d). CD4+ T-cells were also stained for CCR5 to identify this specific target population (Fig. 2.10e). Although memory CD4+ T-cells in the immunized animals appeared to persist at higher levels during acute infection, differences in CD4+ T-cell counts between the groups were not significant (Fig. 2.10c-e). Therefore, although immunization reduced viral loads, this statistically significant reduction did not appear to translate into significant differences in CD4+ T-cell counts in peripheral blood.
Figure 2.10. CD4⁺ T-cell populations after infection. CD4⁺ T-cell counts were synchronized to the last week in which viral RNA was undetectable for each animal. The number of total CD3⁺ CD4⁺ (a), CD3⁺ CD4⁺ CD28⁺ CD95⁻ naïve (b), CD3⁺ CD4⁺ CD28⁺ CD95⁺ central memory (c), CD3⁺ CD4⁺ CD28⁻ CD95⁻ effector memory (d), and CD3⁺ CD4⁺ CCR5⁺ CD95⁺ memory (e) T-cells per μl of whole blood were monitored.

During the challenge phase of this experiment, it was noted that the vaccine protection conferred by scSIV appeared to be greater after infection by intravenous challenge with SIVₘac₂39 than after infection by repeated, low-dose challenge with SIVₘac₂₅₁₇₆. Neutralization by sCD4 is frequently used to evaluate the neutralization resistance properties of different viruses. We therefore compared the ability of a
human sCD4-IgG construct to neutralize SIV_{mac251 UCD} and SIV_{mac239}. We also included the widely used SIV_{mac251 NE} challenge virus, from which the SIV_{mac251 UCD} challenge stock was derived by passage in animals and in rhesus PBMC\textsuperscript{480}, and SIV_{mac316 TM Open}, which is known to be sensitive to sCD4, as a positive control. SIV_{mac239} was neutralized by sCD4-IgG with a 50\% titer of approximately 13 \mu g per ml. Although SIV_{mac251 NE} was neutralized at a similar 50\% titer, 23 \mu g per ml, SIV_{mac251 NE} resisted potent neutralization at high concentrations of sCD4-IgG. Incredibly, SIV_{mac251 UCD} completely resisted neutralization by sCD4-IgG. Although rhesus and human CD4 sequences differ, SIV_{mac251 UCD} is clearly capable of interacting with human CD4 for entry into the human CD4\(^+\) T-cell line used in this neutralization assay. A subset of transmitted/founder HIV-1 strains also appear to be completely resistant to neutralization by sCD4\textsuperscript{209}. Thus, SIV_{mac251 UCD} may possess a level of inherent resistance to neutralization that is comparable to the most neutralization-resistant primary isolates of HIV-1. Greater inherent resistance to neutralization than SIV_{mac239} may contribute to the greater difficulty of conferring protection against this challenge virus.

![Figure 2.11. Neutralization of challenge viruses by sCD4-IgG.](image)

**Figure 2.11. Neutralization of challenge viruses by sCD4-IgG.** The estimated 50\% neutralization titers for sCD4-IgG against SIV_{mac239}, SIV_{mac251 UCD}, SIV_{mac251 NE}, SIV_{smE543-3}, and SIV_{mac316 TM Open} are indicated in brackets.
2. E. DISCUSSION

Stimulation of effective T-cell and antibody responses is likely to be essential for achieving protection against HIV-1. As an experimental vaccine approach designed to elicit broad T-cell responses plus antibodies to the native conformation of the viral envelope glycoprotein as it exists on virions, we developed a technique for producing strains of SIV that are limited to a single cycle of infection\textsuperscript{159,455,456}. Immunization of rhesus macaques with scSIV stimulated robust virus-specific T-cell responses, and significantly reduced viral replication following an intravenous challenge with SIV\textsubscript{mac239}\textsuperscript{159}. While scSIV also elicited low-titer Env-specific antibodies capable of neutralizing T-cell line-adapted SIV\textsubscript{mac251TCLA}, these antibody responses were unable to detectably neutralize the primary isolate SIV\textsubscript{mac239}\textsuperscript{159}. In an effort to enhance Env-specific antibody responses and the extent of protection afforded by scSIV, we immunized macaques with Env-modified strains of scSIV lacking specific structural features thought to interfere with the induction of neutralizing antibodies.

Immunization with Env-modified scSIV facilitated the development of antibodies to epitopes accessible in Envs lacking the 3 N-linked glycans in gp41, or 100 amino acids in the V1V2 region of gp120. However, none of the animals detectably neutralized SIV\textsubscript{mac239} or SIV\textsubscript{mac251UCD}. Therefore, the antibodies responsible for the enhanced neutralization of the g123 and ΔV1V2 Envs may have recognized surfaces that are not accessible on wild-type virions. Alternatively, a subset of these antibodies may have interacted with the wild-type Envs, but bound with an affinity too low to detect neutralization. Likewise, antibodies capable of binding wild-type Envs may have been
present, but were produced at concentrations too low to block infectivity. However, the
induction of antibodies that detectably neutralize these strains, in the absence of
persistent infection by a replicating virus, would have been unprecedented. Although no
animals detectably neutralized the challenge strain, we cannot exclude the possibility that
antibodies may have contributed to protection through various mechanisms other than
neutralization.

Antibodies present in mucosal secretions were measured due to their potential
importance in preventing sexual transmission of HIV-1. Immunization elicited gp120-
specific IgG that was detectable in the CVS of all of the animals, and appeared to be in
equilibrium with the plasma in most cases. Therefore, virus-specific IgG would be
expected to be present in CVS for any immunization strategy that elicits virus-specific
IgG in plasma. In contrast, virus-specific IgA was not efficiently elicited. Prior to
challenge, none of the animals had detectable gp120-specific IgA in CVS, and low-titer
gp120-specific IgA was detectable in the rectal secretions of only one immunized animal,
Mm 305-99. Despite having low to undetectable levels of virus-specific IgA prior to
challenge, all the immunized animals that became infected developed high-titer IgA
responses capable of binding to gp120 and to whole-virus lysate. These high IgA titers
are consistent with post-challenge anamnestic responses primed by immunization.
Alternatively, the capacity of the immunized animals to mount high-titer virus-specific
IgA responses after becoming infected may be an effect of immunological containment of
viral replication.

T-cell responses measured by IFNγ ELISPOT and MHC class I tetramer staining
were similar in magnitude to responses elicited by prime-boost vaccine regimens
employing recombinant DNA and/or viral vectors. Virus-specific CD8\(^+\) T-cell frequencies among lymphocytes isolated from the vaginal mucosa were similar to frequencies in peripheral blood. Thus, immunization with scSIV elicited T cell responses that trafficked to the vaginal mucosa and were present at the site of challenge. Deconvolution epitope mapping identified a mean of 5.4 CD4\(^+\) and 4.3 CD8\(^+\) T-cell epitopes per animal, which was similar to the breadth of responses elicited by persistent infection with live-attenuated SIV. Therefore, immunization with scSIV elicited high-frequency virus-specific T-cells that recognized multiple viral antigens, and trafficked to the vaginal mucosa.

Protection was assessed by repeated, low-dose vaginal inoculation with SIV\(\text{mac251}_{\text{UCD}}\) to model heterosexual transmission of HIV-1. Although there appeared to be a trend towards a greater number of challenge doses required to establish infection for the immunized animals, this trend was not statistically significant (P=0.10; hazard ratio of 0.38, 95% CI=0.12-1.21). However, peak and set-point viral loads were significantly lower in the six immunized animals that became infected relative to the naïve controls. Peak viral loads were reduced by 0.72 log (P=0.0038), and set-point viral loads were reduced by approximately one log for the period between 5 and 67 weeks post-infection (P=0.004; 0.96-log lower, 95% CI=0.31, 1.6). Geometric mean viral loads converged after 23 weeks post-infection, due to the loss and subsequent exclusion of the 2 control animals with the highest viral loads.

The SIV\(\text{mac251}_{\text{UCD}}\) challenge strain appears to be particularly difficult to protect against by immunization, perhaps even more so than SIV\(\text{mac239}\). Differences in peak and set-point viral loads in the immunized animals relative to the naïve controls were not as
great as previously observed following intravenous challenge with SIV\textsubscript{mac}239\textsuperscript{159}.

Moreover, despite the partial containment of viral replication in the immunized animals, we did not observe better preservation of CD4\textsuperscript{+} T-cells. It is possible that genetic differences between SIV\textsubscript{mac}239 and the SIV\textsubscript{mac}251\textsubscript{UCD} challenge stock may have been responsible for this difference in protection. The virus stock of SIV\textsubscript{mac}251\textsubscript{UCD} utilized for repeated, low-dose vaginal challenge in this study was derived from the original SIV\textsubscript{mac}251\textsubscript{NE} isolate by passage of acute-phase virus through animals, and selection of virus cultures with the fastest growth kinetics on rhesus macaque PBMC\textsuperscript{480}. This process may have favored the selection of more fit or more pathogenic variants. SIV\textsubscript{mac}239 was neutralized by sCD4-IgG at 13 \( \mu \)g per ml, whereas SIV\textsubscript{mac}251\textsubscript{UCD} was not neutralized whatsoever at the highest concentration of sCD4-IgG tested, 340 \( \mu \)g per ml. Therefore, the better protection after intravenous infection with SIV\textsubscript{mac}239 versus repeated, low-dose SIV\textsubscript{mac}251\textsubscript{UCD} challenge may, at least in part, be due to differences in inherent resistance to neutralization. Furthermore, 2 amino acids in Env that consistently revert in animals infected with SIV\textsubscript{mac}239\textsuperscript{481} differ from the SIV\textsubscript{mac}251\textsubscript{UCD} challenge stock (data not shown). These, plus additional differences including suboptimal nucleotides at positions elsewhere in the SIV\textsubscript{mac}239 genome\textsuperscript{482}, might explain why it appears to be more difficult to protect against SIV\textsubscript{mac}251\textsubscript{UCD} than SIV\textsubscript{mac}239.

Two immunized animals, Mm 284-99 and Mm 305-99, remained uninfected after 20 weeks of repeated vaginal challenge with SIV\textsubscript{mac}251\textsubscript{UCD}. Inherent genetic differences in the capacity to support SIV replication could explain why these 2 animals remained uninfected. However, there is no evidence that these animals had reduced inherent capacity to support SIV infection, since peak viral loads following the first dose of scSIV
were similar in magnitude among all of the immunized animals. Furthermore, these animals were not resistant to a subsequent intravenous challenge with SIV_{mac251UCD}. Nevertheless, it is possible that genetic factors underlie differences in the T-cell and antibody responses stimulated in these animals by immunization with scSIV. Both animals possessed the protective MHC class I allele Mamu-A*01^{370,483}, and Mm 284-99 had the highest virus-specific T-cell responses in the vaginal mucosa at week 26, and in the peripheral blood at the beginning of the challenge period. Antibody responses in these 2 animals also appeared to differ from the 6 immunized animals that became infected. Mm 284-99 neutralized SIV_{mac239g123} at a titer nearly 200-fold higher than the average for the rest of the group, and 80-fold higher than the next highest animal. Moreover, neutralization of SIV_{mac251UCD} approached a 50% reduction in infectivity at a 1:8 dilution of plasma from Mm 284-99 collected at the beginning of the challenge period. In addition, Mm 284-99 also had the highest anti-gp120 IgG specific activity in cervicovaginal secretions at four of five time points prior to challenge. Both Mm 284-99 and Mm 305-99 also had higher pre-challenge ratios of anti-gp120 IgG specific activity in cervicovaginal secretions versus plasma (ratio (95% CI): 4.0 (2.6, 6.0), P<0.01), perhaps reflecting local overproduction of gp120-specific IgG by B-cells residing in the cervicovaginal mucosa^{484-486}. Also, the only animal that had detectable gp120-specific IgA prior to challenge was Mm 305-99. Hence, there were a number of qualitative and quantitative differences in the T-cell and antibody responses that may have contributed to the absence of SIV infection in Mm 284-99 and Mm 305-99.

The partial containment of SIV replication afforded by T-cell based vaccines has thus far failed to provide adequate protection against immunodeficiency virus
Neutralizing antibodies can prevent the acquisition of viral infection, but eliciting such antibodies is a daunting challenge due to structural and thermodynamic properties of the envelope glycoprotein that render it resistant to antibodies. However, a strategy that elicits effective antibody responses to the viral envelope glycoprotein may be a necessary component of any protective vaccine against HIV-1. In this study, we tested the hypothesis that removal of specific structural features thought to interfere with the induction of neutralizing antibodies might facilitate their development. Despite significantly enhancing antibody responses to epitopes revealed by the removal of the V1V2 loops or the 3 N-linked glycans in gp41, neutralizing activity against SIV<sub>mac</sub>239 and SIV<sub>mac</sub>251<sub>UCD</sub> remained undetectable. Nevertheless, 2 immunized animals remained uninfected after a 20-week period of repeated, low-dose vaginal challenge in which all of the naïve control animals became infected. The 6 immunized animals that ultimately became infected had significantly lower peak and set-point viral loads relative to the naïve control animals. The 2 that remained uninfected appeared to differ in several pre-challenge measures of T-cell and antibody responses, suggesting multiple immune responses may have contributed to the absence of infection after multiple challenges.
CHAPTER 3

An assay for quantifying ADCC based on an NK cell line

and target cells infected by SIV or HIV-1
3. A. ABSTRACT

Biological and technical impediments both hinder progress towards a vaccine for human immunodeficiency virus type 1 (HIV-1). Neutralizing antibody titers are routinely measured in quantitative assays based on cell lines. However, a dependence upon primary natural killer (NK) cells has, thus far, limited the scalability and consistency of assays for antibody-dependent cell-mediated cytotoxicity (ADCC). We now report the development of an ADCC assay based on immortalized NK cell lines that stably express human or rhesus macaque CD16, and a CD4+ target cell line that expresses luciferase from a Tat-inducible promoter. The loss of luciferase from infected target cells in the presence of NK cells and serial antibody dilutions indicates the killing of virus-infected cells by ADCC. The 50% ADCC titers measured using this assay are, on average, 250-fold higher than the 50% neutralizing antibody titers against the same virus. Since antibodies capable of neutralizing primary isolates of HIV-1 and simian immunodeficiency virus (SIV) are often undetectable, particularly in vaccine studies, antibodies capable of neutralizing T-cell line-adapted strains, or binding the gp120 subunit of the viral envelope glycoprotein (Env) in an ELISA, are reported instead. Although ADCC activity was correlated with neutralization and gp120 ELISA titers, these assays may reflect distinct properties of the antibody response. Thus, we have developed a sensitive assay based entirely on cell lines for the routine measurement of ADCC activity against cells infected with HIV-1 or SIV primary isolates.
3. B. INTRODUCTION

The replication of HIV-1 and SIV continues despite the mobilization of vigorous T-cell, antibody, and innate immune responses by infected hosts\textsuperscript{17-20}. The properties of the primate lentiviruses that enable immune evasion and persistent replication frustrate efforts to develop a safe and effective HIV-1 vaccine\textsuperscript{488,489}. Among these properties, the inherent resistance of primary HIV-1 isolates to neutralization by antibodies is perhaps the most significant obstacle\textsuperscript{490,491}.

Thus far, HIV-1 vaccine approaches under consideration for clinical development have only reported antibodies capable of neutralizing the infectivity of neutralization-sensitive strains of HIV-1 and SIV, such as T-cell line-adapted viruses, not the neutralization-resistant primary isolates that are relevant to a vaccine\textsuperscript{166,176,177,209,214,357,358,376,377}. The physiological relevance of neutralization epitopes that emerge during \textit{in vitro} passage remains unclear. Vaccine-elicited antibodies that bind recombinant forms of the viral envelope glycoprotein (Env) can also be detected by enzyme-linked immunosorbent assays (ELISAs). However, even controlling for antigenic variation, many antibodies that bind recombinant forms of Env cannot neutralize, and many that neutralize cannot bind recombinant forms of Env\textsuperscript{178,191,326}. Antibodies in these two categories probably recognize interfaces between Env protein subunits, which are occluded in Env trimers expressed on virions and infected cells, or recognize quaternary epitopes\textsuperscript{182,184,188,195}. Therefore, assays based on T-cell line-adapted viruses or recombinant protein may not be ideal for studying the antibodies that are likely to have antiviral activity against primary isolates \textit{in vivo}. 

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Adaptive and innate immunity have evolved to operate synergistically as an integrated system. Whereas the variable region of an antibody can interact with antigen and neutralize virus infectivity, the constant (Fc) region can activate complement fixation, or interact with cells of the innate immune system\textsuperscript{430,431,492,493}. Crosslinking of Fc receptors on natural killer (NK) cells, dendritic cells, neutrophils, and macrophages stimulates the release of immune modulators including cytokines, chemokines, and interferons\textsuperscript{493,494}. NK cells represent approximately 10% of peripheral blood lymphocytes, are efficient cytotoxic effectors, and can be prompted by crosslinking of the Fc receptor CD16 to kill virus-infected cells by antibody-dependent cell-mediated cytotoxicity (ADCC)\textsuperscript{110,495}. Due to the potential for ADCC to be a potent effector mechanism, and to serve as a proxy for other functions of the same antibodies, we developed an assay for measuring the capacity of antibodies to direct ADCC against HIV-1 or SIV-infected cells.
3. C. METHODS

**Cell lines.** Due to the variability and limited scalability of assays dependent upon primary cells\(^496\), we engineered a pair of cell lines to serve as targets and effectors in ADCC assays. The target cells were derived from NKR.CEM-CCR5 CD4\(^+\) T cells (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, contributed by Dr. Alexandra Trkola). These were transduced with a pLNSX-derived retroviral vector to express Firefly luciferase under the transcriptional regulation of the SIV LTR promoter\(^164\). Target cells were maintained in “R10” consisting of RPMI (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 25 mM HEPES (Invitrogen), 2 mM L-glutamine (Invitrogen), and 0.1 mg/ml Primocin (InvivoGen). The rhesus macaque CD16\(^+\) effector cells were derived from the CD16-negative NK cell line KHYG-1\(^497\) (Japan Health Sciences Foundation) by stable transduction with a pQCXIN-derived retroviral vector expressing macaque CD16 (FCGR3A variant 7)\(^498\). The human CD16\(^+\) effector cells were similarly derived, but using a pQCXIP-derived retroviral vector expressing the human V158 variant of FCGR3A, which has a higher affinity for IgG than the F158 variant\(^499\). The rhesus CD16\(^+\) NK line was selected using neomycin (G418), whereas the human CD16\(^+\) NK line was selected using puromycin. The rhesus CD16\(^+\) NK effector cells were maintained at a density of 1×10\(^5\) to 4×10\(^5\) cells per ml in R10 media supplemented with IL-2 at 10 U per ml (Roche) and cyclosporine A (CsA) at 1 µg per ml (Sigma). The human CD16\(^+\) NK clone requires less IL-2, and was cultured in otherwise identically formulated media containing IL-2 at 5 U per ml.
**ADCC assay.** Target cells were infected by spinoculation\textsuperscript{500}, 4 days prior to each assay, and washed three times with R10 immediately before use. ADCC assays were conducted in round-bottom 96-well plates, with each well containing $10^5$ effector cells and $10^4$ target cells. Relative light units (RLU) indicate luciferase expression by infected targets. Wells containing uninfected targets plus effectors defined 0% RLU, and wells containing infected targets plus effectors with no serum or plasma defined 100% RLU. ADCC activity was measured as the loss of luciferase activity after an 8-hour incubation in the presence of triplicate serial dilutions of heat-inactivated plasma collected in sodium citrate anticoagulant or serum. Luciferase activity was read in black 96-well plates using BriteLite Plus (Perkin Elmer) luciferase substrate.

**Neutralization and ELISA assays.** Neutralization assays were performed as previously described\textsuperscript{164,334}. Maxisorb ELISA plates (NUNC) were coated with recombinant, 6-His tagged SIV\textsubscript{mac239} gp120 protein produced in 239T cells (Immune Technology). Bound antibodies were measured using a horseradish peroxidase-conjugated goat anti-monkey IgG antibody (Santa Cruz Biotechnology). A statistically defined end-point titer was determined by calculating the mean plus 3 standard deviations for pre-immune plasma samples diluted 100-fold, which was the highest dilution tested\textsuperscript{501}.

**Viruses.** SIV\textsubscript{mac239}, scSIV-GFP, HIV-1\textsubscript{NL4-3} and HIV-1\textsubscript{YU2} were produced by transfection of 293T cells. The virus stock of SHIV\textsubscript{KB9} used here was adapted to growth on NKR.CEM-CCR5 CD4\textsuperscript{+} T-cells. SHIV\textsubscript{SF162P3} was expanded in PHA-activated rhesus
PBMC from a stock obtained from the AIDS Research and Reference Reagent Program, which was contributed by Drs. Janet Harouse, Cecilia Cheng-Mayer, Ranajit Pal and the DAIDS, NIAID.

**Depletion of antibodies to human cellular antigens.** Some macaques, especially those immunized with material produced in human cells, have antibodies that direct ADCC against uninfected target cells. These antibodies were measured by reading the background luciferase expression from the SIV LTR promoter of uninfected NKR.CEM-CCR5 CD4+ T-cells on white 96-well plates. Plasma samples from sets containing antibodies reactive against uninfected target cells were all depleted, regardless of anti-target cell activity, to ensure identical treatment. To deplete these antibodies, $10^7$ uninfected target cells were resuspended in the sample and incubated for twenty minutes at room temperature. This procedure was typically repeated multiple times to remove detectable antibodies reactive with uninfected target cells.

**Calcineurin inhibitor experiment.** An aliquot of the Mm CD16+ KHYG-1 clone was thawed and maintained in CsA for 3 weeks, prior to washing the cells, and transferring them to media lacking CsA. These cells were cultured in the absence of CsA for another 3 weeks prior to being frozen in Recovery Cell Culture Freezing Medium (Invitrogen). These cells were thawed the same day they were frozen, at which time additional aliquots of cells were treated with freezing media, with ionomycin at 1 µg per ml, or mock treated. These groups of cells were each divided into 3 aliquots, which were cultured in the presence of CsA at 1 µg per ml, FK-506 at 1 µg per ml, or no drug. The
ADCC assay was performed in the absence of these drugs 3 weeks after to these treatments.

**Laboratory animals.** The animals were Indian-origin rhesus macaques (*Macaca mulatta*) housed in a biocontainment facility at the New England Primate Research Center (NEPRC), and given care in accordance with standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the Harvard Medical School Animal Care and Use Committee. The experiments and procedures were approved by the Harvard Medical Area Standing Committee on Animals, and conducted in accordance to the *Guide for the Care and Use of Laboratory Animals*.466

**Statistical analysis.** Fifty percent titers were calculated as the dilution at which a line connecting the values above and below 50% RLU would intercept the 50% RLU line. Area under the curve (AUC) values for ADCC were calculated such that they would be proportional to 50% ADCC titers, and represent the areas between 100% RLU and the titration curves as they appear in the figures. Whereas %ADCC, defined as 100% minus %RLU, is asymptotic as it approaches 100%, minimum %RLU values are inversely proportional to 50% ADCC titers. Therefore, AUC values for ADCC were calculated from the sum of the values over all dilutions for \( \log_{10} 100 \) minus \( \log_{10} \%RLU \). This sum was multiplied by the \( \log_{10} \)-transformed dilution factor of two to find an area. Correlation coefficients and P values were calculated using Prism version 4.1b (GraphPad Software).
3. D. RESULTS

**CD16 expression on NK cell lines.** Limiting dilution clones of KHYG-1 NK cells were selected that express comparable levels of human or macaque CD16, as determined by staining with the mouse anti-human CD16 monoclonal antibody 3G8, which is cross-reactive with rhesus macaque CD16 (Fig. 3.1a). The level of CD16 expressed on these clones was compared to the level expressed on primary NK cells from 5 humans and from 5 rhesus macaques (Fig. 3.1b and c). To make this comparison, human NK cells were defined as the CD20⁻, CD3⁻, HLA-DR⁻, CD8⁺, CD56⁺, NKG2A⁺ population. Rhesus NK cells were stained in parallel using the same panel of fluorophore-conjugated antibodies, but were defined as the CD20⁻, CD3⁻, HLA-DR⁻, CD8⁺, NKG2A⁺ population. This comparison demonstrates that the level of CD16 expressed on the cloned NK cell lines is similar to, or slightly lower than, the level of CD16 expressed on primary human and macaque NK cells.

![Figure 3.1. CD16 expression on primary NK cells and on NK cell lines.](image)

*Figure 3.1. CD16 expression on primary NK cells and on NK cell lines.* CD16 expression on the parental KHYG-1 cell line and the limiting dilution clones of KHYG-1 cells transduced with human or rhesus macaque CD16 (a). Expression level on the human CD16⁺ KHYG-1 clone (solid red) versus primary NK cells from 5 people (colored lines) (b). Expression level on the rhesus CD16⁺ KHYG-1 clone (solid blue) versus primary NK cells from 5 macaques (colored lines) (c).
The loss of luciferase activity indicates the killing of infected cells. A time course experiment was performed to define the kinetics of luciferase loss. Cells infected with SIV\textsubscript{mac}239 were incubated alone, or with NK cells plus pre-immune plasma, plasma raised against SHIV\textsubscript{KB9}, or plasma raised against SIV\textsubscript{mac}239. Luciferase activity was measured in cells and supernatants collected every hour for 12 hours, and after 24 hours (Fig. 3.2). No differences in luciferase activity were observed in the presence of NK cells plus pre-immune plasma, or plasma raised against SHIV\textsubscript{KB9}. In contrast, plasma from an SIV\textsubscript{mac}239-infected animal caused a loss of luciferase activity in the SIV\textsubscript{mac}239-infected cells, which reached a minimum between the 8- and 12-hour time points. Meanwhile, increases were measured in the luciferase activity in the supernatant. The luciferase activity in the supernatant remained lower than that measured in cells, and returned to baseline levels after 8 hours. Thus, luciferase is released into the supernatant, but its activity rapidly declines. The release of luciferase into the supernatant indicates that the loss of luciferase from the virus-infected cells is associated with a loss of membrane integrity. This experiment also suggests that reading luciferase activity at the 8-hour time point would minimize the contribution of luciferase present in the supernatant while maximizing the sensitivity to detect the loss of luciferase activity from the virus-infected target cells.
Figure 3.2. Changes in luciferase activity over time in the presence of NK cells and plasma. Luciferase activity was measured in terms of percent relative light units (%RLU). The luciferase activity for wells containing infected target cells and NK cells with no plasma at each time point defined 100% RLU, whereas wells containing uninfected target cells and NK cells defined 0% RLU. Cells infected with SIV<sub>mac239</sub> were incubated alone (blue), or in the presence NK cells plus plasma from an uninfected macaque (gray), NK cells plus plasma from a SHIV<sub>KB9</sub>-infected macaque (teal), and NK cells plus plasma from an SIV<sub>mac239</sub>-infected macaque (red). The luciferase activity for cells (solid symbols) and supernatants (open symbols) was measured for samples collected every hour, from 0-12 hours, and once again after 24 hours.

To verify that the loss of luciferase activity from cells and its transient appearance in supernatants indicates cytotoxicity, we performed parallel assays using either luciferase or flow cytometry to measure ADCC activity. Target cells were infected with scSIV that expresses green fluorescent protein (GFP) from the nef position<sup>455</sup>. Prior to the addition of NK cells, the target cells were stained with a nonspecific membrane labeling dye (PKH26) to facilitate discrimination between the effector and target cell populations. Target cells were incubated in the presence of NK cells and serial dilutions of plasma from an SIV<sub>mac239</sub>-infected animal. After 8-hours, luciferase activity was measured for one assay, and cells from an identical assay processed in parallel were stained with an amine-reactive dye that allows living and dead cells to be differentiated by flow cytometry. Living cells with intact cellular membranes exclude amine-reactive dyes. Infected target cells incubated with NK cells but not plasma were used to define
100% luciferase activity, or 100% living, GFP⁺ cells. The percentage of luciferase activity corresponded with the percentage of living, GFP⁺ cells over the range of plasma dilutions measured (Fig. 3.3a). The percentage of dead target cells increased from 1.8 to 2.87% for uninfected versus infected populations in the absence of plasma, due at least in part to the cytopathic effects of SIVmac239 (Fig. 3.3b and c). In the presence of plasma diluted 10²- to 10³.5-fold, the loss of living, GFP⁺ target cells was associated with an increase in the number of dead target cells that stained positive for the amine-reactive dye (Fig. 3.3d-g). Over higher plasma dilutions, 10⁴ to 10⁵.5-fold, the living, GFP⁺ population rose in frequency, nearly reaching the maximum observed in the absence of plasma (Fig. 3.3h-k). Therefore, the loss of luciferase activity in the presence of the NK cell line and serial plasma dilutions indicates the dose-dependent killing of virus-infected cells.
Figure 3.3. Comparison of luciferase activity versus living, virus-infected target cells enumerated by flow cytometry. ADCC activity against target cells infected with scSIV-GFP was quantified by measuring luciferase activity or the frequency of amine-reactive dye-negative, GFP-positive cells (a). Uninfected target cells incubated with NK cells and no plasma were GFP− (b), whereas infected target cells incubated under the same conditions were GFP+ (c). These populations defined the lower and upper limits for the percentage of living, GFP+ cells. The frequencies of dead and living, GFP+ target cells were determined after incubation in the presence of NK cells and plasma diluted 10^2 (d), 10^2.5 (e), 10^3 (f), 10^3.5 (g), 10^4 (h), 10^4.5 (i), 10^5 (j), or 10^5.5 (k) -fold.

Env-specificity of ADCC activity. The ability of plasma samples from macaques infected with SIVmac239 or SHIVKB9 to direct ADCC against cells infected with these viruses was compared to determine the protein specificity of ADCC activity. SHIVKB9 contains HIV-1 env, tat, rev, and vpu genes in a genomic backbone derived from SIVmac239389,502. Plasma raised against SIVmac239 directed ADCC against cells infected with SIVmac239 but not SHIVKB9 (Fig. 3.2). Likewise, plasma raised against SHIVKB9 directed ADCC against cells infected with SHIVKB9 but not SIVmac239. Based on these
results, and the fact that Env is the only viral protein expressed on the cell surface, the ADCC activity measured using this assay is Env-specific.

**Figure 3.4. Reciprocal Env specificity.** Relative light units (RLU) indicating live, luciferase-expressing virus-infected target cells after an 8-hour incubation are lost in the presence of the Mm CD16⁺ NK cell line and serial 2-fold dilutions of plasma raised against the matched virus (solid symbols). ADCC activity was not detected against cells infected with viruses mismatched to the plasma sample (open symbols). The target viruses were SIV<sub>mac</sub>239 (red) and SHIV<sub>KB9</sub> (blue). Dashed lines indicate 50% activity.

**Macaque antibodies cross-reactive with human cellular antigens.** Antibodies reactive with human cellular antigens are a significant technical concern for measuring virus-specific ADCC activity in non-human primate studies. Some macaques have pre-existing antibodies that recognize the human CD4⁺ T-cell line used as targets in these assays, but these antibodies can routinely be depleted (see Materials and Methods) (Fig. 3.5a). Immunization of macaques with material produced in human cells, or with human cells themselves, elicits antibody responses against human proteins<sup>336-343</sup>. Indeed, macaques immunized with scSIV, which is produced by transfection of human 293T cells, potently directed ADCC against cells infected with either SIV<sub>mac</sub>239 or SHIV<sub>SF162P3</sub> (Fig. 3.5b). However, depletion of antibodies reactive with uninfected target cells revealed the SIV Env-specific ADCC activity elicited by immunization (Fig. 3.5b). Multiple
iterations of the depletion procedure do not reduce the ADCC activity measured for animals that lack detectable anti-human antibodies (Fig. 3.5c and d). In contrast to macaques, humans with baseline ADCC activity against these target cells are infrequent (Fig. 3.5e). Of 100 HIV-1 negative human beings tested, plasma from only one appeared to have some detectable baseline activity against cells infected with HIV-1 (Fig. 3.5e). Thus, macaques but not humans frequently have antibodies that interfere with ADCC measurements, and these antibodies can be depleted without significantly affecting virus-specific ADCC activity.

Figure 3.5. Baseline ADCC activity for macaque but not human plasma.
Plasma from some macaques contains antibodies reactive against uninfected target cells (red), but 6 depletions are sufficient to deplete these antibodies from most of 25 representative samples (black) (a). Pooled plasma from a group of animals immunized with scSIV had similarly potent ADCC activity against SIVmac239 (red) or SHIVSF162P3 (solid gray symbols) before depletions (b). However, after 20 depletions of antibody to human cellular antigens, ADCC activity was detectable against SIVmac239 (black) but not SHIVSF162P3 (open gray symbols). Performing 20 depletions on plasma from 2 animals that lack detectable baseline ADCC activity, Mm 382-03 (c) and Mm 333-07 (d), did not significantly reduce ADCC activity against SIVmac239. Baseline ADCC activity was measured for plasma samples from 100 HIV-1 negative people against HIV-1-infected target cells (e).
**ADCC compared with other assays.** Antibody responses in animals chronically infected with SIV\textsubscript{mac}239 were measured using different assays to evaluate the relationships among these assays. Plasma samples from 16 animals infected with SIV\textsubscript{mac}239 were tested for ADCC against cells infected with SIV\textsubscript{mac}239, or SHIV\textsubscript{SF162P3} as a negative control (Fig. 3.6a). The ADCC activity against SIV\textsubscript{mac}239-infected cells was quantified by calculating 50% ADCC titers and area under the curve (AUC) values for ADCC. The relationship between 50% ADCC titers and AUC values for ADCC was evaluated by calculating a Spearman correlation coefficient ($R_s=0.88$, $P<0.0001$) (Fig. 3.6b). These different methods for quantifying ADCC activity are therefore correlated with one another. The same 16 samples were tested for neutralization of SIV\textsubscript{mac}239 under conditions optimized for sensitivity (Fig. 3.6c). The mean 50% titers for ADCC against cells infected with SIV\textsubscript{mac}239 were 250-fold higher than the mean 50% titers for neutralization of SIV\textsubscript{mac}239 infectivity. Therefore, ADCC activity is a considerably more sensitive measure than neutralization against this neutralization-resistant primary isolate. These plasma samples were tested for neutralization of T-cell line-adapted SIV\textsubscript{mac}251\textsubscript{TCLA}, which is highly sensitive to neutralization (Fig. 3.6d). Indeed, all the animals infected with SIV\textsubscript{mac}239 had detectable SIV\textsubscript{mac}251\textsubscript{TCLA} neutralizing antibody titers. Lastly, antibodies capable of binding recombinant SIV\textsubscript{mac}239 gp120 protein were measured by ELISA (Fig. 3.6e). The 50% ADCC titers and 50% neutralization titers against SIV\textsubscript{mac}239 were correlated reasonably well ($R_s=0.53$, $P=0.032$) (Fig. 3.6f). Consistent with the 250-fold greater sensitivity of ADCC versus neutralization, there appeared to be a stronger relationship between neutralization and ADCC for samples with
50% ADCC titers >2x10^3. Although 50% ADCC titers correlated best with neutralization of SIV_{mac251}TCLA \((R_s=0.68, P=0.004)\), samples with a wide range of 50% ADCC titers had highly similar 50% SIV_{mac251}TCLA neutralization titers (Fig. 3.6g). Likewise, gp120 ELISA end-point titers also correlated with 50% ADCC titers \((R_s=0.53, P=0.032)\), but samples with varied 50% ADCC titers mostly had similar gp120 ELISA titers (Fig. 3.6h). AUC values for ADCC were better suited for quantifying weak responses than 50% ADCC titers, and were similarly related to measurements made using the other antibody assays (Fig. 3.6i-k). Since all of these animals were infected with cloned SIV_{mac239}, the individual-to-individual differences were attributable to properties of the antibody response, rather than antigenic variation. Although neutralization of SIV_{mac239} and SIV_{mac251}TCLA, and gp120 ELISA assays generally correlate with ADCC activity, the differences indicate that none are an interchangeable substitute for measuring ADCC itself.
Figure 3.6. Comparison with other antibody assays. ADCC activity against SIV\textsubscript{mac}239 (colors) and SHIV\textsubscript{SF162P3} (gray) was measured for 16 macaques chronically infected with SIV\textsubscript{mac}239 (a). The data were quantified by calculating 50% ADCC titers and area under the curve (AUC) values for ADCC, which are correlated with each other (b). The same samples, plus an additional 4 from uninfected macaques (black), were tested for neutralization of SIV\textsubscript{mac}239 (c), T-cell line-adapted SIV\textsubscript{mac}251\textsubscript{TCLA} (d), and for binding to recombinant SIV\textsubscript{mac}239 gp120 protein in an ELISA (e). The dashed lines indicate 50% RLU, or the absorbance level for a statistically defined end-point ELISA titer. The 50% ADCC titers against SIV\textsubscript{mac}239 were compared to neutralization of SIV\textsubscript{mac}239 (f), neutralization of SIV\textsubscript{mac}251\textsubscript{TCLA} (g), and ELISA titers (h). The AUC values for ADCC against SIV\textsubscript{mac}239 were also compared to neutralization of SIV\textsubscript{mac}239 (i), neutralization of SIV\textsubscript{mac}251\textsubscript{TCLA} (j), and ELISA titers (k).
**ADCC by monoclonal antibodies.** A panel of monoclonal antibodies was tested for ADCC activity against cells infected with the CXCR4-tropic T-cell line-adapted virus HIV-1\textsubscript{NL4-3} (Fig. 3.7). A dengue virus monoclonal antibody (DEN3) was included as a negative control. The gp120-specific monoclonal antibodies 2G12, PG9, PG16, b6, b12, and b13 all had detectable ADCC activity, with b12 directing ADCC at the highest titers. Although the gp41-specific broadly neutralizing monoclonal antibodies 2F5 and 4E10 neutralize HIV-1\textsubscript{NL4-3} \textsuperscript{214}, these antibodies did not direct ADCC against cells infected with HIV-1\textsubscript{NL4-3}. 2F5 and 4E10 are thought to recognize a transient intermediate that exists during fusion \textsuperscript{263,265}, which may not be a common form of Env on virus-infected cells. Thus, ADCC by all the HIV-1-specific monoclonal antibodies except 2F5 and 4E10 suggests that neutralization and ADCC assays can measure antibodies with different specificities.

![Figure 3.7. ADCC by monoclonal antibodies.](image)

**ADCC against an HIV-1 primary isolate.** We have shown that ADCC activity is detectable at high titers against SIV\textsubscript{mac}239, which is a neutralization-resistant primary SIV isolate. Here, we have measured ADCC against target cells infected with the
neutralization-resistant primary HIV-1 isolate, HIV-1\text{YU2} (Fig. 3.8). The majority of 14 plasma samples from American HIV-1 patients directed ADCC against cells infected with HIV-1\text{YU2}. Thus, this assay is capable of measuring ADCC activity against neutralization-resistant primary HIV-1 isolates.

**Figure 3.8. ADCC against cells infected with HIV-1\text{YU2}.** Plasma from an HIV-1 negative control subject (gray) and 14 HIV-1 positive patients was titered for ADCC activity against cells infected by the primary isolate HIV-1\text{YU2}. The dashed line indicates 50% ADCC activity.

**Dose-dependent inhibition of ADCC activity by IL-2.** For reasons that were mysterious at the time, the NK cell line lost its ability to direct ADCC after several months in culture. IL-2 is required for the maintenance of KHYG-1 cells\textsuperscript{497}. The optimal IL-2 concentration for the growth of KHYG-1 cells was reported to be 100 U IL-2 per ml. Since IL-2 is a positive regulator of lymphocyte activation and cytolytic activity, we initially reasoned that increasing the IL-2 concentration from 100 U per ml to 1000 U per ml might reverse the loss of ADCC activity. However, increasing the IL-2 concentration led to a more profound loss of ADCC activity (Fig. 3.9a). The concentration of IL-2 used for growing the cells was therefore titrated downwards, and indeed, culturing the NK cells at a lower concentration of IL-2 resulted in higher ADCC activity (Fig. 3.9b). This effect appeared to be dose-dependent. Lymphocyte activation by IL-2 is mediated through the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, which induces negative feedback regulation by increasing the expression of
suppressor of proteins belonging to the cytokine signaling (SOCS) family. To determine whether the loss of ADCC activity at higher IL-2 concentrations may be explained by the induction of negative feedback regulation, we performed a semi-quantitative RT-PCR experiment. Indeed, we observed that higher concentrations of extracted RNA were necessary to amplify similar levels of SOCS1 and SOCS3 cDNA from NK cells cultured at 10 U IL-2 per ml than 100 U IL-2 per ml. The identities of the SOCS1 and SOCS3 cDNA bands were confirmed by sequencing. Therefore, IL-2 may induce a negative regulatory pathway in KHYG-1 cells. These experiments indicate that it is necessary to culture the NK cell lines in a minimal concentration of IL-2.

**Figure 3.9. Inhibition of ADCC activity by IL-2.** Culturing the Mm CD16+ KHYG-1 line in the presence of high concentrations of IL-2 leads to a loss of ADCC activity (a). The loss of ADCC activity at different concentrations of IL-2 is dose-dependent (b). Dashed lines indicate 50% ADCC activity against cells infected with SIVmac239. RNA extracted from the cells cultured at 100 U IL-2 or 10 U IL-2 per ml in (b) was amplified by RT-PCR using primers for β-actin, perforin, granzyme M, CISH, SOCS1, and SOCS3 (c). For each RT-PCR primer set, the lanes from left to right contain the following template RNA: no RNA, RNA from NK cells cultured 10 U IL-2 per ml diluted 10^-1, 10^-2, or 10^-3, and RNA from NK cells cultured in 100 U IL-2 per ml diluted 10^-1, 10^-2, or 10^-3.
Preventing the loss of ADCC activity after cryopreservation. Cryopreservation of the NK cell line under standard conditions leads to a loss of ADCC activity, but this loss can be prevented by the calcineurin inhibitors cyclosporine A (CsA) and FK-506. Although the NK cell line used in this assay rapidly recovers following a standard cryopreservation protocol, the recovered cells have markedly decreased ADCC activity (Fig. 3.10a). This loss of activity represented a significant impasse in the development of this assay. The addition of freezing media containing 10% dimethyl sulfoxide (DMSO) was sufficient to induce the unresponsive state observed after cryopreservation (Fig. 3.10b). DMSO can induce calcium flux\textsuperscript{504}, which is a ubiquitous second messenger. In the absence of costimulatory signals, calcium flux can induce anergy in T-cells\textsuperscript{84,505}. Indeed, the addition of ionomycin, which mobilizes an influx of calcium into the cytoplasm, recapitulated the loss of ADCC activity observed after cryopreservation or the addition of freezing media (Fig. 3.10c.). In the presence of calcium, calmodulin activates the phosphatase calcineurin, which dephosphorylates nuclear factor of activated T-cells (NFAT), exposing nuclear localization signals that promote the redistribution of NFAT to the nucleus where it regulates gene expression\textsuperscript{505}. Since calcium flux does not render T-cells unresponsive in NFAT1 knockout mice, or in the presence of CsA, this pathway is thought to be important for anergy induction\textsuperscript{506,507}. We observed that two different inhibitors of calcineurin, CsA and FK-506, both preserve ADCC activity by Mm CD16\textsuperscript{+} KHYG-1 cells after cryopreservation or treatment with freezing media or ionomycin (Fig. 3.10a-c). Therefore, calcineurin activity is almost certainly necessary for the induction of unresponsiveness in this NK cell line. These drugs did not affect ADCC activity in mock-treated cells (Fig. 3.10d). However, it was necessary to culture cells in the absence
of CsA prior to this experiment, which may have led to some loss of activity (Fig. 3.10e). Although CsA and FK-506 are used in the clinic to suppress immune function, these drugs can prevent the induction of unresponsiveness by inhibiting calcineurin activity in KHYG-1 cells.

![Figure 3.10](image)

**Figure 3.10. Freezing media causes a loss of killing activity, but this loss can be prevented with calcineurin inhibitors.** Mm CD16-expressing KHYG-1 cells previously cultured in the absence of cyclosporine A (CsA) were taken through a freeze/thaw cycle (a), treated with freezing media but not frozen (b), treated with ionomycin (c), mock treated (d), and compared with cells never taken off of CsA (e). These treatments were conducted in the absence of calcineurin inhibitors, in the presence of CsA, or in the presence of FK-506. The dashed line indicates 50% ADCC activity for a plasma sample from an SIVmac239-infected macaque against SIVmac239-infected target cells.
3. E. DISCUSSION

We have developed a quantitative assay for measuring the capacity of antibodies to direct ADCC. This assay is based entirely on cell lines and thus obviates any requirement for primary cells. The scalability of ADCC assays dependent upon primary cells is limited by the number of NK cells that can be obtained from individual donors. Due to its exclusive usage of immortalized cell lines, the assay reported here is highly scalable for multiple parallel comparisons, and also avoids donor-to-donor variation. Thus, serum, plasma, or monoclonal antibodies can be routinely titrated over triplicate serial 2-fold dilutions in 96-well format. In these ways, this assay is analogous to widely adopted assays for virus neutralization.

This assay is preferable to a variety of common techniques for measuring antibodies against HIV-1 and SIV. Measures of antibody responses, especially in vaccine studies, have often relied upon binding to recombinant forms of Env protein in ELISAs, or neutralization of T-cell line-adapted viruses. However, many antibodies capable of binding recombinant forms of Env are directed against surfaces that are occluded in the native trimer, and cannot neutralize T-cell line-adapted viruses, let alone neutralization-resistant primary isolates. Furthermore, antibodies that recognize quaternary epitopes are not detectable using recombinant gp120. The importance of epitopes exposed by adaptation to growth in T-cell lines remains unclear. These assays are unaffected by differences in the isotype or glycosylation of the Fc region of the antibody, which affect CD16 crosslinking. Although ADCC activity against SIVmac239 was generally correlated with neutralization of T-cell line-adapted
SIV_{mac251 TCLA} and SIV_{mac239} gp120 ELISA titers, these measures were often quite similar for samples with a broad range of ADCC activity. A subset of antibody specificities that are consistently targeted could account for the similarity of most SIV_{mac251 TCLA} neutralization and SIV_{mac239} gp120 ELISA titers. Several recently-described assays for ADCC against HIV-1 and SIV use CD4\(^+\) target cells that are coated with recombinant forms of Env protein \(^{508-517}\), coated with linear peptides \(^{517-519}\), or infected with T-cell line-adapted viruses \(^{509,510,520}\). However, the inherent neutralization resistance of HIV-1 and SIV primary isolates stems from properties of Env that have evolved to limit the access of antibodies \(^{132,188,217,241,244,251,252,254}\). For the principles that confer neutralization resistance to be inapplicable to ADCC would be inconsistent with our understanding of lentiviral immune evasion. Therefore, assays that present primary isolate Env proteins in the native, membrane-bound, trimeric, physiologically relevant form that exists on virus particles and virus-infected cells are preferable for measuring antibody responses against HIV-1 and SIV.

Assays for antibody-dependent cell-mediated virus inhibition (ADCVI) are often used to measure ADCC\(^{422}\). Indeed, ADCC is probably a component of the antiviral activity measured in ADCVI assays. However, the ADCC assay described here has several advantages over ADCVI assays. Although antibodies have numerous functions \textit{in vivo}, the interplay between the multiple antibody-mediated mechanisms that simultaneously contribute to ADCVI may complicate a reductionist experimental approach. The effect of virus neutralization is superimposed and perhaps amplified over the effects mediated by cells bearing Fc receptors. Viral replication in ADCVI assays is typically quantified by measuring HIV-1 p24 or SIV p27\(^{361,422,423}\), but antibodies to p24...
or p27 present in the sample being tested can interfere with antigen capture assays. ADCVI also requires primary cells. Thus, the ADCC assay described here possesses significant advantages over other current methods for measuring ADCC against HIV-1 and SIV.

There appear to be differences between the antibodies that neutralize and those that direct ADCC. Neutralization and ADCC against SIVmac239 correlated moderately well, suggesting an incomplete overlap between the antibodies that mediate these functions. ADCC against cells infected with SIVmac239 was measurable for several plasma samples that did not neutralize SIVmac239 infectivity at detectable levels. The existence of ADCC in the absence of neutralization may largely be accounted for by the 250-fold greater sensitivity of ADCC versus neutralization. A more complete occupation of Env trimers by antibody may simply be required to detect neutralization than ADCC. However, there also appear to be differences in the epitopes responsible for ADCC versus neutralization. The broadly neutralizing monoclonal antibodies 2F5 and 4E10 did not direct ADCC against the T-cell line-adapted virus HIV-1NL4-3, which is sensitive to neutralization by 2F5 and 4E10. The specificity of these antibodies for a fusion intermediate, which may not be a dominant form of Env on infected cells, probably accounts for the absence of ADCC activity. Although 2F5 and 4E10 mediated ADCVI, the observed inhibition could entirely be explained by neutralization. Thus, 2F5 and 4E10 may be prototypical examples of antibodies that neutralize but do not appreciably direct ADCC against virus-infected cells. Since ADCC appears to be important in vivo, measuring both neutralization and ADCC may be optimal.
Antibodies that direct ADCC may relate to the modest protection reported in a recent phase III HIV-1 vaccine clinical trial in Thailand (RV144)\textsuperscript{379}. Although virus-specific CD8\textsuperscript{+} T-cells were largely undetectable, and antibodies capable of neutralizing primary HIV-1 isolates were absent, vaccine recipients consistently had detectable antibodies that bound gp120 in an ELISA. Antibody functions other than neutralization have therefore been postulated as potentially responsible for the protection reported in the RV144 trial\textsuperscript{522}.

The ADCC assay reported here is designed to isolate the interaction between CD16\textsuperscript{+} NK cells, antibody, and virus-infected cells for experiments that specifically address antibody function. This assay is therefore not intended for the study of differences in effector cell function or phenotype, for which primary cells are clearly required. One such difference that may contribute to immunodeficiency in HIV-1 infection is the functional impairment of NK cells\textsuperscript{111-113,523}. However, the effects of IL-2 and calcineurin inhibitors on KHYG-1 cells suggest that this cell line may also be a useful \textit{in vitro} system for understanding the negative regulation of NK cells. Therefore, these cell lines may contribute to studies of effector cell function, in addition to their intended purpose facilitating functional antibody measurements.

We have developed an assay for ADCC that is analogous to modern assays for virus neutralization. Its exclusive usage of immortalized cell lines and ability to measure ADCC against primary isolate Env proteins as they exist on virus-infected cells represent significant advantages over other available assays. Differences between neutralization and ADCC suggest that measuring both activities is optimal. The antibodies that direct
ADCC in this assay would be expected to have a variety of antiviral functions under physiological conditions \textit{in vivo}. 
CHAPTER 4

Antibody-dependent cell-mediated cytotoxicity develops over time during persistent infection with live-attenuated SIV and is a correlate of protection against pathogenic SIV infection.
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4. A. ABSTRACT

Immunization of rhesus macaques with live-attenuated strains of SIV routinely confers complete or apparent sterilizing immunity against challenge with pathogenic SIV. Identifying the mechanisms underlying this protection may provide guidance for HIV-1 vaccine design. Here we investigated the development of antibodies with effector functions against neutralization-resistant SIV strains after inoculation with SIVΔnef, and the potential contribution of these antibodies to protection against SIV infection. In the absence of detectable neutralizing antibodies, Env-specific ADCC titers emerged three weeks after inoculation, increased progressively over time, and were proportional to the extent of SIVΔnef replication. Persistent infection with SIVΔnef elicited significantly higher ADCC activity than transient immunization with SIV limited to single cycle of infection. ADCC titers were higher against viruses matched to the vaccine strain in Env, but were measurable against viruses expressing heterologousEnv proteins from independent SIV isolates. Two separate pathogenic SIV_{mac}251 challenge experiments took advantage of either the strain specificity or the time-dependent development of immunity to overcome complete protection by SIVΔnef. In both experiments, SIVΔnef-inoculated macaques that remained uninfected by SIV_{mac}251 had significantly higher ADCC activity than those that became infected. These results suggest that antibodies with ADCC activity contribute to protection by SIVΔnef.
4. B. INTRODUCTION

Efforts to develop a vaccine against HIV-1 are hindered by our limited knowledge of the types of immune responses that may be capable of preventing HIV-1 infection. Identifying these immune responses and understanding the stimuli required to elicit them may be necessary to design a safe and effective HIV-1 vaccine. Inoculation of rhesus macaques with live-attenuated strains of simian immunodeficiency virus (SIV) often confers complete or apparent sterilizing immunity against pathogenic strains of SIV. Therefore, elucidating the immune responses that mediate protection by live-attenuated SIV may help to guide HIV-1 vaccine development.

Antibody, T cell, and innate immunity have evolved to operate synergistically as an integrated system, and a combination of these immune responses may be necessary for complete protection by live-attenuated SIV. However, the efficacy of at least one of the immune responses necessary for complete protection increases over time, since animals challenged with SIV 251 months after inoculation with live-attenuated SIV are protected from infection, whereas those challenged at early time points become infected. Although live-attenuated SIV elicits virus-specific T-cells, and the quality of these T-cell responses may change over time, the magnitude of virus-specific CD8+ T-cell responses declines after the acute peak of live-attenuated SIV replication. In contrast, antibody responses are known to increase in titer over time through affinity maturation. An essential role for the affinity maturation of antibody responses could account for the time-dependent development of protection by live-attenuated SIV. However, SIV 251 is inherently resistant to neutralization, and antibodies capable of
neutralizing it are typically undetectable among completely protected animals\textsuperscript{345,352}. We therefore hypothesized that functions of antibodies other than neutralization contribute to protection by live-attenuated SIV.

In addition to virus neutralization, the antiviral functions of antibodies include complement fixation and numerous consequences of Fc receptor crosslinking, such as antibody-dependent cell-mediated cytotoxicity (ADCC)\textsuperscript{110,422,431,436,437,444}. Since ADCC represented a plausible effector mechanism and a proxy for other activities by the same functional antibodies, we developed a novel assay for quantifying the ability of antibodies to direct ADCC against virus-infected cells. We use this assay to investigate the induction of antibodies with ADCC activity, and to test the hypothesis that higher ADCC activity against cells infected by the challenge virus correlated with protection. Our results indicate that persistent infection with SIV\textit{Δnef} elicits ADCC titers that develop over time, are specific for the viral envelope glycoprotein (Env), are cross-reactive with Env proteins from heterologous (\textit{i.e.} independently isolated) SIV strains, are proportional to vaccine strain replication, and are correlated with protection against infection by SIV\textit{mac}251.
4. C. METHODS

**ADCC assay.** The ADCC assay has been described in Chapter 3. Antibodies against human cellular antigens were depleted 20 times for the animals immunized with scSIV, and 12 times for the animals in the Env-mismatch and vaginal challenge studies.

**Neutralization assay.** Neutralization was measured as previously described\textsuperscript{164,334}. The sensitivity of the virus neutralization assay was maximized by minimizing the amount of virus input required to obtain consistent levels of infection. These amounts were 0.5 ng p27 SIV\textsubscript{mac239}, 0.5 ng p27, 5 ng p27 SIV\textsubscript{mac251,NE}, and 0.5 ng p27 SIV\textsubscript{mac251,UCD} per well containing 15,000 C8166-secreted alkaline phosphatase (SEAP) reporter cells. The reciprocal neutralization experiment by week 22 sera against SIV\textsubscript{mac239} and SIV\textsubscript{mac239/E543-3env} was performed under different conditions, with 2 ng p27 and 5,000 C8166-SEAP cells per well. Plasma or serum dilutions were pre-incubated with virus for 1 hour at 37°C before adding C8166-SEAP cells. After three days, SEAP activity was determined using a luminescent assay (Applied Biosystems).

**Animal experiments and sample preparation.** The animals were Indian-origin rhesus macaques (Macaca mulatta) housed in a biocontainment facility at the New England Primate Research Center (NEPRC), and given care in accordance with standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the Harvard Medical School Animal Care and Use Committee. The experiments and procedures were approved by the Harvard Medical Area Standing Committee on Animals,
and conducted in accordance to the *Guide for the Care and Use of Laboratory Animals*\textsuperscript{466}. When necessary, animals were anesthetized by ketamine-HCl at fifteen mg/kg.

**Viruses.** The SIV\textsubscript{mac}239 and SIV\textsubscript{mac}239/E543-3env challenge doses used on week 22 consisted of 20 animal-infectious doses of virus produced by transfection of 293T cells. The week 33 challenge with SIV\textsubscript{mac}239 contained 10 animal-infectious doses of a rhesus PBMC-derived virus stock used in various previous studies\textsuperscript{159}. The intravenous SIV\textsubscript{mac}251\textsubscript{NE} challenge was 10 animal-infectious doses (32 pg p27) of a rhesus PBMC stock prepared in February 1991, used in other studies\textsuperscript{345,347,352}. Vaginal challenges consisted of 2 inoculations on 1 day of 1 ml undiluted SIV\textsubscript{mac}251\textsubscript{UCD}\textsuperscript{480} (100 ng p27), prepared at the California National Primate Research Center in June 2004. Neutralization and ADCC assays were done using SIV\textsubscript{mac}239 and SIV\textsubscript{mac}239/E543-3env produced by transfection of 293T cells, and SIV\textsubscript{mac}251\textsubscript{NE} and SIV\textsubscript{mac}251\textsubscript{UCD} expanded from the corresponding challenge stocks in rhesus PBMC. SHIV\textsubscript{SF162P3} was also expanded in rhesus PBMC (AIDS Research and Reference Reagent Program, NIAID, NIH, contributed by Drs. Janet Harouse, Cecilia Cheng-Mayer, Ranajit Pal and the DAIDS, NIAID). Neutralization was measured as previously described\textsuperscript{164,334}.

**Plasma viral RNA load measurements.** Challenge viruses were detected using primers specific for the *nef* sequences of SIV\textsubscript{mac}239 or SIV\textsubscript{mac}251 within the deletion in SIV\textsubscript{mac}239\textsubscript{Δnef}\textsuperscript{344}. Complete or apparent sterilizing protection was defined as the absence of detectable wild-type viral RNA using a real-time RT-PCR assay with a nominal threshold of detection of 10-30 copies of RNA per ml\textsuperscript{473}.
**Statistical analysis.** Fifty percent titers were calculated as the dilution at which a line connecting the values above and below 50% RLU would intercept the 50% RLU line. AUC values for ADCC were calculated such that they would be proportional to 50% ADCC titers, and represent the areas between 100% RLU and the titration curves as they appear in the figures. Whereas %ADCC, defined as 100% minus %RLU, is asymptotic as it approaches 100%, minimum %RLU values are inversely proportional to 50% ADCC titers. Therefore, AUC values for ADCC were calculated from the sum of the values over all dilutions for log_{10}100 minus log_{10}%RLU. This sum was multiplied by the log_{10}-transformed dilution factor of 2 to find an area. Statistical significance was evaluated in Prism version 4.1b (GraphPad Software) using 2-tailed Mann-Whitney U tests, 2-tailed Fisher’s exact tests, 2-tailed Wilcoxon matched pairs tests, and Spearman correlation coefficients.
D. RESULTS

**Time-dependent maturation of antibody responses.** Plasma samples drawn at longitudinal time points after inoculation with SIV\textsubscript{mac}239\Delta nef were tested for their ability to neutralize SIV\textsubscript{mac}239 and to direct ADCC against SIV\textsubscript{mac}239-infected cells. Only 4 of 10 macaques developed neutralizing antibody titers, and these were not detectable until 13 weeks after inoculation with SIV\textsubscript{mac}239\Delta nef (Fig. 4.1a). In contrast, ADCC titers were detectable in all animals just 3 weeks after inoculation with SIV\textsubscript{mac}239\Delta nef (Fig. 4.1b). These ADCC titers were Env-specific, since none of the plasma samples directed ADCC against target cells infected with SHIV\textsubscript{SF162P3}, which expresses the Env protein of HIV-1\textsubscript{SF162} in an SIV\textsubscript{mac}239 genetic background. To quantify ADCC titers, we calculated the plasma dilution that reduces the luciferase signal from virus-infected cells by 50%, and to measure differences in the extent of target cell elimination over all dilutions tested, we calculated values for the area under the curve (AUC). By both measures, progressive increases in ADCC activity were observed over 21 weeks. Thus, antibody titers capable of directing ADCC against SIV\textsubscript{mac}239-infected cells increased over time, but unlike neutralizing antibodies, emerged early and were detectable in all animals.
Figure 4.1. Development of neutralizing antibody and ADCC titers in macaques inoculated with SIV$_{mac}$239Δnef. Plasma collected from 10 animals at 0, 3, 5, 7, 13 or 15, and 21 weeks after inoculation with SIV$_{mac}$239Δnef was evaluated for its capacity to neutralize SIV$_{mac}$239 (a) and to direct ADCC against SIV$_{mac}$239-infected cells (b). The loss of relative light units (RLU) indicates the loss of virus-infected cells during an 8-hour incubation in the presence of plasma and an NK cell line. Target cells infected by SHIV$_{SF162P3}$ served as a negative control for all ADCC assays (gray). Dashed lines indicate 50% activity. All of these animals were completely protected against i.v. challenge with SIV$_{mac}$239. The top 5 animals were challenged on week 5, and the bottom 5 were challenged on week 15.

**Persistent replication required to elicit high ADCC titers.** The contribution of ongoing vaccine strain replication to the development of ADCC activity was evaluated by comparing SIV$_{mac}$239Δnef to scSIV. Plasma samples collected 2 or 12 weeks after a series of inoculations with scSIV$_{159}$ were tested for ADCC against SIV$_{mac}$239-infected cells (Fig. 4.2a). Five weeks after inoculation with SIV$_{mac}$239Δnef, 50% ADCC titers were on average 43-fold higher than those elicited by scSIV, and this difference
expanded to 250-fold by week 21 (**Fig. 4.2b**). The 50% ADCC titers (**Fig. 4.2b**) and the AUC values for ADCC (**Fig. 4.2c**) at any time point after inoculation with SIV\textsubscript{mac239Δnef} were significantly higher than at either time point after inoculation with scSIV (2-tailed Mann-Whitney U tests, P=0.002 to P<0.001). Since the geometric mean peak viral RNA loads in plasma for SIV\textsubscript{mac239Δnef} and scSIV were within 2-fold of each other, 1.3x10\textsuperscript{5} and 7.4x10\textsuperscript{4} copies per ml respectively (**Fig. 4.3**), the considerable differences in ADCC activity probably reflect differences in the persistence of SIV\textsubscript{mac239Δnef} versus scSIV.

**Figure 4.2. ADCC titers elicited by SIV\textsubscript{mac239Δnef} versus scSIV.** Plasma samples drawn on weeks 2 and 12 after inoculation with scSIV were titered for ADCC against SIV\textsubscript{mac239}-infected cells (a). Week 12 was the day of i.v. challenge with SIV\textsubscript{mac239}, when 11 of 12 of the scSIV-immunized animals became infected\textsuperscript{[159]}. Target cells infected with SHIV\textsubscript{SF162P3} served as a negative control (gray). Dashed lines indicate 50% activity. The 50% ADCC titers (b) and the AUC values for ADCC (c) elicited by SIV\textsubscript{mac239Δnef} were significantly higher than those elicited by scSIV (2-tailed Mann-Whitney U tests, P=0.002 to P<0.001).
Figure 4.3. SIV\textsubscript{mac}239Δ\textit{nef} versus scSIV vaccine strain viral loads. Geometric mean vaccine strain viral loads reflecting virus particles produced in vivo after inoculation with SIV\textsubscript{mac}239Δ\textit{nef} or scSIV are shown. The scSIV experiments are described in detail by Jia \textit{et al.}\textsuperscript{159}. The scSIV recipients in Group A were inoculated 3 times with scSIV that was trans-complemented with the vesicular stomatitis virus glycoprotein (VSV G), whereas the animals in Group B were inoculated 6 times with scSIV that was not trans-complemented.

**Antibody recognition of heterologous Env proteins.** The ADCC activity against SIV strains that were matched or mismatched with the vaccine strain in Env was compared. Sera were collected from 12 macaques inoculated with SIV\textsubscript{mac}239Δ\textit{nef} (Fig. 4.4a), and 12 inoculated with a recombinant form of SIV\textsubscript{mac}239Δ\textit{nef} containing the \textit{env} gene of SIV\textsubscript{sm}E543-3\textsuperscript{206} designated SIV\textsubscript{mac}239Δ\textit{nef}/E543-3\textit{env} (Fig. 4.4b). Sera from all 24 animals were tested for ADCC activity against target cells infected with SIV\textsubscript{mac}239 or SIV\textsubscript{mac}239/E543-3\textit{env}. On average, the 50% ADCC titers were 7-fold higher when the vaccine and test viruses were matched in Env than when they were mismatched (2-tailed Wilcoxon matched pairs test, P<0.0001). The 50% ADCC titers were also approximately 7-fold higher at week 22 than at week 6 (2-tailed Wilcoxon matched pairs test, P<0.0001). Thus, the 50% ADCC titers against the Env-matched virus at week 6 and the Env-mismatched virus at week 22 were comparable. Therefore, ADCC titers against Env-
mismatched viruses were lower and required more time to develop than ADCC titers against Env-matched viruses.

Figure 4.4. ADCC against target cells infected by viruses matched or mismatched to the vaccine strain in Env. Sera drawn 0, 6, or 22 weeks after inoculation with SIV$_{mac}$239Δnef (a) or with the recombinant vaccine strain SIV$_{mac}$239Δnef/E543-3env (b) were tested for ADCC against target cells infected with SIV$_{mac}$239 (black), SIV$_{mac}$239/E543-3env (green), or SHIV$_{SF162P3}$ (gray). Dashed lines indicate 50% activity.

Sera collected on week 22 were also tested for neutralization of SIV$_{mac}$239 and SIV$_{mac}$239/E543-3env. Although the majority of animals could neutralize the Env-matched viruses, neutralization of the Env-mismatched viruses was low to undetectable (Fig. 4.5). Thus, despite being low to undetectable in assays for virus neutralization, antibodies that cross-react with heterologous Env proteins could be measured using assays for ADCC.
Figure 4.5. Neutralization of viruses matched or mismatched to the vaccine strain in Env. Sera drawn 22 weeks after inoculation with SIV\textsubscript{mac239}\Delta\text{nef} (a) or the recombinant vaccine virus SIV\textsubscript{mac}\Delta\text{nef/E543-3\textit{env}} (b) were titered for neutralization of SIV\textsubscript{mac239} (black) or SIV\textsubscript{mac239/E543-3\textit{env}} (green). Dashed lines indicate 50% activity.

ADCC activity is proportional to the extent of vaccine strain replication. The extent of vaccine strain replication was estimated by calculating AUC values for log\textsubscript{10}-transformed SIV\Delta\text{nef} viral RNA loads in plasma over the first 21 or 22 weeks after inoculation. AUC values for viral loads among animals inoculated with SIV\textsubscript{mac239}\Delta\text{nef} and SIV\textsubscript{mac239}\Delta\text{nef/E543-3\textit{env}} were similar, averaging 65 and 67 log\textsubscript{10}-transformed RNA copies per ml × weeks, respectively. The relationships between AUC values for viral loads and ADCC activity at the end of this time period were evaluated by calculating Spearman correlation coefficients (\(r_s\)). Vaccine strain replication correlated with 50% ADCC titers against Env-matched (\(r_s=0.68, P<0.0001\)) and Env-mismatched (\(r_s=0.55, P=0.006\)) viruses (Fig. 4.6a), and also with AUC values for ADCC against Env-matched (\(r_s=0.64, P<0.0001\)) and Env-mismatched (\(r_s=0.42, P=0.0421\)) viruses (Fig. 4.6b). These
relationships suggest that the development of antibodies that direct ADCC is driven by the extent of antigenic stimulation provided by vaccine strain replication.

Figure 4.6. Relationship between the extent of vaccine strain replication and ADCC activity. The extent of SIV\textsubscript{mac}239\textDelta neo or SIV\textsubscript{mac}239\textDelta neo/E543-3env replication was estimated from the area under the curve (AUC) of log\textsubscript{10}-transformed vaccine strain viral loads over weeks 0 through 21 or 22, and compared to ADCC activity at week 21 or 22. Vaccine strain viral load AUC values were correlated with 50% ADCC titers (a) against Env-matched ($r_s=0.68$, $P<0.0001$) and Env-mismatched ($r_s=0.55$, $P=0.006$) SIV strains, and also with AUC values for ADCC activity (b) against Env-matched ($r_s=0.64$, $P<0.0001$) and Env-mismatched ($r_s=0.42$, $P=0.0421$) SIV strains. Linear regression lines are drawn.

**Challenge with viruses matched or mismatched in Env.** Half the animals inoculated with each vaccine strain were challenged intravenously with SIV\textsubscript{mac}239 on week 22, and half were challenged with SIV\textsubscript{mac}239/E543-3env. Three naïve control animals challenged with each virus all became infected, whereas only 2 of 24 immunized animals became infected. Although both were in the group inoculated with SIV\textsubscript{mac}239\textDelta neo and challenged with SIV\textsubscript{mac}239/E543-3env, mismatch in Env did not significantly affect protection (2-tailed Fisher’s exact test, $P=0.48$). The 2 animals that became infected had lower ADCC activity against SIV\textsubscript{mac}239/E543-3env-infected cells than most of the animals that remained uninfected (Fig. 4.7). However, significant differences cannot be detected with only 2 infected animals.
Figure 4.7. Neutralization and ADCC on the day of intravenous challenge with SIV\textsubscript{mac239/E543-3env}. Twelve macaques were challenged with an intravenous dose of SIV\textsubscript{mac239/E543-3env} on week 22 after inoculation with SIV\textsubscript{mac239Δnef} or SIV\textsubscript{mac239Δnef/E543-3env}. The 50% neutralization titers against SIV\textsubscript{mac239/E543-3env} (a), and the 50% ADCC titers (b) and AUC values for ADCC (c) against cells infected by SIV\textsubscript{mac239/E543-3env} were compared for infected versus uninfected animals. The significance of differences could not be determined (ND) since only 2 animals became infected.

The animals that remained uninfected were subsequently re-challenged. All 12 that remained uninfected after intravenous challenge with SIV\textsubscript{mac239} on week 22 again remained uninfected after re-challenge with SIV\textsubscript{mac239} on week 33. The 10 that had remained uninfected after challenge with SIV\textsubscript{mac239/E543-3env} on week 22 were re-challenged with an intravenous dose of SIV\textsubscript{smE543-3} on week 46, and 3 of these animals became infected. However, infection by SIV\textsubscript{smE543-3} appeared to be related to TRIM5 genotype\textsuperscript{39-41} (Fig. 4.8).
Figure 4.8. Neutralization and ADCC on the day of intravenous challenge with SIV<sub>sm</sub>E543-3. Ten macaques were challenged with an intravenous dose of SIV<sub>sm</sub>E543-3 on week 46 after inoculation with SIV<sub>mac</sub>239Δnef or SIV<sub>mac</sub>239Δnef/E543-3env. Sera collected on the day of challenge were tested for neutralization of SIV<sub>mac</sub>239/E543-3env (a) and for ADCC of cells infected by SIV<sub>mac</sub>239/E543-3env (b). Solid black symbols indicate animals that became infected by SIV<sub>sm</sub>E543-3. Dashed lines indicate 50% activity. Target cells infected with SHIV<sub>SF162P3</sub> served as a negative control for ADCC assays (gray). Differences in 50% ADCC titers (c) and in AUC values for ADCC activity (d) were not significant. The only animal with the non-protective Q/Q TRIM5 genotype became infected, whereas all 4 animals with the protective TFP/TFP genotype remained uninfected<sup>39-41</sup>.

**ADCC correlates with protection against SIV<sub>mac</sub>251.** The group of 6 animals inoculated with SIV<sub>mac</sub>239Δnef and 6 inoculated with SIV<sub>mac</sub>239Δnef/E543-3env that exhibited complete protection against 2 intravenous challenges with SIV<sub>mac</sub>239 were subsequently re-challenged with an intravenous dose of SIV<sub>mac</sub>251<sub>NE</sub> on week 46. Three of these 12 animals became infected by SIV<sub>mac</sub>251<sub>NE</sub>, as did both naïve controls.
challenged at the same time. Although all three immunized animals that became infected were among those inoculated with SIV_{mac239Δnef/E543-3env}, the trend toward more infections in this group was not significant (2-tailed Fisher’s exact test, P=0.18). Neither vaccine strain viral loads among the animals that became infected versus those that remained uninfected (Fig. 4.9a and b), nor among those immunized with SIV_{mac239Δnef} versus SIV_{mac239Δnef/E543-3env} differed significantly (Fig. 4.9c and d).

**Figure 4.9.** SIVΔnef viral loads among animals challenged with SIV_{mac251NE}. There were no significant differences in vaccine strain viral loads among the animals that became infected versus those that remained uninfected after intravenous challenge with SIV_{mac251NE} in terms of peak log_{10} RNA copies per ml (2-tailed Mann-Whitney U test, P=0.8636) (a), or AUC log_{10} RNA copies per ml \times weeks for the period of weeks 0-46 after inoculation (2-tailed Mann-Whitney U test, P=0.2091) (b). SIV_{mac239Δnef} and SIV_{mac239Δnef/E543-3env} did not differ significantly in peak viral loads (2-tailed Mann-Whitney U test, P=0.8182) (c), or in AUC viral loads over weeks 0-46 (P=0.9372) (d).

Sera drawn the day of challenge with SIV_{mac251NE} were tested for neutralization of SIV_{mac251NE} (Fig. 4.10a). Neutralizing antibody titers were low to undetectable, and differences among the infected versus uninfected animals were not significant at the
highest serum concentration tested (2-tailed Mann-Whitney U test, P=0.3727). These serum samples were also tested for ADCC against SIV_{mac251NE}-infected cells (Fig. 4.10b), and all had measurable ADCC activity. Although the titration curves for the animals that became infected by SIV_{mac251NE} grouped together at the low end of the range of ADCC activity (Fig. 5.10b), differences in 50% ADCC titers were not significant (Fig. 4.10c). However, the animals that remained uninfected by SIV_{mac251NE} had significantly higher AUC values for ADCC than those that became infected (2-tailed Mann-Whitney U test, P=0.0091) (Fig. 4.10d). Therefore, more complete elimination of the SIV_{mac251NE}-infected target cell population by ADCC correlated with protection against infection by intravenous challenge with SIV_{mac251NE}. 
Figure 4.10. Neutralization and ADCC on the day of intravenous challenge with SIV\textsubscript{mac}251\textsubscript{NE}. Macaques were challenged with an intravenous dose of SIV\textsubscript{mac}251\textsubscript{NE} on week 46 after inoculation with SIV\textsubscript{mac}239\textsubscript{Δnef} or SIV\textsubscript{mac}239\textsubscript{Δnef/E543-3env}. Sera collected the day of challenge were evaluated for neutralization of SIV\textsubscript{mac}251\textsubscript{NE} (a) and ADCC against SIV\textsubscript{mac}251\textsubscript{NE}-infected cells (b). Solid black symbols indicate animals that became infected by SIV\textsubscript{mac}251\textsubscript{NE}. Dashed lines indicate 50% activity. Target cells infected with SHIV\textsubscript{SF162P3} served as a negative control for ADCC assays (gray). Differences in 50% ADCC titers were not significant (c). However, AUC values for ADCC were higher among the animals that remained uninfected versus those that became infected (2-tailed Mann-Whitney U test, \(P=0.0091\)) (d). None of these macaques had the MHC class I alleles Mamu-A*01, -B*08 or -B*17 associated with reduced viral replication\textsuperscript{379,483,528}.

To address the temporal association between the development of antibody responses and protective immunity, we measured neutralization and ADCC titers in animals challenged at different time points after inoculation with SIV\textsubscript{mac}239\textsubscript{Δnef}. Groups of 6 female macaques each were challenged by high-dose vaginal inoculation of SIV\textsubscript{mac}251\textsubscript{UCD} at weeks 5, 20, or 40 after inoculation with SIV\textsubscript{mac}239\textsubscript{Δnef} (Reeves \textit{et al.}, manuscript in preparation). All 6 animals challenged at week 5 became infected, as did 3
of 6 animals challenged at week 20, and 4 of 6 animals challenged at week 40. Three naïve control animals challenged at each time point all became infected, except for one challenged at week 20. A trend towards higher SIV_{mac}239\Delta nef AUC viral loads through 5, 20, or 40 weeks after inoculation among the animals that remained uninfected versus those that became infected was not significant (Fig. 4.11).

Figure 4.11. SIV_{mac}239\Delta nef viral loads among animals challenged with SIV_{mac}251_{UCD}. Peak \log_{10} SIV_{mac}239\Delta nef viral loads did not correlate with protection against infection by SIV_{mac}251_{UCD} (2-tailed Mann-Whitney U test, P=0.8437) (a). SIV_{mac}239\Delta nef AUC \log_{10} RNA copies per ml × weeks for the period through the day of challenge with SIV_{mac}251_{UCD} at 5, 20, or 40 weeks after inoculation appeared higher among the animals that remained uninfected, but this difference was not significant (2-tailed Mann-Whitney U test, P=0.0939) (b).

Sera collected on the day of challenge with SIV_{mac}251_{UCD} were evaluated for neutralization of SIV_{mac}251_{UCD} (Fig. 4.12a-c). However, neutralization of SIV_{mac}251_{UCD} was not detectable for any of these serum samples (Fig. 4.12a-c). The capacity of the same sera to direct ADCC against SIV_{mac}251_{UCD}-infected cells was evaluated (Fig. 4.12d-f). In contrast to neutralization, all had measurable ADCC activity (Fig. 4.12d-f). Statistically significant outcomes could not be reached at individual time points, or for a group of animals that combines just those challenged on weeks 20 and 40 (Table 4.1). However, when the animals challenged 5, 20 and 40 weeks after inoculation with SIV_{mac}239\Delta nef were analyzed together, those that remained uninfected had higher 50%
Figure 4.12. Neutralization and ADCC on the day of high-dose vaginal challenge with SIV\textsubscript{mac251UCD}. At 5, 20, or 40 weeks after inoculation with SIV\textsubscript{mac239\Delta nef}, groups of 6 macaques each were challenged by high-dose vaginal inoculation with SIV\textsubscript{mac251UCD}. Serum collected the day of challenge was evaluated for neutralization of SIV\textsubscript{mac251UCD} (a-c) and ADCC against SIV\textsubscript{mac251UCD}-infected cells (d-f). Symbols appear in color for immunized macaques that remained uninfected by SIV\textsubscript{mac251UCD}, and in black for those that became infected. Target cells infected with SHIV\textsubscript{SF162P3} served as a negative control for ADCC assays (gray). Dashed lines indicate 50% activity. The groups challenged on weeks 5, 20, and 40 were combined for statistical analysis (g-j). The macaques remaining uninfected by SIV\textsubscript{mac251UCD} had higher 50% ADCC titers than those that became infected (2-tailed Mann-Whitney U test, $P=0.0487$) (g). A similar but non-significant trend was observed AUC values for ADCC (2-tailed Mann-Whitney U test, $P=0.0761$) (h). Non-significant trends were in the direction of lower peak SIV\textsubscript{mac251UCD} viral loads for animals with higher 50% ADCC titers ($r_s=-0.4615$, $P=0.1124$) (i) and higher AUC measurements of ADCC activity ($r_s=-0.4560$, $P=0.1173$) (j). Linear regression lines are shown. The macaque with the lowest ADCC titers among those remaining uninfected was the only animal possessing the protective combination of MHC class I alleles Mamu-\textit{A*01} and -\textit{B*17.43}.

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ADCC titers on the day they were challenged than those that became infected (2-tailed Mann-Whitney U test, P=0.0487) (Fig. 4.12g). A similar pattern was observed using AUC values for ADCC, although the difference was not significant (Fig. 4.12h). Also, among the animals that became infected, there was a trend towards higher ADCC activity in animals with lower peak SIV$^{mac251}_{UCD}$ viral loads (Figs. 4.12i and j). Therefore, higher ADCC titers present at late time points after inoculation with SIV$^{mac239\Delta nef}$ correlated with protection against infection by high-dose vaginal challenge with SIV$^{mac251}_{UCD}$.

<table>
<thead>
<tr>
<th>Time Points Included (Weeks)</th>
<th>5</th>
<th>20</th>
<th>40</th>
<th>20, 40</th>
<th>5, 20, 40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak SIV$^{\Delta nef}$ Viral Load</td>
<td>ND</td>
<td>P=0.4</td>
<td>ND</td>
<td>P=0.4318</td>
<td>P=0.8437</td>
</tr>
<tr>
<td>AUC SIV$^{\Delta nef}$ Viral Load</td>
<td>ND</td>
<td>P=0.1</td>
<td>ND</td>
<td>P=0.7551</td>
<td>P=0.0939</td>
</tr>
<tr>
<td>50% ADCC Titer</td>
<td>ND</td>
<td>P=0.1</td>
<td>ND</td>
<td>P=0.6389</td>
<td>P=0.0487</td>
</tr>
<tr>
<td>AUC Values for ADCC</td>
<td>ND</td>
<td>P=0.1</td>
<td>ND</td>
<td>P=0.4318</td>
<td>P=0.0761</td>
</tr>
</tbody>
</table>

ND = Could not be determined due to < 3 animals in one of the comparison groups.

Table 4.1. 2-tailed Mann-Whitney U tests for the significance of differences among animals challenged vaginally with SIV$^{mac251}_{UCD}$. 
4. E. DISCUSSION

Identifying the immune responses that mediate protection by live-attenuated SIV and understanding their induction may inspire strategies for engineering a safe and effective HIV-1 vaccine. We hypothesized that antibody functions other than neutralization contribute to the protective immunity provided by live-attenuated SIV against pathogenic SIV challenge. We now show that ADCC activity correlates with complete or apparent sterilizing protection against SIV\textsubscript{mac251} infection. Furthermore, properties of the antibody response reflected in ADCC titers mirror hallmarks of protection by live-attenuated SIV. The protective immunity conferred by live-attenuated SIV increases over time\textsuperscript{345,347}, is usually incomplete against challenge with a heterologous SIV strain\textsuperscript{418,420}, and is greater for vaccine strains that replicate at higher levels\textsuperscript{347,352}. In accordance with these observations, our data indicates that ADCC titers increase progressively over time, are lower against viruses expressing heterologous Env proteins, and are proportional to the extent of vaccine strain replication. Therefore, antibodies that direct ADCC may contribute to complete protection by live-attenuated SIV.

In two separate challenge experiments, we observed a relationship between higher ADCC activity and protection against infection with SIV\textsubscript{mac251}. In one experiment, macaques inoculated with SIV\textsubscript{mac239Δnef} or SIV\textsubscript{mac239Δnef}/E543-3\textsubscript{env} that remained uninfected after intravenous challenge with SIV\textsubscript{mac251\textsubscript{NE}} had higher AUC values for ADCC than those that became infected. In another experiment, animals that remained uninfected after high-dose vaginal challenge with SIV\textsubscript{mac251\textsubscript{UCD}} at different time points
after inoculation with SIV\textsubscript{mac}239\Delta nef had higher 50\% ADCC titers than those that became infected. Although differences in AUC values for ADCC were significant in one experiment, whereas 50\% ADCC titers were significant in the other, our power to detect correlates was limited by the small number of animals. Also, these two measures of ADCC activity may not respond equally to differences in the vaccine strains, the challenge viruses, and the time of challenge. The animals that became infected by SIV\textsubscript{mac}251\textsubscript{NE} were immunized with SIV\textsubscript{mac}239\Delta nef/E543-3env, whereas those challenged with SIV\textsubscript{mac}251\textsubscript{UCD} were all immunized with SIV\textsubscript{mac}239\Delta nef. Animals inoculated with SIV\textsubscript{mac}239\Delta nef/E543-3env had higher AUC values for ADCC against the Env-matched virus at week 6 than against the Env-mismatched virus at week 22, despite having similar 50\% ADCC titers. Thus, a greater sensitivity of AUC to detect differences in ADCC against Env-mismatched viruses may account for the significance of differences in AUC against SIV\textsubscript{mac}251\textsubscript{NE} in the first study. More complete elimination of cells infected by SIV\textsubscript{mac}251\textsubscript{NE} versus SIV\textsubscript{mac}251\textsubscript{UCD} may have also favored differences in AUC over 50\% titers. This higher ADCC activity may be due to the longer time period for the maturation of antibody responses before challenge with SIV\textsubscript{mac}251\textsubscript{NE} (i.e. 46 versus 5, 20, and 40 weeks after inoculation), or to an increase in the antibody resistance of SIV\textsubscript{mac}251\textsubscript{UCD} as a result of its distinct passage history\textsuperscript{480}. Although differences between the two SIV\textsubscript{mac}251 challenge experiments may have favored one measure of ADCC activity over the other, ADCC activity correlated with protection against infection with SIV\textsubscript{mac}251 in both experiments.

While the relationship between ADCC activity and the outcome of challenge suggests that these antibodies contribute to protection, correlation does not establish
causation. In addition to ADCC, Fc receptor crosslinking stimulates the secretion of molecules that promote lymphocyte homing and activation, and that inhibit virus replication\textsuperscript{422,444}. The antibodies that direct ADCC may also mediate effector functions through complement fixation\textsuperscript{431,436}. Furthermore, ADCC assays may measure antibodies that block virus infection at concentrations present \textit{in vivo}, but are undetectable in conventional neutralization assays. Other mechanisms of immunity not mediated by antibodies may also covary with ADCC activity. T-cell, antibody, and innate immune responses may all be affected by the extent of antigenic stimulation. It is conceivable that the observed relationships are due to differences that exist among animals inoculated with SIV\textsubscript{mac}239Δ\textit{nef} versus SIV\textsubscript{mac}239Δ\textit{nef}/E543-3\textit{env}, or among animals challenged 5, 20, and 40 weeks after inoculation with SIV\textsubscript{mac}239Δ\textit{nef}. Thus, while our findings implicate antibodies in protection by live-attenuated SIV, they do not preclude a role for other immune responses.

More than one type of immune response elicited by live-attenuated SIV may be necessary for protection against infection with SIV\textsubscript{mac}251. Passive transfer experiments in different live-attenuated SIV vaccine models have yielded mixed results on the ability of antibodies alone to protect against SIV infection, demonstrating complete protection in one study\textsuperscript{409}, and no protection in another\textsuperscript{408}. In contrast to the sub-neutralizing polyclonal antibody responses that correlated with protection against SIV\textsubscript{mac}251 infection in this study, relatively high concentrations of neutralizing monoclonal antibodies were necessary to protect macaques against SHIV infection\textsuperscript{410-413,447}. T-cell responses present in macaques inoculated with SIVΔ\textit{nef}\textsuperscript{527}, but absent in macaques that received antibodies passively, may help to explain this apparent difference in the neutralizing antibody titer.
required to prevent infection. Therefore, antibodies and T-cells elicited by live-attenuated SIV may act in concert to mediate complete protection.

Reports of protection against SIV\textsubscript{mac}239 by vaccination with SHIV\textsubscript{89.6}\textsuperscript{529-534} have not ruled out a role for antibodies in complete or apparent sterilizing protection by live-attenuated SIV. Protection was defined in these studies as control of viral loads to less than 10\textsuperscript{4} copies of viral RNA per ml\textsuperscript{529,530,533}, which we would define as partial protection, not complete or apparent sterilizing protection. The limit of detection for SIV\textsubscript{mac}239-specific sequences was 100-1000 copies of proviral DNA per 10\textsuperscript{5} cells, or 0.1-1\% of lymphocytes\textsuperscript{529}, which was a relatively high threshold. Although some macaques had viral loads under 500 copies of RNA per ml\textsuperscript{529}, they all had detectable viral RNA at necropsy\textsuperscript{532}. SIV\textsubscript{mac}239 RNA was present in tissues from 11 of 12 SHIV\textsubscript{89.6}-vaccinated macaques 14 days post-challenge\textsuperscript{531,534}. Moreover, infection of macaques with plasmid DNA containing the infectious molecular clone of SHIV\textsubscript{89.6} did not confer as strong protection against SIV\textsubscript{mac}239\textsuperscript{535,536}, suggesting the cell culture conditions in which the vaccine virus was produced may have contributed to protection. Furthermore, in the reciprocal experiment, Wyand \textit{et al.} showed that SIV\textsubscript{mac}239\Delta3 does not protect against SHIV\textsubscript{89.6P} infection\textsuperscript{420}. Therefore, reports of protection by SHIV\textsubscript{89.6} against SIV\textsubscript{mac}239 challenge have not demonstrated complete protection in the absence of an Env-specific antibody response.

Our observations are in agreement with other reports that have associated antibody responses with vaccine protection. Studies from the Robert-Guroff laboratory, and most recently from Barouch \textit{et al.}, have correlated lower viral loads after infection with higher ADCC activity against target cells coated with monomeric gp120.
protein\textsuperscript{508,515,537}, or infected with T-cell line-adapted SIV\textsuperscript{509}. However, these studies did not correlate ADCC activity with protection from infection, or measure ADCC using target cells infected with neutralization-resistant SIV strains. Nevertheless, antibodies capable of binding recombinant forms of gp120 and neutralizing neutralization-sensitive SIV strains correlated with protection from infection\textsuperscript{537}. In the context of vaccination with different live-attenuated strains of SIV, Johnson \textit{et al.} found that antibody avidity correlated with control or protection from infection after vaginal challenge with SIV\textsubscript{mac251}\textsubscript{NE}\textsuperscript{342}. Similarly, Wyand \textit{et al.} reported that neutralization of SIV\textsubscript{mac251}\textsubscript{NE} at a 1:4 dilution of serum correlated with protection in a combined group of animals that remained uninfected or strongly controlled SIV\textsubscript{mac251}\textsubscript{NE} viral loads\textsuperscript{347}. However, low-titer neutralization of SIV\textsubscript{mac251}\textsubscript{NE} only appeared to be detectable in CEMx174 cells, and therefore may reflect a minor variant that enters this CCR5-negative T-cell line more efficiently\textsuperscript{538}. Taken together, these studies suggest that antibody responses contribute to protection.

Interest in antibody functions other than neutralization has recently increased, due to a modest reduction in the rate of HIV-1 infection among recipients of a recombinant canarypox vector prime and protein boost vaccine in Thailand (RV144)\textsuperscript{379}. This approach elicited binding antibodies, but virus-specific CD8\textsuperscript{+} T cells and neutralizing antibodies were largely undetectable. Functions of antibodies other than neutralization have therefore been postulated as potentially responsible for protection in the RV144 trial\textsuperscript{522}.

Persistent expression of Env may be essential to elicit effective antibody responses. The progressive increases in ADCC activity over time, and the considerably
higher ADCC activity elicited by SIV\textsubscript{mac}239\Delta\textit{nef} versus scSIV, imply that the persistent antigenic stimulation provided by the ongoing replication of SIV\textsubscript{mac}239\Delta\textit{nef} stimulated the development of high ADCC activity. These differences may also contribute to the better protection provided by SIV\Delta\textit{nef} in comparison to scSIV\textsuperscript{159}. The SIV\textsubscript{mac}239\Delta\textit{nef}-inoculated animals used for the comparison with scSIV were all completely protected against i.v. challenge with SIV\textsubscript{mac}239, half on week 5 and half on week 15. In contrast, 11 of 12 scSIV-immunized animals became infected after i.v. challenge with the same dose of SIV\textsubscript{mac}239, albeit on a different day, 12 weeks after the last inoculation with scSIV. Thus, in a comparison between vaccines that afford complete versus incomplete protection, complete protection was associated with significantly higher ADCC activity. In addition, a longer period of persistent infection with SIV\Delta\textit{nef} was required to reach comparable ADCC titers against SIV strains expressing heterologous Env proteins. Persistent Env expression may therefore be required to induce high and broadly reactive ADCC titers against naturally transmitted HIV-1 strains with diverse Env sequences.

A vaccine for HIV-1 must contend with sequence variation that typically renders neutralizing sera ineffective against heterologous HIV-1 isolates\textsuperscript{125,285}. The Env proteins of SIV\textsubscript{mac}239 and SIV\textsubscript{sm}E543-3 differ in amino acid sequence by 18\%, which approximates the median difference between the Env proteins of individual HIV-1 isolates within a clade\textsuperscript{125}. Since sera poorly neutralized viruses mismatched in Env, the ADCC titers against these viruses suggest that antibodies may have broader efficacy than is generally revealed by neutralization assays. The complete protection in 10 of 12 animals challenged with Env-mismatched viruses on week 22 is not incompatible with a role for antibody responses in protection. The ADCC titers against the Env-mismatched
viruses on week 22 were comparable to those against the Env-matched viruses at week 6, but SIV\textsubscript{mac239}\textDelta\textit{nef}-inoculated macaques were completely protected against i.v. challenge with SIV\textsubscript{mac239} on week 5. The difference in time required for the development of protective immunity against SIV\textsubscript{mac251} versus SIV\textsubscript{mac239} may relate to differences in the inherent antibody resistance of these strains. The extent of cross-reactivity in ADCC activity suggests that it may be possible to elicit antibody responses by vaccination that are capable of directing ADCC against diverse primary isolates of HIV-1.

These results have implications not only at the basic level of supporting a protective role for antibodies when neutralization is low or undetectable, but also at the applied level of understanding the kind of stimulation that is necessary for a vaccine to induce effective antibody responses. We show that properties of the ADCC titers elicited by SIV\textDelta\textit{nef} mirror hallmarks of protection by live-attenuated SIV, and that ADCC activity correlates with protection against SIV\textsubscript{mac251} infection. These observations suggest that the antibodies responsible for ADCC activity contribute to complete protection by live-attenuated SIV. Although our results imply that antibodies have greater breadth than is generally revealed by neutralization assays, they also suggest that persistent Env expression may be necessary to elicit high and broadly reactive ADCC titers by vaccination. Therefore, strategies to persistently stimulate Env-specific antibody responses may improve the efficacy of vaccines against HIV-1.
CHAPTER 5

ADCC in the immune correlates analysis of the ALVAC-AIDSVAX HIV-1 vaccine efficacy trial in Thailand (RV144)
Acknowledgements

We are very grateful to Peter Gilbert, Ying Huang, and their colleagues at The Statistical Center for HIV/AIDS Research & Prevention (SCHARP) for the multitude of statistical analyses performed, and for their clear presentation. We would also like to thank the leadership of the RV144 correlates analysis group for their roles in organizing this study and explaining the analyses, especially Barton Haynes, David Montefiori, Jerome Kim, and Nelson Michael.

Data is included in this chapter that was produced in the laboratories of Susan Zolla-Pazner, Georgia Tomaras, David Montefiori, and Guido Ferrari. The ELISA data against gp70-V1V2 are from Susan Zolla-Pazner. The IgG and IgA binding data against gp120, gp140, and gp41 are from Georgia Tomaras. The neutralizing antibody assays were performed in David Montefiori’s laboratory. Guido Ferrari supplied the gp120-coated cell ADCC assay data.

Jackson Harvey assisted the execution of the RV144 pilot study experiments. Lisa Heyer and David Williams assisted with the case-control assays.
5. A. ABSTRACT

The RV144 trial was the first HIV-1 vaccine clinical trial to report protection against HIV-1 infection. It therefore offers the first opportunity to identify correlates of vaccine protection against HIV-1. Due to the availability of many different immunological assays, and the potential for a high false discovery rate if many hypotheses were tested, a pilot study was conducted to identify 6 primary variables for an immune correlates analysis. The criteria evaluated were low background, a wide dynamic range for vaccine-elicited immune responses, reproducibility, and non-redundancy with other assays. Our assay for ADCC, which is based on an NK cell line and HIV-1-infected target cells, was selected based on its performance in these criteria. Significant relationships were found with 2 of the other 6 assays. Antibodies against an HIV-1 V1V2 loop structure correlated with protection from HIV-1 infection, and Env-specific IgA correlated with risk of infection. Although the ADCC activity measured among RV144 vaccinees was weak, there was a non-significant trend suggesting that those with higher relative ADCC activity had a lower risk of HIV-1 infection. Moreover, when IgA was eliminated as a risk factor by excluding most vaccinees with detectable IgA responses, there was a borderline significant inverse correlation between ADCC activity and HIV-1 infection. Thus, among vaccinees without detectable IgA responses, ADCC activity may have contributed to vaccine protection. These results suggest that ADCC activity should be measured in future HIV-1 vaccine clinical trials.
5. B. INTRODUCTION

The RV144 trial provides the first opportunity to identify correlates of vaccine protection in an HIV-1 vaccine efficacy trial. Among 8197 vaccine trial participants who received at least 1 of 4 scheduled doses of a canarypox vector (ALVAC-vCP1521) encoding a recombinant Env protein composed of a CRF01_AE gp120 engrafted onto a clade B gp41, and 2 boosts with CRF01_AE and clade B recombinant gp120 proteins formulated with adjuvant (AIDSVAX B/E), 51 became infected with HIV-1 over a 42 month follow-up period\textsuperscript{379}. In contrast, 74 of 8198 volunteers who received at least one dose of a placebo became infected with HIV-1 over the same time period. Vaccine efficacy was calculated to be 31\% (95\% CI=1.1 to 52.1\%, P = 0.04). Identifying immune responses that correlate with vaccine protection in the RV144 trial may generate hypotheses on the nature of the protective immune responses, and suggest parameters for the analysis of future HIV-1 vaccine studies.

Correlates analyses must be designed to minimize the potential for false positives. To minimize the false discovery rate, P-values or significance thresholds need to be adjusted in proportion to the number of comparisons\textsuperscript{539}. Therefore, to maximize the potential for identifying significant correlates of vaccine protection in the RV144 trial, the primary correlates analysis was designed to minimize the number of hypotheses tested. A pilot study was conducted to select a small number of assays for this analysis. Based on the criteria of having a low false positive rate, a large dynamic range for vaccine-induced immune responses, high reproducibility, and non-redundancy (\textit{i.e.} low correlation among assays), 6 assays were selected for the primary analysis. Holm-
Bonferroni P- and Q-values adjusted for testing 6 hypotheses were reported. Since this study had the exploratory objective of generating hypotheses, relationships with a P-value of 0.05 and a Q-value of 0.2 were deemed acceptable. Significant correlations may be interpreted as a protective immune response, a surrogate for an unidentified but covarying protective immune response, or a marker for individual-to-individual variation in susceptibility to HIV-1 infection.

Based on the metric properties of data we generated for the pilot study, ADCC against target cells infected with the CRF01_AE virus HIV-192TH023 was selected as one of the 6 primary immune variables. The 5 other primary variables, which were measured by different groups, were IgA against recombinant clade B gp140 and CRF01_AE gp120 protein, IgG avidity for recombinant CRF01_AE gp120, neutralization of several neutralization-sensitive CRF01_AE viruses and T-cell line-adapted clade B viruses, binding to a recombinant protein with a clade B V1V2 loop sequence engrafted into a mouse retrovirus envelope glycoprotein (gp70-V1V2), and cytokine expression by CD4+ T-cells stimulated with CRF01_AE gp120 peptides. The focus on antibody and CD4+ T-cell responses reflects the observation that CD8+ T-cell responses were not distinguishable between vaccine and placebo recipients in the RV144 trial.

Relationships between the primary variables and vaccine protection were examined using 2 types of regression models. The relationship between each primary variable and infection status at the end of a 42-month follow-up period was determined by estimating an odds ratio (OR) by logistic regression. Any potential relationship with infection rate was evaluated using Cox proportional hazards to calculate a hazard ratio (HR) that is based on the estimated time of infection. An OR or HR of 1 indicates no
relationship, whereas >1 indicates a positive correlation and <1 indicates an inverse correlation. Two primary variables correlated with protection or risk. Indexed IgA binding scores positively correlated with infection status (OR=1.54 per standard deviation (SD) increase, 95% CI=1.05 to 2.25, P=0.03, Q=0.08). Binding to gp70-V1V2 correlated with protection (OR=0.57 per SD increase, 95% CI=0.36 to 0.90, P=0.02, Q=0.08). Interaction analyses conducted among IgA scores and the other primary variables suggested that all the other parameters have borderline significant correlations with protection among vaccinees with low IgA. Thus, the pilot study and primary analysis suggested parameters for the evaluation of candidate HIV-1 vaccine approaches, and generated hypotheses on the types of immune responses associated with protection or risk of infection in the RV144 trial.
5. C. METHODS

**Plasma samples.** Among the 51 vaccines who became infected with HIV-1, week 0 and 26 plasma samples were available for 41. Five vaccine recipients who remained uninfected after 42 months were paired with each of the 41 who became infected, for a total of 205 uninfected vaccinees. This pairing was conducted based on gender, self-reported behavioral risk, and number of immunizations. For example, for 1 woman who received 2 immunizations and became infected, 5 women who received 2 immunizations and reported similar behavioral risk factors but remained uninfected were included as controls. Plasma samples from 20 placebo recipients who became infected and 20 placebo recipients who remained uninfected were included as well. Thus, samples from 286 participants (572 samples total) were tested for ADCC against cells infected by HIV-1\textsubscript{92TH023} in the case-control study. As of this writing, we remain blinded to the placebo versus vaccine and infected versus uninfected status of each sample.

**Viruses.** ADCC was evaluated using 2 CRF01_AE viruses. HIV-1\textsubscript{CM235}, which is a molecular clone, was prepared by transfection of 293T cells. HIV-1\textsubscript{92TH023} was selected for testing of the RV144 samples, since the gp120 component of the recombinant Env protein expressed by the canarypox vector was derived from HIV-1\textsubscript{92TH023}. The uncloned HIV-1\textsubscript{92TH023} virus stock was obtained from the AIDS Research Reference Reagent Program (Division of AIDS, NIAID, NIH), and was originally contributed by UNAIDS. This virus was first expanded in human PBMC, but due to a relatively low yield, was
expanded in the CEM.NKR-CCR5 cell line that was used for the ADCC assays in this study.

**ADCC assay.** The ADCC assay was conducted as described in “Chapter 3: An assay for quantifying ADCC based on an NK cell line and target cells infected by SIV or HIV-1.” The NK effector cells expressed the V158 variant of human CD16 (FCGR3A). The target cells are a limiting dilution clone derived from the previously described cell line CEM.NKR-CCR5, by transduction with a pQCXIP-derived vector expressing human CCR5, due to relatively low CCR5 expression by the original clone. Target cells were spinoculated with 300 ng p24 HIV-1,92TH023 per 5x10^5 cells 4 days prior to use. Week 0 and week 26 plasma samples from individual trial volunteers were always tested on the same 96-well plate.

**Statistical analysis.** The primary variable evaluated was a measure of partial area between the curves (pABC). To calculate pABC, \( \log_{10} \) RLU values for the week 26 sample were subtracted from \( \log_{10} \) RLU values for the week 0 (baseline) sample. A log scale was used due to the linear relationship between \( \log_{10} \) RLU values and 50% ADCC titers in a large sample set with a wide range of activity. Areas were calculated by the trapezoidal method. Negative values were replaced with zeroes, since negative areas are forbidden. The sum of the differences over the 4 highest plasma dilutions tested was multiplied by \( \log_{10}2 \), the dilution factor on a log scale. Fifty percent ADCC titers were not used since the only samples to reach 50% were those with high baseline activity.
Statistical analyses were conducted by Peter Gilbert and colleagues at The Statistical Center for HIV/AIDS Research & Prevention (SCHARP).
5. D. RESULTS

**Measurement of ADCC by RV144 plasma samples for the pilot study.** A pilot study was performed to select a small number of assays to be included in the primary correlates analysis. The first two criteria for assay selection were that assays must have a low false positive rate and a large dynamic range for measuring vaccine-elicited immune responses. To address these criteria, we measured ADCC by blinded samples from 80 vaccine recipients and 20 placebo-immunized controls. The vaccine recipients chosen for the pilot study had received all 4 immunizations with the canarypox vector ALVAC-vCP1521 and 2 boosts with AIDSVAX B/E. Uncloned HIV-1\textsubscript{192TH023} was used as a target virus, since this strain is the source of the gp120 in the ALVAC-vCP1521 component of the vaccine. As a measure of quality control, a plasma sample from a Thai HIV-1-positive donor was tested in parallel with all RV144 samples (Fig. 5.1). The ADCC activity and reproducibility for the positive control sample appeared to be quite robust. In parallel, plasma samples from RV144 participants collected on week 0 (baseline) and week 26 (2 weeks after the last boost) were tested for their capacity to direct ADCC against HIV-1\textsubscript{192TH023}-infected cells (Fig. 5.2). Based on an initial visual inspection, a subset of the week 26 plasma samples clearly had detectable ADCC activity over baseline. However, only one plasma sample reached 50% ADCC, and this was the one with the highest baseline activity. Although a range of vaccine-elicited ADCC activity was observed, it was relatively weak in comparison to the positive control sample from an HIV-1-infected donor.
Figure 5.1. ADCC by positive control plasma. A positive control sample from a Thai HIV-1 patient was tested along side the RV144 plasma samples in every assay run. The dashed line indicates 50% activity. This positive control plasma robustly directs ADCC against cells infected with HIV-1_92TH023.

Figure 5.2. For the pilot study, plasma samples collected at week 0 (baseline) and week 26 (2 weeks after last boost) were tested for ADCC against cells infected with HIV-1_92TH023. This figure depicts the primary data for blinded samples and continues on the next page. The data displayed on this page were collected on 1.3.11. The plasma samples tested for this component of the pilot study belonged to 80 vaccine recipients and 20 placebo recipients. The dashed lines indicate 50% activity.
Figure 5.2 continued. The data on this page were collected on 1.4.11. This figure continues on the next page.
**Figure 5.2 continued.** The data on this page were collected on 1.6.11. This figure continues on the next page.
Figure 5.2 continued. The data on this page were collected on 1.10.11.
The level ADCC activity at the highest concentration of plasma tested was selected for analysis. The vaccine and placebo recipients were ranked by the difference in ADCC activity observed at week 0 versus 26 (Fig. 5.3). This analysis revealed a range of ADCC activity. A cut-off value of just 9% ADCC (91% RLU) was chosen to demarcate a positive response. For an analysis using this relatively low threshold, 2 of the 20 placebo recipients had >9% ADCC activity at week 26, and 10 of 100 total had >9% ADCC activity at week 0. Nevertheless, nearly all placebo and baseline samples had approximately 0% ADCC activity. One sample had particularly high ADCC activity at week 26, but this was the vaccine recipient with the highest baseline signal. Thus, aside from a single outlier, the week 0 and placebo samples scored close to 0% ADCC activity, and a range of activity was observed at week 26.

![Figure 5.3. Vaccine and placebo recipients ranked by ADCC activity. Plasma samples for 20 placebo and 80 vaccine recipients were tested for ADCC activity against HIV-1<sub>92T</sub>H023 as part of the pilot study.](image)

**Analysis of replicate samples for the pilot study.** The third criterion for the selection of assays for the primary analysis was reproducibility. To address the reproducibility of this assay, we measured blinded quadruplicate aliquots of plasma
samples from 6 HIV-1-infected donors and 2 HIV-1-negative controls (Fig. 5.4). The data from the 6 HIV-1-infected donors were used to calculate an intra-assay coefficient of variation (CV). The CV values for different assays related to ADCC were compared (Fig. 5.5). These assays included ADCC and ADCVI assays using fresh and frozen cells, and Biacore assays for Fc receptor binding. Our assay had an optimal CV score for reproducibility.

**Figure 5.4. Blinded replicate ADCC assays using HIV-1 positive and negative plasma.** ADCC activity was measured against target cells infected with HIV-1\textsubscript{CM235}. Samples from 6 HIV-1 patients are gray, yellow, cyan, green, pink and blue. The 2 negative samples are black and red.

**Figure 5.5. Coefficient of variation (CV) across the 6 HIV-1-positive plasma samples.** ADCC, ADCVI, and Fc receptor binding data from different labs were quantified by partial area under the curve (pAUC), net percent activity, and \(\log_{10}\) raw readout. Our assay had an optimal CV score for reproducibility (yellow).
**Correlation among assays.** The fourth parameter evaluated in the pilot study was redundancy among assays. Given the small number of hypotheses that could be tested in the primary analysis, it was reasonable to exclude assays that were correlated with one another. To control for the possibility that a low correlation could be due to poor intra-assay reproducibility, the analysis of correlations among assays used the quadruplicate data sets from the replicates analysis. Although many correlations were initially explored, a subset of comparisons using pAUC values for ADCC are shown (Fig. 5.6). The pAUC values for ADCC against target cells infected with HIV-1CM235 were compared against assays for IgG binding to recombinant gp120 (Fig. 5.6a), gp140 (Fig. 5.6b), and gp41 (Fig. 5.6c). ADCC activity was also compared with a magnitude-breadth (MG) index for the ability of these plasma samples to neutralize 27 different CRF01_AE viruses (Fig. 5.6d). Of particular interest was the relationship between ADCC assays that use infected versus recombinant gp120-coated CD4+ T-cells as targets (Fig. 5.6e). However, none of these comparisons generated significant correlations. Thus, measuring ADCC against virus-infected cells appears to be non-redundant with assays for IgG to recombinant forms of Env protein, including ADCC directed against gp120-coated cells, and virus neutralization.
Figure 5.6. Relationships between ADCC against infected target cells and other assays. The pAUC values for ADCC activity by blinded quadruplicate samples from HIV-1-infected people against target cells infected with the CRF01_AE virus HIV-1CM235 were compared against assays for IgG binding gp120 (a), gp140 (b), and gp41 (c), MB indices for neutralization of 27 different CRF_01AE isolates (d), and against an ADCC assay that uses CD4+ target cells coated with recombinant gp120 protein (e).

Measurement of ADCC for the primary analysis. Based on the pilot study data, the assay for ADCC against virus-infected cells was selected to be one of the 6 primary variables for the RV144 immune correlates analysis. The assays were performed over 10 days, each in parallel with a positive control sample from an HIV-1-infected Thai donor (Fig. 5.7). The average 50% ADCC titer for the positive control sample was 51,577, and the range was 31,237 to 81,954. Blinded samples collected at week 0 and week 26 from
41 vaccinees who became infected, 205 vaccinees who remained uninfected, 20 placebo recipients who became infected, and 20 placebo recipients who remained uninfected were tested for ADCC against cells infected with HIV-1<sub>92TH023</sub> (Fig. 5.8). In comparison to the HIV-1-positive donor plasma, the ADCC activity elicited in the RV144 is relatively weak. However, a subset of samples had vaccine-induced ADCC activity at week 26 that was detectable over baseline.

**Figure 5.7.** ADCC by positive control plasma tested in parallel with the RV144 case control plasma samples. The dashed line indicates 50% activity.
Figure 5.8. **ADCC by RV144 case-control plasma samples.** Plasma samples collected on week 0 (baseline) or week 26 (approximately 2 weeks after the last immunization) in the RV144 trial were evaluated for their ability to direct ADCC against target cells infected by HIV-192TH023. The dashed line indicates 50% ADCC activity. As of this writing, we remain blinded to the identities of the placebo versus vaccine recipients who became infected or remained uninfected. This figure is continued on the next page.
Figure 5.8 continued. This figure is also continued on the next page.
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**Figure 5.8 continued.** This is the last page containing the primary data for the case-control plasma samples.
**ADCC activity in infected versus uninfected vaccinees.** Measures of vaccine-induced immune responses for ADCC and the other 5 primary variables were ranked into high, medium, and low tertiles. The partial area between the curves (pABC) at weeks 0 and 26 for each RV144 participant was the primary variable for ADCC data. The low and medium pABC values for the vaccine group overlapped with the pABC values for the placebo group, suggesting that it would be reasonable to consider the low and medium responders negative for ADCC activity (Fig. 5.9). The univariate logistic regression analysis revealed a non-significant trend towards a lower risk of being infected at the end of the 42-month follow-up period in the high versus low ADCC groups (OR=0.60, 95% CI=0.24 to 1.48, P=0.27). Similar numbers were computed for a multivariate logistic regression analysis (OR=0.59, 95% CI=0.22 to 1.59, P=0.30). Although there was a trend towards a lower risk of HIV-1 infection in the high versus low ADCC groups, there was no statistical evidence for protection in RV144 participants with high versus low ADCC activity.

Rather than considering high versus low responders, the extent of ADCC activity was instead treated as a quantitative variable for univariate and multivariate logistic regression analyses of infection status at the end of the follow-up period. The univariate logistic regression analysis revealed no relationship (OR=0.92 per SD increase, 95% CI=0.62 to 1.37, P= 0.68, Q=0.68). The multivariate logistic regression analysis produced a similar outcome (OR=0.96 per SD increase, 95% CI=0.68 to 1.35, P=0.81, Q=0.81). Thus, treated as a quantitative variable, the ADCC activity measured in RV144 participants was unrelated to risk of infection.
Figure 5.9. ADCC activity for infected versus uninfected vaccine and placebo recipients. The pABC values for ADCC activity were ranked into low (open symbols), medium (shaded symbols), and high (solid black center) tertiles. Women (red outlines) and men (blue outlines) are indicated separately.

ADCC activity and HIV-1 infection rate. The incidence of HIV-1 infection over time was charted separately for placebo recipients, and for vaccinees in the low, medium, and high tertiles of ADCC activity (Fig. 5.10). Cox proportional hazards were computed to evaluate the significance of any relationship between ADCC activity and HIV-1 infection incidence over time. For the univariate Cox regression analysis, there was a non-significant trend towards a lower hazard ratio (HR) for HIV-1 infection among the high versus low ADCC tertiles (HR=0.60, 95% CI=0.24 to 1.46, P=0.26). Similar results were obtained for the multivariate Cox regression analysis (HR=0.59, 95% CI=0.22 to 1.59, P=0.30). Thus, there was a non-significant trend towards a lower infection rate for RV144 participants in the high versus low ADCC tertiles.

ADCC was treated as a quantitative variable in additional Cox regression analyses. However, neither the univariate (HR=0.94 per SD increase, 95% CI=0.67 to 1.33, P=0.74, Q=0.77) nor the multivariate (HR=0.92 per SD increase, 95% CI=0.62 to 1.37, P=0.69,
analyses suggested a relationship between infection rate and pABC values for ADCC as a quantitative variable. As observed for the logistic regression analysis of infection status at the end of the 42-month follow-up period, the rate of HIV-1 infection over this time period was also unrelated to ADCC activity when it was treated as a quantitative variable.

![Figure 5.10](image)

**Figure 5.10. Incidence curves for HIV-1 infection by ADCC activity tertile.** HIV-1 infection rates are shown for vaccinees with pABC values for ADCC in the high, medium, and low tertiles, and for placebo recipients. For vaccinees in the high versus low tertiles, HR=0.60 to 0.61, P=0.26 to 0.32.

**Interaction analyses with Env-specific IgA scores.** Since Env-specific IgA was correlated with risk, ADCC and the other 5 primary variables were analyzed in terms of IgA score. Statistically significant interactions were observed between IgA score and ADCC activity for both a logistical regression analysis, and for an analysis of HIV-1 incidence using Cox proportional hazards (for both, P=0.03, Q=0.1). Among participants above the 80th percentile for ADCC activity, higher IgA score increased the number of
infections over the full follow-up period (OR=1.96 per SD, 95% CI=1.22 to 3.14, 
P=0.01), and increased the HIV-1 infection rate (HR= 2.11 per SD, 95% CI=1.27 to 3.51, 
P<0.01). Furthermore, among participants with low IgA scores below 20th the percentile, 
those with higher ADCC activity (treated as a quantitative variable) had a lower number 
of infections (OR=0.55 per SD, 95% CI=0.31 to 1.00, P=0.05), and had a decreased rate 
of infection (HR=0.58 per SD, 95% CI=0.34 to 0.99, P=0.05). Therefore, with higher 
IgA eliminated as a risk factor, a borderline significant correlation between ADCC 
activity and protection was observed.

**Relationships among the primary variables.** Although one of the objectives of 
the pilot study was to identify assays that were not redundant with each other, these 
analyses were based upon samples from 6 HIV-1-infected donors. The potential 
redundancy of the 6 primary variables was therefore re-examined using the considerably 
larger data set of samples from RV144 vaccine recipients (Fig. 5.11). This analysis also 
serves the purpose of determining which immune responses were related among RV144 
vaccinees. In ascending order from the weakest relationship, indicated by the lowest 
Spearman rank coefficient, the \( R_s \)-values between ADCC activity and each other primary 
variable were IgA \( R_s=0.15 \), gp120 \( R_s=0.25 \), CD4\(^+\) T-cells \( R_s=0.27 \), gp70-V1V2 \( R_s=0.27 \), and neutralization \( R_s=0.44 \). Thus, in line with the objectives of the pilot study, weak 
correlations were observed between ADCC and the other primary variables. The weakest 
relationship was with IgA score, which was correlated with risk. The strongest 
relationship was with virus neutralization, another measure of antibodies that react with 
membrane-bound, trimeric Env.
Infected versus gp120-coated CD4⁺ target cells. The results of ADCC assays using CD4⁺ target cells that are infected with virus or coated with recombinant gp120 protein appear to differ. Although the analysis of correlates of infection and risk in the RV144 trial evaluated just 6 primary variables that were selected on the basis of results from the pilot study, the plasma samples were tested in numerous other assays. One of these was the recombinant gp120-coated CD4⁺ target cell ADCC assay⁵¹⁴. Whereas the multivariate Cox proportional hazards analysis revealed a non-significant trend towards a lower infection rate for vaccinees in the high versus low infected target cell ADCC tertiles (HR=0.59, 95% CI=0.22 to 1.59, P=0.30), the gp120-coated CD4⁺ target cell assay revealed a non-significant trend towards a higher infection rate for vaccinees in the...
high versus low ADCC tertiles (HR=2.00, 95% CI=0.69 to 5.78), P=0.20). Thus, in relation to the HIV-1 incidence curves, the trends for the virus-infected versus gp120-coated target cell ADCC assays were in opposite directions.

Antibodies that compete with the HIV-1 gp120-specific monoclonal antibody A32 appear to be responsible for a significant proportion of the ADCC activity measured using gp120-coated target cells\(^5\). The A32 Fab fragment (\textit{i.e.} no Fc region to interact with CD16) can block most ADCC activity by plasma from HIV-1-infected donors in the gp120-coated cell assay. The A32 Fab fragment also blocks a significant proportion of the ADCC activity by RV144 plasma samples against gp120-coated target cells (Guido Ferrari, unpublished observations). To address the hypothesis that A32 would block ADCC activity against HIV-1\(^{92TH023}\)-infected target cells in our ADCC assay, we tested week 0 and 26 plasma samples from 15 RV144 participants diluted 1:50 for ADCC in the presence of 10 \(\mu\)g per ml of the A32 Fab or a negative control Fab against respiratory syncytial virus (RSV) “Synagis” (Fig. 5.12). For quality control, the Thai HIV-1-infected donor plasma was titered in parallel, and its 50% ADCC titer of 52,250 was comparable to previous observations (Fig. 5.12 inset). The ADCC activity by this plasma sample diluted 1:50 (almost 90%) was not appreciably different in the presence of the A32 blocking Fab versus Synagis (Fig. 5.12). Since the positive control sample was known to have a 50% ADCC titer of approximately 50,000, we also tested the ability of A32 to block ADCC by a 1:50,000 dilution of the plasma. However, even when the antibody concentration was brought down to a level that mediated 50% ADCC activity, which may optimize the dynamic range for a blocking experiment, the A32 Fab had no appreciable effect. A moderate level of apparent blocking of ADCC by sCD4-IgG was
surprising, since A32 appears to enhance sCD4-IgG binding in the context of an ELISA\textsuperscript{181}. Only one RV144 plasma sample appeared to be blocked by the A32 Fab (614844). However, among the 10 samples selected for having detectable ADCC activity against HIV-1\textsubscript{92TH023}-infected target cells in the pilot study, 732807-603237, there was no evidence that A32 blocked ADCC activity. Thus, different epitopes appear to be important for ADCC activity against virus-infected versus gp120-coated target cells.

Figure 5.12. A32 Fab blocking experiment. Target cells infected with HIV-1\textsubscript{92TH023} were pre-incubated with the A32 Fab fragment, or with the Synagis Fab as a negative control. Fifteen plasma samples collected at weeks 0 or 26 during the RV144 trial (participant identification numbers 614844-603237) were tested for ADCC at a 1:50 dilution in the presence of each Fab. After addition of plasma, the final concentrations of each Fab were 10 μg per ml. A plasma sample from a Thai HIV-1 patient with a 50% ADCC titer of approximately 1:50,000 was titered in parallel as a positive control (inset). Assays with A32 or Synagis Fab fragments with no plasma, the positive control plasma at 1:50, the positive control plasma at 1:50,000, and sCD4-IgG at 10 μg per ml were also included. ADCC activity was calculated as 100 minus %RLU. Error bars indicate 1 SD for triplicate tests.
5. E. DISCUSSION

ADCC activity was evaluated as a primary variable in the immune correlates analysis of the RV144 HIV-1 vaccine efficacy trial. The rationale for including an ADCC assay as one of the 6 primary variables stemmed from the consistent detection of antibody responses in the absence of detectable neutralization of primary HIV-1 isolates\textsuperscript{379}. Thus, antibody activities other than neutralization have been suggested as potentially responsible for any vaccine protection in RV144\textsuperscript{522}. However, the ADCC assay based on an NK cell line and virus-infected target cells was selected over other candidate assays due to having low or zero baseline activity, the ability to detect a range of vaccine-elicited immune responses, high reproducibility, and non-redundancy with other assays included in the pilot study. Indeed, the data set generated in the case-control experiment replicated these characteristics.

The vaccine-elicited ADCC activity appeared to be relatively low. Whereas none of the RV144 samples reached 50% ADCC at the highest plasma concentration tested (a 1:32 dilution) in the absence of a high baseline, the positive control plasma from a Thai HIV-1-infected donor had a mean 50% ADCC titer of 51,577. Thus, the ADCC activity by the positive control plasma exceeded that of the highest vaccine-induced ADCC activity by at least 3 orders of magnitude. However, we have not surveyed the capacity of plasma from Thai HIV-1 patients to direct ADCC against cells infected with HIV-1\textsubscript{92TH023}, and the positive control plasma used here may represent an outlier. Extensive overlap was observed between the pABC values for ADCC measured in the vaccine versus placebo groups. However, this overlap was not attributable to high background.
Rather, it was observed since the vaccine-elicited ADCC activity was quite near the bottom of the dynamic range for detectable responses. The low level of ADCC activity by RV144 vaccine recipients against virus-infected cells is perhaps consistent with this assay measuring a physiologically relevant immune response. The reduction in infections among vaccine versus placebo recipients in RV144 was relatively modest (31%, 95% CI=1.7 to 51.8%, P=0.04)\textsuperscript{379}. Furthermore, no protection was observed in prior vaccine efficacy trials that included the recombinant protein component of the vaccine regimen tested in RV144\textsuperscript{357,358}. These vaccines did not elicit antibodies capable of neutralizing primary HIV-1 isolates. Thus, a low response for a modestly protective vaccine may be the expected outcome for a physiologically relevant assay.

The gp70-V1V2 and IgA binding assays may have been better suited for detecting antibody responses elicited in RV144 vaccinees than the assay for ADCC against virus-infected cells. In contrast to the overlap observed between placebo and vaccine recipients for ADCC against virus-infected target cells, only one placebo recipient was placed in the middle tertile for the gp70-V1V2 and IgA binding assays. Thus, these assays may have had greater power to measure differences among RV144 vaccinees than the ADCC assay.

Usage of different methods for analyzing the data affected the relationships observed among the primary variables and HIV-1 infection risk. Whereas a non-significant trend was noted towards a lower risk of HIV-1 infection among vaccinees in the high versus low tertiles of ADCC activity, similar trends were not observed in the corresponding analyses that treated ADCC activity as a quantitative variable. It is possible that attempting to estimate the change in risk of infection per standard deviation increase in ADCC activity was futile since the majority of the data points were negative.
Consequently, differences between the high versus low tertiles may have been more meaningful. However, there was a borderline significant relationship between ADCC activity treated as a quantitative variable and protection among the vaccinees with the bottom 20% of IgA scores (P=0.05), suggesting that the lack of any relationship in the other analyses that treated ADCC as a quantitative variable may be due to the inclusion of IgA responders. Therefore, there was a trend towards fewer infections among the RV144 participants with detectable versus negative ADCC activity, and a borderline significant increase in protection per increase in ADCC among vaccinees with low IgA scores.

Several of the assays used in the immune correlates analysis appear to measure distinct immune responses. Whereas gp70-V1V2 binding correlated with protection, IgA scores correlated with risk. Although vaccines with higher IgA did not have higher infection rates than placebo recipients, IgA or an associated response may have counterbalanced protective immune responses, or may be a surrogate for a non-protective immune response. It is possible that distinct cues that promote class switching to IgA may have led to the stimulation of Env-specific IgA at the expense of IgG, even though the immunizations in the RV144 trial were intramuscular, not mucosal. However, another plausible explanation is that IgA is a marker for previous exposure to HIV-1. Virus-specific IgA responses have been detected in HIV-1-exposed, uninfected people. It is possible that previous HIV-1 exposure may have primed IgA responses that were boosted in the RV144 trial. Indeed, IgA binding to HIV-1 gp120 correlated with behavioral risk (P=0.034). Also, whereas we noted a non-significant trend towards lower risk of infection among vaccinees in high versus low tertiles of ADCC activity against
virus-infected cells, another group noted a non-significant trend towards increased risk of infection associated with higher ADCC against gp120-coated CD4$^+$ target cells. One potential explanation for this surprising result could be that gp120 binds to CD4 on the target cells, thus occluding the CD4-binding site. RV144 participants who made antibodies to the CD4-binding site may have had lower scores for ADCC against gp120-coated CD4$^+$ target cells than against virus-infected cells. Also in agreement with the existence of differences between ADCC assays based on virus-infected versus gp120-coated CD4$^+$ target cells, the A32 Fab was capable of blocking most ADCC activity in the gp120-coated cell assay but not in the virus-infected cell assay. Furthermore, A32 targets an epitope in constant region 1 (C1)$^{515}$, and there was a non-significant trend towards greater risk of infection among vaccinees with higher antibody responses to C1. The opposing trends exhibited by ADCC against virus-infected cells versus IgA scores and ADCC against gp120-coated target cells suggest that these assays measure distinct antibody responses.

Although the ADCC activity by RV144 vaccinees against virus-infected cells was relatively low, there were non-significant trends towards lower infection risk in vaccinees in the high versus low tertiles of ADCC activity. Among vaccines who were low or negative for the risk factor of Env-specific IgA index, there was a borderline significant correlation between ADCC activity and protection. Therefore, when higher IgA is eliminated as a risk factor, ADCC activity may be a correlate of protection. These observations, and the metric properties of the ADCC assay that led to its selection as one of the primary variables, including its non-redundancy with other assays, suggest that immune responses elicited in future HIV-1 vaccine clinical trials ought to be measured
using this assay. Taken together with the observation that antibodies against gp70-V1V2 correlated with protection, these results suggest that antibody functions other than neutralization may have contributed to vaccine protection in RV144.
CHAPTER 6

Conclusions
6. Conclusions

*Data support a role for antibodies in vaccine protection*

Evidence presented in this dissertation supports a role for antibodies in vaccine protection, even when neutralization is not detectable. We have generated data suggesting that antibodies contribute to vaccine protection against SIV and HIV-1 infection in the absence of detectable virus neutralization. The degree of immune protection afforded by live-attenuated SIV against SIV_{mac251} challenge increases over time\textsuperscript{345,347}, coinciding with the increase over time in ADCC activity. SIV_{mac239\Delta nef} routinely provides complete or apparent sterilizing protection, whereas scSIV does not\textsuperscript{159}, and we show that SIV_{mac239\Delta nef} elicits antibodies with significantly higher ADCC activity than scSIV. Live-attenuated SIV strains that replicate at higher levels confer greater protection than those that replicate at lower levels\textsuperscript{347,352}. Consistent with this inverse relationship between the degree of attenuation and protection, we show positive correlations between the extent of SIV\Delta nef replication and ADCC activity. Furthermore, the greater protection provided by live-attenuated SIV against homologous versus heterologous SIV challenge\textsuperscript{418,420} may be due, at least in part, to the reduced cross-reactivity we observed against Env-mismatched SIV strains. These differences in ADCC activity mirror differences in protection by live-attenuated SIV. However, most compellingly, in two different animal experiments, we show that measures of ADCC activity correlate with complete protection against SIV_{mac251} in macaques inoculated with live-attenuated SIV. Antibodies capable of neutralizing the challenge viruses were low to undetectable among these animals. These observations suggest that the antibodies that direct ADCC contribute to protection by live-attenuated SIV.
The results of the immune correlates analysis of the RV144 trial are consistent with a role for antibodies in vaccine protection against HIV-1 infection, despite the absence of detectable virus neutralization. We observed a non-significant trend favoring RV144 participants in the high versus low tertile of ADCC activity for lower risk of HIV-1 infection. Furthermore, there was borderline significant protection for vaccinees with higher ADCC activity when vaccinees with high Env-specific IgA are eliminated. Taken together with the significant correlation of higher V1V2-specific antibodies with lower risk of HIV-1 infection, these data support a role for antibodies in vaccine protection against HIV-1 infection. Since neutralizing antibodies were undetectable, these results suggest that functions of antibodies other than neutralization may have contributed to any modest vaccine protection against HIV-1 infection in the RV144 trial.

**Previous evidence antibodies contribute to protection in the absence of detectable neutralization.** Previous observations provided the rationale for embarking on the study of antibodies in the absence of detectable neutralization. HIV-1 appears to preemptively escape neutralizing antibody responses, suggesting that sub-neutralizing antibody titers select for escape mutants. However, the time-dependence of protection by live-attenuated SIV against SIV$_{mac251}$ infection provided the most important suggestion of antibody-mediated protection in the absence of detectable neutralization. The affinity maturation of antibody responses over time is a well-characterized phenomenon. Thus, although most animals protected from infection with SIV$_{mac251}$ lack detectable antibodies capable of neutralizing this challenge virus, the maturation of immunity on the timescale of months nonetheless appeared to be
consistent with an antibody response. In addition, the notion that neutralizing antibodies were perhaps less cross-reactive than T-cell responses against a heterologous virus, coupled with the incomplete protection against challenge with this heterologous virus, were consistent with a role for antibodies in protection by live-attenuated SIV. Although the presence of antibody responses in the absence of significant CD8⁺ T-cell responses in RV144 was also consistent with a protective role for antibodies, RV144 did not contribute to the rationale for this project. We had developed the ADCC assay to address the hypothesis that ADCC is a correlate of protection by live-attenuated SIV before the results of the RV144 trial were announced. Indeed, in July of 2009 we submitted our abstract for the 2009 Symposium on Nonhuman Primate Models for AIDS, where we first publically presented the ADCC assay, whereas the RV144 results were first announced in September of 2009. Other published observations, including studies that emerged after we began work on this project, support a role for antibody responses in protection against SIV and HIV-1 in the absence of detectable neutralizing antibodies.

*Difficulty of eliciting antibodies functional against SIV and HIV-1*

**Observations on the development of antibody responses.** We have coupled studies aimed at identifying an immune response that contributes to vaccine protection with experiments that gain insight into its induction. The progressive increases in ADCC activity over 21 weeks after inoculation with SIVmac239Δnef suggest that the stimulation of antibody responses is continuous, and stand in contrast to the relatively low ADCC activity elicited by the transient stimulation of antibody responses by scSIV. A
comparison of the vaccine-strain viral loads for SIV\textsubscript{mac239Δnef} versus scSIV suggest that sustained antigenic stimulation is necessary to elicit high ADCC titers by vaccination. The ALVAC-AIDSVAX B/E vaccine regimen used in RV144 may be analogous to scSIV in providing transient antigenic stimulation rather than the persistent stimulation that appears to be required to develop high ADCC titers. These differences are probably due to a requirement for extensive somatic hypermutation over a period of months. Furthermore, a longer time period (\textit{i.e.} 6 versus 22 weeks) was required to elicit antibodies with similar ADCC titers against heterologous SIV strains. Thus, our observations suggest that, going forward, experimental HIV-1 vaccines that provide only transient antigenic stimulation will fail to elicit high and broadly reactive ADCC titers against primary HIV-1 isolates. However, our ability to detect an antibody activity that is functional at high titers against divergent neutralization-resistant viruses suggests that it may be possible to elicit antibodies that are effective against circulating HIV-1 isolates through novel vaccine strategies.

The magnitude of antigenic stimulation appears to affect the induction of antibody responses. Due to animal-to-animal variation, differences were observed in the extent of SIVΔnef replication over a fixed time period. The positive correlation between AUC values for SIVΔnef viral loads over weeks 21-22 after inoculation and the ADCC activity measured at the end of this time period suggests that the magnitude of antigenic stimulation drives the development of Env-specific antibody responses. Furthermore, the 50\% ADCC titers against the Env-mismatched virus among the animals with the highest SIVΔnef replication were equivalent to the 50\% ADCC titers against the Env-matched virus among the animals with the lowest SIVΔnef replication. Therefore, a higher dose of
antigen may elicit higher ADCC titers, and may help to overcome the reduction in antibody cross-reactivity associated with antigenic variation in Env.

**Difficulty of eliciting neutralizing antibodies.** Several passive transfer studies have clearly demonstrated that neutralizing antibodies can prevent SHIV infection in macaques. Therefore, a vaccine capable of eliciting broadly neutralizing antibodies could potentially end the HIV-1 pandemic. However, these passive transfer studies suggest that a relatively high concentration that is capable of neutralizing 100% of viral infectivity at low dilutions (i.e. close to the undiluted concentration present in vivo), or neutralizing 50% of virus infectivity at a 1:100 dilution, would be the minimum necessary to consistently prevent infection. Thus, high titer broadly neutralizing antibodies could theoretically provide consistent protection against HIV-1 infection. However, the induction of antibodies able to potently neutralize a broad range of neutralization-resistant t/f HIV-1 isolates seems unrealistic at the present time.

The difficulty of eliciting neutralizing antibodies stems from the inherent resistance of primate lentiviruses to neutralization. These viruses possess Env proteins have evolved structural and thermodynamic properties that enable continuous replication and transmission to new hosts in the face of intense antibody responses. No vaccine approach under consideration for use in humans has successfully elicited antibodies capable of neutralizing primary isolates of HIV-1 or SIV. Nevertheless, antibodies capable of neutralizing autologous HIV-1 primary isolates at titers over 100 often develop months after infection. However, antibodies capable of neutralizing a broad range of heterologous HIV-1 isolates are
considerably less frequent, and arise over a time scale of years\(^{191,285,305,306,310,490}\). Therefore, in the context of natural HIV-1 infection, a considerable period of time is required to develop neutralizing antibody titers that may be capable of conferring protection against infection by circulating primary HIV-1 isolates.

HIV-1 Env-specific antibody responses in general, but especially antibodies capable of neutralizing a broad range of HIV-1 isolates, exhibit unusually extensive somatic hypermutation\(^{191,326-328,330,331}\). An estimated nucleotide substitution rate for somatic hypermutation of \(10^{-3}\) mutations per round of cell division\(^{313}\) appears consistent with highly mutated neutralizing antibodies emerging over the observed time frame of months to years. Therefore, the apparent requirement for extensive somatic hypermutation over an extended period of time represents a significant obstacle to the induction of broadly neutralizing antibodies by a vaccine.

*The problem with correlating antibody responses with protection*

**Counterbalancing factors on viral loads and antibody responses.** Measuring an immune response or genotype, and attempting to correlate it with lower viral loads, higher CD4\(^+\) T-cell counts, or other clinical markers, is a typical premise for pathogenesis studies aimed at identifying correlates of protection\(^{551}\). However, our observation that higher vaccine strain viral loads are associated with higher ADCC titers may complicate studies based on the above premise. Whereas these antibodies may contribute to the control of viral loads, their stimulation also appears to be proportional to virus replication. Clearly, there may be a point of diminishing returns in the context of pathogenic SIV, HIV-1 or SHIV infection, beyond which higher viral loads interfere with the induction of
antibody responses\textsuperscript{366,369,389-393}. In the context of live-attenuated SIV, in which viral replication is decoupled from CD4\textsuperscript{+} T-cell depletion\textsuperscript{58,525,526}, we did not observe such an inflection point. Others have also observed a positive correlation between antibody responses and viral loads\textsuperscript{309,310,552}. This dynamic interaction between viral replication and its suppression by or stimulation of antibody responses may obfuscate attempts to correlate antibody responses with protection in pathogenesis studies.

\textit{Sensitivity of assays for ADCC versus neutralization}

\textbf{Proportion of Env bound by antibody.} The simplest explanation for the sensitivity of ADCC versus neutralization assays is probably the most significant. Neutralization of SIV and HIV-1 may be related to antibody occupancy of Env trimers\textsuperscript{122,553}. Wild-type SIV\textsubscript{mac}239 has been estimated to possess 7 to 16 Env trimers per virion\textsuperscript{443}. It is possible that in a state of dynamic equilibrium between antibodies and Env, most or all of the trimers need to be constantly bound by antibody in order to detect virus neutralization. Perhaps consistent with this premise, increasing the number of spikes per virion by truncating the cytoplasmic tail of Env increases the neutralization resistance of SIV\textsubscript{mac}239\textsuperscript{249}. During a neutralization assay, HIV-1 or SIV have many hours to achieve attachment and the steps necessary for entry. Therefore, occupying 50\% of Env trimers at any moment may not be sufficient to observe neutralization. Although the number of crosslinked CD16 molecules necessary to trigger ADCC is unknown, it seems reasonable that the minimum proportion required to crosslink CD16 may be considerably lower than is required to detect neutralization. Therefore, the most significant factor contributing to
the greater sensitivity of assays for ADCC versus neutralization is probably that lower antibody occupancy is necessary for ADCC than neutralization.

**The special case of CD4 and coreceptor binding site antibodies.** The CD4 binding site may be the most important antibody epitope for an HIV-1 vaccine. It must be available to some extent for receptor engagement, and also is relatively conserved in sequence. These factors alone make the CD4 binding site an ideal antibody epitope to target in a vaccine. However, antibodies that bind the CD4-binding site often do not neutralize virus infectivity, and soluble forms of CD4 poorly neutralize primary isolates, if at all\textsuperscript{209,217}. Kwong et al. suggest that the poor neutralization of primary isolates by CD4 binding site antibodies and sCD4 owes to a neutralization resistance mechanism that has evolved to discriminate between antibodies in solution versus membrane-bound CD4 molecules on the surface of a CD4\textsuperscript{+} T-cell\textsuperscript{217}. The observation that inspired this model was that dodecameric sCD4 neutralized the infectivity of primary isolates at titers that were least 2 orders of magnitude higher than monomeric sCD4. Kwong et al. propose that high avidity interactions are necessary to overcome the entropy cost associated with adopting the CD4-bound conformation. Thus, avid interactions with dodecameric CD4, or membrane-bound CD4 molecules restricted to a 2-dimensional plane on the surface of a CD4\textsuperscript{+} T-cell, overcome the thermodynamic cost of the conformational rearrangements necessary for stable CD4 binding, whereas soluble CD4 or antibodies directed to the CD4 binding site diffuse away more readily and do not efficiently induce these conformational changes.
The mechanism proposed by Kwong et al. for HIV-1 to discriminate between soluble antibodies versus CD4 molecules on CD4\(^+\) T-cells may not confer comparable resistance to ADCC. CD4 binding site antibodies bound by CD16 on the surface of an NK cell would similarly be localized to a 2-dimensional plane, and may mimic membrane-bound molecules on the surface of a CD4\(^+\) T-cell. Thus, a mechanism that has evolved to discriminate between soluble antibodies versus CD4 localized to a 2-dimensional plane may not effectively discriminate between a CD4\(^+\) T-cell and an NK cell armed with CD4 binding site antibodies. In addition, coreceptor binding site antibodies bound by CD16 may cooperatively facilitate conformational changes in gp120. Thus, the thermodynamically unfavorable conformational changes necessary for CD4 and coreceptor binding may confer greater resistance to neutralization than ADCC. Therefore, in comparison to antibodies targeting other epitopes, CD4 and coreceptor binding site antibodies may have disproportionately greater capacity to direct ADCC than to neutralize viral infectivity.

**Viral debris, shed gp120, or misfolded forms of Env.** At first, we were concerned that the sensitivity of the ADCC assay relative to neutralization assays was potentially problematic. It might be reasonable to expect neutralization to be detectable for any antibody capable of binding the native, trimeric form of Env that exists on virions and virus-infected cells. Therefore, detection of ADCC at dilutions of antibody a couple of orders of magnitude higher than neutralization, in a range that would be more typical for ELISA assays using recombinant gp120 protein, might suggest the ADCC assay measures misfolded forms of Env, shed gp120, or viral debris. However, other
explanations appear likely to account for our ability to detect intact Env trimers at lower antibody concentrations than are capable of neutralizing virus infectivity. Vaccine recipients in the RV144 trial were immunized with recombinant gp120, and consistently made antibody response capable of binding to recombinant forms of gp120, but did not have particularly robust ADCC activity against virus-infected cells. Therefore, the relatively low ADCC activity in RV144 vaccinees suggests that shed gp120 may not be a significant target for ADCC activity. In assays using plasma from macaques, our use of SHIV-infected cells controls for ADCC activity against viral debris, since SIV-infected macaques make antibodies against Gag and other non-Env viral proteins. Indeed, ADCC activity can be observed by SIV plasma against SHIV-infected cells if the target cells are not washed prior to the ADCC assay (data not shown). It is possible for ADCC targeting viral debris on bystander infected and uninfected cells may have a role in vivo, but we are not measuring it. Although it is possible that misfolded forms of Env contribute to ADCC activity, the presence of antibody-sensitive targets on cells infected with neutralization-resistant viruses would be inconsistent with our understanding of the immune evasion strategies of SIV and HIV-1. It seems likely that properly folded, trimeric forms of Env account for most of the ADCC activity.

Closing remarks

**Antibodies in vaccine protection.** Our data on the ADCC activity in the contexts of live-attenuated SIV and the immune correlates analysis of the RV144 trial converge in support of a role for antibodies in vaccine protection against SIV and HIV-1 infection. Previous SIV and HIV-1 vaccine studies have typically focused on neutralizing
antibodies as the only measured activity of functional antibodies. Various vaccine approaches that fail to elicit antibodies detectable in assays for neutralization of primary isolates are able to elicit antibodies detectable in binding assays or that neutralize T-cell line-adapted viruses. However, it is unclear whether antibodies detected in these assays are capable of mediating any antibody functions against neutralization-resistant primary isolates, since recombinant forms of gp120 display conserved epitopes absent from intact trimers, and the overlap between antibodies that neutralize T-cell line-adapted viruses versus those that mediate activities other than neutralization against neutralization-resistant primary isolates seems likely to be partial. In contrast, we have developed and employed an assay that measures antibodies functional against neutralization-resistant primary isolates, in the absence of detectable neutralization. Using this assay, we show that the ADCC activity elicited by SIVΔnef mirrors features of protection by live-attenuated SIV, and correlates with protection against SIVmac251 infection. Due to the metric properties of the ADCC assay, it was selected as one of the primary variables for the immune correlates analysis of the RV144 trial. Non-significant trends were observed in the direction of RV144 participants having a lower risk of HIV-1 infection if they were in the high versus low tertiles of ADCC activity. Other laboratories participating in this collaborative study identified IgG antibodies to V2 as a correlate of protection, and IgA antibodies to Env as a correlate of risk (Haynes et al., in review). A borderline significant correlation between ADCC and protection was observed when vaccinees with Env-specific IgA responses were excluded. These data support a role for antibodies in vaccine protection against SIV and HIV-1 in instances when neutralization is undetectable.
**Multiple immune responses in vaccine protection.** The induction of T-cell and antibody responses in combination is probably necessary to confer vaccine protection against HIV-1 infection. Although an extraordinarily high concentration of neutralizing antibodies appears to be required to prevent infection by passive transfer of antibody to naïve macaques\(^{410-414}\), animals immunized with live-attenuated SIV are protected from infection by SIV\(_{mac251}\) despite having low to undetectable neutralizing antibody titers\(^{345,352}\). The immune system is highly integrated, and different arms of the immune response confer vaccine protection in combination\(^{407}\). Therefore, the contrast between the high titer of neutralizing antibodies that is required to confer complete protection in naïve macaques, and the low to undetectable neutralization by antibody responses we have correlated with protection by live-attenuated SIV, suggests that a combination of antibodies and T-cells elicited by live-attenuated SIV mediate protection.

**Nature, quality, and quantity of antibodies necessary for vaccine protection.**

The 2010 scientific strategic plan of the Global HIV Vaccine Enterprise articulated the fundamental problem that we lack basic insights into the nature, quality, and quantity of the immune responses necessary to confer vaccine protection\(^{400}\). The data presented in this dissertation permit a commentary on this topic. Live-attenuated SIV suggests that in the context of a vaccine that also elicits both CD4\(^+\) and CD8\(^+\) T-cell responses\(^{525-527}\), detectable virus neutralization may not be a requirement for vaccine protection. This observation appears to be encouraging, since years of persistent infection elicits broadly neutralizing antibodies in only a minority of HIV-1 patients\(^{305,306,309-311}\). However, we
observed a wide range of ADCC activity for samples with sub-neutralizing antibody titers. Persistent stimulation of Env-specific antibody responses with SIVΔnef elicited significantly higher ADCC activity than transient stimulation of antibody responses with scSIV. Although antibodies may contribute to vaccine protection in the absence of detectable virus neutralization, those that correlated with protection against SIVmac251 were relatively potent after a 20-46 week maturation period. Antibody responses appear to correlate with vaccine protection from HIV-1 infection in the RV144 trial, although the vaccine-elicited immune responses did not reliably prevent HIV-1 infection. Thus, the level of ADCC activity observed in the RV144 trial is below the potency that may be necessary to consistently confer vaccine protection against HIV-1 infection. Therefore, antibody responses with ADCC activity that is at least as high as that observed 20 to 46 weeks after inoculation with SIVΔnef, and that is significantly higher than that elicited by scSIV or in the RV144 trial, may contribute to reliable vaccine protection against HIV-1 infection.

**Induction of effective antibody responses.** The data presented here provide guidance for HIV-1 vaccine development by helping to understand the type of stimulation that is necessary to elicit antibody responses that are functional against neutralization-resistant lentiviruses. The progressive increases in ADCC activity over time after inoculation with SIVΔnef, the significantly higher ADCC activity elicited by SIVΔnef versus scSIV, and the longer time period required to develop comparable ADCC titers against Env-mismatched viruses suggest that the persistent stimulation of Env-specific antibody responses may be essential for the induction of high ADCC activity.
against circulating HIV-1 isolates. Higher magnitude antigenic stimulation also appeared to increase the ADCC activity elicited over a fixed time period. The transient stimulation of Env-specific antibody responses in the RV144 trial elicited low to undetectable levels of ADCC activity. Therefore, the induction of antibodies that remain functional in the face of antigenic diversity requires Env-specific antibody responses to be stimulated for a relatively long period of time. In addition to being essential for their induction, persistent antigenic stimulation is also likely to be necessary to prevent the waning of vaccine-elicited T-cell and antibody responses over time. The data presented in this dissertation suggest that the antibodies capable of directing ADCC may contribute to vaccine protection against SIV and HIV-1 infection, but also suggest that persistent antigenic stimulation is necessary to elicit antibodies with high and broadly reactive ADCC activity.
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Envelope-Modified Single-Cycle Simian Immunodeficiency Virus Selectively Enhances Antibody Responses and Partially Protects against Repeated, Low-Dose Vaginal Challenge

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