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Dissecting the Humoral Immune Response to Simian Immunodeficiency Virus

Mechanisms of Antibody-Mediated Virus Neutralization

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Abstract

The ultimate goal of an AIDS vaccine is to elicit potent cellular and humoral immune responses that will result in broadly enduring protective immunity. During the past several years, we have focused on characterizing the quantitative and qualitative properties of the antibody response, principally working to define the mechanism(s) of antibody-mediated neutralization in vitro. We have utilized a panel of monoclonal antibodies generated from monkeys infected with attenuated SIV for more than 8 mo to dissect the early events of virus infection involved in antibody-mediated neutralization. Presented herein are highlights from our studies that have identified potential mechanisms by which antibodies neutralize SIV in vitro.

Key Words

Neutralization
Antibody
Surface plasmon resonance
SIV envelope
Vaccine

Introduction

Human immunodeficiency virus (HIV) infection of humans results in destruction of the immune system, specifically CD4+ T cells, ultimately leading to acquired immunodeficiency syndrome (AIDS). Efforts to study HIV infection in humans are hampered by the relatively mild symptoms associated with initial infection; as a result, the majority of individuals are not immediately aware of their HIV status, making analysis of the

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early events of infection difficult. SIV infection of rhesus macaques provides a model that closely resembles human immunodeficiency virus type 1 (HIV-1) infection of humans regarding modes of transmission, the pattern of virus replication, the clinical course of disease progression, and the nature of the virus-specific immune response *(1,2).* The SIV/macaque model provides the needed advantage over HIV infection of humans such that monkeys can be experimentally infected with well-characterized strains of virus and the progression of infection/disease can be followed by sampling blood and/or tissues at proscribed timepoints, especially early post-infection, over a shorter time period.

One of the highest priorities in AIDS research is the development of a vaccine to elicit potent cellular and humoral immune responses that will result in enduring, broadly protective immunity. Much effort has focused on elucidating the mechanisms and specificity of cellular immune responses in the SIV/macaque model, demonstrating the role of cytotoxic T lymphocytes (CTLs) in mediating viral control during the primary infection and in maintaining the viral setpoint in chronic infection *(3,4).* While less is known about virus-specific antibody responses, passive protection experiments have demonstrated the protective efficacy of antibodies against virus exposure in the SIV/monkey *(5–8)* and SHIV/monkey models *(9–15),* demonstrating the ability of antibody alone to mediate sterile protection against pathogenic virus challenge. This review highlights the work in our lab to understand the quantitative and qualitative properties of antibody responses associated with the development of protective immunity in the SIV/macaque model for AIDS vaccine development, specifically focusing on defining the mechanism(s) by which antibody neutralizes SIV in vitro.

Identification of Prolonged Antibody Maturation in SIV Infected Rhesus Macaques

Our initial studies built on previous work which demonstrated that inoculation of rhesus macaques with attenuated SIV resulted in broadly protective immunity when challenged with pathogenic virus at 8 mo post-vaccination but not when challenged at 5 mo post-vaccination *(6).* Additionally, with limited assessment of serological parameters, Clements et al. identified an evolution of SIV-specific antibody that appeared to parallel the development of protective immunity. Following these insights, we designed studies to extensively characterize, both quantitatively and qualitatively, the antibody response raised by rhesus macaques in response to infection with SIV. To this end, we chose a panel of assays with which to characterize the antibody response, including (i) antibody titer to native viral envelope glycoproteins, (ii) stability of the antibody-envelope interaction to treatment with 8 *M* urea (i.e., antibody avidity), (iii) reactivity of antibody to conformational and linear envelope determinants (i.e., conformational dependence), and (iv) antibody neutralization. The application of this panel of assays to longitudinal serum samples taken from macaques infected with attenuated SIV led to the identification of a lengthy, complex maturation of the viral envelope-specific antibody response (Fig. 1) *(16).* This antibody maturation profile was characterized by a rapid increase in antibody titer concomitant with a gradual increase in antibody avidity and decrease in antibody conformational dependence. In addition, early peak levels in virus neutralization at 2–3 mo were followed by a

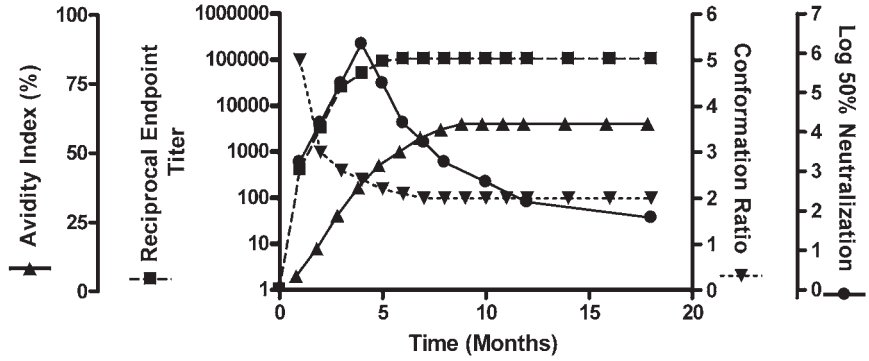

Fig. 1. Envelope-specific antibody maturation profile in monkeys infected with SIV. Representative schematic of the evolution of envelope-specific antibody responses in SIV infection determined by a panel of serological assays that include the quantitative measurement of antibody titer and the qualitative measurements of antibody avidity, conformational dependence, and neutralization. Antibody endpoint titers emerge and reach peak, steady-state levels by 2–3 mo post-infection. In contrast, antibody avidity and conformation ratios gradually evolve during the first 6–8 mo, achieving steady-state levels that are then sustained throughout infection. Neutralizing antibody responses to the attenuated strain emerge and peak early in infection, then gradually decrease over time. Antibody responses early in infection that are still undergoing changes in qualitative properties are defined as immature, while antibody responses later in infection that have achieve a steady-state level in qualitative properties have been defined as mature *(16,21).*

decrease to steady-state levels. The trend in neutralization elicited by the attenuated vaccine strain was similar for both the homologous and the heterologous challenge viruses, and did not generally correlate with the ability of the animal to be protected from pathogenic challenge *(6,16).* Most important, this antibody maturation profile is associated with the generation of broadly protective immunity to pathogenic challenge, and the assays were also able to identify striking differences between protective and non-protective immune responses *(6,8,16–19).* Similar antibody maturation profiles have been observed in other lentiviral systems including equine infectious anemia virus (EIAV) *(20),* simian/human immunodeficiency virus (SHIV) *(21),* and human immunodeficiency virus (HIV) *(21,22).* Based on this observed maturation of the immune system associated with protective immunity, we isolated monoclonal antibodies (MAbs) from protected monkeys in an effort to further characterize the protective SIV-specific antibody response *(23,24).*

Characterization of Monoclonal Antibodies Derived from Rhesus Macaques Infected with Attenuated SIV

We generated a unique panel of MAbs derived from the peripheral blood of four rhesus macaques infected with attenuated SIV/17E-CL for more than 8 mo, the timepoint at which the envelope-specific polyclonal antibody response had achieved maturation and the monkeys were protected from a pathogenic virus challenge *(6,16, 23,24).* Thus, these MAbs are reflective to some degree of the protective immune response generated to attenuated SIV infection. While the majority of these antibodies recognize conformational determinants on gp120, linear epitopes in V1, V2, V3, and the

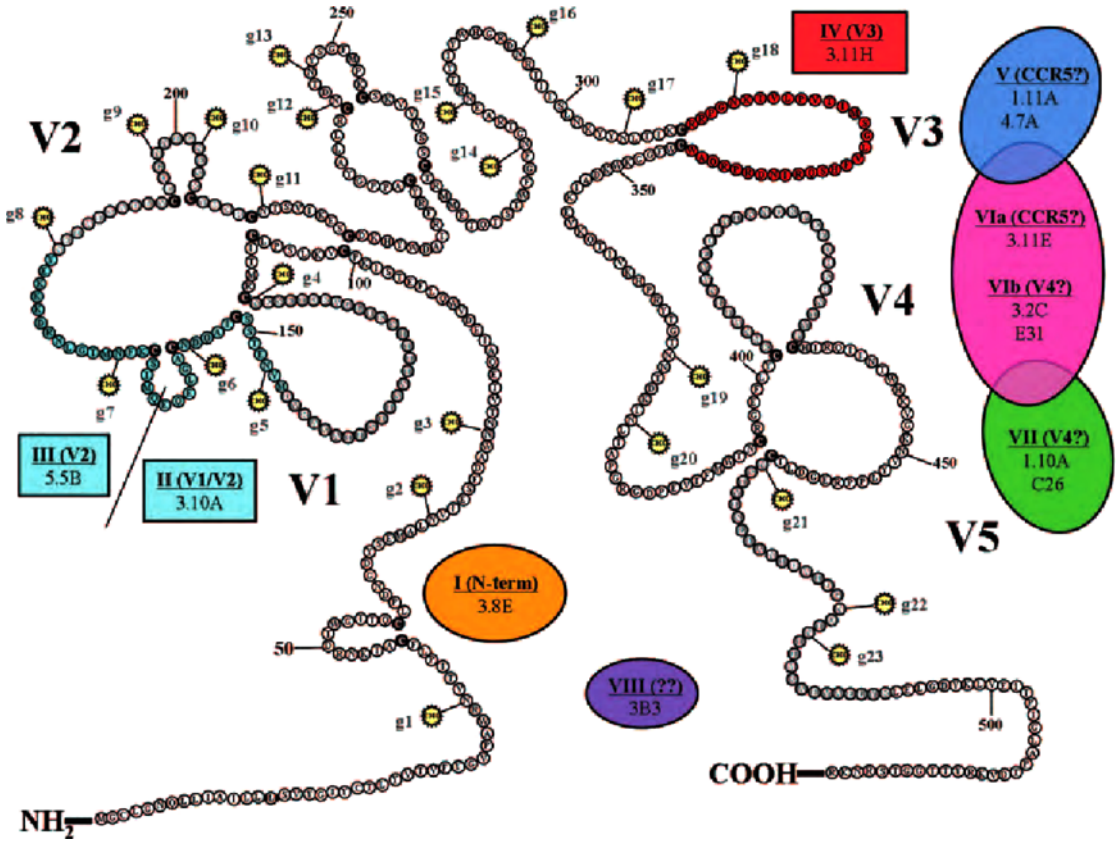

Fig. 2. Schematic representation of rhesus monoclonal antibody binding to SIV gp120. The known/proposed binding regions for rhesus MAbs are shown superimposed on a predicted secondary structure of SIV/17E-CL gp120. Drawing of the disulfide bonds is based on the hypothetical assignment of intrachain disulfide bonds for HIV-2 and SIV *(39),* the known disulfide bonds for HIV-1 *(40),* and the structural model for SIV envelope glycoproteins *(41).* The variable regions were based on the designations defined by Choi et al. *(25),* and the V3 region corresponds to the analogous V3 loop of HIV-1. Potential N-linked (N-X-S/T) glycosylation sites are indicated by the addition of a stalked-CHO symbol at the asparagines (N) residue, and are numbered g1–g23. MAbs reacting to linear epitopes were defined by reactivity to peptides in ELISA are indicated in rectangles and are shaded in the linear sequence *(24);* MAbs recognizing conformational epitopes are indicated in ovals.

C-terminal region were identified using synthetic, overlapping 20mer peptides. Cross-competition analysis using whole MAbs revealed that this panel identified at least nine binding domains on gp120, with several of these domains likely representing closely related but distinct binding epitopes (Fig. 2). Attempts to map the conformational epitopes recognized by these antibodies revealed several competition groups that bound a protease digestion fragment of gp120 containing the V3 through V5 regions. In addition, two competition groups demonstrated sensitivity to defined mutations in the V4 region previously identified to be associated with neutralization resistance *(25).* Finally, we identified three competition groups (two of which demonstrated V4 sen-

sitivity) that exhibited potent neutralizing activity against the homologous SIV/17E-CL strain using a standard neutralization assay, monitoring for CPE or p27 antigen at d 10 after infection *(24).* Recent analysis using a more sensitive assay identified the ability of an additional MAb, 3.11H, to neutralize SIV/17E-CL in vitro *(26).*

Following the initial MAb characterization, we performed analyses to tease out the method by which the MAbs neutralize SIV in vitro. It has been established that one common mechanism of neutralization exhibited by envelope-specific antibodies is the ability to block the primary receptor, CD4 *(27–32).* While several antibodies capable of inhibiting CD4 binding have been derived from immunizing mice for relatively short periods of time with recombinant SIV envelope proteins *(33,34),* none of the rhesus MAbs generated to date have blocked SIV gp120-CD4 binding by ELISA *(23,24).* Because the MAbs do not block CD4 binding yet manage to neutralize virus, we performed assays to determine if neutralizing MAbs inhibited fusion. We found that neutralizing MAbs inhibited cell-mediated fusion in a CD4-independent manner (i.e., MAbs inhibited envelope-coreceptor-mediated cell-to-cell fusion in the absence of CD4), suggesting that neutralization may be related to inhibition of the envelope-coreceptor and fusion interactions (unpublished data). To address this, we directly demonstrated the ability of neutralizing MAbs to inhibit gp120 binding to CCR5 expressing cells by flow cytometry (unpublished data). Together, these analyses suggest that one mechanism of neutralization by these MAbs lies at the level of fusion. Furthermore, neutralizing MAbs, including V3-specific MAbs, are likely to bind a cluster of closely related, potentially overlapping epitopes in or near the coreceptor binding site outside the V3 region of gp120.

Binding Rates, not Affinity, Distinguish Between Phenotypically Distinct Strains of SIV

The panel of rhesus MAbs described above bound with similar efficiency to envelope proteins from various strains of SIV in a number of solid phase assays, including (i) viral envelope proteins expressed on cells infected with several strains of SIV (including SIV/17E-CL, SIVmac239, SIVmac251 and SIVdeltaB670) *(23,24),* (ii) SIV/17E-CL soluble gp140 trimers produced in a mammalian system in a ConA ELISA, and (iii) soluble gp120 monomers produced in a baculovirus expression system (SIVmac251) or viral gp120 (SIV/17E-CL) in nonreducing Western blot analyses. Using a selected panel of MAbs representing each of the competition groups, we performed real-time binding studies using surface plasmon resonance (SPR) as measured on a Biacore 3000 to determine the kinetics of MAb binding to recombinant, trimeric SIV gp140 proteins *(26).* Owing to the observed lack of correlation between the ability of MAbs to bind envelope and their ability to neutralize SIV/17E-CL in vitro, we proposed that differences might exist in the binding kinetics between neutralizing and nonneutralizing MAbs that would correlate with the ability of a given MAb to neutralize SIV. However, results from these analyses failed to distinguish between neutralizing and nonneutralizing MAbs based on rates or affinity of binding.

Additionally, we sought to determine whether sensitivity to neutralization of SIV strains in vitro could be related to differences in MAb binding. To accomplish this, we compared properties of rhesus MAb binding to the neutralization-resistant SIVmac239 rgp140 with the neutralization-sensitive SIV/17E-CL rgp140 in kinetic SPR analyses *(26).* In general, we observed significant differences in

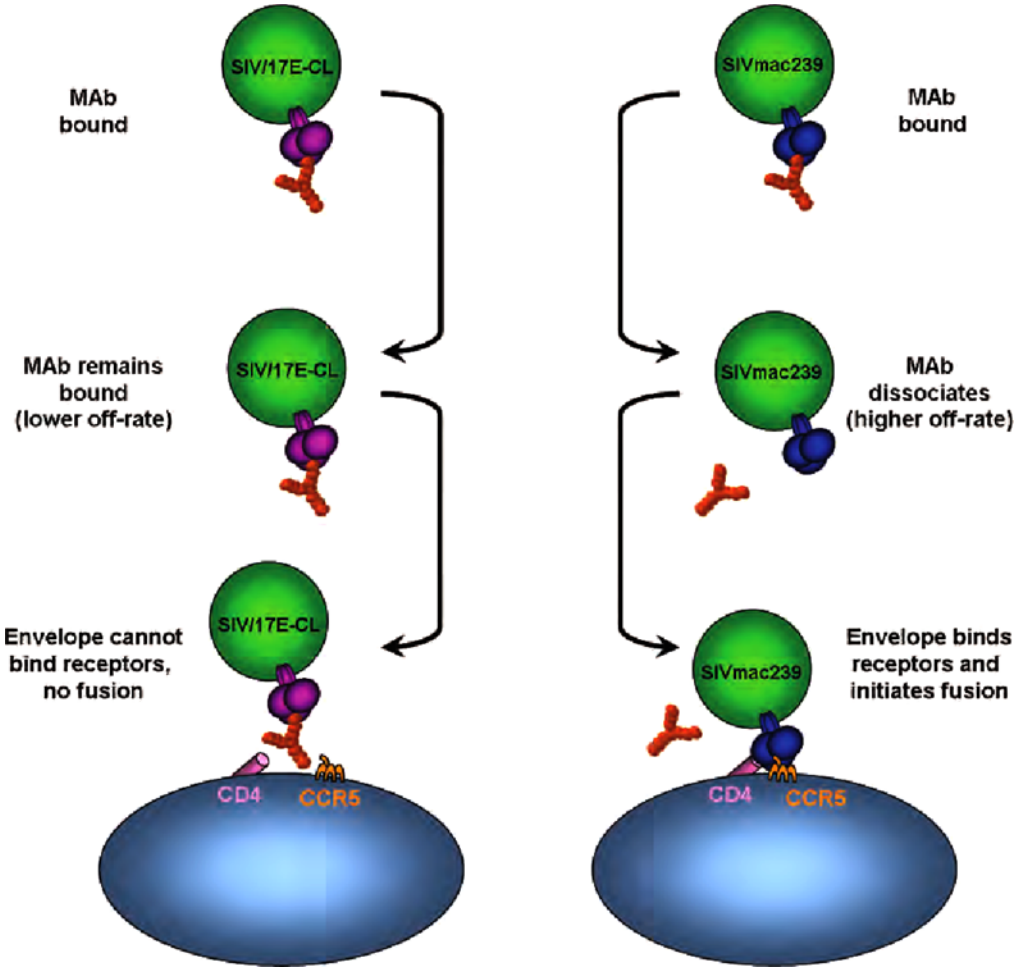

Fig. 3. Schematic representation of the potential influence of MAb dissociation rates on virus neutralization. Neutralization-sensitive SIV/17E-CL is shown on the left and neutralization-resistant SIVmac239 in shown on the right. We propose that the decreased stability (exhibited by higher dissociation rates) of MAb binding to SIVmac239 affords increased propensity for the viral envelope to interact unimpeded with cellular CD4 receptor and coreceptor (CCR5). This results in initiation of the fusion process and subsequent infection, thus avoiding neutralization. For neutralization-sensitive SIV/17E-CL MAb binding is more stable (lower dissociation rates), decreasing the ability of envelope to interact with the cellular receptors, ultimately leading to neutralization.

the rates of MAb binding between the two envelope proteins with the median association and dissociation rates for the MAbs being one-half log higher to SIVmac239 than was observed for SIV/17E-CL. We did not, however, identify significant differences in the affinity of the MAbs for SIVmac239 and SIV/17E-CL. These results demonstrated for the first time the ability of differences in antibody binding kinetics to predict in vitro neutralization-sensitivity of SIV. Furthermore, we proposed that the relative instability of MAb binding to SIVmac239 rgp140 compared to SIV/17E-CL rgp140 may provide

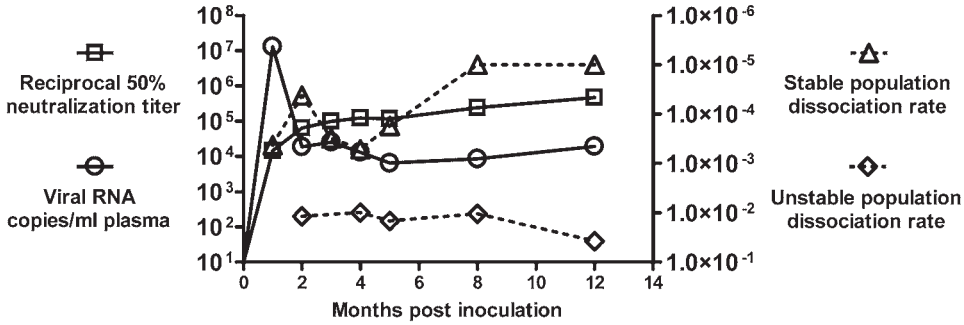

Fig. 4. Relationship of apparent dissociation rates with neutralization and viral load from a rhesus macaque infected with SIV/17E-CL. The plasma viral load peaked at 1 mo and dropped to a steady-state level of approx 10^4 copies/mL by 5 mo. Neutralization titers increased over the course of infection. The apparent dissociation rate of the stable antibody population was identified by SPR and fluctuated over the course of the infection, with a lower dissociation rate at 12 mo than was exhibited at 1 mo. In contrast, the apparent dissociation rate of the unstable population remained constant over the entire time period measured.

one mechanism for the neutralization resistance of SIVmac239 virus in vitro (Fig. 3).

The Polyclonal Antibody Response to SIV Infection Undergoes Dynamic Changes Early in Infection

In an effort to translate our findings with MAb binding kinetics to the study of polyclonal serum antibody responses generated in rhesus macaques infected with attenuated SIV, we analyzed the quantitative and qualitative binding properties of well-characterized, longitudinal serum samples to trimeric, recombinant SIV/17E-CL gp140 envelope proteins using SPR *(35).* We initiated this study in an attempt to identify a reliable measure of the strength of antibody binding without resorting to treatment with 8 *M* urea as in the avidity ELISA, thus removing the potential limitations involved in measuring binding stability under partially denaturing conditions. Results from these SPR studies indicated that two measurable antibody populations existed in the majority of the samples analyzed. The first antibody population identified exhibited relatively slow apparent association/dissociation rates ("stable") and its magnitude increased steadily during the time period analyzed, while the second population demonstrated fast apparent association/dissociation rates ("unstable"). The presence of the unstable population was sporadic over the course of the first few months. The unstable population was first identified at 2 mo post-infection, was not present at 3 mo post-infection, and then reappeared at 4 mo post-infection and remained throughout the final timepoints measured. This evolution of the antibody response was consistent with the observed avidity maturation as defined using our standard panel of serological assays (Fig. 1). However, SPR provided a higher-resolution analysis of polyclonal antibody-binding properties, particularly with respect to the early timepoints post-infection, that is not possible with standard serological assays. Importantly, we observed that stability of the antibody population with time post-infection corresponded with potent neutralization of homologous SIV in vitro. Furthermore, the dissociation rate of the stable antibody population appeared to fluctuate in response to viral load while the unstable population dissociation rate remained constant (Fig. 4).

Similar to the results for MAb binding described above, these results with polyclonal antibody suggest that the stability of the antibody–envelope interaction may be an important mechanism of serum antibody virus neutralization. Additionally, measuring the apparent rates of association and dissociation using SPR provides unique numerical descriptors to characterize the level of antibody maturation achieved by candidate vaccine strategies capable of eliciting broadly neutralizing antibody responses.

Concluding Remarks and Future Directions

The humoral immune response to pathogenic SIV infection exists as a paradox of the response to attenuated SIV infection; despite similar antibody maturation profiles, the outcome of disease is markedly different, with pathogenically infected animals ultimately progressing to simian AIDS (SAIDS) *(6,16,36).* The difference(s) between the two responses is unclear, although it is known that evolution of high levels of antibody able to neutralize homologous virus in vitro is absent during pathogenic infection *(37.)* In an effort to better understand the contribution of neutralizing antibodies to the outcome of infection, the present studies aim to identify the mechanism(s) by which antibody can mediate neutralization of SIV. An extensive characterization of MAbs derived from rhesus macaques exhibiting protective immunity has allowed us to grossly identify functional envelope determinants targeted as part of an effective immune response in vivo. Specifically, we have identified regions in the V4 loop and, putatively, the coreceptor binding site as those areas targeted by neutralizing antibodies during attenuated SIV infection (unpublished data). Additionally, we have identified binding properties of antibodies that allow us to distinguish between neutralization-sensitive (SIV/17E-CL) and neutralization-resistant (SIVmac239) virus strains *(26).* We propose that the higher antibody dissociation rate (lower antibody stability) to SIVmac239 envelope may provide CD4 the opportunity to bind and recruit coreceptor, thus initiating the fusion process and evading neutralization even in the presence of antibody *(26,38).*

We also believe that differences between the immune responses to attenuated and pathogenic SIV are likely to lie in the initial negotiation between the virus and the immune system. To address these differences, we are embarking on a series of studies designed to focus on the early antibody–virus interactions in the SIV/macaque model. First, we will expand our previous work by generating novel rhesus MAbs from monkeys infected with pathogenic as well as attenuated SIV during the entire course of infection, not solely during the late stage associated with protective immunity. This will allow us to examine the evolution in envelope determinants targeted by the immune system over the entire course of infection instead of providing a snapshot of the mature antibody response. Additionally, this will allow us to identify the differences in antigenic determinants or mechanism associated with the early virus/immune system interaction events that are likely to predict the long-term outcome to infection. Second, we will correlate in vitro assays of antibody mechanism and function with antibody-mediated virus inhibition in vivo. These studies will involve moving our studies of antibody maturation to the cellular level, where we plan to characterize and define the role of the B cells in SIV infection. We also will attempt to identify critical residues involved in rhesus antibody binding to SIV envelope proteins using a combination of alanine scanning mutagenesis, computational modeling, and X-ray crystallography. Together, these structural studies will provide

us with a high-resolution analysis of the envelope determinants targeted during SIV infection and allow for the rational design of immunogens capable of eliciting broadly effective immune responses.

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