



# Targeting the CD4- and Coreceptor-Binding Sites of the HIV-1 Envelope Glycoprotein

## Citation

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**TARGETING THE CD4- AND CORECEPTOR-BINDING SITES  
OF THE HIV-1 ENVELOPE GLYCOPROTEIN**

A dissertation presented

by

**Matthew Ryan Gardner**

to

**The Division of Medical Sciences**

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Virology

Harvard University

Cambridge, Massachusetts

April 2014

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## **Targeting the CD4- and Coreceptor-Binding Sites of the HIV-1 Envelope Glycoprotein**

### Abstract

The HIV-1 envelope glycoprotein, Env, facilitates the translocation of the viral capsid across the cellular membrane. Env is a trimer of hetero-dimers composed of a gp120 subunit and gp41 transmembrane protein. The gp120 subunit binds the primary receptor, CD4, leading to conformational changes of Env that then promote binding to the coreceptor, principally CCR5 or CXCR4. As the sole protein on the surface of the virion, Env is under continuous pressure from the host's antibody response. Two classes of antibodies target the highly conserved receptor-binding sites of gp120: CD4-binding site (CD4bs) and CD4-induced (CD4i) antibodies.

Here we focus on inhibiting HIV-1 entry by targeting both the CD4- and coreceptor-binding sites. In Chapter 2 we show that an improved CCR5-mimetic peptide, CCR5mim2-Ig, used in combination with CD4-Ig, neutralized HIV-1 isolates more potently than either inhibitor alone. Similarly, greater neutralization was achieved by combining a CD4i antibody, E51, with a CD4bs antibody, VRC01. We show that this increased potency derives from the ability of E51 to induce quaternary changes of the Env trimer, facilitating greater access of VRC01 to its epitope. Our studies also underscore key mechanistic differences between CD4-Ig and CD4bs antibodies.

Chapter 3 describes the usefulness of targeting both receptor-binding sites by characterizing a dual-acting inhibitor: eCD4-Ig. eCD4-Ig consists of CD4-Ig fused at its

C-terminus with CCR5mim1. eCD4-Ig bound Env with higher avidity than CD4-Ig and its sulfopeptide-fusion inhibited the tendency of CD4-Ig to enhance infection. The potency of eCD4-Ig is similar to or better than the best HIV-1 broadly neutralizing antibodies (bNAbs). eCD4-Ig is also more broadly neutralizing than any current HIV-1 bNAb, and it efficiently neutralized a panel of 38 HIV-1 isolates resistant to the best CD4bs bNAbs as well as SIV and HIV-2 isolates. Lastly, eCD4-Ig retained its efficacy *in vivo*: it protected humanized mice from an HIV-1 challenge and was expressed by an adeno-associated virus (AAV) vector in rhesus macaques at levels greater than 100 µg/mL. Taken together, our studies highlight the advantages of targeting both receptor-binding sites simultaneously and describe a new HIV-1 entry inhibitor with potential clinical use.

To my parents, David and Gay Gardner.

## **Acknowledgements**

Over the course of my graduate student career, I have come across many individuals that have helped me out along the way. First are the lab members of the Farzan lab. I am very grateful for the assistance provided by Kai Chan, Christoph Fellingner, Hema Kondur, and Vinita Joshi to maintain various tasks like splitting cells, changing medium, harvesting protein, etc for when I was unable to be in lab. I would like to thank Jessica Chiang for help introducing me to the lab and techniques I needed to know in order to get started. I cannot estimate the amount of time Brian Quinlan set aside to help teach me details about molecular biology, structure modeling, and show me how to perform experimental techniques. I would like Brian for the confidence he has given me to develop and optimize experimental procedures. I would also like to thank Charles Bailey for always giving me quick answers to questions and help with computer programs.

There are many words that I could use to describe Mike Farzan. The one at the top of the list is friend. There was never a time when my relationship with Mike didn't feel like equals standing on the same ground. I would like to thank Mike for giving me the confidence to be an independent thinker and a better scientist as I move forward towards being an independent investigator. Mike has always treated my ideas with respect, even from when I first joined the lab. His constant excitement for science keeps me enthusiastic about the work I do.

I would not be here today if it were not the continual love and support from my family members – my dad, David Gardner, my mom, Gay Gardner, my sister, Denise

Gardner, and my brother, Christopher Gardner. My family continues to motivate and inspire me to think harder and always do my best.

Lastly, and certainly not least, I would like to give many thanks to my girlfriend, Meredith Davis. She has set aside her emotions to allow me to follow my goals. Her constant love and support continue to drive me to pursue my goals with great passion. The many beer festivals, sushi nights, and Sam Adams Open Houses have been the highlights of my graduate school career and I cannot imagine spending my time with someone else. I look forward to our scientific endeavors and future life together.

## Table of Contents

<b>Abstract</b>	iii
<b>Acknowledgments</b>	vi
<b>List of Figures</b>	xi
<b>Chapter 1: Introduction - The HIV-1 Pandemic and the Role of</b>	
<b>Entry Inhibitors</b>	1
1.A. The HIV-1 Pandemic	2
1.B. HIV-1 Replication Cycle	3
<i>Viral entry</i>	3
<i>Reverse transcription</i>	5
<i>Nuclear import</i>	6
<i>Integration</i>	6
<i>Transcription of viral genes</i>	7
<i>Assembly and budding</i>	7
<i>Accessory proteins</i>	8
1.C. HIV-1 Envelope Glycoprotein	9
<i>Biosynthesis of HIV-1 Env</i>	9
<i>Receptor binding causes tertiary and quaternary conformational changes</i>	10
1.D. Sequence Diversity of Env	14
<i>HIV-1 isolates are categorized into clades based on env gene sequence</i>	14
<i>HIV-1 isolates are categorized into tiers based on neutralization resistance</i>	14
1.E. Mechanisms of Neutralization Resistance	14
<i>Sequence diversity</i>	14
<i>The glycan shield</i>	15
<i>Conserved areas of Env are difficult to access</i>	15
<i>Entropy cost for binding the receptor-binding sites</i>	16
1.F. HIV-1 Entry Inhibitors	16
<i>Clinical entry inhibitors</i>	17
<i>Soluble CD4</i>	18
<i>HIV-1 broadly neutralizing antibodies</i>	19
<i>CD4-binding site antibodies</i>	22
<i>Glycan-dependent antibodies</i>	24
<i>MPER antibodies</i>	25
<i>The gp120-gp41 epitope</i>	26
<i>CD4-induced antibodies</i>	26
<i>Peptide inhibitors</i>	28
<i>Dual-acting inhibitors</i>	29
1.G. Antibody Combinations	30
1.H. Summary	31

<b>Chapter 2: Combinations of HIV-1 Entry Inhibitors that Target the CD4- and Coreceptor-Binding Sites Efficiently Neutralize HIV-1 Isolates</b>	32
Acknowledgements	33
2.A. Abstract	34
2.B. Introduction	34
2.C. Materials and Methods	36
2.D. Results	40
<i>Mixtures of CD4-Ig and CCR5mim2-Ig neutralize isolates more efficiently than either inhibitor alone</i>	40
<i>Mixtures of E51 and VRC01 neutralize HIV-1 more efficiently than either antibody alone</i>	42
<i>E51 does not enhance CD4bs antibody binding to monomeric gp120</i>	44
<i>E51 enhances binding of CD4bs bNAbs to cell-surface expressed Env trimers</i>	46
<i>Unlike CD4-Ig, CD4bs bNAbs do not promote HIV-1 infection of CD4-negative cells</i>	49
2.E. Discussion	50
<b>Chapter 3: A CCR5-mimetic sulfopeptide converts CD4-Ig to an Exceptionally broad and potent inhibitor of HIV-1 entry</b>	54
Acknowledgements	55
3.A. Abstract	56
3.B. Introduction	57
3.C. Materials and Methods	59
3.D. Results	67
<i>A C-terminal sulfopeptide markedly enhances the neutralizing activity of CD4-Ig</i>	67
<i>eCD4-Ig utilizes both sulfopeptides to avidly bind Env trimers</i>	69
<i>eCD4-Ig can bind three Env receptor-binding sites without steric interference</i>	71
<i>eCD4-Ig and its variants potently neutralize a diverse panel of tier 2 and tier 3 isolates</i>	73
<i>eCD4-Ig retains its efficacy in vivo</i>	84
3.E. Discussion	91
<b>Chapter 4: Discussion – Implications for the Development of an HIV-1 Vaccine</b>	95
4.A. The state of HIV-1 vaccine efforts	96
4.B. Developing antigens that will generate HIV-1 bNAbs	98
4.C. Soluble trimeric Env as an antigen	100
4.D. Antibody cocktails as a new HIV-1 therapy	100
4.E. Translating bNAb <i>in vitro</i> properties	102
4.F. The role of CD4i antibodies in an HIV-1 vaccine	103

4.G. Using eCD4-Ig as a vaccine alternative	104
4.H. Predicting escape pathways of eCD4-Ig	106
4.I. Effectively using AAV vectors for HIV-1 therapy	108
4.J. Other gene therapy strategies to consider	110
4.K. Closing remarks	111
References	113
Appendix: Permissions	134

## List of Figures

### Chapter 1

Figure 1.1. The HIV-1 entry process	5
Figure 1.2. Crystal structure of a soluble trimeric Env	10
Figure 1.3. The closed and open states of Env	13
Figure 1.4. The epitopes of HIV-1 Env	21
Figure 1.5. Footprints of CD4-binding site antibodies	24

### Chapter 2

Figure 2.1. CCR5mim2-Ig synergizes with CD4-Ig to neutralize HIV-1 infection	42
Figure 2.2. <i>In vitro</i> neutralization by combinations of CD4bs Antibodies with E51	44
Figure 2.3. Promotion of monomeric gp120 association by CD4bs Antibodies and E51	46
Figure 2.4. Promotion of E51 or CD4bs antibody association to Trimeric HIV-1 Env	48
Figure 2.5. Enhancement of HIV-1 entry into Cf2Th CCR5+/CD4- Cells by CD4bs antibodies	50
Figure 2.6. Model of Env induction by E51 and CD4bs antibodies	51

### Chapter 3

Figure 3.1. Characterization of CD4-Ig/CCR5-mimetic peptide fusion proteins	68
Figure 3.2. Mechanistic studies of eCD4-Ig	70
Figure 3.3. A model of eCD4-Ig bound to HIV-1 envelope glycoprotein trimer	72
Figure 3.4. eCD4-Ig variants efficiently neutralize Tier 2 and Tier 3 HIV-1 and SIV isolates	75
Figure 3.5. IC <sub>80</sub> values for eCD4-Ig variants assayed in Figure 3.4	76
Figure 3.6. Comparison of eCD4-Ig variants and HIV-1 neutralizing antibodies	78
Figure 3.7. Extended neutralization assays, IC <sub>90</sub> values, and standard error for the studies of Figure 3.6	80
Figure 3.8. A summary of HIV-1, IHV-2, and SIV neutralization studies	83
Figure 3.9. <i>In vivo</i> activity of eCD4-Ig in humanized mice	84
Figure 3.10. Characterization of rh-eCD4-Ig <sup>mim2</sup> in rhesus macaques	86
Figure 3.11. Characterization of rh-eCD4-Ig <sup>mim2</sup> variants to gain sensitivity against SIV and SHIV isolates	89
Figure 3.12. rh-eCD4-Ig <sup>I39N,mim2</sup> expression with ssAVV vectors	91

### Chapter 4

Figure 4.1. Modeling Env escape from eCD4-Ig	107
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## **CHAPTER 1: INTRODUCTION**

### **The HIV-1 Pandemic and the Role of Entry Inhibitors**

## 1.A. The HIV-1 Pandemic

Human Immunodeficiency Virus Type 1 (HIV-1) was identified as the cause of what is now known as Acquired Immunodeficiency Syndrome (AIDS) in 1981<sup>1-3</sup>. HIV-1 is classified in the genus *Lentivirus* and in the family of *Retroviridae*. Current data from the World Health Organization suggests there are approximately 35.3 million people worldwide living with AIDS<sup>4</sup>. In that same year, there were 2.3 million people newly infected with HIV-1 and 1.3 million AIDS related deaths<sup>4</sup>. In sub-Saharan Africa alone, prevalence rates exceed 10% in numerous countries totaling 22 million HIV-1 infections<sup>4,5</sup>.

HIV-1 infection is usually characterized with an initial acute primary infection where there is an infection of CD4+ T cells, dendritic cells, and macrophages and a spike in viral RNA load around  $10^5$  to  $10^6$  copies/mL of plasma around two weeks post infection<sup>6</sup>. The spike of viral RNA is followed by the reduction of free virus in the blood by CD8+ T cells and antibodies neutralizing virus. The decrease in viral load reaches a set point of around  $10^4$  to  $10^5$  RNA copies/mL around four months post infection<sup>7,8</sup>. In untreated individuals, latent infection can last an average of 10 years as the virus continues to replicate in lymphoid organs. However, the number of CD4+ T cells will decrease to levels below 200 cells/mL, indicating the disease has progressed to AIDS. At these levels, the immune system cannot control opportunistic infections. For example, the HIV-1 pandemic has led to the resurgence in tuberculosis infections. The average survival rate for untreated individuals is about three years.

Current anti-viral cocktail therapies are efficient at suppressing viral replication and increasing life expectancy of infected individuals. However, of the 35 million people

infected, less than 10 million of those individuals had access to anti-retroviral therapy. Individuals that are on the anti-retroviral therapy must take their medication daily with an average annual cost ranging from \$11,000 to \$23,000<sup>9</sup>. Until a conventional HIV-1 vaccine is available, new therapies are continually examined in an effort to decrease treatment costs.

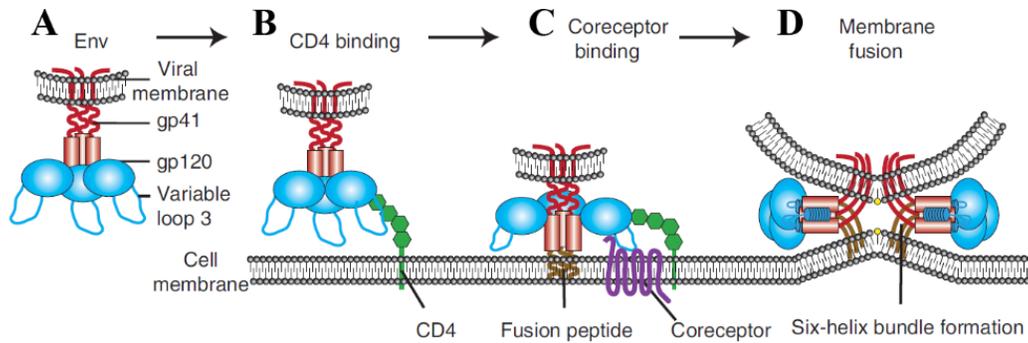
## **1.B. HIV-1 Replication Cycle**

### *Viral entry*

HIV-1 entry is the first phase of the viral life cycle in which the viral capsid core translocates across the host cell membrane into the cytosol. Entry is mediated by the viral envelope glycoprotein (Env), a trimer of non-covalently bound hetero-dimers of gp120 and gp41. The precursor protein of Env, gp160, trimerizes and is cleaved by furin into gp120, the binding domain, and gp41, the transmembrane domain<sup>10</sup> (Figure 1.1A).

Entry initiates when the virus associates with the host cell. This initial viral attachment to the host cell can be a low specificity binding event. A number of attachment factors have been classified that help HIV-1 entry into host cells. These attachment factors include the negatively charged heparan sulfate proteoglycans, which have been shown to bind Env nonspecifically<sup>11</sup>. Other attachment factors that have been identified are alpha-4-beta-7 integrins and dendritic cell-specific intercellular adhesion molecular 3-grabbing non-integrin (DC-SIGN)<sup>12-14</sup>. Unlike an actual receptor, these attachment factors are not required for the virus to gain entry and do not confer permissibility on non-permissive cell types. Attachment factors are used for bringing the virus into closer proximity of the primary receptor necessary for entry<sup>15</sup>.

HIV-1 uses CD4 as its primary receptor<sup>16-18</sup> (Figure 1.1B). It was recently suggested that an Env cluster binds a patch of CD4 on the surface of the host cell<sup>19</sup>. CD4 contacts the gp120 subunit of Env at a specific CD4-binding site<sup>20,21</sup>. Env then undergoes conformational changes that rearranges its variable domains including the V1/V2 loop and subsequently the V3 loop<sup>22-24</sup>. CD4 binding was initially thought to lead to the creation of the bridging sheet<sup>25,26</sup>, but recent structural data suggests the bridging sheet is present in the unliganded gp120<sup>27</sup>. Once in the CD4-bound state, Env is primed to bind its coreceptor, principally the chemokine receptors CCR5 or CXCR4<sup>28-31</sup> (Figure 1.1C). Binding of the coreceptor triggers viral fusion with the host cell membrane. CD4 and coreceptor binding exposes gp41, and in particular its hydrophobic fusion peptide, which inserts into the target cell membrane and forms the pre-hairpin intermediate (PHI). Following insertion of the fusion peptide, the gp41 amino-terminal helical region (HR-N or HR1) that is inserted into the target cell can then fold at the hinge with the carboxy-terminal helical region (HR-C or HR2) that is tethered to the virus. The folding of the three HR1 and HR2 regions forms the six-helix bundle (Figure 1.1D) and brings the target cell membrane into close proximity of the viral membrane, promoting mixing of the lipids from each bilayer. Lipid mixing of the two membranes ultimately leads to the formation of the fusion pore, providing access of the viral capsid to the cellular cytosol.



**Figure 1.1. The HIV-1 entry process**<sup>32</sup>. (A) Env is expressed on the viral membrane as a trimer of gp120 and gp41 heterodimers. (B) HIV-1 enters the cell by Env binding its primary receptor, CD4. (C) Env then undergoes conformational changes that promote binding to its coreceptor, principally CCR5 or CXCR4. (D) The fusion peptide of gp41 inserts into the target cell membrane and begins the formation of the six-helix bundle. This brings the host cell membrane in close proximity to the viral membrane for membrane fusion to occur. (Adapted from Wilen CB, Tilton JC, and Doms RW, 2012<sup>32</sup>).

### *Reverse transcription*

After entry into the cell, the virus initiates reverse transcription of its genome in the cytoplasm of newly infected cells. Completion of reverse transcription requires the disassembly of the capsid core and formation of the reverse transcriptase complex (RTC)<sup>33,34</sup>. HIV-1 encodes and packages its own reverse transcriptase which uses the HIV-1 genomic RNA as its template and tRNA(Lys3) as its primer. The HIV-1 primer binding site is about 180 nucleotides starting at the 5' end of the genome. During reverse transcription, RNase H actively degrades RNA that associates with transcribed DNA in an RNA-DNA complex<sup>35,36</sup>. The degradation of the RNA template exposes the newly synthesized minus-strand DNA. Minus-strand DNA transfer occurs and acts as a second primer binding the purine-rich polypurine tract (ppt) at the 3' end of the viral RNA genome<sup>37</sup>. The complementary DNA (cDNA) is synthesized while RNase H cleaves the majority of the viral RNA. The ppt acts as the primer for plus-strand DNA synthesis which continues until the first 18 tRNA nucleotides are copied. Once copied, RNase H degrades the tRNA and the plus-strand is moved to the R region of the 5' end. Extension

of both the minus- and plus-strand DNA generates the double-stranded viral DNA necessary for integration<sup>38</sup>.

### *Nuclear import*

After the RTC has transcribed the double-stranded viral DNA, the virus forms a stable pre-integration complex (PIC) consisting of the viral integrase protein (IN) as well as Vpr and Matrix (MA). The PIC is trafficked to the nuclear periphery along microtubules<sup>39</sup>. Too large to be transported through nuclear pores, the actual mechanism by which the PIC enters the nucleus remains unclear. HIV-1 infects non-dividing cells implying that the PIC is actively transported across the nuclear membrane. In contrast, simpler retroviruses cannot infect non-dividing cells and rely on the breakdown of the nuclear membrane during mitosis to enter the nucleus<sup>40</sup>. The PIC creates extensive changes to the nuclear membrane in order to be actively transported into the nucleus<sup>41,42</sup>. There is evidence that HIV-1 utilizes the importin pathway by recruiting numerous host proteins to the nuclear membrane that facilitate nuclear import. These cellular factors include importin- $\alpha$ , importin- $\beta$ , and importin  $\gamma$ <sup>43-45</sup>.

### *Integration*

Once in the nucleus, the integration process is mediated by the virus IN protein, which enters the nucleus with the viral DNA as part of the PIC. Integration occurs directly at the termini of the viral DNA which can be inserted into semi-nonrandom locations in the host cell chromosomal DNA. Association with the host protein LEDGF<sup>46,47</sup> usually results in integration towards areas of active transcription<sup>48</sup>. IN catalyzes the 3' processing of the viral DNA. IN then joins the 3' hydroxyl to the 5' hydroxyl ends of the target DNA by a nucleophilic reaction on the target DNA

phosphodiester backbone at the site of integration. This is known as strand transfer<sup>49</sup>. Finally, gap repair of the integrated DNA is completed by enzymes of the host cell DNA repair synthesis pathway. These include a flap endonuclease which trims the 5' end, a polymerase which fills the gap, and a ligase to close the nicked DNA. Once the DNA is fully repaired, the chromatin is remodeled back into its nucleosomal form<sup>50,51</sup>.

### *Transcription of viral genes*

Transcription from the integrated viral genome is regulated in both cell-type and differentiation-dependent manner through the binding of cellular and viral proteins to the 5' LTR, which serves as the promoter<sup>52</sup>. HIV-1 uses numerous host cell proteins, along with viral proteins, to form extensive transcriptional units. HIV-1 uses complex alternative splicing to produce the wide range of transcripts needed to generate all the viral proteins. The numerous transcripts are achieved through the use of four different splice donor and eight different splice acceptor sites. Transcription begins with the processing of the early genes: *tat*, *rev*, and *nef*. Tat binds transcription complexes at the 5' LTR for transcriptional regulation<sup>53</sup>. Rev binds a specific motif in the *env* gene termed the rev response element (RRE)<sup>54-56</sup>. Rev binding to the RRE<sup>57,58</sup> is essential for the viral replication cycle as it promotes the transport of the unspliced 9-kb and partially spliced 4-kb viral mRNA out of the nucleus and into the cytoplasm. The longer transcript serves as a genome for packaging and other transcripts are used for producing the structural viral proteins needed for assembly: Gag, Pol, and Env.

### *Assembly and budding*

In the last stage of the virus life cycle, the virus assembles all the elements necessary to produce an infectious virus. Assembly occurs at the plasma membrane and

is mediated by the Gag polyprotein<sup>59</sup>. Gag binds to the plasma membrane, concentrates Env to the membrane, and uses its RNA packaging sequence to ensure that two viral genomic RNA copies are packaged into the virion. The Gag polyprotein assembles in spherical particles and as budding occurs, the viral protease (PR) acts on the polyprotein to produce Gag maturation<sup>60</sup>. The N-terminal domain of Gag is called MA, the domain that binds the plasma membrane and recruits Env<sup>61</sup>. The middle domain of Gag is the Capsid domain (CA), which forms the capsid shell of the mature viral core. The nucleocapsid (NC) domain has two zinc finger motifs that hold the viral genome during assembly. At the C-terminal end of Gag is the p6 region which recruits necessary parts of the cellular ESCRT pathway, like ALIX, as well as HIV-1 accessory proteins. The viral protease cleaves the Gag precursor, inducing major changes in the virion to complete the maturation process. Budding requires recruitment of components of the host cell ESCRT pathway to complete the release of viral particles from the host cell plasma membrane<sup>62</sup>.

### *Accessory proteins*

For successful infection, HIV-1 utilizes accessory proteins to evade a host immune response. The viral protein Nef can downregulate surface expression of CD4<sup>63,64</sup> to avoid Env-CD4 interactions when the virus is budding, MHC-I to evade CD8+ effector T-cell function<sup>65</sup>, and HLA-A and HLA-B to avoid natural killer (NK) cell function<sup>66</sup>. Vif is used to target APOBEC3, a host restriction factor, for polyubiquitination and degradation<sup>67</sup>. HIV-1 uses Vpu to counteract immune responses, such as down regulation of Bst2/Tetherin<sup>68</sup> to keep viral particles from being tethered to the cell surface. Like Vif, both Vpr and Vpu also target host cell factors for degradation by ubiquitination. Vpr

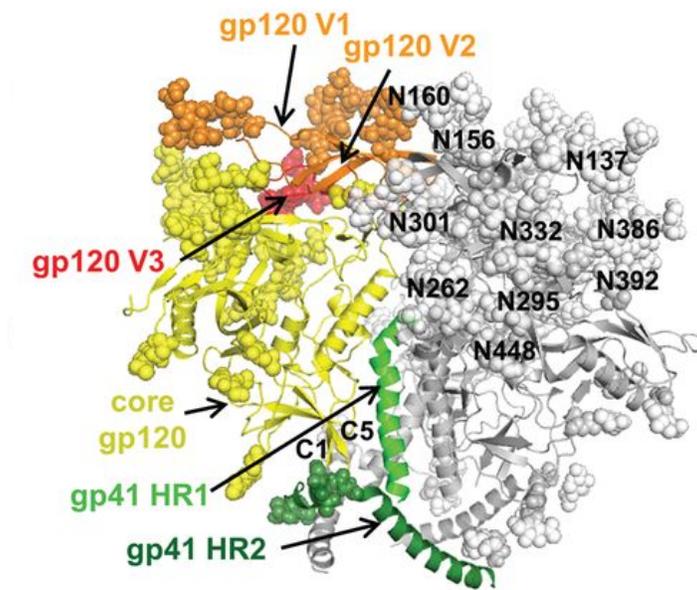
also functions in creating cell cycle arrest during the G2 phase. In short, retroviruses have evolved numerous mechanisms to evade the host's immune response.

### **1.C. HIV-1 Envelope Glycoprotein**

#### *Biosynthesis of HIV-1 Env*

HIV-1 Env is generated from the *env* gene encoded by the virus. Env is synthesized as an approximately 845- to 870-amino acid precursor polypeptide<sup>69</sup>. An N-terminal signal peptide traffics Env to the endoplasmic reticulum (ER) where it acquires numerous N-linked, high-mannose glycans<sup>70,71</sup>. Transport of Env out of the ER is delayed to facilitate proper folding and oligomerization through disulfide bonds<sup>72,73</sup>. Env oligomerizes into a trimeric complex<sup>74</sup>, a common quaternary structure found in viruses that use type 1 fusion for entry. Trimeric Env is then transported to the Golgi apparatus where it is furin-cleaved into its gp120 subunit and gp41 transmembrane domain<sup>10,75,76</sup>, the components of mature Env. The gp120 subunit contains most of the surface-exposed elements of Env including approximately 24 N-linked glycosylated residues<sup>70</sup>. The gp41 domain consists of a membrane-proximal ectodomain region (MPER), a transmembrane domain responsible for anchoring Env to the viral membrane, and a long cytoplasmic tail at the C-terminus, which is necessary for Env incorporation into the virion. There are about three to four N-linked glycans found on gp41<sup>71,77</sup>. Noncovalent interactions keep gp120 and gp41 together in the assembled trimer<sup>78,79</sup>. Env trimers are trafficked to the plasma membrane and incorporated into virions where about 7-14 of mature, intact Envs are actually expressed on an infectious particle<sup>80,81</sup>. Initially, Env is sparsely distributed over the virion and has little lateral movement because of the rigidity of the gag lattice. Following the maturation of gag, specifically when MA separates from CA, Env

overcomes its movement restriction and clusters on the virion, a process mediated by its cytoplasmic domains<sup>19</sup>. Figure 1.2 represents the crystal structure of a soluble Env trimer highlighting the gp120 core, V1/V2 loops, V3 loop, gp41, and important glycosylation residues on gp120<sup>82</sup>.



**Figure 1.2. Crystal structure of a soluble trimeric Env<sup>82</sup>.** Presented is the crystal structure of a soluble, cleaved trimeric Env. The arrangements of the V1/V2 loops (orange) and V3 loop (red) as well as the location of gp41 (green) are highlighted. The gp120 core of one gp120 monomer is in yellow. The second and third gp120 monomers and gp41 proteins are shaded in grey. Labeled are the key N-linked glycosylations on gp120 by which antibodies can bind to. (Adapted from Julien JP *et al.*, 2013<sup>82</sup>).

*Receptor binding causes tertiary and quaternary conformational changes*

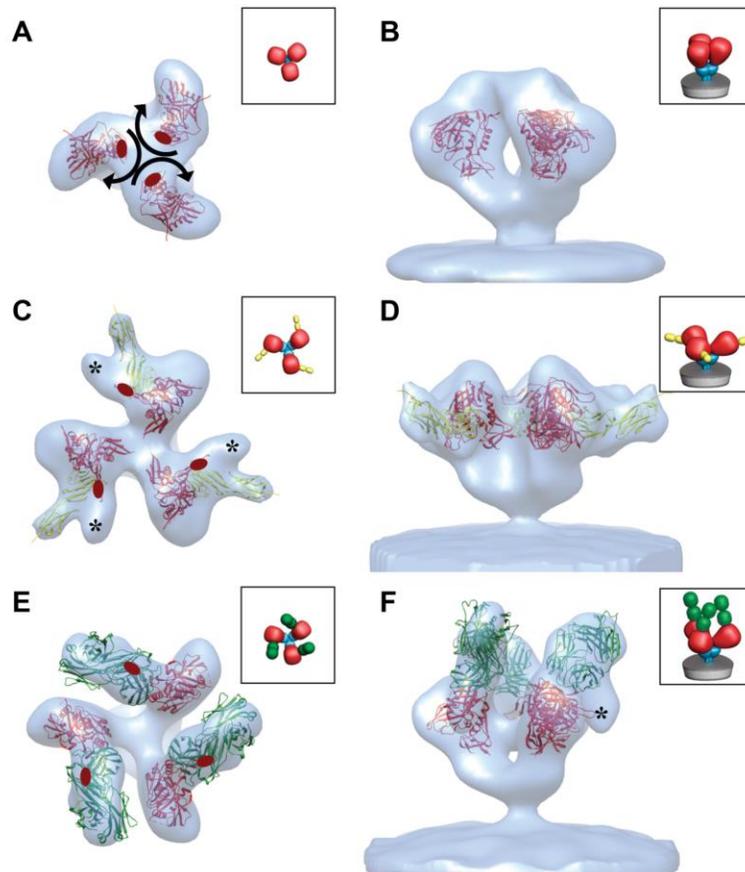
As the sole protein on the viral membrane, Env is necessary to mediate fusion of target cell and virion membranes, enabling translocation of the capsid core into the cytosol. To gain entry into a target cell, Env binds a series of receptors. Following identification of HIV-1 as the etiological agent of AIDS, it was soon recognized that the

primary target cell was CD4+ T-helper cells<sup>16</sup>. Later studies confirmed that the primary receptor for HIV-1 was CD4 itself<sup>17,18</sup>. CD4 is obligate but not sufficient for viral entry, due to the requirement of a coreceptor. Nearly a decade passed until coreceptors for HIV-1 were identified. Cocchi *et al.*, showed that macrophage tropic HIV-1 isolates could be inhibited by the  $\beta$ -chemokines RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$ <sup>83</sup>. At the same time, Feng *et al.* showed that a chemokine-like receptor, now known as CXCR4, was necessary for infection of laboratory adapted strains of HIV-1<sup>31</sup>. A number of groups subsequently showed that HIV-1 uses chemokine receptors as coreceptors that are necessary to drive viral fusion. These chemokine receptors belong to the family of G protein-coupled receptors (GPCRs) and include CCR2b, CCR3, CCR5, CXCR4, GPR1, GPR15 (Bob), and STRL33 (Bonzo)<sup>28-30,84-86</sup>. Principally, HIV-1 isolates use CCR5 as its coreceptor, while lab-derived isolates and isolates found late in infection acquire the ability to use CXCR4. Isolates that use CCR5 as their coreceptor are termed R5 viruses while isolates that use CXCR4 as their coreceptor are termed X4 viruses and those isolates that can use both are R5X4 viruses<sup>87</sup>. A key commonality of HIV-1 coreceptors, relevant to the work described herein, is that they are tyrosine sulfated at their N-termini<sup>88,89</sup>, a property which facilitates efficient HIV-1 entry.

As stated, Env consists of a gp120 subunit and gp41 transmembrane domain. The gp120 subunit is involved in receptor- and coreceptor-binding and is heavily glycosylated. There are five highly variable regions termed V1-5 as well as five more conserved regions<sup>90</sup>. V1-4 are bounded by disulfide bonds and described as loops. The architecture of gp120 consists of an inner domain formed by the conserved regions, the glycosylated outer domain, and bridging sheet that comprises four anti-parallel  $\beta$ -strands.

The gp120 core interacts with the gp41 stalk while a portion of the gp41 stalk is exposed on the viral membrane called the membrane-proximal external region (MPER).

The states of Env under different conditions has been extensively studied. In particular CD4-bound and CD4-free states of gp120 and of Env have been described and characterized. The binding site of CD4 is within a recessed pocket of gp120 that is somewhat protected by the variable loops<sup>91</sup>. One of the critical contacts between CD4 and gp120 is in a deep cavity of gp120, containing a highly conserved GGD(P/L)E motif, which is filled by CD4 phenylalanine 43<sup>25,91</sup>. Conserved contact residues critical for CD4 binding surround this pocket. Unbound, trimeric Env assumes a “closed” conformation with the V1/V2 loop stumps positioned near the center of the trimer (Figure 1.3A-B)<sup>22-24</sup>. Binding of CD4 to gp120 rotates gp120 out from the central access of the trimer and the V1/V2 and V3 loops are repositioned creating an “open” conformation (Figure 1.3C-D)<sup>22-24</sup>. Repositioning of the V3 loop affords greater access to the coreceptor binding site, as is visible in the structure of the coreceptor-binding antibody 412d complexed with gp120<sup>92</sup>. Early evidence suggested that the bridging sheet did not form until post-CD4 binding<sup>25,26</sup>. However, newer data suggests that the bridging sheet may exist even in the absence of CD4 binding and that it may be considered its own unique domain of gp120<sup>27</sup>.



**Figure 1.3. The closed and open states of Env<sup>22</sup>.** Shown are the cryo-EM diagrams of Env in various states. **(A and B)** When unbound, Env assumes a closed conformation. **(C and D)** Upon CD4 binding, Env is changed into an open conformation with a distinct repositioning of the V1/V2 loops (red dots) and V3 loops (asterisk). **(E and F)** Binding of 17b, whose epitope is the coreceptor-binding site, induces the same open conformation produced by CD4 binding with the same repositioning of the V1/V2 loops and V3 loop. (Adapted from Tran EE *et al.*, 2012<sup>22</sup>).

Although binding of CD4 induces an open conformation advantageous for coreceptor binding, antibodies binding the coreceptor-binding site also put the trimer in the same open conformation (Figure 1.3E-F)<sup>22</sup>. However, the coreceptor binding site is protected by the V1/V2 loops, limiting access to this site<sup>93</sup>. The co-crystal structure of the 412d in complex with gp120 localized two sulfated tyrosines of 412d to a highly-conserved sulfotyrosine-binding pocket of gp120<sup>92</sup>. It is presumed that the sulfotyrosines of CCR5 and other coreceptors target this pocket in a similar manner.

## **1.D. Sequence Diversity of Env**

### *HIV-1 isolates are categorized into clades based on env gene sequence*

The pandemic HIV-1 strains are all classified as part of the “main group” (Group M), which derive from an ancestral strain closely related to SIV chimpanzee<sup>94</sup>. Non-pandemic HIV-1 strains have been assigned into group O (for “outlier”) and group N (for “non-M, non-O”). Group M strains can be further subtyped into clades A through K based on the *env* gene sequence. Isolates that have recombined are designated as circulating recombinant forms (CRF) with the clades used to form the isolate. Certain clades are predominate in different regions of the world. For example, Sub-Saharan Africa and India have a majority of clade C isolates while clade B is found in Europe and the Western Hemisphere.

### *HIV-1 isolates are categorized into tiers based on neutralization resistance*

The clade format organizes HIV-1 isolates based on sequence. However, neutralization resistance varies from isolate to isolate within a clade. Recently, HIV-1 isolates have been categorized into a 3-tier system based on neutralization sensitivity against plasma pooled from HIV-1(+) individuals<sup>95</sup>. Tier 1 isolates are the most neutralization sensitive and tend to be lab-adapted strains. Most HIV-1 isolates are defined as tier 2, while a small group – those most neutralization resistant to pooled sera – falls into the tier 3 category.

## **1.E. Mechanisms of Neutralization Resistance**

### *Sequence diversity*

The properties of Env complicate efforts to develop an effective HIV-1 vaccine. The virus has a high mutation rate because of its low-fidelity RT. RT introduces

approximately one new mutation per copied viral genome<sup>96</sup>. Diversity can also be generated through insertions, deletions, and recombinations between the copackaged genomes, all of which can be observed in Env. The constant mutation of Env, along with its structural features that promote immune evasion, has created a challenge for developing a vaccine that will induce broadly neutralizing antibodies (bNAbs) against HIV-1. The variable loops in particular exhibit very high sequence diversity, including variation in their length<sup>97</sup>. Because the variable loops are the most exposed parts of Env, mutations in these domains can quickly lead to antibody escape<sup>98</sup>, without loss of fitness to the virus.

#### *The glycan shield*

In addition to sequence diversity of the variable loops, the numerous glycosylations on Env present another obstacle. There are approximately 24 N-linked glycosylation sites on gp120 and 3-4 glycosylation sites on gp41<sup>70,71</sup>. The density of glycans on the surface of Env creates a “glycan shield” that limits access of antibodies to Env<sup>99</sup>. HIV-1 antibodies target glycans on gp120, however, the virus can readily escape these antibodies through continual mutation of glycosylated residues<sup>100-102</sup>.

#### *Conserved areas of Env are difficult to access*

The complex structure of Env creates problems for accessing the conserved areas even without the presence of glycans. The receptor- and coreceptor-binding sites are two of the most conserved sites on Env. Not only is the CD4-binding site difficult to access because of the V1/V2 loops<sup>21,103</sup> and nature of the closed trimeric structure, but it is also in a recessed cavity of gp120<sup>20</sup>. Another challenge is the sequence diversity around the cavity. These residues, not important for CD4-binding, are part of the epitopes of most

CD4-binding site antibodies, and can easily mutate, leading to viral escape. The coreceptor-binding site is also blocked by the variable loops and has a much smaller conserved pocket at the base of the V3 loop. A final relatively conserved epitope is the MPER region. In contrast to influenza hemagglutinin (HA), whose gp41 analogue HA2 has a long accessible stalk, gp41 has a short and relatively inaccessible gp41 epitope. Antibodies that bind a highly conserved region on gp41 tend to be broad, but they also bind the viral membrane<sup>104-106</sup>. The epitopes of these MPER antibodies tend to be most accessible during the process of fusion, and it is thought that smaller peptides might be more efficient than antibodies<sup>69</sup>.

#### *Entropy cost for binding the receptor-binding sites*

As mentioned, binding of both CD4 and coreceptor antibodies induces conformational changes to Env, and there is a free energy barrier to these changes. Antibodies targeting either receptor-binding site region might stabilize Env in the CD4-bound state, imposing an entropy cost similar to that seen with CD4 (~50 kcal/mol)<sup>91</sup>. This entropy cost diminishes antibody affinity, and has been termed “conformational masking” whereby Env uses energetics to “hide” critical conserved epitopes.

### **1.F. HIV-1 Entry Inhibitors**

Env is the sole viral protein expressed on the viral membrane, and thus the only one that can be targeted by proteins to prevent entry into host cells. As mentioned, Env uses a number of strategies that allow the virus to escape inefficient pressure from a single inhibitor. With numerous domains, multiple distinct glycosylation sites, and two receptor-binding sites, there are many epitopes on Env to inhibit viral entry. Described

below are different categories of HIV-1 entry inhibitors that target various epitopes on Env.

### *Clinical entry inhibitors*

There is currently an FDA approved peptide that is part of a combinational drug therapy, T20 (Fuzeon/Enfuvirtide), a 36-amino acid peptide that modeled after HR2 of gp41<sup>107</sup>. Because of its similarity to HR2, T20 competes with HR2 in binding HR1 and block viral fusion to the host cell membrane. The heptad repeats of gp41 are hidden within the trimeric complex and thus, T20 can only access HR1 during the fusion process. It is worth noting that the T20 epitope can be induced by other inhibitors, like sCD4 and CCR5mim1-Ig<sup>108,109</sup>.

Another way to block entry is to target the receptors. Maraviroc (UK 427,857) is another FDA approved drug that is a CCR5 antagonist<sup>110</sup>. Individuals infected with R5 virus treated with maraviroc over 10 days saw a decline in viral titers at levels greater than 1.6 log<sub>10</sub> in plasma HIV-1 RNA<sup>111</sup>. The efficacy of maraviroc was examined in two phase 3 trials that resulted in a drop in plasma HIV-1 RNA around 0.8 log<sub>10</sub> copies/mL in those individuals receiving treatment<sup>112,113</sup>. However, there was a tendency individuals treated with maraviroc to see coreceptor switching as X4 viruses started to emerge<sup>113</sup>. Other CCR5 antagonists currently being evaluated for FDA approval include vicriviroc<sup>114</sup> and cenicriviroc<sup>115</sup>. There are currently two small molecules that are being evaluated as potential CXCR4 drugs, AMD3100 and AMD11070. However, the problem with CXCR4 inhibitor treatment is that X4 viruses are normally present with R5 viruses<sup>116,117</sup>. Treating with combinations of CCR5 and CXCR4 inhibitors might be more effective.

Antibodies targeting CD4 and coreceptors have also proven effective. A humanized anti-CD4 antibody, ibalizumab (iMab), binds to domain 2 of CD4 and decreases the flexibility of CD4<sup>118</sup>. iMab was able to reduce viral load in treated individuals up to 1.5 log<sub>10</sub> RNA copies/mL<sup>119</sup>. However, resistance emerged about 9 weeks post treatment<sup>120</sup>. Like iMab, PRO 140 is a humanized antibody but it binds CCR5, blocking gp120 attachment to the coreceptor<sup>121,122</sup>. Individuals treated with PRO 140 saw an average reduction of 1.8 log<sub>10</sub> HIV-1 RNA copies and no toxicity was observed<sup>123</sup>. Both PRO 140 and iMab have undergone phase 2 clinical studies.

### *Soluble CD4*

Identification of CD4 as the HIV-1 primary receptor led to initial studies showing that a soluble form of CD4 (sCD4) could bind gp120 and prevent syncytia formation<sup>124</sup>. Numerous *in vitro* studies showed that sCD4 could also prevent viral entry<sup>125-128</sup>. Importantly, it was shown that, unlike cell-expressed CD4, sCD4 did not have high affinity for MHC class II, and therefore it did not interfere with MHC class II association with the T cell receptor<sup>124</sup>. More recently, N4, a rhesus form of two-domain sCD4 fused to an immunoglobulin G2 (IgG2) Fc region, showed its effectiveness *in vivo* as it protected 2 of 3 rhesus macaques from an SIVmac316 challenge<sup>129</sup>. However, sCD4 enhanced SIV infection when used as a treatment<sup>130</sup>. In human patients, it was shown that antibodies are generated against sCD4<sup>131</sup>, which can hinder its ability to bind and neutralize virus. sCD4 has an advantage over other inhibitors in that it binds only the conserved sites on gp120. However, sCD4 binds most HIV-1 isolates with lower affinity than neutralizing antibodies, and high concentrations of sCD4 are necessary to completely neutralize primary HIV-1 isolates<sup>132</sup>. Large doses of sCD4-IgG2 were

necessary to reduce viral loads in HIV-1(+) children and adults, and in some cases suppression to below detectable levels was observed. Although efficacy of sCD4-Ig treatments was limited, no adverse effects of sCD4-Ig treatment were observed in these trials.

### *HIV-1 broadly neutralizing antibodies*

After HIV-1 is transmitted to a new host, there is a rapid peak in viral load. At this stage (the acute stage), CD4+ T cell levels decline but a CD8+ T-cell response drops viral loads down to a set point. As early as 12 days post infection, the host starts to mount an antibody response against the virus. However, these are only HIV-1 specific binding antibodies that do not have neutralizing capability. These binding antibodies usually develop around four to six weeks post infection. The first anti-Env antibodies detected are IgM anti-gp41 antibodies. Next, non-neutralizing antibodies targeting the V3 loop are generated and followed by weak neutralizing anti-V3 antibodies. Viremia is normally held in check during the asymptomatic portion of the infection and allows for neutralizing antibodies (NAbs) to develop. Most NAbs target the variable regions of Env with others targeting the CD4-binding site and MPER regions. HIV-1 broadly neutralizing antibodies (bNAbs) can take from months to years to develop. Continual generation and development of HIV-1 antibodies allows the virus to escape the host immune response, leading to CD4+ T cell depletion to the point of AIDS.

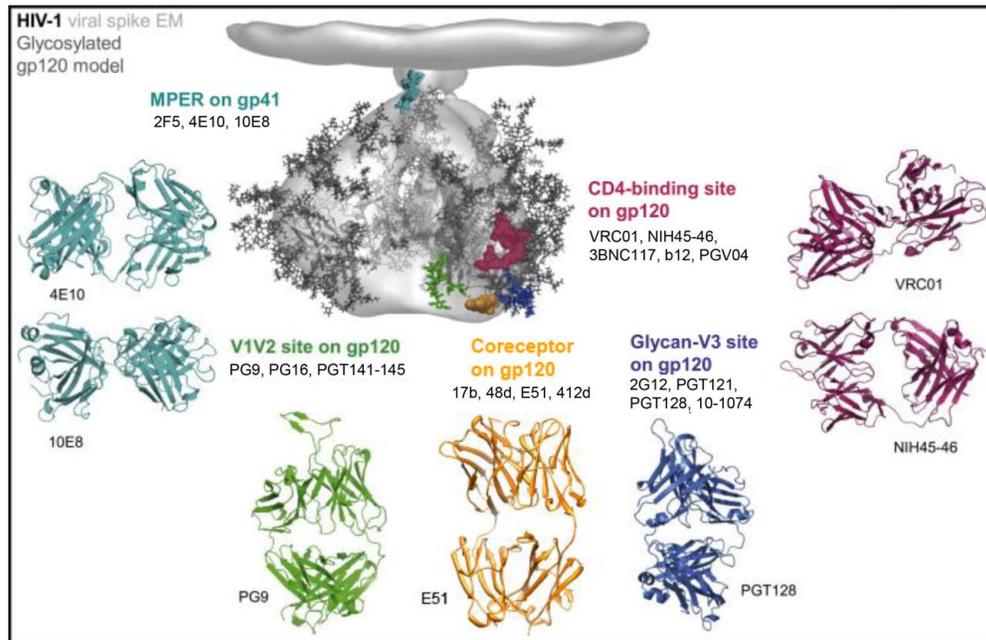
Many antibodies are generated against Env, but very few are efficient NAbs and many of those that lack breadth. HIV-1 broadly neutralizing antibodies (bNAbs) are believed to take from months to years to develop. HIV-1 bNAbs have been under intense focus in recent years as new technologies and techniques have helped identify a few

classes as bNAbs. Most groups looking to screen for bNAbs have used patient sera that shows high neutralization reactivity against HIV-1 isolates. In the early 1990s, phage display technology was used for combining heavy chains and light chains from one elite controller and it was through this method that the first bNAb was identified<sup>133,134</sup>. More recent techniques have used gp140 trimers for selecting antibodies based on binding<sup>135</sup>. The problem with this technique is that most antibodies identified by trimer binding are efficient neutralizers of tier 1 isolates but lack potency against many tier 2 and most tier 3 isolates.

A newer screen technique was used by Walker *et al.* to identify what was the first of many broader and more potent bNAbs<sup>100</sup>. This screen obtained and stimulated a large amount of B cells from an infected patient, and the resulting antibodies produced by the B cells were tested for neutralization against 2-4 HIV-1 isolates. The best neutralizers were then cloned out and further characterized against a larger panel of HIV-1 isolates. Another novel technique was described by Wu *et al.* who started with a YU2 gp120 core (lacking variable loops 1 to 3) and resurfaced the outer domain of Env with SIV residues except for the CD4-binding site<sup>136</sup>. With this resurfaced stabilized core (RBC3), antibodies directed against the CD4bs were identified, with some as bNAbs. This was followed by Scheid *et al.* who developed a 2CC core where a YU2 gp140 trimer lacking variable loops 1 to 3 was used for binding memory B cells from patients with highly efficient sera<sup>137</sup>. New primer sets made to have a larger coverage were then used to clone out the heavy and light chains of the antibodies from the B cells selected in this screen. The 2CC core differed from the RBC3 core in that, excepting the variable loop deletions,

gp140 was unaltered. This allowed Sheid *et al.* to identify antibodies targeting sites other than the CD4bs.

These newly identified HIV-1 bNAbs have encouraged efforts to develop an HIV-1 vaccine. Identifying these bNAbs demonstrated that there are a few sites of vulnerability of Env. These sites include the CD4-binding site (CD4bs), glycosylation of N332 on the V3 loop, glycosylation of N160 on the V1/V2 loop, and the MPER region (Figure 1.4). Characterizing HIV-1 bNAbs has also revealed key features of these antibodies, for example long CDR-H3s (heavy chain complementarity determining region 3) and high amounts of somatic hypermutation. The different classes of HIV-1 antibodies are discussed below.



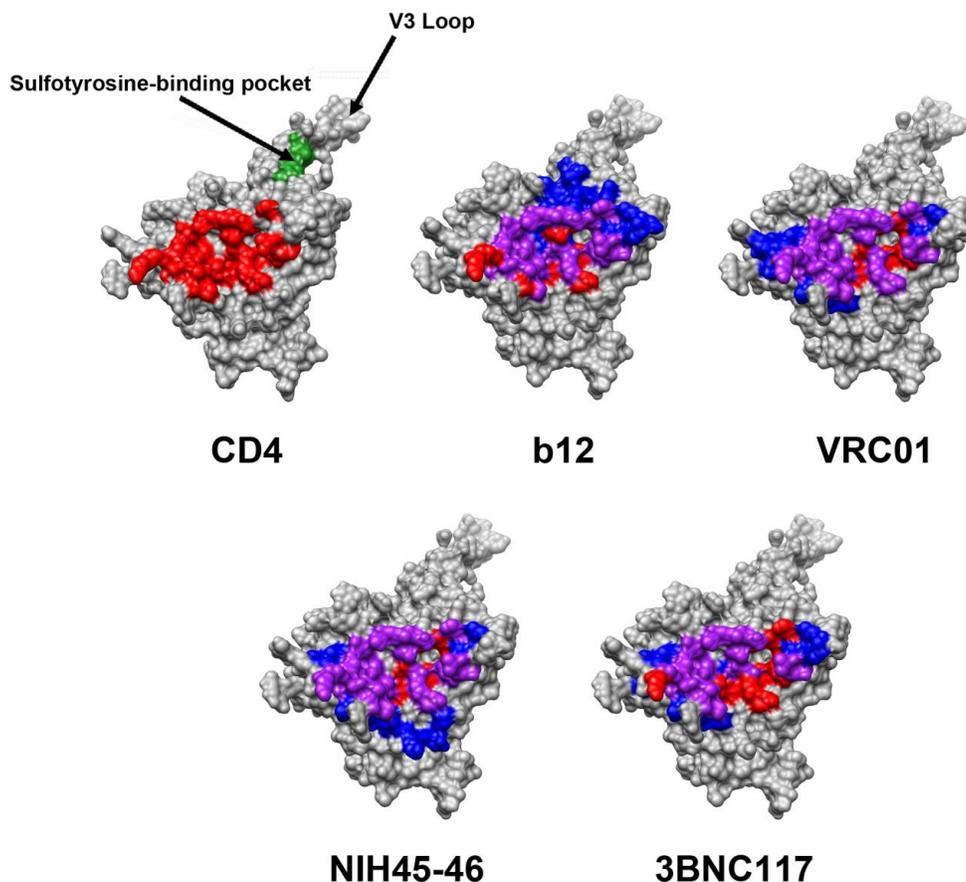
**Figure 1.4. The epitopes on HIV-1 Env<sup>138</sup>.** In grey is the cryo-EM tomogram of HIV-1 Env isolate Bal<sup>105</sup> fitted with gp120 monomers in the CD4-bound state<sup>139</sup> and modeled with glycosylated residues. The numerous epitopes on Env are diagramed: red – the CD4-binding site; blue – N322 on V3; green – N160 on V1/V2; cyan – the MPER region; and orange – the sulfotyrosine-binding pocket. (Adapted from Kwong PD and Mascola JR, 2012<sup>138</sup>).

### *CD4-binding site antibodies*

Of the epitopes mapped on HIV-1 Env, the CD4-binding site (CD4bs) is an especially important target because it is conserved and antibodies directed against this region directly block CD4 binding. The bNAb b12 was first identified in 1994 from a 31 year-old male who had been HIV seropositive but showed no signs of symptoms for six years<sup>134</sup>. Of note, b12 bound the CD4-binding site but structural data showed that it did not induce the CD4-bound state<sup>26</sup>. Although it doesn't induce the CD4-bound state, b12 does produce conformational changes of gp120 which causes it to have a high perturbation factor for binding Env<sup>140</sup>. Binding of less potent CD4bs antibodies, F015 and b13, also produce substantial conformational changes on Env but are different than the changes produced by b12<sup>141</sup>. Interestingly, the heavy chain of b12 contains a large CDR-H3 that binds gp120 in close proximity to the coreceptor-binding site. As a possible artifact of phage display, b12 does not use its light chain for any contacts on gp120<sup>26</sup>. b12 is extremely efficient against clade B isolates, neutralizing ~75% of isolates tested. However, the breadth of b12 is limited to neutralizing ~35% of all isolates assayed<sup>136</sup>.

A more potent CD4bs antibody, VRC01, was isolated in 2010. Wu *et al.* used a “bait” YU2 scaffold gp120 where the CD4 binding site was conformationally stable and resurfaced without glycosylation. Among the antibodies identified, VRC01 neutralized ~90% of the isolates it was tested against with a mean  $IC_{50}$  of 0.3 ug/uL<sup>136</sup>. In common with b12, VRC01 shows a high affinity for both the bound and unbound conformations of gp120<sup>142</sup>. Structural analysis indicates that VRC01 covers ~98% of the CD4 binding site<sup>142</sup>, as seen in Figure 1.5.

Since the discovery of VRC01, more neutralizing antibodies have been isolated from HIV-1 infected patients. A more potent CD4bs antibody, PGV-04, has been characterized and shown to share the same VH1-2 germline origin as VRC01<sup>143</sup>. Using the 2CC core, Scheid *et al.* identified NIH45-46 and 3BNC117, both of which have shown to be more potent than VRC01<sup>137</sup>. NIH45-46 may be more potent than VRC01 because of its ability to bind the bridging sheet of gp120<sup>144</sup>. An engineered NIH45-46 variant containing a G54W mutation in its CDR-H2, bound gp120 with higher affinity, presumably by occupying the Phe43 pocket of gp120<sup>144</sup>. The G54W mutation also increased the potency of the NIH45-46, gaining the ability to neutralize previously resistant viruses. Further mutations of the NIH45-46 light chain have improved NIH45-46<sup>G54W</sup> and were shown to neutralize NIH45-46 escape isolates<sup>145</sup>. Numerous other antibodies were characterized through these studies including 12A12, HJ16, VRC03, and 8ANC195. The proliferation of CD4bs antibodies identified offers the promise that CD4bs antibodies can be generated even with all the strategies Env employs to hide the CD4-binding site.



**Figure 1.5. Footprints of CD4-binding site antibodies.** The footprints of CD4, b12, VRC01, NIH45-46, and 3BNC117 are modeled onto a gp120 monomer in the CD4-bound state. The CD4 footprint is in red, antibody footprint is in blue, and overlapping contact residues are purple. Also labeled on the CD4 footprinting model are the sulfotyrosine-binding pocket (green) and V3 loop. Coordinates for the models are as follows: CD4 – 2QAD; b12 – 2NY7; VRC01 – 4LST; NIH45-46 – 3U7Y; 3BNC117 – 4JPV. Modeling was done using Chimera.

### *Glycan-dependent antibodies*

The isolation and characterization of the monoclonal antibody 2G12 identified a novel glycosylation epitope on Env<sup>146</sup>. 2G12, originally described as a broadly neutralizing antibody, was found to bind to N-linked carbohydrates on the C2, C3, C4, and V4 regions of gp120. In 2009, Walker *et al.* described two novel bNAbs, PG9 and PG16, that neutralized 79% and 74% of isolates tested, respectively<sup>100</sup>. Structure data indicated that PG9 binds two N-glycans, N156 and N160, as well as a strand on the

V1/V2 loop of gp120 and the large CDR-H3 of PG16 forms a “hammer head” shape that is important for its potency<sup>139,147</sup>. Interestingly, PG9 and PG16 have a unique neutralization profile where they do not completely neutralize certain HIV-1 isolates, presumably through incomplete glycosylation of Env<sup>148</sup>. Another feature of PG9 and PG16 is that they bind trimeric Env efficiently but not monomeric gp120<sup>100</sup>. Since the discovery of PG9 and PG16, other bNAbs targeting the V1/V2 loop have been identified, including CH01-04 and PGT141-145<sup>102,149</sup>.

Walker *et al.* followed up their PG9 and PG16 with a PGT series of antibodies. The most potent from this group, including PGT121, PGT122, and PGT128, targeted the N332 glycan at the base of the V3 loop<sup>82,102,150</sup>. Separately, Mouquet *et al.*, described a different series of N332 antibodies based on the antibody 10-1074<sup>151</sup>. The difference between the PGT121-series and 10-1074 is that the 10-1074 series is completely dependent on a glycosylated N332 residue whereas the PGT121-series uses a more complex epitope. The crystal structure of a soluble, cleaved HIV-1 Env in complex with PGT122 determined this epitope to be at the base of the V1 and V3 loops as well as the glycosylated residues N137, N156/173, N301, and N332<sup>82</sup>. Although there are differences between PG9/PG16, PGT-series, and 10-1074-series of antibodies, it is important to note that these glycan-dependent antibodies show that the glycan shield itself can be efficiently targeted for neutralization.

#### *MPER antibodies*

The conserved MPER region of gp41 serves as another vulnerable site for antibodies. Notable MPER antibodies include 2F5<sup>104</sup>, 4E10<sup>105</sup>, and 10E8<sup>152</sup>. Of these, 10E8 showed great breadth, neutralizing 98% of the isolates tested. 10E8 was initially

described to be different than historical MPER antibodies in that it did not bind lipid and was not autoreactive. However, it was recently reported that 10E8 does bind lipid bilayers through its CDR-H3<sup>106</sup>. Like 4E10 and 2F5, 10E8 binds a fusion-intermediate of gp41 indicating a common mechanism by which MPER antibodies bind gp41. MPER antibodies are of considerable scientific and therapeutic interest because among bNAbs, they uniquely target a gp41 epitope, and they block gp41 conformational changes necessary for fusion.

#### *The gp120-gp41 epitope*

A relatively new antibody epitope was defined to include both gp120 and gp41. bNAbs targeting this site include 8ANC195<sup>137</sup>, PGT151<sup>153</sup>, and PGT152<sup>153</sup>. The gp120-gp41 epitope was first described through the characterization of m43, an antibody derived from phage display that neutralized 34% of the isolates assayed<sup>154</sup>. However, it was unclear whether m43 contacted both the gp120 and gp41 subunits simultaneously. The crystal structures of 8ANC195 and PGT151 definitively showed that these antibodies interact with both subunits on the Env trimer<sup>155,156</sup>. The breadth of these antibodies is slightly less than the CD4bs, glycan-dependent, and MPER antibodies as 8ANC195, PGT151, and PGT152 neutralized 66%, 66%, and 64% of a panel of HIV-1 isolates, respectively<sup>137,153</sup>. Similar to PG9 and PG16, PGT151 and PGT152 both have incomplete neutralization behavior<sup>153</sup>. Yet, the gp120-gp41 epitope could be another target for vaccine design.

#### *CD4-induced antibodies*

Before the discovery of the HIV-1 coreceptors, a group of HIV-1 antibodies was identified, whose binding to gp120 is increased by the binding of CD4<sup>157</sup>. The epitope of

these antibodies was later identified as the coreceptor-binding region of gp120. Because the binding of CD4 to gp120 induces the binding of these antibodies, this class of antibodies was called CD4-induced antibodies (CD4i). These antibodies bind the coreceptor region of gp120 and some do so using sulfated tyrosines<sup>158</sup>, presumably mimicking the coreceptor. CD4i antibodies commonly have long CDR-H3s and most originate from the germline precursor variable gene VH1-69<sup>159</sup>. The best characterized CD4i antibodies are 17b and 48d, two antibodies with weak neutralizing capabilities which neutralize X4 lab-adapted strains rather than most primary R5 strains<sup>157</sup>. The CD4i antibody E51 was shown to block CCR5 binding site and epitope mapping located the E51-binding site to the bridging sheet and the base of the V3 loop<sup>160</sup>. Like E51 and the CCR5 coreceptor, the CD4i antibody 412d also uses sulfated tyrosines to achieve binding to gp120<sup>161</sup>, and binds an epitope similar to that of E51. Both E51 and 412d are more potent against primary HIV-1 isolates than the previously characterized 17b and 48d<sup>161</sup>. The sulfated-tyrosines of 412d have been localized to a conserved pocket of the coreceptor binding site<sup>162</sup>. Though CD4i antibodies target a critical epitope of gp120, binding to gp120 comes at a substantial entropy cost. CD4i antibodies have some of the highest perturbation factors – the amount of conformational changes Env undertakes in order for the antibody to bind – which limit its ability to bind Env with high affinity<sup>140</sup>. Efficient HIV-1 bNAbs have a low PF and bind Env with high affinity. The high perturbation factor explains why CD4i antibodies are less potent than other HIV-1 bNAbs.

### *Peptide inhibitors*

As mentioned, because Env has recessed and hidden its receptor-binding sites, these sites can be more readily accessed by smaller peptides, single chain variable fragments (scFv), or Fab (fragment antigen-binding) constructs than by full-length antibodies. Vita *et al.* developed a CD4-mimetic peptide built from scorpion toxin scaffold containing CD4 residues 40-43 (QSGF)<sup>163</sup>. These CD4 residues target the Phe43 pocket of the gp120 CD4-binding site. This CD4-mimetic peptide could induce 17b binding, indicating that it promotes the CD4-bound state of Env<sup>164</sup>. Quinlan *et al.* created soft-randomized libraries of the CD4-mimetic peptide and were able to improve its affinity for gp120 using phage display. CD4-mim6 could neutralize HIV-1 isolates greater than the predecessor – CD4mim1 – but overall, was not as broad or potent as CD4-Ig.

Because the sulfotyrosine-binding pockets of gp120 are smaller than the CD4-binding cavity, the epitope can be targeted by linear peptides. As stated above, the CDR-H3s of numerous CD4i antibodies contain sulfated-tyrosines that mimic those found at the N-terminus of CCR5. The CDR-H3 of E51 has five tyrosines modified by sulfates that are critical for it to bind gp120<sup>158</sup>. Accordingly, it was shown that a CCR5-mimetic (CCR5min) based on the 15 amino-acid portion of the CDR-H3 of E51 with the Fc of IgG1, CCR5mim1-Ig, could bind to gp120 molecules and neutralize a panel of HIV-1 pseudoviruses<sup>165</sup>. The dimer form of CCR5mim1-Ig can bind to trimeric envelope better than the monomer form presumably because it can bind two gp120 monomers of the Env trimer<sup>109</sup>. Newer CCR5min peptides have been developed based on CDR-H3 of PG16 and CCR5mim1<sup>166</sup>. The CDR-H3 of PG16, like most CD4i antibodies, contains sulfated

tyrosines. Out of seven swap variants of PG16 and CCR5mim2, two variants - pSwap3-Ig and pSwap7-Ig (CCR5mim2-Ig) - bound gp120 and Env trimers better than CCR5mim1-Ig. Like CD4i antibodies, binding of Env by CCR5mim peptides can be induced by sCD4 binding.

### *Dual-acting inhibitors*

New HIV-1 entry inhibitors are being developed that target multiple epitopes of Env. Such inhibitors have been designed to target the receptor-binding sites on Env. One of these inhibitors fused sCD4 with the scFv form of 17b (sCD4-17b)<sup>167,168</sup>. This construct was able to neutralize a diverse panel of isolates covering a range of different clades. Similar to that of sCD4-17b, when a double-mimetic peptide was created by Quinlan and Joshi *et al.* by fusing CD4mim6 with CCR5mim3 and expressed as an immunoadhesin, it could neutralize HIV-1 isolates better than CD4-Ig<sup>169</sup>. Both the sCD4-17b and double-mimetic peptide constructs have the ability to bind both receptor-binding sites on gp120. Chen *et al.* developed similar fusion constructs (4Dm2m and 6Dm2m) that target the receptor-binding sites by fusing a single domain form of sCD4-Ig with m36.4, which targets the coreceptor binding site<sup>170</sup>. Both 4Dm2m and 6Dm2m neutralized HIV-1 isolates more efficiently than VRC01. Lu *et al.* engineered an effective bivalent HIV-1 entry inhibitor composed of sCD4 with the fusion inhibitor T1144<sup>171</sup>. Similar to the previous groups, Falkenhagen *et al.* developed fused constructs of sCD4 with scFV-17b or FI<sub>T45</sub>, a fusion inhibitor similar to T20<sup>172</sup>. Along with a tri-acting construct of sCD4 with scFV-17b and FI<sub>T45</sub>, these constructs could neutralize HIV-1 isolates better than CD4-Ig and T20. Taking a somewhat different approach, West *et al.* generated bivalent inhibitors by fusing domains 1 and 2 of CD4 to 17b, E51, and 2G12

antibodies<sup>173</sup>. Again, these constructs increased the neutralization efficiencies compared to the original constructs. Taken together, dual-acting constructs targeting multiple epitopes of Env inhibit HIV-1 more efficiently than their separate components that target single epitopes.

Targeting multiple epitopes on Env can also be achieved by creating bivalent antibodies. Bivalent antibodies are created through a “knobs and holes” design by mutating residues in the antibody Fc domain. The knobs and holes method can achieve >90% heterodimer formation of bivalent antibodies *in vitro*<sup>174</sup>. Although bivalent anti-Env antibodies have not yet been described, Pace *et al.* have created an antibody combining iMab, with the scFv forms of PG9 and PG16 (termed PG9-iMab and PG16-iMab, respectively)<sup>175</sup>. iMab localizes the fused PG9 or PG16 scFv domains to CD4. This localization of the scFvs keeps them in close proximity to gp120 when the Env binds CD4. Despite relatively lower breadth of PG9 and PG16 compared to other bNAbs, both PG9-iMab and PG16-iMab neutralized 100% of 118 HIV-1 isolates tested. The concept of targeting the HIV-1 receptors is unique and can be broadened towards targeting a coreceptor using an antibody like PRO 140.

### **1.G. Antibody Combinations**

Another way of targeting multiple epitopes of Env is to combine HIV-1 antibodies. Early studies showed that combinations mixing CD4bs antibodies with antibodies directed to the V2 or V3 loops could be synergistic<sup>176-181</sup>. A few groups looked at combinations of b12, 2G12, 2F5, and 4E10<sup>182-185</sup>, as these were the best NAbs available until the discovery of PG9 and PG16. A more recent report examined a combination of PG9 with VRC01 and found there to be no synergy, yet it was shown that

the antibodies could complement each other to increase their overall breadth<sup>186</sup>. Synergy was also observed between two classes of antibodies targeting the C1 and V2 regions of Env that were derived from vaccine recipients in the RV144 Thai trial<sup>187</sup>. *In vivo* studies have shown that antibody combinations are able to suppress infection in HIV-1 infected humanized mice<sup>188</sup> and SHIV infected rhesus macaques<sup>189,190</sup>. Further work describing how different classes of bNAbs work together will be critical to the development of antibody cocktails for therapy.

## **1.H. Summary**

HIV-1 Env is a structurally complex viral protein that contains numerous obstacles hindering the development or elicitation of potent bNAbs. HIV-1 can be more efficiently neutralized with fewer routes of escape by targeting multiple conserved epitopes on Env with HIV-1 bNAbs or peptide inhibitors. In the following chapters, we will explore the utility of targeting the CD4- and coreceptor-binding site simultaneously. In Chapter 2, we demonstrate that we can achieve more potent neutralization with combinations of CD4-Ig and CCR5mim2-Ig or CD4bs antibodies with CD4i antibodies. In Chapter 3, we characterize a newly developed HIV-1 entry inhibitor that fuses CCR5mim1 to the C-terminus of CD4-Ig. In Chapter 4, we discuss our findings in the context of current efforts to develop antibody-based therapeutics or an HIV-1 vaccine.

## **CHAPTER 2**

**Combinations of HIV-1 Entry Inhibitors that Target the CD4-  
and Coreceptor-Binding Sites Efficiently Neutralize HIV-1 Isolates**

## Acknowledgements

The data presented in Figure 2.1 is derived from the published work:

Chiang JJ, Gardner MR, Quinlan BD, Dorfman T, Choe H, and Farzan M.  
Enhanced recognition and neutralization of HIV-1 by antibody-derived CCR5-mimetic peptide variants. *J Virol.* 2012 Nov 86(22):12417-21.

I would like to Jessica J. Chiang for her contributions to Figure 2.1B.

Experiments were designed and performed for Figures 2.2-5 by MRG.

This work was supported by the National Institutes of Health (R01 AI080324; R01 AI0914767; and P01 AI100263 awarded to MF).

## **2.A. Abstract**

The HIV-1 envelope glycoprotein (Env) is a trimer of gp120/gp41 heterodimers that mediates viral entry. Env binds cellular CD4, an association which stabilizes a conformation favorable to its subsequent association with a coreceptor, typically CCR5 or CXCR4. The CD4- and coreceptor-binding sites serve as epitopes for two classes of HIV-1 neutralizing antibodies – CD4 binding site (CD4bs) and CD4-induced (CD4i) antibodies, respectively. Here we observed that, at a fixed total concentration, mixtures of the CD4i antibody E51 and the CD4bs antibody VRC01 neutralized the HIV-1 isolates ADA and 89.6 more efficiently than either antibody alone. Using a wider range of isolates, we found that E51 promoted association of four CD4bs antibodies to the Env trimer, but not to monomeric gp120. Interestingly, and in contrast to CD4-Ig, the relationship is not reciprocal: CD4bs antibodies do not efficiently promote E51 binding to Env trimer. Consistent with these observations, CD4-Ig, but none of the CD4bs antibodies tested, substantially increased HIV-1 infection of a CD4-negative, CCR5-positive cell line. We conclude that E51 and VRC01 are more potent together largely because E51 promotes access of VRC01 to the Env trimer. Our data also suggest that potent CD4bs antibodies avoid inducing Env conformations that bind E51 or CCR5.

## **2.B. Introduction**

Human immunodeficiency virus type 1 (HIV-1) uses its envelope glycoprotein (Env) to gain entry into host cells. Env is synthesized as precursor gp160 proteins which assemble as trimers before they are cleaved into gp120 and gp41 subunits<sup>191</sup>. The gp120 subunit binds the primary HIV-1 receptor, CD4<sup>16-18</sup>, which then induces tertiary and quaternary conformational changes in Env that promote association with a coreceptor,

usually CCR5 or CXCR4<sup>28,31</sup>. The CD4- and coreceptor-binding sites are the two most conserved regions of gp120<sup>20,21</sup>. Two classes of antibodies, corresponding to these regions have been defined: CD4-binding site (CD4bs) antibodies, and CD4-induced (CD4i) antibodies. The latter are so named because CD4 binding induces a conformation that promotes their association with gp120.

The antibody b12, isolated from a patient library by phage display, was the first potent CD4bs antibody described<sup>134</sup>. However, its breadth was limited to 35% against a broad panel of HIV-1 isolates<sup>136</sup>. More recently, broader and more potent antibodies have been identified, notably VRC01, 3BNC117, and NIH45-46<sup>136,137,142</sup>. These antibodies neutralize more than 90% of HIV-1 isolates assayed. The breadth and potency of NIH45-46 was increased through both a G54W mutation – the added tryptophan targeting a cavity in the CD4-binding site of gp120 occupied by phenylalanine 43 of CD4 – and through additional light-chain modifications<sup>144,145</sup>. These highly potent broadly neutralizing antibodies (bNAbs) can protect from HIV-1 challenge and reduce viral loads in infected humanized mice and rhesus macaques<sup>188-190,192</sup>.

Relative to CD4bs antibodies, well characterized CD4i antibodies like 17b and 48d, are substantially less broad and potent<sup>157,193,194</sup>. This inefficiency is largely a consequence of their recognition of an Env conformation usually unavailable in the absence of CD4, coinciding with the inaccessibility of their epitope to antibody-sized molecules due to the proximity of the viral and cellular membranes<sup>93</sup>. Some CD4i antibodies, including E51 and 412d, mimic CCR5 by incorporating sulfotyrosines into their heavy-chain CDR3 regions<sup>89,158</sup>. These sulfotyrosines bind highly conserved pockets on gp120 that recognize the CCR5 amino-terminus. Perhaps as a consequence,

E51 and 412d typically bind Env and neutralize HIV-1 more efficiently than 17b or 48d. The structure of gp120 in complex with 412d localizes two sulfotyrosine binding pockets at the base of the third variable loop and in the fourth conserved domain<sup>92</sup>.

Because CD4 and CD4i antibodies bind the envelope glycoprotein cooperatively, we explored the relationship between the CD4i antibody E51 and a panel of CD4bs bNAbs. We first showed that we could get greater neutralization of four HIV-1 isolates when targeting the receptor-binding sites with CD4-Ig and CCR5mim2-Ig. We then observed that, at the same total concentrations, mixtures of E51 and the CD4bs antibody VRC01 were more potent than either antibody alone. However, unlike CD4-Ig, CD4bs antibodies did not promote E51 binding to the Env trimer. Consistent with this observation, CD4-Ig, but not CD4bs antibodies, could promote infection of CCR5-positive, CD4-negative cells. In contrast, E51 promoted quaternary changes in Env that increased its binding to several highly potent CD4bs antibodies, including VRC01. Thus, CD4i antibodies can enhance the potency of CD4bs antibodies, presumably by increasing access to the CD4-binding site.

## **2.C. Materials and Methods**

**Cells and plasmids.** All cell lines were grown in DMEM with 10% fetal bovine serum at 37°C. TZM-bl cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc<sup>195-199</sup>. Cf2-Th CCR5-positive, CD4-negative cells were provided by Dr. Hyeryun Choe. The variable heavy and light chains of E51 were cloned into human and murine IgG1 expression vectors. 89.6, ADA, JRFL, SA32, and ConC gp160 expression vectors have been previously described<sup>109,166</sup>. 89.6, ADA, SA32, and ConC gp160-Δcytoplasmic tail

expression vectors have been previously described<sup>166</sup>. pNL4-3.luc.R<sup>-</sup>.E<sup>-</sup> was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Nathaniel Landau<sup>200,201</sup>. CD4-Ig has been previously described<sup>166</sup>. Vectors expressing VRC01 heavy and light chains were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. John Mascola<sup>136,202</sup>. 10E8 expression vectors were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Mark Connors<sup>152</sup>. Purified b12 and 2G12 antibodies were obtained from Polymun Scientific. Vectors expressing NIH45-46 and 3BNC117 were provided by Dr. Michel Nussenzweig. The NIH45-46<sup>G54W</sup> heavy-chain expression vector was provided by Dr. Pamela Bjorkman.

**Antibody and CD4-Ig production and purification.** HEK293T cells (ATCC) seeded in T-175 flasks (Falcon) were co-transfected with 40 µg of both heavy chain vector and light chain vector per flask of the antibodies VRC01, NIH45-46, NIH45-46W, 3BNC117, E51, 10E8 or with 80 µg per flask CD4-Ig vector with Calcium Phosphate transfection kit (Clontech). At 12-16 hours post transfection, cells were washed with PBS and grown in FreeStyle 293 medium (Invitrogen). 48 hours post-transfection, medium was collected, centrifuged, and filtered with a 0.45 µm filter flask (Millipore). Protein A sepharose beads (GE Healthcare) and Complete protease inhibitor cocktail (Roche) were added to the medium and agitated overnight at 4°C. Beads were collected in a gravity flow through column (Bio-Rad), washed once with 0.5M NaCl in PBS, washed once with PBS, and eluted with IgG Elution Buffer (Thermo Scientific) into 1M Tris-HCl Buffer, pH 9.0 (G Biosciences). Buffer was exchanged with PBS and protein concentrated to 1

mg/mL with Amicon Ultra Centrifugation Filters (Millipore). Antibodies were stored at 4°C.

**TZM-bl neutralization assays.** Pseudotyped HIV-1 was produced by co-expression of envelope glycoproteins of the indicated HIV-1 isolates with NL4-3ΔEnv, an HIV-1 expression vector lacking a functional *env* gene. 293T cells, grown to 50% confluency in T175 (Falcon) flasks, were transfected with 25 μg of plasmid encoding envelope glycoprotein, 45 μg of NL4-3ΔEnv, and 5 μg each of plasmids expressing the HIV-1 *tat* and *rev* genes, by the calcium phosphate technique. 10% FBS-DMEM was changed at 12 h, and medium was collected at 48 h. Viral supernatants were cleared by centrifugation for 10 min at 1500 × g, passed through a 0.45-μm syringe filter (Millipore), and stored at -80 °C.

TZM-bl neutralization assays were performed as previously described<sup>203</sup>. Briefly, HIV-1 pseudoviruses were pre-incubated with titrated amounts of antibody or CD4-Ig in DMEM (10% FBS) for 1 hour at 37°C. TZM-bl cells were detached by trypsinization and diluted in DMEM (10% FBS) to 100,000 cells/mL and added to the pseudovirus/inhibitor mixture. Cells were then incubated for 48 hours at 37°C. Viral entry was analyzed using Britelite Plus (Perkin Elmer) and luciferase was measured using a Victor X3 plate reader (Perkin Elmer). Combination Index (CI) values for 50% inhibition (CI<sub>50</sub>) to determine synergy were calculated as previously described<sup>204-207</sup>.

**GHOST cell neutralization assay.** GHOST-CCR5 or -CXCR4 cells were plated into 12-well plates at 50,000 cells per well. HIV-1 pseudovirus was diluted in RPMI and titrated amounts of CCR5mim2-Ig, pSwap3-Ig, CCR5mim1-Ig, and pCa5R-Ig were added. Virus and inhibitor were incubated at room temperature for 20 minutes and added

to the cells for 2 hours at 37°C. Cells were then washed with serum free medium and then incubated in 1 mL of DMEM (10% FBS) for 48 hours at 37°C. Cells were then trypsinized and resuspended in PBS (2% FBS), pelleted, and resuspended in a fixative solution (1% paraformaldehyde in PBS). Viral entry was determined by flow cytometry based on GFP expression.

**ELISA studies of monomeric HIV-1 gp120.** ELISA plates (Costar) were coated with 5 µg/mL HIV-1 gp120 (Immune Technolog Corp.) overnight at 4°C. Plates were washed with PBS-T (PBS + 0.05% Tween-20) twice and blocked with 5% milk in PBS for 1 hour at 37°C. Dilutions of murine-E51, CD4-Ig or CD4bs antibodies blocked with 5% milk in PBS were added to the plate and incubated for 1 hour at 37°C. Samples were washed five times with PBS-T and fixed concentrations of CD4-Ig or CD4bs antibodies (in the case of samples pre-incubated with murine-E51) or murine-E51 (in the case of samples pre-incubated with CD4-Ig or CD4bs antibodies) were added. Plates were incubated for 1 hour at 37°C. Samples were washed five times with PBS-T and labeled with a horseradish peroxidase-conjugated secondary antibody (Jackson Immuno Research) recognizing human IgG1. Plates were incubated for 1 hour at 37°C and then washed ten times with PBS-T. TMB solution (Fisher) was added for 10 minutes at room temperature and then stopped with TMB Stop Solution (Southern Biotech). Absorbance was measured at 450 nm by a Victor X3 plate reader (Perkin Elmer).

**Flow cytometric analysis of cell-expressed envelope glycoprotein trimers** HEK293T cells were transfected with plasmids expressing the indicated envelope glycoproteins lacking cytoplasmic residues 732 to 876 (HXBc2 numbering) together with plasmid encoding the tat protein. Transfection medium was replaced after an overnight

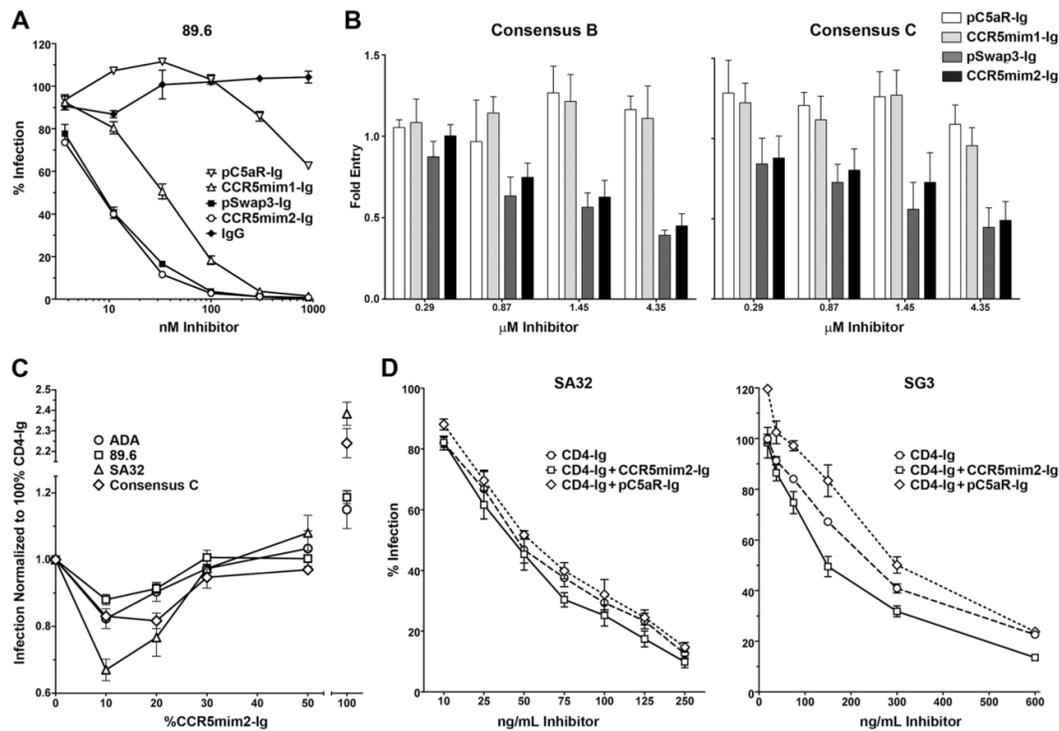
incubation and cells were harvested 48 hours post-transfection. Harvested cells were washed twice in flow cytometry buffer (PBS with 2% goat serum, 0.01% sodium azide). Cells were incubated with serially diluted CD4bs antibody, CD4-Ig, or murine-E51 on ice for 1 hour and then washed twice with flow cytometry buffer. If cells were pre-incubated with murine-E51, cells were then incubated with a 10 ng/mL of CD4bs antibody or CD4-Ig on ice for 1 hour. If cells were pre-incubated with CD4bs antibodies or CD4-Ig, cells were then incubated with 25 ng/mL murine E51 on ice for 1 hour. After incubation, cells were washed twice with flow cytometry buffer. APC or FITC-labeled secondary antibodies recognizing human or murine Fc (Jackson Immuno Research), respectively, were incubated with cells for 30 minutes. Cells were washed twice with flow cytometry buffer, twice with PBS, and resuspended in 1% paraformaldehyde solution. Binding was analyzed with an Accuri C6 Flow Cytometer (BD Biosciences) and data was analyzed with the C6 Software (BD Biosciences).

**Infection of CD4-negative cells.** HIV-1 pseudovirus expressing firefly luciferase was pre-incubated with titrated amounts of antibodies or CD4-Ig in DMEM (10% FBS) for 1 hour at 37°C. CD4-negative Cf2Th-CCR5 cells were harvested and diluted in DMEM (10% FBS) to 100,000 cells/mL and added to the pseudovirus/inhibitor mixture. Cells were then incubated for 48 hours at 37°C. Viral entry was analyzed using Britelite Plus (Perkin Elmer) and luciferase was measured by a Victor X3 plate reader (Perkin Elmer).

## **2.D. Results**

**Mixtures of CD4-Ig and CCR5mim2-Ig neutralize HIV-1 isolates more efficiently than either inhibitor alone.** Having shown that new CCR5mim peptides bind Env, we wanted to test their ability to neutralize HIV-1 isolates. We analyzed pC5aR-Ig,

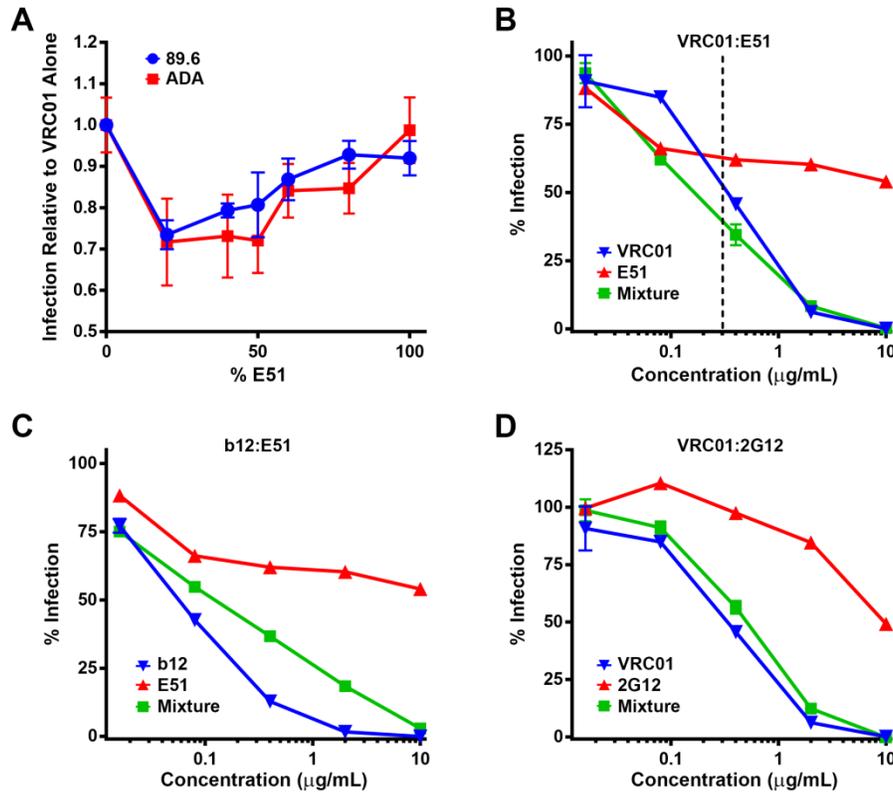
CCR5mim1-Ig, pSwap3-Ig, and CCR5mim2-Ig using a previously described entry inhibition assay. Both pSwap3-Ig and CCR5mim2-Ig neutralized the dualtropic, clade B isolate 89.6 more efficiently than CCR5mim1-Ig, with 50% inhibitory concentrations ( $IC_{50}$ s) of approximately 10 nM. They also neutralized consensus B and C isolates more efficiently, but with  $IC_{50}$ s of approximately 4  $\mu$ M. In an effort to show that targeting the minimum receptor binding epitopes would be more efficient than targeting either epitope alone, we next investigated the ability of CCR5mim2-Ig to enhance CD4-Ig-mediated neutralization of HIV-1. When total protein was kept constant at the  $IC_{50}$  of CD4-Ig alone, a 9:1 ratio of CD4-Ig to CCR5mim2-Ig (4.5:1 on a molar basis) neutralized all pseudoviruses assayed more efficiently than CD4-Ig alone. This effect was evident at a 9:1 ratio for a range of total protein concentrations, more so for the neutralization sensitive SG3 isolate.



**Figure 2.1.** CCR5mim2-Ig synergizes with CD4-Ig to neutralize HIV-1 infection. **(A)** The inhibitory activities of the indicated peptide-Fc fusions were measured with a TZM-bl neutralization assay using the envelope glycoprotein of the dualtropic clade B isolate 89.6. Infectivity is represented as a percentage of luciferase activity in the absence of inhibitor. **(B)** The indicated peptide-Fc fusions were assayed for their ability to limit infection of retroviruses pseudotyped with consensus B and C envelope glycoproteins in GHOST-CCR5 cells. Infection was measured as green fluorescent protein (GFP) activity by flow cytometry. **(C)** Relative infection of HIV-1 pseudotyped with the indicated envelope glycoproteins was measured with a TZM-bl neutralization assay in the presence of various ratios of CD4-Ig and CCR5mim2-Ig in which the total amount of protein was held constant. Horizontal axis indicates percentage of CCR5mim2-Ig. The total amount of protein for each isolate was chosen to be approximately that of the IC<sub>50</sub> of CD4-Ig alone: 89.6 (clade B, R5X4), 50 ng/ml; ADA (clade B, R5), 40 ng/ml; SA32 (clade C, R5), 75 ng/ml; and consensus C (clade C, R5), 4 μg/ml. **(D)** A TZM-bl cell neutralization assay was performed at various concentrations of CD4-Ig and CCR5mim-Ig at a 9:1 ratio. The figure shows infection of cells with the SA32 (clade C, R5) and SG3 (clade B, X4) pseudoviruses in the presence of the CD4-Ig or CD4-Ig/peptide-Ig mixtures at various concentrations.

**Mixtures of E51 and VRC01 neutralize HIV-1 more efficiently than either antibody alone.** Having shown we can get greater neutralization through mixtures of CD4-Ig and CCR5mim2-Ig in Figure 2.1C-D, we sought to determine whether an approach using HIV-1 antibodies would yield a similar result. We chose to study the CD4-binding site

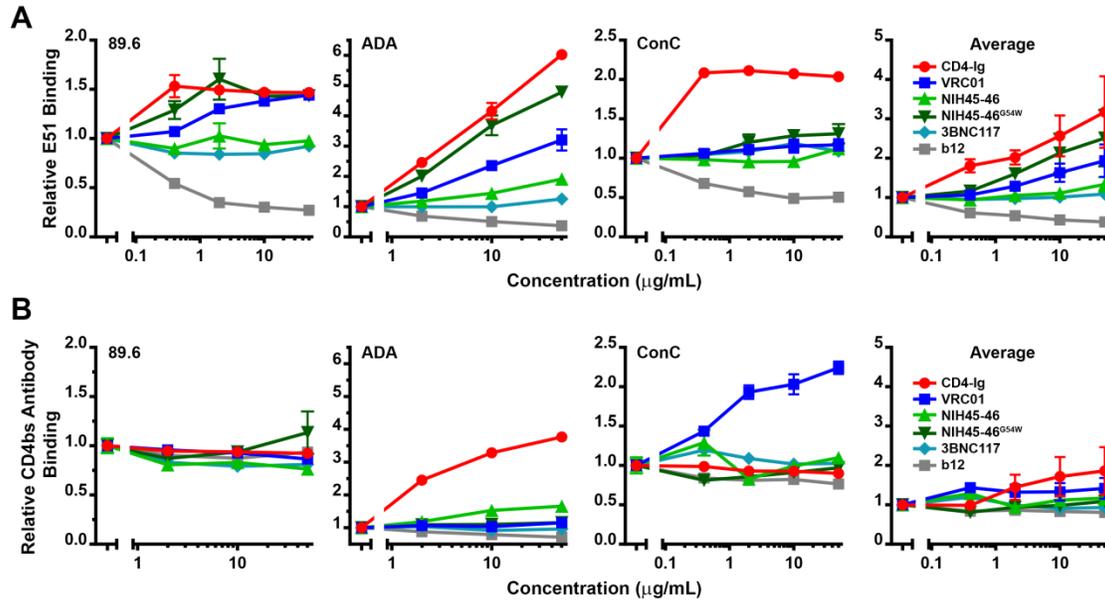
antibody VRC01 and the CD4i antibody E51, because they have similar potencies against a number of clade B HIV-1 isolates. Fixing the total antibody concentration, we measured neutralization of HIV-1 using varying antibody ratios. We observed that 80:20, 60:40, and 50:50 ratios of VRC01 to E51 more potently neutralized the HIV-1 isolates 89.6 and ADA than either antibody alone (Figure 2.2A). A 50:50 ratio of these antibodies also neutralized the ADA isolate more efficiently than either antibody across several concentrations between 0.08 and 2  $\mu\text{g/ml}$  (Figure 2.2B). Similar mixtures of the CD4bs antibody b12 with E51, or of the glycosylation-dependent antibody 2G12 with VRC01 were assayed in parallel. These antibody mixtures did not outperform the more potent antibody at any concentration (Figs. 2.2C-D). Chou-Talalay analysis indicated that the Combination Index at 50% neutralization ( $CI_{50}$ ) for VRC01/E51 mixture was 0.54, indicating that this combination was synergistic. b12/E51 and VRC01/2G12 mixtures yielded  $CI_{50}$  values greater than 1.0, indicating that these antibody mixture exhibited no synergy. Thus, under some conditions and with some HIV-1 isolates, synergy between E51 and VRC01 can be observed. Note however, that mixtures of E51 with the CD4bs antibodies NIH45-46 or 3BNC117 neutralized less efficiently than either CD4bs antibody alone, due to their greater potency relative to E51 (not shown).



**Figure 2.2. *In vitro* neutralization by combinations of CD4bs antibodies with E51.** (A) HIV-1 isolates 89.6 and ADA were pre-incubated for 1h with a fixed concentration (0.3 μg/mL) but varying ratios of VRC01:E51 mixtures. TZM-bl cells were then added and incubated for 48h. Infection was measured by the percentage luciferase expression in the absence of inhibitor. (B) Similar set up as (A) except HIV-1 isolate ADA was pre-incubated with varying concentrations of VRC01, E51, or a 50:50 mixture of VRC01 and E51. The dashed line indicates the fixed concentration used in (A). (C) Similar set up as (B) except HIV-1 isolate ADA was pre-incubated with varying concentrations of b12, E51, or a 50:50 mixture of b12 and E51. (D) Similar set up as (B) except HIV-1 isolate ADA was pre-incubated with varying concentrations of VRC01, 2G12, or a 50:50 mixture of VRC01 and 2G12.

**E51 does not enhance CD4bs antibody binding to monomeric gp120.** To understand the mechanism by which VRC01 and E51 combine to more potently neutralize ADA and 89.6, we sought to determine whether either antibody enhanced association of the other to gp120 monomers and to cell expressed Env trimers. We first examined by ELISA whether binding of E51 to immobilized gp120 was improved in the presence of increasing amounts of four CD4bs bNAbs or the CD4bs antibody b12, whose epitope extends into a region proximal to the E51 epitope. To distinguish E51 from CD4bs

antibodies, its Fc region was exchanged with that of murine IgG1, and anti-human or anti-murine secondary antibodies were used to detect CD4bs antibodies and E51 binding, respectively. As expected, CD4-Ig consistently enhanced E51 binding, while b12 inhibited E51 binding, to all three gp120 proteins tested. The ability of the CD4bs bNAbs to promote E51 association was isolate and antibody dependent. Specifically, VRC01 and NIH45-46<sup>G54W</sup>, but not NIH45-46 or 3BNC117, enhanced binding of E51 to clade B gp120 proteins, but not to a consensus clade C (ConC) gp120 (Figure 2.3A). We also assayed the ability of E51 to promote gp120 association with each of these antibodies. In general, E51 had little effect on most of these antibodies, but did promote CD4-Ig association with ADA gp120, and VRC01 association with ConC gp120 (Figure 2.3B). Thus, for clade B isolates, VRC01 modestly enhanced E51 association with gp120 monomers, but E51 had no effect on VRC01 binding to these monomers.



**Figure 2.3. Promotion of gp120 monomeric association by CD4bs antibodies and E51.** (A) ELISA plates were coated with 5 µg/mL of indicated gp120 molecules. Immobilized gp120 was pre-incubated with serially diluted CD4-Ig or CD4bs antibodies as indicated starting at 50 µg/mL. Wells were washed and incubated with a constant amount of E51. Binding of E51 was measured by absorbance of an anti-HRP secondary antibody recognizing murine-Fc at 450 nm and data was normalized to E51 binding without pre-incubation of CD4-Ig or CD4bs antibody. (B) Similar experiment as (A) except gp120 molecules indicated were pre-incubated with serially diluted E51 starting at 50 µg/mL. Wells were washed and incubated with a constant concentration CD4-Ig or indicated CD4bs antibody. Data was analyzed same as in (A) except normalized to binding of CD4-Ig or CD4bs without E51 pre-incubation.

### E51 enhances binding of CD4bs bNAb to cell-surface expressed Env trimers. We

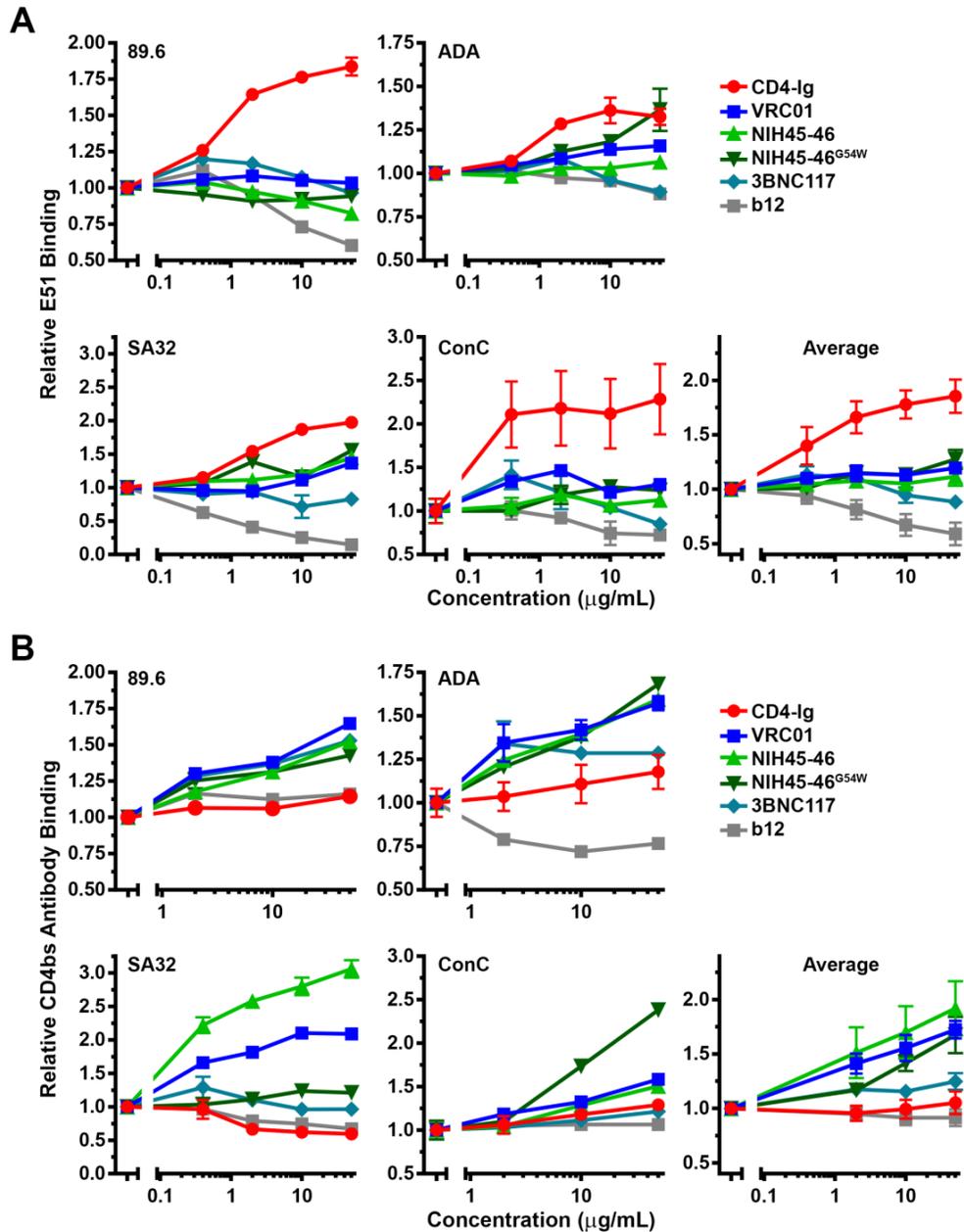
next explored whether changes in the quaternary structure of Env may be more relevant

to the synergy observed in Figure 2.2A-B. Using flow cytometry, we examined the

ability of E51 to bind Env trimers expressed on the surface of HEK293T cells in the presence of increasing amounts of CD4bs antibodies pre-bound to Env (Figure 2.4A).

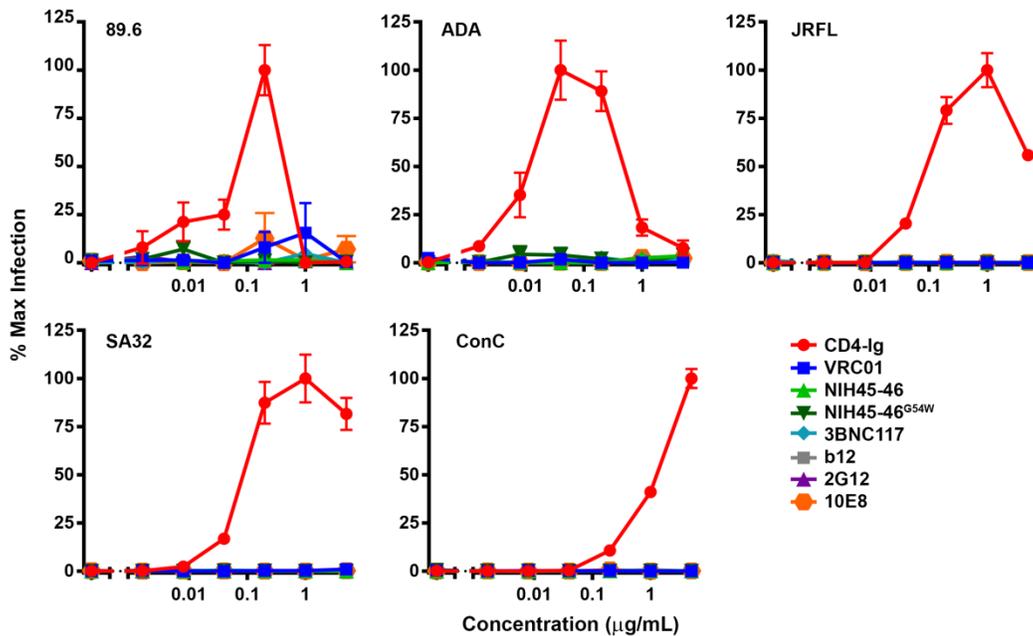
We observed that, at varying concentrations, CD4-Ig could promote binding of E51 to Env, but that no CD4bs bNAb did so efficiently. b12 interfered with E51 binding to Env as it did with monomeric gp120. Surprisingly, and in contrast, varying concentrations of E51 enhanced binding of all four CD4bs bNAbs to Env, but had only very modest effects

on CD4-Ig or b12 (Figure 2.4B). We conclude that E51 facilitates binding of VRC01 and other CD4bs bNAbs to the Env trimer, but that, unlike CD4-Ig, CD4bs bNAbs do not increase binding of E51. Taken together with the monomeric gp120 ELISA data (Figure 2.3), we conclude that E51 induces quaternary conformations in Env favorable to its association with CD4bs bNAbs. Finally, we infer that the synergy observed in Figure 2 is largely due to the ability of E51 to promote these quaternary conformations.



**Figure 2.4. Promotion of E51 or CD4bs antibody association to trimeric HIV-1 Env.** (A) HEK293T cells were transfected to express indicated envelope glycoproteins that lack a portion of the cytoplasmic domain. Cells were pre-incubated with concentrations of CD4-Ig and indicated antibodies at 50  $\mu\text{g/mL}$  and serially diluted by 5-fold to 0.4  $\mu\text{g/mL}$ . Cells were washed and then incubated with 0.25  $\mu\text{g/mL}$  E51. Binding was analyzed by flow cytometry. Mean fluorescent intensity (MFI) data are normalized to value of E51 binding without CD4-Ig or CD4bs antibodies pre-bound. (B) Similar experiment as (A) except cells were pre-incubated with E51 at serially diluted concentrations starting at 50  $\mu\text{g/mL}$ . Cells were washed and then incubated with 0.01  $\mu\text{g/mL}$  of indicated antibody or CD4-Ig. Analysis was performed same as (A) except MFI data were normalized to the value of each antibody or CD4-Ig binding without E51 pre-incubation.

**Unlike CD4-Ig, CD4bs bNAbs do not promote HIV-1 infection of CD4-negative cells.** As shown in Figure 2.4, CD4-Ig clearly behaves differently from CD4bs bNAbs. It promotes E51 association to Env trimers but its own association with Env is largely unaffected by the presence of E51. In contrast, CD4bs bNAbs do not significantly improve E51 binding to Env, but E51 promotes association of CD4bs bNAbs. To determine if these differences had functional consequences, we incubated five HIV-1 isolates with CCR5-positive, CD4-negative Cf2Th cells in the presence of CD4-Ig or CD4bs bNAbs (Figure 2.5). As expected, CD4-Ig substantially enhanced infection of these cells, with maximum enhancement peaking at 0.1-1  $\mu\text{g/ml}$ . In contrast, little or no enhancement of infection was observed with CD4bs bNAbs antibodies, or with the neutralizing antibodies b12, 2G12, or 10E8. Thus the inability of CD4bs bNAbs to promote E51 association is consistent with their corresponding inability to promote infection of CCR5-positive cells when cellular CD4 is limiting or absent. We infer that, unlike CD4-Ig, these antibodies do not promote binding of viral Env to CCR5.

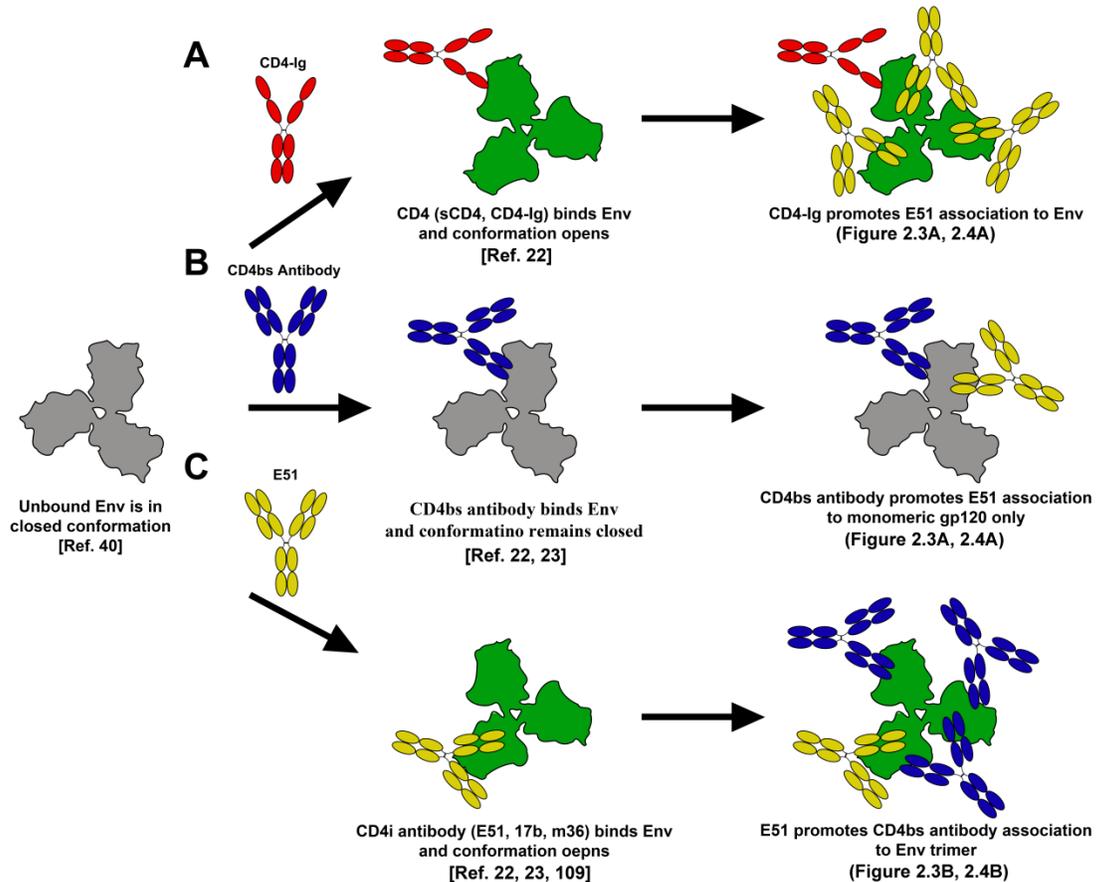


**Figure 2.5. Enhancement of HIV-1 entry into Cf2Th CCR5+/CD4- cells by CD4bs antibodies.** Luciferase-expressing HIV-1 pseudotyped with the indicated Env was pre-incubated with the antibodies indicated. Dog thymus Cf2Th cells expressing CCR5, but not CD4, were added to the virus-antibody mixture and incubated for 48 hrs. Infection was measured by the percentage luciferase expression in the absence of inhibitor.

## 2.E. Discussion

Here we observed a modest synergy in two cases when targeting the HIV-1 receptor-binding sites: in one instance CD4-Ig and CCR5mim2-Ig was combined and in a second case, mixtures of the CD4bs antibody VRC01 and the CD4i antibody E51 were assayed. In an effort to understand the basis of this synergy, we studied how E51 interacts with several VRC01-like bNAbs antibodies, as well as the CD4bs antibody b12. We observed with monomeric gp120 that CD4-Ig induced a conformation preferred by E51, but in general VRC01-like bNAbs did so less efficiently. Differences between CD4-Ig and VRC01-like antibodies were more pronounced with cell-expressed trimeric Env. CD4-Ig robustly enhanced E51 binding to Env trimers (Figure 2.6A), but VRC01-

like antibodies were much less efficient in promoting E51 binding (Figure 2.6B). Consistent with this observation, CD4-Ig, but not CD4bs antibodies enhanced HIV-1 infection of a CD4-negative, CCR5-positive cell line.



**FIG 2.6. Model of Env induction by E51 and CD4bs antibodies.** Shown is the model for induction that combines our data with the findings of Kwong *et al.* [Ref. 109], Tran *et al.* [Ref. 22], and Bartesaghi *et al.* [Ref. 23]. The unbound trimeric Env starts in a closed conformation (left). (A) When CD4 (or soluble CD4, CD4-Ig) binds Env, the conformation is opened [Ref. 22]. This conformation promotes CD4i binding, like E51 or 17b, both on monomeric gp120 and trimeric Env. (B) In contrast, the binding of a CD4bs antibody (VRC01, NIH45-46, etc.) keeps the conformation in a closed state [Ref. 23]. Though this is the case, these antibodies have the ability to moderately promote CD4i binding on monomeric gp120 as seen in Figure 2.3A and described in Scheid *et al.*<sup>137</sup> (C) Like CD4, when E51 (or other coreceptor inhibitors like 17b, m6, CCR5mim1-Ig, etc.) bind Env, the Env conformation opens [Ref. 22, 22, 109]. This conformation induces CD4bs antibody binding on trimeric Env, but not monomeric gp120 as seen in Figure 2.4B and Figure 2.5B, respectively.

The most striking difference, however, between CD4-Ig and CD4bs bNAbs was their ability to bind trimeric Env in the presence or absence of E51. E51 had almost no

effect on the ability of CD4-Ig to bind trimers. In contrast, E51 markedly enhanced Env binding of VRC01, NIH45-46, NIH45-46<sup>G54W</sup>, and to a lesser extent 3BNC117 (Figure 2.6C). This effect was not observed with gp120 monomers. We therefore infer that E51 can promote or stabilize quaternary conformational changes in Env that facilitate access of CD4bs bNAbs. The quaternary changes do not affect CD4-Ig binding and, indeed, are likely induced by CD4-Ig binding. These functional observations are consistent with cryo-electron microscopy studies by Tran *et al.* who observed that the CD4i antibody 17b, but not VRC01, induced an open conformation in Env<sup>22</sup>. When a mixture of VRC01 and 17b was added to Env, the conformation was open. The current study makes clear that CD4bs bNAbs preferentially bind this open conformation. Bartesaghi *et al.* show that in the open conformation of Env, the variable loops of Env are shifted in a way that further exposes the Env's CD4-binding site, as well as, presumably, the coreceptor binding site<sup>23</sup>. The CDR-H3 of E51 is a close mimic of CCR5, so it is not surprising that E51 preferentially binds and induces or stabilizes this open conformation.

This study also highlights other differences between CD4-Ig and VRC01-class bNAbs. As mentioned, CD4-Ig more efficiently promotes E51 binding to monomeric gp120 and trimeric Env. This difference may be a necessary property of potent CD4bs antibodies for two possible reasons. First, an antibody selected for potency would not be expected to promote infection at low concentrations or when cellular CD4 was limiting. Second, there is likely to be an energy and/or an entropy barrier limiting access to the open conformation. If an antibody can avoid the free energy penalty of inducing this conformation, it can bind with greater affinity<sup>140</sup>. In contrast, cellular CD4 and therefore CD4-Ig must necessarily pay this penalty to facilitate coreceptor binding. CD4i

antibodies, which bind gp120 and Env similarly to CCR5, must also pay this penalty, perhaps explaining the lower potency of these antibodies relative to CD4bs bNAbs. Of course, when Env is bound to CD4, this energetic cost is paid, and CD4i antibodies bind efficiently.

We show here that the synergy between VRC01 and E51 is likely the result of the ability of E51 to induce the open state, and facilitate access to the CD4-binding site. Highly potent VRC01-like bNAbs are rare. Most CD4bs antibodies in the sera of infected individuals neutralize Env less efficiently, in part because they cannot access their epitopes on the closed Env trimer and in part because they pay an energetic price for inducing the CD4-bound state on Env. Weaker CD4bs antibodies F105, b6, and b13 have been shown to have a high perturbation factor on Env which has the consequence of being less reactive towards a broad range of HIV-1 isolates<sup>140</sup>. Thus the presence of relatively potent CD4i antibodies in the sera might complement both limitations of these less potent CD4bs antibodies. It is further possible that CD4i antibodies, by promoting exposure to the CD4-binding site, might enhance the generation of more potent CD4bs antibodies. It may therefore be useful to elicit CD4i antibodies as part of a stepwise effort to elicit VRC01-class antibodies, or to enhance the potency of less efficient CD4bs antibodies.

## **CHAPTER 3**

**A CCR5-mimetic Sulfopeptide Converts CD4-Ig to an Exceptionally**

**Broad and Potent Inhibitor of HIV-1 Entry**

## Acknowledgements

I would like to thank the following for their contributions to the work presented in this chapter:

Jessica J. Chiang and Tatyana Dorfman for their contributions to Figure 3.1.

Hema R. Kondur for cloning the CD4-Ig/eCD4-Ig heterodimer used in Figure 3.2C-D.

Michael S. Seaman and his lab for running the neutralization panels seen in Figure 3.4-5.

Julie M. Decker for her contributions to Figure 3.6B and Figure 3.7B-C.

Michael D. Alpert for running the ADCC assays in Figure 3.6C.

Kevin G. Haworth for handling the mice for the experiments in Figure 3.9.

Lisa M. Kattenhorn for her contributions to Figure 3.10A-C and Figure 3.12A-B.

This work was supported by the National Institutes of Health grants R01 AI091476 and R01 AI080324 (MF), P01 AI100263 (GG, RCD, MF), RR 000168 (MRG, LMK, DTE, RCD, MF), R37 (BHH).

### 3.A. Abstract

Soluble CD4 and its more bioavailable form, CD4-Ig, neutralize HIV-1 less potently than many broadly neutralizing antibodies (bNAbs) because they bind the HIV-1 envelope glycoprotein with lower affinity, and they promote infection when cellular CD4 is limiting. Here we show that a fusion of CD4-Ig with a small CCR5-mimetic sulfopeptide (eCD4-Ig) overcomes both of these shortcomings: eCD4-Ig bound the envelope glycoprotein with high avidity and its sulfopeptide inhibited infection promoted by CD4-Ig. eCD4-Ig efficiently neutralized a diverse panel of antibody-resistant tier 2 and tier 3 HIV-1 isolates without exception and with  $IC_{50}$ s ranging from 0.002 to 1.137  $\mu\text{g/ml}$  (0.02-11 nM) – values comparable to or better than those of the best HIV-1 bNAbs. Because it binds only conserved regions of the HIV-1 envelope glycoprotein, it is also broader than any bNab. Underscoring this breadth, eCD4-Ig potently neutralized HIV-2 and antibody-resistant SIV isolates, as well as a comprehensive panel of 38 HIV-1 isolates resistant to the current best CD4-binding site (CD4bs) bNAbs. Further, eCD4-Ig induced markedly more efficient antibody-dependent killing of HIV-1- or SIV-infected cells than did CD4-Ig. Finally, eCD4-Ig retained its efficacy *in vivo*: it protected humanized mice from a high-dose HIV-1 challenge, and a rhesus macaque analogue could be expressed to peak levels greater than 100  $\mu\text{g/ml}$  in three of four macaques inoculated with an adeno-associated virus (AAV) vector. eCD4-Ig is thus a highly potent, exceptionally broad and mechanistically distinct inhibitor of HIV-1 entry with the potential to prevent or treat an HIV-1 infection.

### 3.B. Introduction

Entry of the human immunodeficiency virus type 1 (HIV-1) requires the cell-surface expression of CD4<sup>16-18</sup> and a coreceptor, principally CCR5 or CXCR4<sup>28,31</sup>. CCR5-using (R5) viruses predominate in the early years of an untreated infection. Coincident with declining immune function, viruses sometimes emerge that utilize CXCR4, either in addition to CCR5 (R5X4 isolates) or alone (X4 isolates). Association of the HIV-1 envelope glycoprotein gp120 with CD4 promotes its subsequent association with CCR5 or CXCR4<sup>208,209</sup>. The CCR5 amino terminus contains sulfotyrosines critical for this association<sup>89</sup>. Some CD4-induced (CD4i) HIV-1 neutralizing antibodies, for example E51 and 412d, are similarly sulfated in their heavy-chain CDR3 regions, and the sulfotyrosines of these antibodies bind the same highly conserved coreceptor-binding region of gp120 as the CCR5 N-terminus<sup>92,158</sup>. Envelope glycoproteins of R5X4 and X4 isolates also retain these sulfotyrosine-binding pockets and are efficiently recognized by tyrosine-sulfated CD4i antibodies<sup>158</sup>.

We have previously shown that a 15-amino acid sulfopeptide homologous to the CCR5 amino terminus and derived from heavy-chain CDR3 region of the CD4i antibody E51 (CCR5mim1), bound gp120 proteins of a range of R5, R5X4 and X4 isolates, even in the absence of CD4<sup>165</sup>. CCR5mim1 bound gp120 and neutralized HIV-1 more efficiently than sulfopeptides based directly on CCR5<sup>165</sup>. Like CCR5 and tyrosine-sulfated CD4i antibodies, binding of CCR5mim1 to the HIV-1 envelope glycoprotein was dependent on residues that compose the gp120 sulfotyrosine-binding pockets and was enhanced by soluble CD4 (sCD4). Unlike most CD4i or CD4-binding site (CD4bs)

antibodies, however, Fc-dimerized forms of CCR5mim1 (CCR5mim1-Ig) can bind two gp120 monomers of the HIV-1 envelope glycoprotein trimer.

Soluble forms of CD4 have been extensively studied as potential therapeutics<sup>210,211</sup>. Soluble CD4 and the more bioavailable immunoadhesin, CD4-Ig, bind gp120 of all HIV-1 isolates, can irreversibly inactivate the HIV-1 envelope glycoprotein, and are demonstrated to be safe for use in humans<sup>212,213</sup>. However, the affinity of CD4 for gp120 is lower than that of efficient neutralizing antibodies<sup>214</sup>. In addition, the neutralizing potency of CD4-Ig is further reduced by its parallel ability to enhance infection by promoting direct association with CCR5 or other coreceptors<sup>211,215</sup>. This effect is most visible at low concentrations or when cell-expressed CD4 is limiting, but it likely attenuates the potency of CD4-Ig at most concentrations and conditions.

In recent years, a number of highly potent broadly neutralizing antibodies (bNAbs) have been characterized including, among others, the CD4-binding site (CD4bs) bNAbs VRC01, PGV-04, NIH45-46, and 3BNC117 (reviewed in<sup>216</sup>). These antibodies can be used to protect humanized mice from infection, and can suppress replication of specific simian-human immunodeficiency viruses (SHIV) in macaques<sup>188-190</sup>. They bind Env with high affinity and typically do not promote infection at lower concentrations. However their epitopes are invariably larger than the receptor-binding domain of Env, and include variable residues that limit antibody breadth and permit escape of the virus without loss of fitness<sup>142,188</sup>. These antibodies also are extensively hypermutated and include multiple T- and B-cell epitopes that can be recognized as non-self<sup>217</sup>. Both properties limit their utility as therapeutics or in long-term prophylaxis strategies.

Here we show that a fusion of CD4-Ig and CCR5mim1 binds the Env trimer cooperatively and with high avidity. This fusion, eCD4-Ig, simultaneously occludes the CCR5-binding sites of two gp120 monomers as well as the CD4-binding site of one of these monomers, inhibiting the ability of CD4 to promote infection. Moreover, it is at least as potent as the best neutralizing antibodies, is broader than any bNAb or entry inhibitor described to date, and, as we discuss, has additional properties that make it useful as a complement to current anti-retroviral therapies, or as part of an alternative vaccine.

### 3.C. Materials and Methods

**Plasmids and Cells.** Plasmid expressing CD4-Ig was previously described<sup>218</sup>. Fusion constructs were created by adding sequence encoding CCR5mim1 and tetra-glycine linker to N-terminus (fusion1) or between domain 2 and human Fc (fusion2) of CD4-Ig by inverse PCR. eCD4-Ig and eCD4-Ig<sup>mim2</sup> were created by adding sequence encoding a tetra-glycine linker and CCR5mim1 or CCR5mim2, respectively, to the C-terminus of CD4-Ig by inverse PCR. Glutamine 40 to alanine mutations were generated in eCD4-Ig and eCD4-Ig<sup>mim2</sup> mutants by Quickchange PCR. The following reagent was obtained through the NIH AIDS Reagent Program (Division of AIDS, NIAID, NIH): CMVR VRC01 H, CMVR VRC01 L, from Dr. John Mascola<sup>136,202</sup>; PVO, clone 4 (SVPB11) from Dr. David Montefiori and Dr. Feng Gao<sup>203</sup>; ZM109F.PB4 SVPC13 from Dr. C. A. Derdeyn and Dr. E. Hunter<sup>219</sup>, pNL4-3.Luc.R-E- from Dr. Nathaniel Landeau<sup>200,201</sup>, TZM-bl cells from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc<sup>195-199</sup>. The variable heavy and light chains of b12 and PG16 were cloned into the CMVR VRC01 H and L plasmids. SHIV-SF162P3N clone 8 and SHIV-SF162P3N clone 11 Env

expression plasmids was a generous gift from Cecilia Cheng-Mayer. Plasmids encoding TPST-2 and the envelope glycoproteins pNL4-3 $\Delta$ env, 89.6, ADA, SG3, SA32, YU2, JRFL, KB9, VSV-G, HIV-2 ST, SIVmac239, SIVmac316, and replicative 89.6 and SG3 viruses were previously described<sup>28,88,158,165,220</sup>. Human embryonic kidney HEK293T cells were obtained from ATCC. Cf2Th-CD4+.CCR5+ and CfTh-CCR5+ cells were a generous gift from Dr. Hyeryun Choe.

**Purification of CD4-Ig, eCD4-Ig variants, and antibodies.** Production of CD4-Ig, eCD4-Ig variants and antibodies was performed as previously described<sup>169</sup>. Briefly, HEK293T cells in 140 mm plates were transfected with 25 ug/plate at 50% confluency by the calcium phosphate transfection method. Plasmids encoding sulfated proteins were cotransfected with a plasmid encoding human tyrosine protein sulfotransferase 2 (TPST2). At 12 hrs post-transfection, 10% FBS-DMEM media was replaced with serum-free 293 Freestyle media (Invitrogen). Media was collected after 48 hrs, debris was cleared by centrifugation for 10 min at 1,500 g and filtered using 0.45  $\mu$ m filter flasks (Millipore). Complete protease inhibitor cocktail (Roche) was added to the filtered supernatants. 500  $\mu$ l bed volume of Protein A sepharose beads (GE Healthcare) were added and were agitated 4°C overnight. The bead/media mixture was collected by gravity flow column (Biorad) and was washed with 30 mL PBS (Lonza) + 0.5M NaCl (0.65M NaCl final) followed by 10 mL PBS. Protein was eluted with 3M MgCl<sub>2</sub> in phosphate buffered saline (PBS). Buffer was exchanged for PBS and protein was concentrated to 1 mg/ml by Ultrafiltration (Amicon Ultra) at 4,000 g.

**Flow Cytometry Analysis of Envelope Glycoprotein Binding.** HEK293T cells were transfected with plasmids expressing envelope glycoprotein lacking cytoplasmic residues

732 to 876 (HXBc2 numbering) together with plasmid encoding the tat protein.

Transfection medium was replaced after an overnight incubation and cells were harvested 48 hours post transfection. Harvested cells were washed twice in flow cytometry buffer (PBS with 2% goat serum, 0.01% sodium azide). Cells were incubated with CD4-Ig or eCD4-Ig on ice for 1 hour and then washed twice with flow cytometry buffer. A secondary antibody recognizing human Fc (Jackson Immuno Research) was added to the cells for 30 minutes. Cells were washed twice with flow cytometry buffer, twice with PBS, and resuspended in 1% paraformaldehyde solution. Binding was analyzed with an Accuri C6 Flow Cytometer (BD Biosciences) and data analyzed with the C6 Software (BD Biosciences).

**Viral enhancement assay.** HIV-1 pseudovirus expressing firefly luciferase was pre-incubated with titrated amounts of CD4-Ig or eCD4-Ig variants in DMEM (10% FBS) for 1 hour at 37°C. CD4-negative Cf2Th-CCR5 cells were harvested and diluted in DMEM (10% FBS) to 100,000 cells/mL and added to the pseudovirus/inhibitor mixture. Cells were then incubated for 48 hours at 37°C. Viral entry was analyzed using Britelite Plus (Perkin Elmer) and luciferase was read using a Victor X3 plate reader (Perkin Elmer).

**HIV-1 Neutralization Assays.** GHOST-CCR5 or -CXCR4 cells were plated into 12-well plates at 50,000 cells per well. HIV-1 pseudovirus was diluted in RPMI and titrated amounts of CD4-Ig, fusion1, fusion2, or eCD4-Ig were added. Virus and inhibitor were incubated at room temperature for 20 minutes and added to the cells for 2 hours at 37°C. Cells were then washed with serum free medium and then incubated in 1 mL of DMEM (10% FBS) for 48 hours at 37°C. Cells were then trypsinized and resuspended in PBS

(2% FBS), pelleted, and resuspended in a fixative solution (1% paraformaldehyde in PBS). Viral entry was determined by flow cytometry based on GFP expression.

For studies of infectious virus, unstimulated PBMCs were harvested and resuspended in RPMI medium (15% FBS, 20 U/mL IL-2). Cells were plated in a 12-well plate at  $10^6$  cells per well. HIV-1 virus was diluted in RPMI and varying amounts of inhibitor were added. The virus and inhibitor was incubated at room temperature for 20 minutes and added to the cells for 3 hours at 37°C. Cells were then washed with serum-free medium and resuspended in fresh RPMI medium (15% FBS, 20 U/mL IL-2). At 3-day intervals post infection, supernatants were collected and fresh RPMI medium (15% FBS, 20 U/mL IL-2) was added to the cells. Supernatants were analyzed for viral infection by ELISA with Alliance HIV-1 p24 antigen ELISA kit (Perkin Elmer).

TZM-bl neutralization assays were performed as previously described<sup>203</sup>. Briefly, HIV-1 pseudoviruses were pre-incubated with titrated amounts of CD4-Ig or eCD4-Ig variants in DMEM (10% FBS) for 1 hour at 37°C. TZM-bl cells were harvested and diluted in DMEM (10% FBS) to 100,000 cells/mL and added to the pseudovirus/inhibitor mixture. Cells were then incubated for 48 hours at 37°C. Viral entry was analyzed using Britelite Plus (Perkin Elmer) and luciferase was read using a Victor X3 plate reader (Perkin Elmer).

**Antibody-dependent cell-mediated cytotoxicity assays.** ADCC activity was performed as previously described<sup>221</sup>. Briefly, CEM.NKR CCR5 CD4+ T cells were infected for 4 days with infectious HIV-1 NL4.3, SHIV-KB9, or SIVmac239. After 4 days, KHYG-1 effector cells were co-incubated with infected cells in the presence of titrated CD4-Ig,

eCD4-Ig variants, or the b12 antibody for 8 hours. ADCC activity was measured by luciferase activity as above.

**Production of HIV-1<sub>NL4-3</sub> stocks.** A molecular clone of HIV-1<sub>NL4-3</sub> were obtained from the AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS, NIAID, NIH from material deposited by Suzanne Gartner, Mikulas Popovic, Robert Gallo and Malcolm Martin. Virus stocks were produced in 293T cells by transient transfection using TurboFect (Thermo Scientific) and 12 µg of proviral plasmid. Supernatants were harvested at 40 hours, filtered through 0.45 µm filters, and dispensed into single use doses and frozen at -80°C. Viruses were quantified by p24 ELISA (Zeptometrix, Buffalo, NY) and by GHOST cell titer<sup>222</sup> to determine infectious units per mL (IU/mL). Titering was performed per the GHOST cell line protocol obtained through ARRRP.

**Hematopoietic stem cell isolation and NSG mouse transplantation.** Human CD34+ hematopoietic stem cells (HSC) were isolated from fetal livers obtained from Advanced Bioscience Resources, INC (ABR, Alameda, CA). Tissue was disrupted and incubated with 1mg/mL Collagenase/Dispase (Roche Applied Sciences) for 15 min at 37°C. Cells were isolated by passing the disrupted tissue through a 70 µm filter. Red blood cells were lysed in BD Pharm Lyse (BD Biosciences, San Jose, CA), with CD34+ cells being isolated using CD34 MACS microbeads (Miltenyi) according to manufacturer's instructions with an additional purification step using a second column. NOD.Cg-Prkdc scid Il2ry tm1Wj/Szj (NOD/SCID/IL2ry<sup>null</sup>, NSG) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Neonatal mice received 150 cGy radiation, and 2-4 hours later 1x10<sup>6</sup> CD34+ HSCs in 1% heparin (Celgene, Summit, NJ) via intrahepatic

injection. Mice were monitored for engraftment levels of human CD45+ cells and development of T cells and B cells at 8, 10, and 12 weeks post engraftment.

**Mouse infections, treatment, and analysis.** Humanized mice with evidence of human CD4+ T cell development in blood were infected with  $5 \times 10^4$  IU of HIV-1<sub>NL4.3</sub> by intraperitoneal injection. Mice were administered with 65 µg of eCD4-Ig once weekly for the first 2 weeks, starting at 8 day prior to the HIV-1 challenge, and then twice weekly starting week 3 by retro-orbital injection while under anesthetization by 2.5% isofluorane. Mock treated mice received a retro-orbital injection of PBS one and eight days preceding HIV-1 challenge, and were anesthetized in parallel with eCD4-Ig mice throughout. Every week post-infection the mice were anesthetized by inhalation of 2.5% isoflourane and blood was collected retro-orbitally for analysis. At week 6, three eCD4-Ig treated mice and one mock treated mouse (who had not become infected) were challenged a second time with  $5 \times 10^4$  IU HIV-1<sub>NL4-3</sub>. Mouse blood was blocked for 20 minutes at room temperature in FBS (Denville) and stained with appropriate antibodies for 15 minutes at room temperature. Red blood cells were removed by incubation in BD FACS Fix/Lysing Solution (BD Biosciences), which was removed by dilution with PBS prior to analysis by flow cytometry. HIV-1 levels in peripheral blood were determined by extracting viral RNA from mouse plasma at each blood draw using a viral RNA isolation kit (Qiagen, Germantown, MD) followed by Taqman One-Step RT-PCR (Life Technologies, Carlsbad, CA) using a primer and probe set targeting the HIV-1 LTR region, as previously described<sup>223,224</sup>. Reactions were performed and analyzed using a 7500 Fast Realtime PCR System (Life Technologies).

**Flow cytometry.** Stained cells were acquired on a FACS Canto II (BD Biosciences) and analyzed using FlowJo software v7.6.5 (Tree Star Inc., Ashland, OR). Blood samples were stained using human-specific antibodies at a 1:20 dilution for CD4-V450 (RPA-T4), CD8-APC (RPAT8), CD3-PE (UCHT1), and CD45-PerCP (TUI16) (BD Bioscience). Up to 10,000 events were recorded for viable cell populations and gated based on fluorescence minus one control as previously described<sup>224</sup>.

**eCD4-Ig antibody concentrations in NSG mice.** *In vivo* concentrations of eCD4-Ig antibodies were measured by ELISA as previously described<sup>129</sup>. Briefly, to measure NSG mouse and macaque serum concentrations, ELISA plates (Costar) were coated with 5 µg/mL HIV gp120 overnight at 4°C. Plates were washed with PBS-T (PBS + 0.05% Tween-20) twice and blocked with 5% milk in PBS for 1 hour at 37°C. Titrations of sera blocked with 5% milk in PBS were added to the plate and incubated for 1 hour at 37°C. Samples were washed five times with PBS-T and a horseradish peroxidase secondary antibody (Jackson Immuno Research) recognizing human IgG1. Plates were incubated for 1 hour at 37°C and then washed ten times with PBS-T. TMB solution (Fisher) was added for 10 minutes at room temperature and then stopped with TMB Stop Solution (Southern Biotech). Absorbance was read at 450 nm by a Victor X3 plate reader (Perkin Elmer), and compared with a standard curve generated using a eCD4-Ig mixed with pre-inoculation sera.

**AAV inoculation of rhesus macaques.** Three two-year old AAV1-negative male Indian-origin rhesus macaques were housed at the New England Primate Research Center in accordance with standards set forth by the American Association for Accreditation of Laboratory Animal Care. These macaques were inoculated with 1 ml saline containing 2

$\times 10^{13}$  AAV1 particles delivering a self-complementary rh-eCD4-Ig<sup>mim2</sup> transgene (IgG2 isotype) into both quadriceps (0.5 ml per quadriceps). 1 ml of sera was obtained at -4, -2, 0, 2, 4, 6, 8, and 10 weeks post-inoculation.

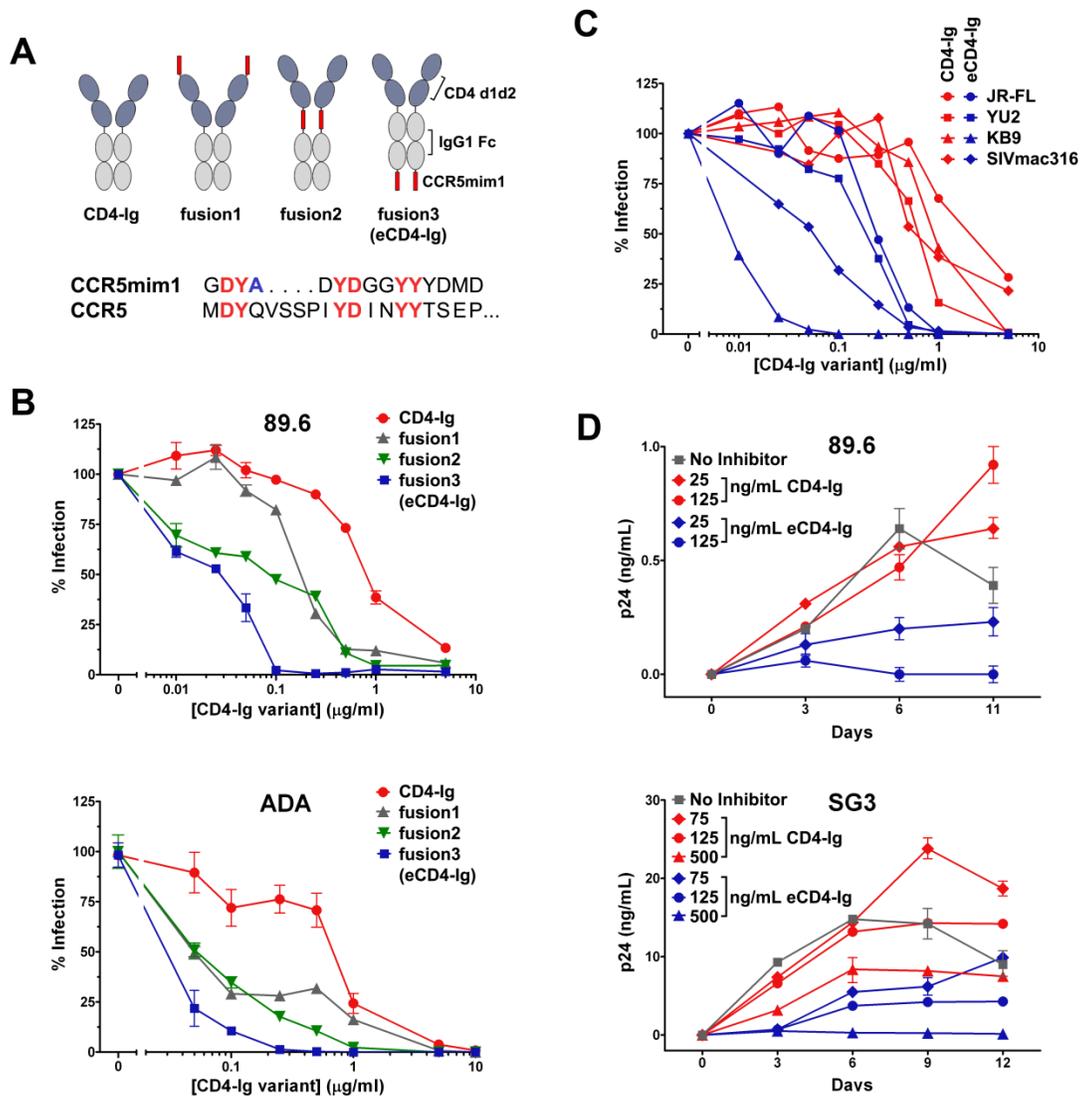
**eCD4-Ig, rh-eCD4-Ig<sup>mim2</sup> and anti-rh-eCD4-Ig<sup>mim2</sup> antibody concentrations in NSG mice and rhesus macaque sera.**

*In vivo* concentrations of eCD4-Ig, rh-eCD4-Ig<sup>mim2</sup> and anti-rh-eCD4-Ig<sup>mim2</sup> antibodies were measured by ELISA as previously described<sup>129</sup>. Briefly, to measure NSG mouse and macaque serum concentrations, ELISA plates (Costar) were coated with 5 $\mu$ g/mL SIV gp120 overnight at 4°C. Plates were washed with PBS-T (PBS + 0.05% Tween-20) twice and blocked with 5% milk in PBS for 1 hour at 37°C. Titrations of sera blocked with 5% milk in PBS were added to the plate and incubated for 1 hour at 37°C. Samples were washed five times with PBS-T and a horseradish peroxidase secondary antibody (Jackson Immuno Research) recognizing human IgG1 or rhesus CD4 was added. Plates were incubated for 1 hour at 37°C and then washed ten times with PBS-T. TMB solution (Fisher) was added for 10 minutes at room temperature and then stopped with TMB Stop Solution (Southern Biotech). Absorbance was read at 450 nm by a Victor X3 plate reader (Perkin Elmer), and compared with a standard curve generated using a rh-eCD4-Ig<sup>mim2</sup> mixed with pre-inoculation sera. Anti-rh-eCD4-Ig<sup>mim2</sup> antibodies were measured in the same way except that ELISA plates were coated with rh-eCD4-Ig<sup>mim2</sup> and anti-rh-eCD4-Ig<sup>mim2</sup> antibodies were measured using an anti-rhesus light-chain antibody, and compared with a standard curve generated using an anti-rhesus CD4 antibody.

### 3.D. Results

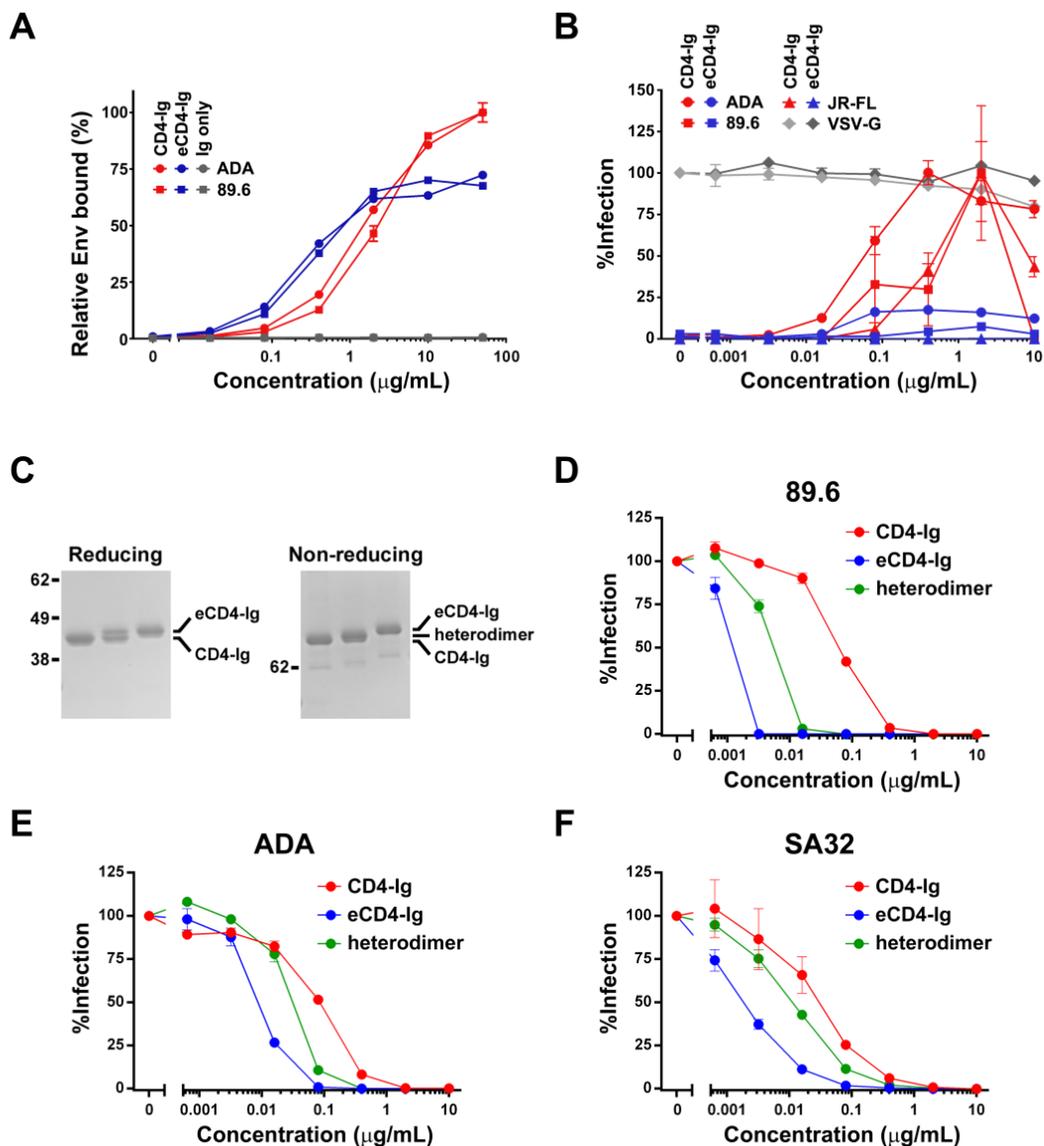
#### **A C-terminal sulfopeptide markedly enhances the neutralizing activity of CD4-Ig.**

We reasoned that, due to the cooperativity between CD4-Ig and CCR5mim1<sup>109,166</sup>, a fusion of the two would bind the envelope glycoprotein with higher affinity and avidity than either molecule alone. We further hypothesized that inclusion of the CCR5mim1 sulfopeptide would prevent association of CD4-Ig-bound virions with CCR5 or CXCR4, thereby limiting the ability of CD4-Ig to promote infection. Finally, we anticipated that this fusion would retain or improve the ability of CD4-Ig to irreversibly inactivate virion-associated Env<sup>213</sup>. To test these possibilities, three fusion proteins were initially generated (Figure 3.1A). In fusion1, CCR5mim1 was fused to the amino-terminus of CD4-Ig. In fusion2, the sulfopeptide was inserted at the junction between domain 2 of CD4 and the hinge region of the CD4-Ig Fc domain. In fusion3, the sulfopeptide was fused to the C-terminus of the Fc domain. Fusion3 was subsequently renamed eCD4-Ig for its enhanced potency. In each case, a tetraglycine linker connects the sulfopeptide to CD4-Ig. Each of these CD4-Ig variants neutralized R5, R5X4, and X4 isolates more efficiently than CD4-Ig (Figure 3.1B and not shown). eCD4-Ig consistently outperformed fusion1 and fusion2, and neutralized an initial panel of clade B isolates and SIVmac316 with IC<sub>50</sub>s 10- to 100-fold lower than CD4-Ig (Figure 3.1C and not shown). Consistent with its potency, eCD4-Ig, but not CD4-Ig, halted replication of 89.6 and SG3 viruses in PBMC at low nanomolar concentrations: 125 ng/mL (1.25 nM) and 500 ng/mL (5 nM), respectively (Figure 3.1D).



**Figure 3.1. Characterization of CD4-Ig/CCR5-mimetic peptide fusion proteins.** (A) CD4-Ig and fusion constructs with CCR5mim1 peptide are represented. CD4-Ig is comprised of CD4 domains 1 and 2 (blue) fused to a human IgG1 Fc domain (grey). In fusion1, the CCR5mim1 sulfopeptide (red) is fused to the amino-terminus of CD4-Ig with a tetraglycine linker. In fusion2, the tetraglycine linker and CCR5mim1 are inserted between the carboxy-terminus of CD4 domain 2 and the IgG1 hinge region. In fusion3 (subsequently renamed eCD4-Ig), the sulfopeptide is fused to the carboxy-terminus of CD4-Ig, again preceded by a tetraglycine linker. Figure also shows the sequence of CCR5mim1 aligned with that of the CCR5 amino-terminus. Common residues, including four CCR5 sulfotyrosines, are shown in red. An alanine in CCR5mim1 (blue) is substituted with a tyrosine in CCR5mim2, described below. (B) HIV-1 pseudotyped with the envelope glycoproteins of the 89.6 or ADA isolates was incubated with GHOST-CCR5 cells and the indicated concentrations of CD4-Ig variants. Infection was measured by flow cytometry 2 days later. (C) Experiments similar to those in panel B using HIV-1 pseudotyped with the envelope glycoproteins of the JR-FL, YU2, KB9, or SIVmac316 isolates. (D) Infectious 89.6 or SG3 HIV-1 was incubated with PBMC in the presence of the indicated concentrations of CD4-Ig or eCD4-Ig. Supernatants were collected on the day indicated and viral p24 levels were measured by ELISA.

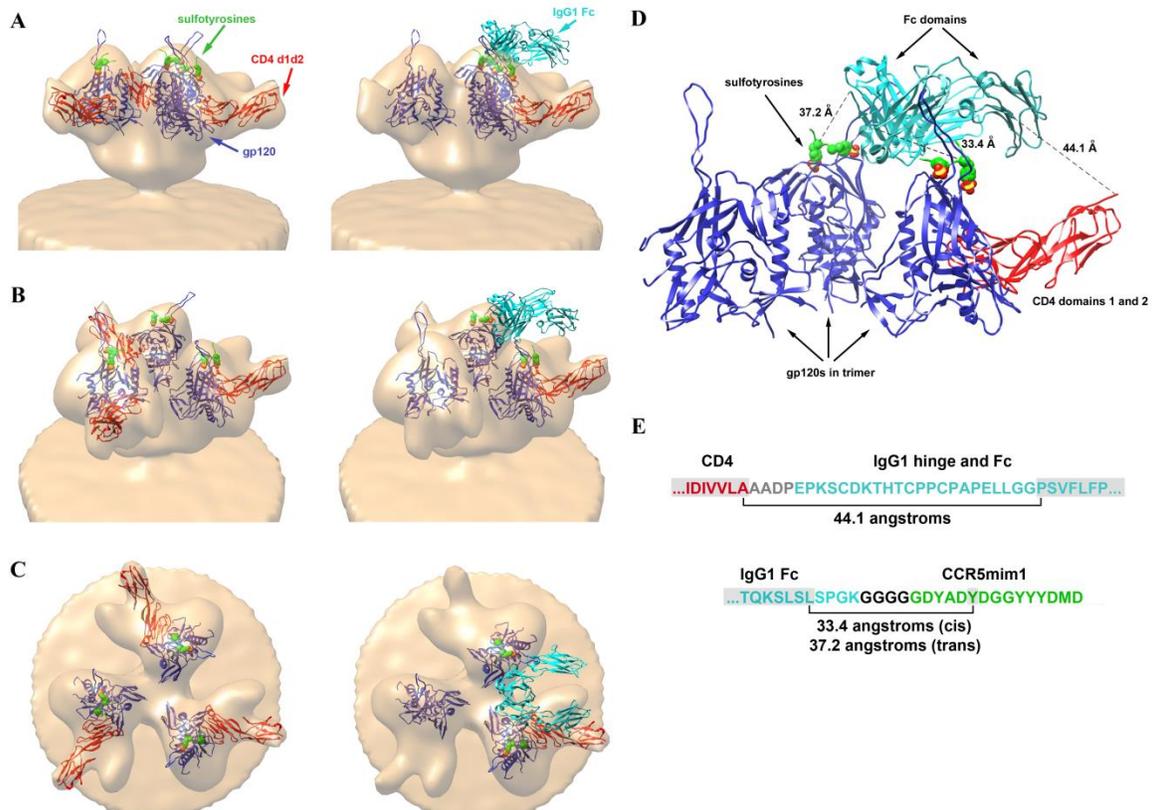
**eCD4-Ig utilizes both sulfopeptides to avidly bind Env trimers.** To better understand the markedly greater potency of eCD4-Ig relative to CD4-Ig, we compared the ability of each to bind cell-surface expressed HIV-1 envelope glycoprotein trimers (Figure 3.2A). eCD4-Ig bound these trimers more efficiently than did CD4-Ig, with half-maximum binding at 0.35  $\mu\text{g/ml}$ , compared with 1.0  $\mu\text{g/ml}$  for CD4-Ig. eCD4-Ig saturated trimer-expressing cells with approximately one-third less bound protein than CD4-Ig, indicating that eCD4-Ig associates with greater avidity than CD4-Ig, and that one-third of the CD4-binding sites were occluded due to the presence of the sulfopeptide. Although eCD4-Ig binds the envelope glycoprotein with higher avidity than CD4-Ig, comparison of Figures 3.1B and 3.2A suggests that improved avidity does not fully account for the greater potency of eCD4-Ig in neutralization assays. Consistent with this, eCD4-Ig was much less prone to promote HIV-1 infection in CCR5-positive, CD4-negative cells than was CD4-Ig (Figure 3.2B). Thus the marked increase in potency of eCD4-Ig relative to CD4-Ig can be explained, at least in part, by the higher avidity with which it binds HIV-1 envelope glycoprotein trimers and by its decreased ability to promote infection. It is also possible that eCD4-Ig is more efficient than CD4-Ig at irreversibly inactivating Env. To explore the contribution of each sulfopeptide to the improved neutralization of eCD4-Ig, we made a “knobs and holes” heterodimer of CD4-Ig and eCD4-Ig. Relatively pure heterodimers could be generated (Figure 3.2C). These CD4-Ig/eCD4-Ig consistently neutralized more efficiently than CD4-Ig, but less efficiently than eCD4-Ig (Figures 3.2D-F). Thus, consistent with our studies of sulfopeptide Fc fusions<sup>109</sup>, eCD4-Ig utilizes both of its sulfopeptides to neutralize HIV-1.



**Figure 3.2. Mechanistic studies of eCD4-Ig.** (A) 293T transfected to express 89.6 or ADA envelope glycoprotein were incubated on ice with indicated concentrations of CD4-Ig, eCD4-Ig, or IgG. Binding was analyzed by flow cytometry using a secondary antibody recognizing the IgG1 Fc domain of these proteins. (B) HIV-1 expressing luciferase and pseudotyped with enveloped glycoproteins of the ADA, JR-FL, or 89.6 isolates or with VSV-G was incubated with Cf2Th-CCR5 cells in the presence of the titrated concentrations of CD4-Ig or eCD4-Ig. Luciferase activity was measured two days post infection to assess viral infection, and normalized to the maximum value observed for each pseudovirus. Experiments in panels A and B are representative of at least four with similar results. (C) CD4-Ig, eCD4-Ig, and a CD4-Ig/eCD4-Ig heterodimers were analyzed by SDS-PAGE and stained with Coomassie blue. (D-F) HIV-1 pseudotyped with the indicated envelope glycoprotein was incubated in triplicate with TZM-bl cells and varying concentration of CD4-Ig, eCD4-Ig, or a CD4-Ig/eCD4-Ig heterodimer shown in panel C. Luciferase activity was determined two days post-infection.

### **eCD4-Ig can bind three Env receptor binding sites without steric interference.**

We have previously observed enhancement of neutralization when CCR5mim1-Ig was mixed with CD4-Ig, but this effect was modest, implying that the CD4 domain of eCD4-Ig and at least one sulfopeptide binds in *cis*, that is, from the same eCD4-Ig molecule. Figures 3.2C-F show that a heterodimer of eCD4-Ig and CD4-Ig neutralizes less efficiently than eCD4-Ig itself, indicating that both eCD4-Ig sulfopeptides contribute to neutralization. To determine whether eCD4-Ig could access two CCR5-binding sites on Env while occupying one CD4-binding site, we built a model based on the cryo-electron structure of CD4-bound Env (Figure 3.3A-C), and fitted into this density the crystal structure of gp120 (blue) complexed to CD4 (red) and the tyrosine-sulfated antibody 412d (sulfotyrosines in green)<sup>22,24,92,103</sup>. The 412d heavy-chain sulfotyrosine 100 occupies the same pocket bound by one CCR5 sulfotyrosine, presumed to be CCR5 tyrosine 10<sup>92</sup>. We then positioned the structure of an Fc domain close to sulfotyrosine 100 residues bound to two gp120 monomers and to the C-terminus of CD4 domain 2, while avoiding steric interference with the electron density defining CD4-bound Env (Figure 3.3D). We observed that the Fc domain could be positioned so that residues absent from the crystal structures could readily bridge the distance from the Fc domain to CD4 domain 2, as well as the distances to each of the 412d sulfotyrosines (Figure 3.3E). Thus eCD4-Ig can simultaneously bind all three receptor-binding sites without steric interference. Combining data from Figure 2 and our previous studies, we infer that eCD4-Ig binds Env with a unique three point, claw-like grip.



**Figure 3.3. A model of eCD4-Ig bound to the HIV-1 envelope glycoprotein trimer.** (A) On the left, the structure (2QAD) of gp120 (YU2 isolate) bound to the tyrosine-sulfated CD4i antibody 412d and CD4 domains 1 and 2<sup>92</sup>, was fitted into a cryoelectron micrograph of HIV-1 envelope glycoprotein (Bal isolate) bound to CD4<sup>22</sup>. gp120 and CD4 are shown in blue and red, respectively. 412d sulfotyrosines are represented as green (carbon), red (oxygen), and yellow (sulfur) spheres. On the right, the Fc domain of human IgG1 (1FCC, cyan)<sup>225</sup> is oriented to avoid steric interaction with the envelope glycoprotein while minimizing distances between the carboxy terminus of domain 2 of CD4 and the amino-terminus of one Fc domain monomer, and between the carboxy-termini of the two Fc domain monomers and sulfotyrosine 100 of the 412d heavy chain (Tys 100). This sulfotyrosine occupies a pocket in gp120 thought to bind CCR5 sulfotyrosine 10<sup>159</sup>. This pocket is also critical for binding CCR5mim1 and CCR5mim2<sup>165,166</sup>. Consistent with previous studies<sup>109</sup>, the resulting distances are short enough to permit each sulfopeptide to bind a gp120 monomer (see panels d and e), whereas only one CD4 can bind. For clarity only the bound CD4 is shown. (B) The same structures shown in panel a, rotated 30 degrees about the horizontal axis. (C) The same structures shown in panel a, rotated 90 degrees about the horizontal axis. (D) The same structure shown in panel a is rotated 30 degrees about the trimer axis to highlight distances between the carboxy-terminus of CD4 and the amino-terminus of one Fc domain monomer (44.1 angstroms), between the carboxy-terminus of the Fc domain monomer fused to the bound CD4 and Tys 100 bound in *cis* to the same gp120 monomer (33.4 angstroms), and the carboxy-terminus of the Fc domain monomer fused to the unbound CD4 and Tys 100 bound in *trans* to an adjacent gp120 monomer (37.2 angstroms, unbound CD4 not shown). (E) Residues not shown in the crystal structures that must span these distances are represented, bounded by residues visible in the crystal structures (boxed in gray). Note that the distances spanned are well under the extension of a typical beta strand.

### **eCD4-Ig and its variants potently neutralized a diverse panel of tier 2 and tier 3**

**isolates.** We further explored the breadth and potency of eCD4-Ig using a panel of neutralization resistant tier 2 and 3 viruses<sup>95</sup> from multiple HIV-1 clades, as well as SIVmac251 (Figure 3.4A and 3.5A). In parallel, we assayed three additional eCD4-Ig variants. In the first, eCD4-Ig<sup>mim2</sup>, CCR5mim1 was replaced by CCR5mim2. CCR5mim2 differs from CCR5mim1 by single alanine 4 to tyrosine substitution (Figure 3.1A), which increased its affinity for a number of HIV-1 envelope glycoproteins<sup>166</sup>. We also introduced a previously characterized glutamine 40 to alanine mutation into CD4 domain 1 of eCD4-Ig (eCD4-Ig<sup>Q40A</sup>)<sup>214</sup>. This modification has been shown to increase the affinity of CD4 for the gp120 molecules of some isolates. Both mutations were combined into a final variant (eCD4-Ig<sup>Q40A,mim2</sup>). Each of these eCD4-Ig variants, including eCD4-Ig itself, substantially outperformed CD4-Ig for all viruses in the panel, typically improving neutralization potency of tier 2 and 3 isolates by 20 to >200-fold (Figure 3.4A and 3.5A). Moreover, in contrast to CD4-Ig and bNAbs, eCD4-Ig efficiently neutralized every isolate tested. For example IC<sub>80</sub> value of 3BNC117 for the clade B isolate AC10.0.29 is reported >50 µg/ml<sup>152</sup>, whereas eCD4-Ig and eCD4-Ig<sup>mim2</sup> neutralized this isolate with IC<sub>80</sub>s of 0.7 and 0.5 µg/ml, respectively (7 and 5 nM; Figure 3.5A). Similarly, the reported IC<sub>80</sub> of NIH45-46 for the clade D isolate 3016.v5.c45 was again >50 µg/ml<sup>152</sup>, whereas eCD4-Ig and eCD4-Ig<sup>mim2</sup> both neutralized this isolate with IC<sub>80</sub>s of 0.1 µg/ml (1 nM). Underscoring its exceptional breadth, eCD4-Ig also neutralized SIVmac251 with an IC<sub>50</sub> of 0.006 µg/ml (60 pM), 33 times more efficiently than CD4-Ig. In general, the more neutralization resistant a virus, the better eCD4-Ig and its variants performed relative to CD4-Ig. In most cases, replacement of CCR5mim1

with CCR5mim2 modestly improved neutralization. Similarly, the Q40A mutation in CD4 domain 1 further improved eCD4-Ig and eCD4-Ig<sup>mim2</sup> neutralization of most HIV-1 isolates, but not of SIVmac251. The IC<sub>50</sub> and IC<sub>80</sub> values shown in Figure 3.4 and Figure 3.5 compare favorably with those of previous studies of bNAbs using the same isolates (compare the final columns of Figure 3.4A).

We then evaluated eCD4-Ig, eCD4-Ig<sup>mim2</sup>, eCD4-Ig<sup>Q40A,mim2</sup> and the bNAb NIH45-46 with a panel that included nearly every isolate previously shown to be resistant to either or both of the two best CD4bs antibodies, NIH45-46 or 3BNC117 (Figure 3.4B and 3.5B). Again both eCD4-Ig variants efficiently neutralized every isolate assayed and substantially more efficiently than CD4-Ig, with IC<sub>50</sub>s ranging from <0.001 µg/ml to 1.453 µg/ml. In contrast, 26 isolates were resistant to NIH45-46, and 29 isolates have been previously reported resistant to 3BNC117. Thus, eCD4-Ig variants but not CD4-Ig efficiently neutralized tier 2 and 3 isolates with potencies comparable to those reported for highly potent bNAbs, as well as all of 38 isolates resistant to one or both of the best CD4-binding site bNAbs.

**A**

Virus	Clade	Tier	eCD4-Ig variants					Fold	Geometric Mean:	
			CD4-Ig	eCD4-Ig	mim2	Q40A	Q40A, mim2		eCD4-Ig variants	CD4bs bNAbs
SF162.L5	B	1A	<0.001	<0.001	<0.001	<0.001	<0.001	1.0	0.001	0.048
BaL.26	B	1B	0.006	<0.001	<0.001	0.001	<0.001	>6	0.001	0.015
DJ263.8	AG	1B	0.018	0.001	0.001	0.004	0.001	12.7	0.001	0.032
ZM109F.P84	C	1B	0.042	0.002	0.002	0.003	0.002	19.0	0.002	0.063
TV1.21	C	1B	0.045	0.002	0.001	0.008	0.001	22.5	0.002	ND
3365.v2.c20	A	2	0.066	0.002	0.002	0.016	0.002	19.6	0.003	0.020
SIVmac251.30	SIV	3	0.197	0.006	0.007	0.144	0.025	10.0	0.020	>50
QH0692.42	B	2	0.396	0.013	0.013	0.004	0.002	65.3	0.006	0.681
THRO4156.18	B	2	0.508	0.018	0.017	0.009	0.004	49.6	0.010	2.855
Q769.d22	A	2	0.661	0.021	0.028	0.021	0.018	30.4	0.022	0.011
3016.v5.c45	D	2	0.681	0.028	0.029	0.19	0.026	15.2	0.045	0.268
Q259.d2.17	A	2	2.141	0.076	0.079	0.081	0.048	30.8	0.070	0.034
T33-7	AG	3	3.512	0.053	0.053	0.005	0.003	245.1	0.014	0.023
T25118	AG	3	6.071	0.275	0.206	0.125	0.016	58.8	0.103	0.996
AC10.0.29	B	2	6.284	0.179	0.181	0.443	0.331	23.9	0.263	1.466
ZM135M.PL10a	C	2	6.373	0.42	0.272	0.094	0.042	43.7	0.146	0.289
PV0.4	B	3	9.506	0.212	0.214	0.032	0.025	122.5	0.078	0.162
CH115.12	B	3	25.676	0.786	0.554	0.586	0.255	50.8	0.505	ND
Du156.12	C	2	26.267	0.782	0.694	0.173	0.067	93.3	0.782	0.044
T257-31	AG	2	40.001	0.509	0.416	3.429	0.507	51.4	0.779	0.560
X1193_C1	G	2	40.218	0.367	0.476	0.111	0.028	263.5	0.153	ND
TRJ04551.58	B	3	>50	0.717	0.581	0.12	0.052	>221.4	0.226	0.052
TRO.11	B	2	>50	0.917	0.759	0.225	0.131	>132.1	0.378	0.227
R1166.c1	AE	2	>50	1.137	0.983	1.246	0.226	>66.8	0.749	0.890

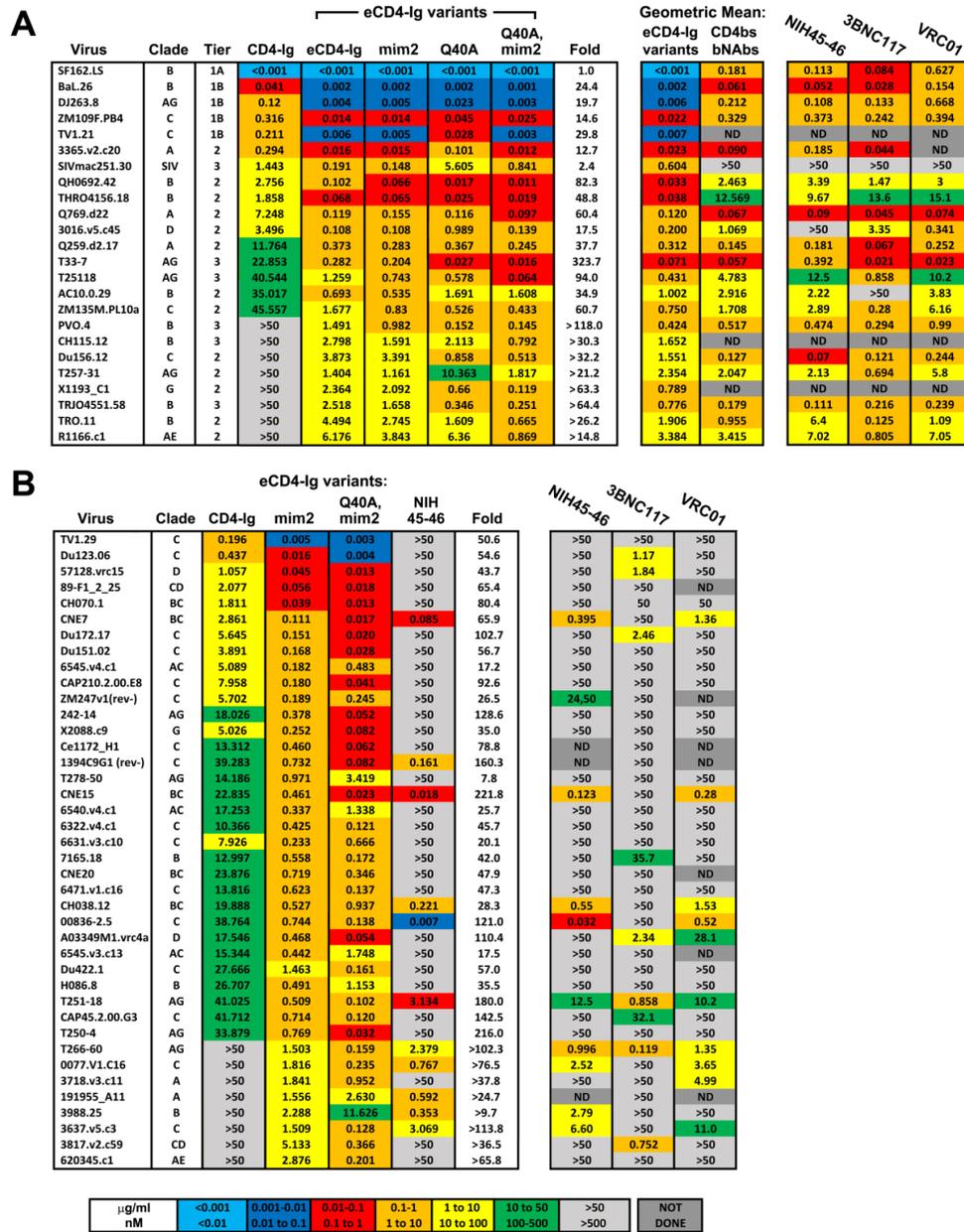
**B**

Virus	Clade	CD4-Ig	eCD4-Ig variants:				Fold	NIH45-46	3BNC117	VRC01
			mim2	Q40A, mim2	NIH 45-46	Fold				
TV1.29	C	0.055	0.001	<0.001	>50	>55.0	>50	>50	>50	
Du123.06	C	0.082	<0.001	<0.001	>50	>82.0	>50	0.183	13.6	
57128.vrc15	D	0.243	0.007	0.004	>50	45.9	>50	0.432	>50	
89-F1_2_25	CD	0.491	0.022	0.008	>50	37.0	>50	>50	ND	
CH070.1	BC	0.507	0.010	0.003	>50	92.6	>50	7.89	18.7	
CNE7	BC	0.576	0.028	0.005	0.014	48.7	0.147	>50	0.54	
Du172.17	C	0.821	0.022	0.003	>50	101.1	>50	1.19	>50	
Du151.02	C	0.823	0.046	0.011	>50	36.6	>50	>50	7.7	
6545.v4.c1	AC	0.835	0.043	0.123	>50	11.5	>50	>50	>50	
CAP210.2.00.E8	C	1.033	0.029	0.010	>50	60.7	>50	8.16	>50	
ZM247.v1(rev-)	C	1.079	0.043	0.056	2.185	22.0	2.94	>50	ND	
242-14	AG	1.192	0.042	0.006	>50	75.1	>50	>50	>50	
X2088.c9	G	1.437	0.075	0.028	>50	31.4	>50	>50	>50	
Cell172_H1	C	1.619	0.127	0.014	>50	38.4	ND	>50	ND	
1394C9G1 (rev-)	C	1.738	0.086	0.011	0.027	56.5	ND	>50	ND	
T278-50	AG	1.826	0.203	0.665	>50	5.0	>50	>50	>50	
CNE15	BC	1.848	0.112	0.006	0.005	71.3	0.052	>50	0.08	
6540.v4.c1	AC	1.987	0.073	0.192	>50	16.8	>50	>50	>50	
6322.v4.c1	C	2.282	0.100	0.029	>50	42.4	>50	>50	>50	
6631.v3.c10	C	2.396	0.102	0.197	>50	16.9	>50	>50	>50	
7165.18	B	2.469	0.143	0.038	>50	33.5	>50	6.54	>50	
CNE20	BC	2.583	0.123	0.050	3.682	32.9	7.83	>50	ND	
6471.v1.c16	C	2.662	0.164	0.036	>50	34.6	>50	>50	>50	
CH038.12	BC	3.490	0.139	0.176	0.059	22.3	0.183	>50	0.379	
00836-2.5	C	3.566	0.083	0.025	<0.001	74.0	0.015	>50	0.128	
A03349M1.vrc4a	D	3.874	0.176	0.018	>50	68.8	>50	0.512	4.66	
6545.v3.c13	AC	3.960	0.092	0.501	>50	18.4	>50	>50	ND	
Du422.1	C	4.268	0.157	0.036	>50	56.8	>50	>50	>50	
H086.8	B	6.522	0.071	0.088	>50	82.5	>50	>50	>50	
T251-18	AG	7.626	0.188	0.035	0.863	94.0	1.36	0.203	3.58	
CAP45.2.00.G3	C	7.661	0.140	0.029	>50	120.2	>50	0.589	9.47	
T250-4	AG	8.961	0.218	0.006	>50	247.8	>50	>50	>50	
T266-60	AG	10.982	0.377	0.029	0.363	105.0	0.219	0.032	0.353	
0077.V1.C16	C	12.703	0.245	0.042	0.160	125.2	0.308	>50	1.04	
3718.v3.c11	A	15.392	0.676	0.401	2.049	29.6	0.857	>50	0.218	
191955_A11	A	20.979	0.454	0.757	0.116	35.8	ND	>50	ND	
3988.25	B	21.728	0.623	1.332	0.071	23.9	0.358	>50	2.1	
3637.v5.c3	C	28.616	0.655	0.046	0.799	164.9	1.77	>50	4.09	
3817.v2.c59	CD	30.770	1.453	0.130	>50	70.8	>50	0.216	>50	
620345.c1	AE	46.395	0.515	0.051	>50	286.3	>50	>50	>50	

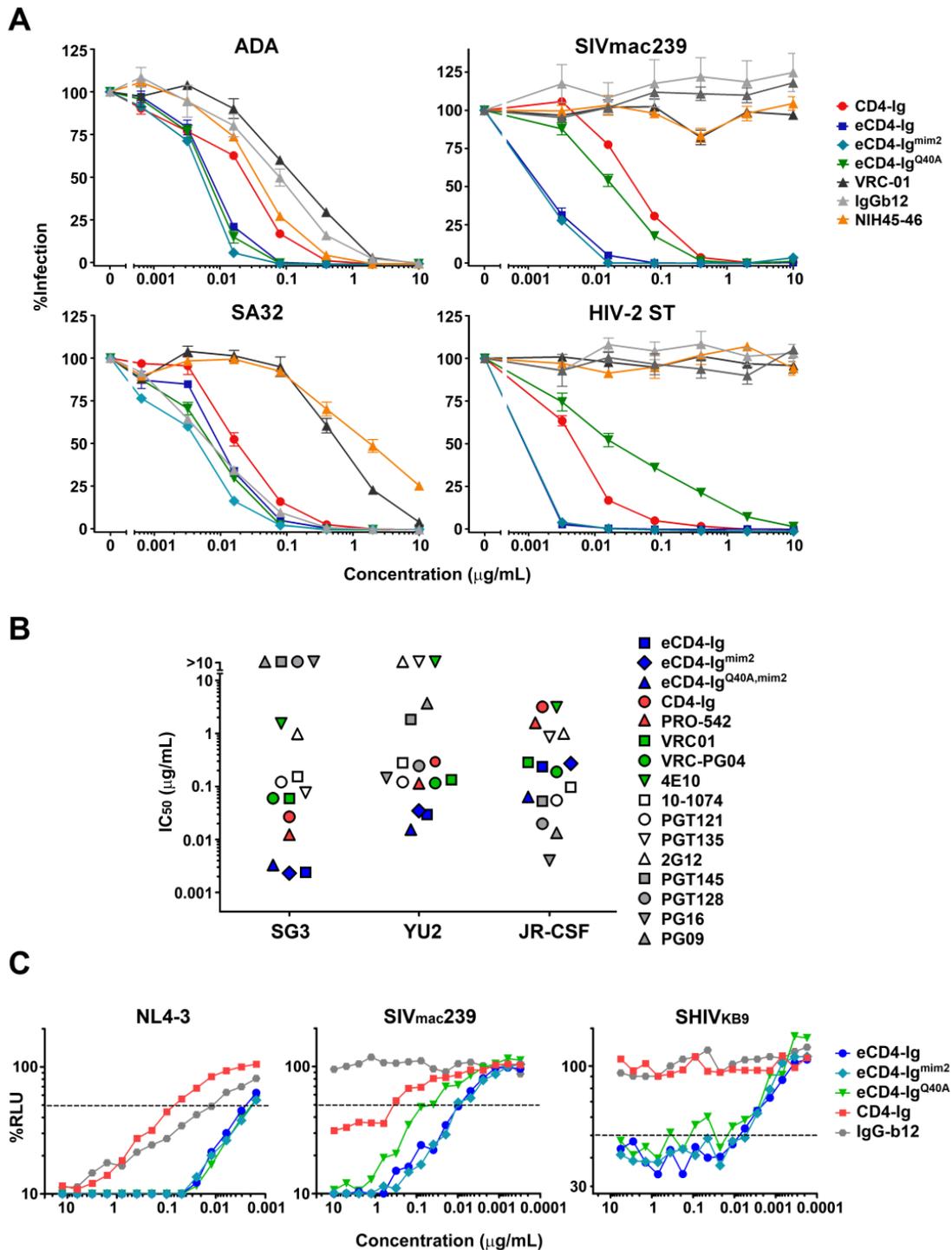
µg/ml	<0.001	0.001-0.01	0.01-0.1	0.1-1	1 to 10	10 to 50	>50	NOT DONE
nM	<0.01	0.01 to 0.1	0.1 to 1	1 to 10	10 to 100	100-500	>500	

**Figure 3.4. eCD4-Ig variants efficiently neutralize Tier 2 and Tier 3 HIV-1 and SIV isolates.**  
 (A) The IC<sub>50</sub> values (µg/mL) of CD4-Ig, eCD4-Ig, eCD4-Ig<sup>mim2</sup>, eCD4-Ig<sup>Q40A</sup>, and eCD4-Ig<sup>Q40A,mim2</sup> against 24 viral isolates selected for their neutralization resistance are shown. HIV-1 pseudotyped with the indicated envelope glycoprotein was incubated in triplicate with TZM-bl cells and varying concentrations of CD4-Ig or the indicated eCD4-Ig variant. Luciferase activity was determined two days post-infection. ‘Fold’ indicates the ratio of the IC<sub>50</sub> value of CD4-Ig to the IC<sub>50</sub> value of the geometric mean of the assayed eCD4-Ig variants. The geometric mean of CD4bs antibodies 3BNC117, NIH45-46, and VRC01 was calculated from values reported in Huang *et al.* and Sheid *et al.*<sup>137,152</sup>  
 (B) Experiments similar to those in panel a except that the IC<sub>50</sub> values of CD4-Ig, eCD4-Ig<sup>mim2</sup>, eCD4-Ig<sup>Q40A,mim2</sup> and NIH45-46 were determined for a panel of 40 viral isolates selected for resistance to the CD4bs bNAbs 3BNC117 and NIH45-46. IC<sub>50</sub> values of the CD4bs antibodies VRC01, NIH45-46, and 3BNC117 listed in the three rightmost columns were reported in Huang *et al.* and Scheid *et al.*<sup>137,152</sup>



**Figure 3.5. IC<sub>80</sub> values of for eCD4-Ig variants assayed in Figure 3.4.** (A) IC<sub>80</sub> values (μg/mL) of CD4-Ig, eCD4-Ig, eCD4-Ig<sup>mim2</sup>, eCD4-Ig<sup>Q40A</sup>, and eCD4-Ig<sup>Q40A,mim2</sup> against 24 viral isolates selected for their neutralization resistance are shown, determined as described in Figure 3.4 legend. Right panel includes published IC<sub>80</sub> values<sup>152</sup> for the CD4bs antibodies VRC-01, NIH45-46, and 3BNC117, and the geometric mean of the IC<sub>80</sub> values of eCD4-Ig variants and these CD4bs antibodies. (B) IC<sub>80</sub> values (μg/mL) of CD4-Ig, eCD4-Ig<sup>mim2</sup>, and eCD4-Ig<sup>Q40A,mim2</sup>, and NIH45-46 against 40 viral isolates, 38 of which are resistant to either NIH45-46 or 3BNC117. Right panel includes published IC<sub>80</sub> values<sup>152</sup> for the CD4bs antibodies VRC-01, NIH45-46, and 3BNC117. 'Fold' indicates the ratio of the IC<sub>80</sub> value of CD4-Ig to the IC<sub>80</sub> value of the geometric mean of the assayed eCD4-Ig variants.

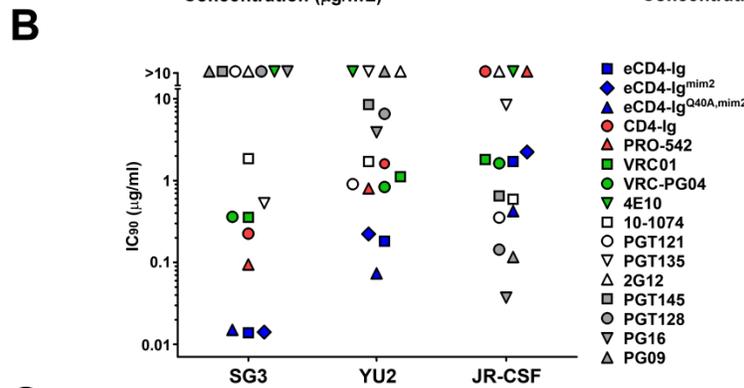
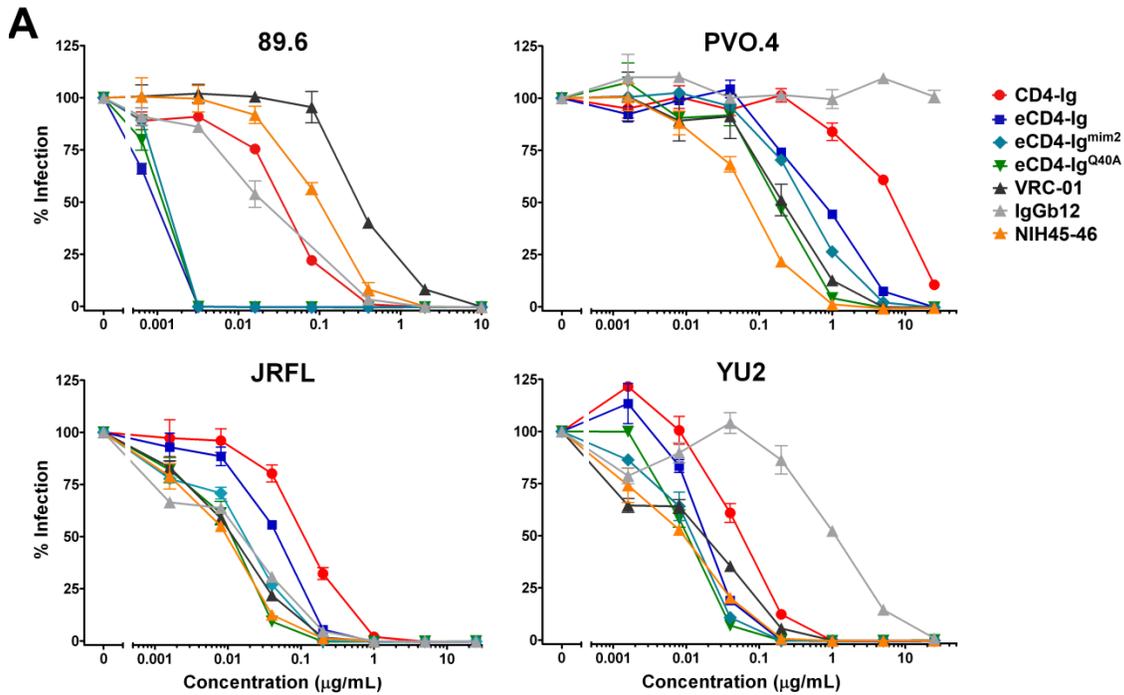
We then directly compared ability of CD4-Ig, the eCD4-Ig variants eCD4-Ig<sup>mim2</sup>, eCD4-Ig<sup>Q40A</sup>, and the bNAbs VRC01, NIH45-46, and IgGb12 to neutralize the neutralization resistant SIV isolate SIVmac239 as well as HIV-2 ST and several additional clade B and C HIV-1 isolates (Figure 3.6A and Figure 3.7A)<sup>226</sup>. Again, eCD4-Ig and its variants neutralized all six HIV-1 isolates with efficiencies comparable to or better than these bNAbs. Moreover, eCD4-Ig and its variants, but not any of these bNAbs, efficiently neutralized HIV-2 ST and SIVmac239. As was observed with SIVmac251, the variant bearing the Q40A mutation was less efficient at neutralizing SIVmac239 and HIV-2 ST than those without this mutation.



**Figure 3.6. Comparison of eCD4-Ig variants and HIV-1 neutralizing antibodies.**

(A) HIV-1 pseudotyped with the envelope glycoproteins of the indicated HIV-1, HIV-2 or SIV isolates were incubated in triplicate with TZM-bl cells and the indicated concentrations of CD4-Ig, eCD4-Ig variants, or the CD4bs antibodies IgGb12, VRC-01 or NIH45-46. Error bars represent standard errors. (B) Experiments similar to those in panel a except that a wider array of entry inhibitors (continued on p. 79)

(continued) was characterized with HIV-1 pseudotyped with the SG3, YU2, or JR-CSF isolates, and  $IC_{50}$  values are plotted. eCD4-Ig variants are represented with blue, CD4-Ig and the tetrameric CD4-Ig construct PRO-542 with red, and HIV-1 neutralizing antibodies with green, grey, and white. (C) Antibody-dependent cell-mediated cytotoxicity (ADCC) was measured using CEM.NKR-CCR5 target cells incubated with infectious HIV-1<sub>NL4-3</sub>, SHIV<sub>KB9</sub> or SIVmac239 for four days. Cells were then incubated with KHYG-1 NK effector cells<sup>227</sup> for 8 hours in the presence of the indicated concentrations of CD4-Ig, eCD4-Ig variants, or the HIV-1 neutralizing antibody IgGb12, and ADCC activity was measured as loss of luciferase activity from the target cells. Experiments are representative of two (panels A and C) or three (panel B) with similar results.



**C**

IC<sub>50</sub> values

Virus	eCD4-Ig variants				VRC01	VRC-PG04	PGT145	PG09	PG16	4E10	2G12	PGT121	PGT128	PGT135	10-1074	PRO542
	CD4-Ig	eCD4-Ig	mim2	Q40A, mim2												
SG3	0.027	0.002	0.002	0.003	0.060	0.060	> 10	> 10	> 10	1.540	0.981	0.121	> 10	0.076	0.154	0.012
+/-	0.002	0.000	0.000	0.001	0.009	0.014	0.000	0.000	0.000	0.499	0.153	0.023	0.000	0.020	0.021	0.002
YU2	0.293	0.030	0.035	0.015	0.134	0.116	1.842	3.702	0.145	> 10	> 10	0.121	0.244	> 10	0.280	0.115
+/-	0.043	0.003	0.004	0.000	0.005	0.002	0.279	0.481	0.009	0.000	0.000	0.021	0.059	0.000	0.028	0.014
JR-CSF	3.175	0.237	0.270	0.064	0.285	0.188	0.052	0.013	0.004	3.103	0.995	0.055	0.020	0.859	0.097	1.588
+/-	0.635	0.023	0.043	0.006	0.074	0.061	0.004	0.002	0.000	0.120	0.096	0.020	0.008	0.066	0.014	0.115

IC<sub>90</sub> values

Virus	eCD4-Ig variants				VRC01	VRC-PG04	PGT145	PG09	PG16	4E10	2G12	PGT121	PGT128	PGT135	10-1074	PRO542
	CD4-Ig	eCD4-Ig	mim2	Q40A, mim2												
SG3	0.225	0.014	0.014	0.015	0.356	0.360	> 10	> 10	> 10	> 10	> 10	> 10	> 10	0.529	1.860	0.094
+/-	0.023	0.001	0.000	0.001	0.018	0.031	> 10	0.000	0.000	0.000	0.000	0.000	0.000	0.178	0.012	0.013
YU2	1.607	0.182	0.223	0.074	1.113	0.836	8.519	> 10	3.896	> 10	> 10	0.906	6.544	> 10	1.717	0.802
+/-	0.040	0.013	0.017	0.001	0.012	0.030	0.992	0.000	1.341	0.000	0.000	0.157	1.125	0.000	0.074	0.096
JR-CSF	> 10	1.709	2.237	0.423	1.812	1.629	0.651	0.117	0.037	> 10	> 10	0.353	0.144	8.429	0.593	> 10
+/-	0.000	0.087	0.401	0.077	0.134	0.255	0.303	0.006	0.005	0.000	0.000	0.038	0.042	0.137	0.096	0.000

**Figure 3.7. Extended neutralization assays, IC<sub>90</sub> values, and standard errors for the studies of Figure 3.6.** (A) TZM-bl neutralization studies of the indicated isolates as described in Figure 3.6A. (B) IC<sub>90</sub> values for the experiments shown in Figure 3.6B, presented in the same format. (continued on p.81)

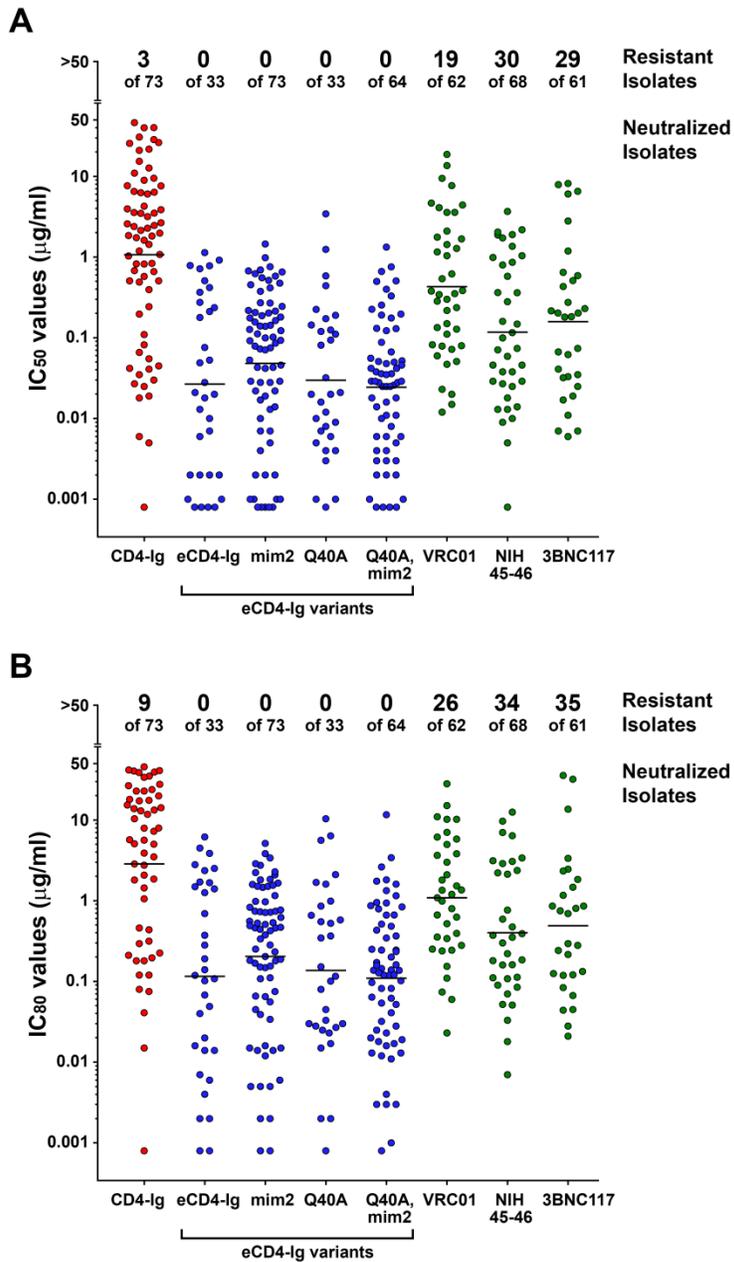
(continued) (C) Numeric  $IC_{50}$  and  $IC_{90}$  values of the experiment shown in Figure 3.6B, using the same color coding of Figure 3.4 and Figure 3.5. Standard errors are also indicated below their  $IC_{50}$  and  $IC_{90}$  values.

We further compared eCD4-Ig, eCD4-Ig<sup>mim2</sup> and eCD4-Ig<sup>Q40A,mim2</sup> using three additional HIV-1 isolates and a broader set of antibodies and inhibitors (Figure 3.6B, 3.7B and 3.7C). eCD4-Ig and variants neutralized SG3 and YU2 more efficiently than any members of this broader panel. However five antibodies – PG9, PG16, PGT128, PGT121, and PGT145 – neutralized JR-CSF more efficiently than any eCD4-Ig variant. Note, however, that four of the antibodies more potent against JR-CSF (grey in Figure 3.6B) did not neutralize SG3 at the maximum concentration of 10  $\mu$ g/ml. All eCD4-Ig variants neutralized these isolates with  $IC_{50}$ s less than 0.3  $\mu$ g/ml (3 nM) and all neutralized more efficiently than CD4-Ig, the tetrameric CD4-Ig variant PRO-542<sup>212</sup>, or the antibodies 2G12, 4E10, and VRC01. The potency of eCD4-Ig and its variants was reflected in the strikingly greater efficiency with which they mediated antibody-dependent cell-mediated cytotoxicity (ADCC). eCD4-Ig, eCD4-Ig<sup>mim2</sup>, and eCD4-Ig<sup>Q40A,mim2</sup> each facilitated more efficient killing of infected cells by CD16+ natural killer cells<sup>227</sup> than did CD4-Ig or the antibody IgGb12 (Figure 3.6C). All eCD4-Ig variants performed similarly, approximately 30-40 times more efficiently than CD4-Ig, except that the variant bearing the Q40A mutation was again less efficient against SIVmac239. Thus the carboxy-terminal modification of eCD4-Ig did not interfere with the ADCC effector function of its Fc domain.

Figure 3.8 presents a comparison of the neutralization studies compiled from the experiments shown in Figures 3.4-3.7 and from previous studies. It shows that, even excluding SIV, HIV-2, and antibody resistant HIV-1 viruses, the geometric means of the

IC<sub>50</sub> and IC<sub>80</sub> values of eCD4-Ig and its variants is roughly 3-4 times lower than that of VRC01, NIH45-46, or 3BNC117, and is 20-50 times lower than that of CD4-Ig.

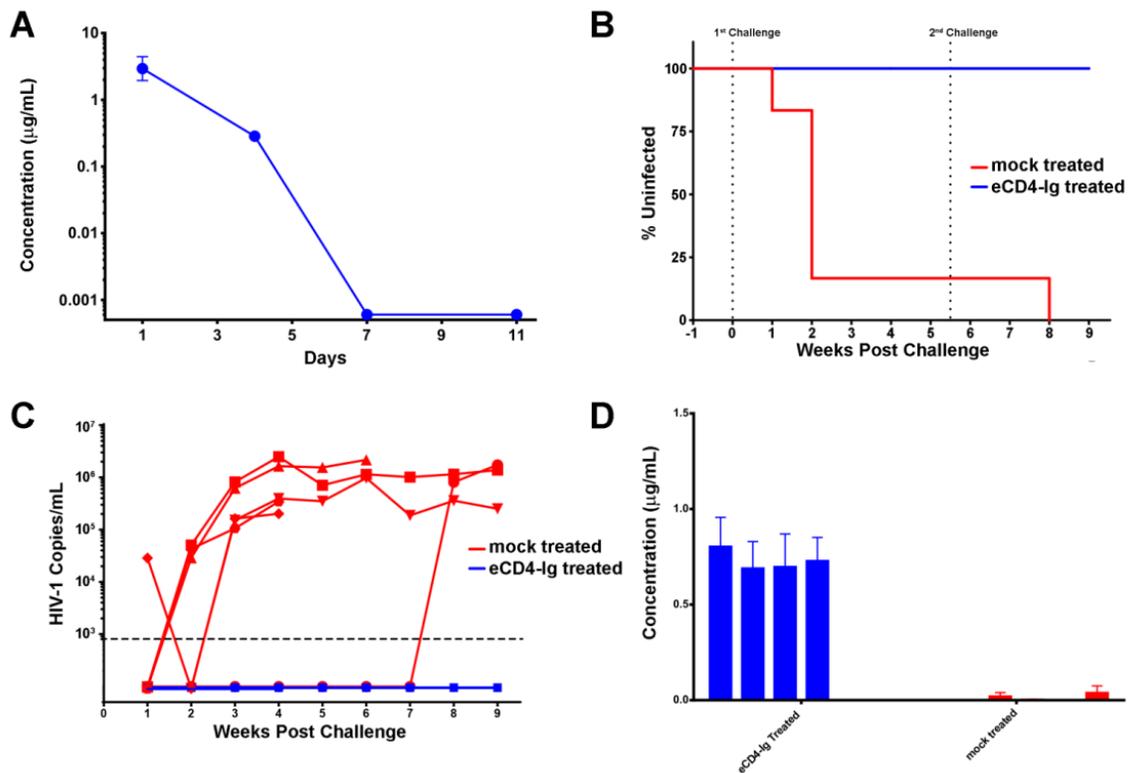
Moreover, each eCD4-Ig variant neutralized 100% of the isolates assayed. In fact we have yet to identify or select an HIV-1, HIV-2 or SIV isolate that could not be efficiently (IC<sub>50</sub> < 1.5 µg/ml) neutralized by eCD4-Ig. We conclude that eCD4-Ig is at least as potent as the best HIV-1 bNAbs and is broader than any bNAb.



**Figure 3.8. A summary of HIV-1, HIV-2 and SIV neutralization studies.**

The  $IC_{50}$  and  $IC_{80}$  values from studies of Figures 3.4, 3.5, 3.6 and 3.7 are summarized. The numbers of isolates that were not 50% (A) or 80% (B) neutralized by 50  $\mu\text{g/ml}$  of the indicated inhibitors are listed on top. Dots indicate  $IC_{50}$  and  $IC_{80}$  values of the remaining neutralized isolates. Geometric means are calculated for neutralized isolates only and are indicated with a horizontal line. Note that these data include SIV and HIV-2 isolates as well as 38 HIV-1 isolates selected to be resistant to NIH45-46 or 3BNC117, so that isolates resistant to these antibodies are over-represented. Data for VRC01 and 3BNC117 were reported in Huang *et al.* and Scheid *et al.*<sup>137,152</sup> Data for NIH45-46 includes data from these sources as well as from Figures 3.4 and 3.5.

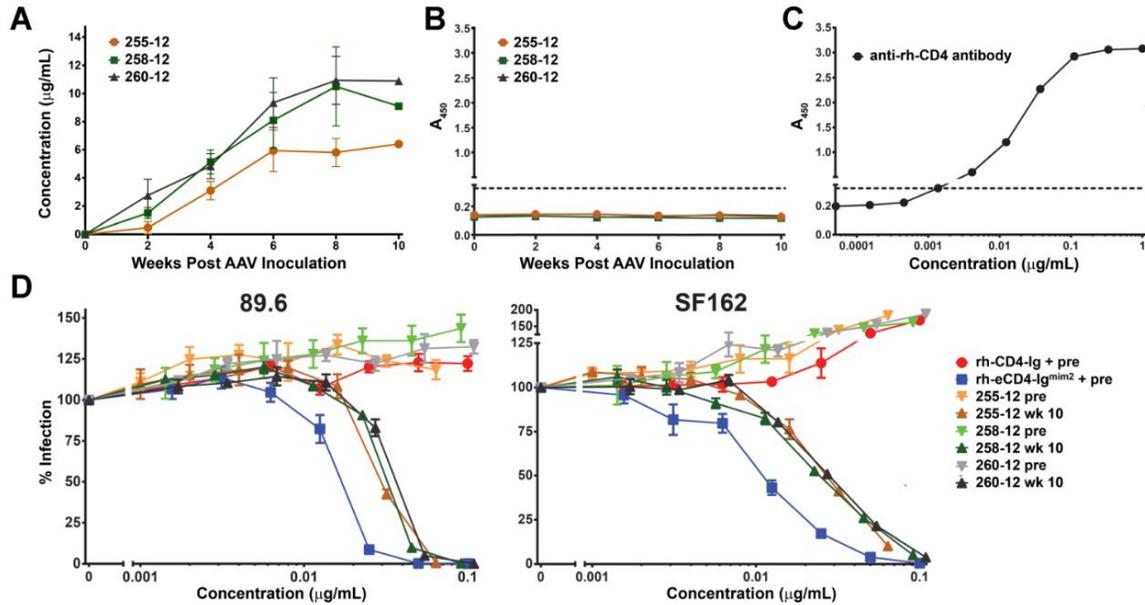
**eCD4-Ig retains its efficacy *in vivo*.** To determine if eCD4-Ig retained its potency *in vivo*, we inoculated four humanized NOD/SCID/ $\gamma$ c (NSG) mice with sufficient eCD4-Ig<sup>mim2</sup> to maintain serum titers of 2-4  $\mu$ g/ml (Figure 3.9A). These mice, as well as six humanized control NSG mice were then challenged intravenously with  $5 \times 10^4$  p24 infectious units of HIV-1<sub>NL4-3</sub> (Figures 3.9B-D). No virus could be detected in any of the four eCD4-Ig<sup>mim2</sup> inoculated mice, whereas 5 of 6 control animals were infected. Five weeks later, we rechallenged three of the previously challenged eCD4-Ig<sup>mim2</sup>-treated mice, and the single uninfected control mouse. Again, no eCD4-Ig<sup>mim2</sup>-treated mouse was infected, whereas the uninfected control mouse became infected. Thus eCD4-Ig<sup>mim2</sup> can protect a humanized mouse from a high-dose HIV-1 challenge.



**Fig 3.9. *In vivo* activity of eCD4-Ig in humanized mice.** (A) eCD4-Ig stability in NSG mice measured over the course of 11 days. 65  $\mu$ g of eCD4-Ig was administered to 8 NSG mice via retro orbital injection. Samples were taken at days 1, 4, 7, and 11 post injection and titers were analyzed by ELISA. (B) Infection curves of eCD4-Ig treated (blue line, n = 4) or mock treated with PBS (red line, n = 6) (continued on p.85)

(continued) humanized NSG mice challenged with HIV-1<sub>NL4-3</sub>. Mice were monitored weekly over the course of nine weeks. Three uninfected eCD4-Ig treated mice and one uninfected mock treated mouse were challenged a second time with the same virus and titers, as indicated. Values indicate percent of mice detectably infected. (C) Viral loads in RNA copies/mL are shown for each humanized mouse of panel B. Mice treated with eCD4-Ig are indicated with blue lines and mice treated with PBS are indicated with red lines. The 800 copies/mL limit of detection of this assay is indicated by a dashed line. (D) Average eCD4-Ig titers for each mouse in both the eCD4-Ig- and mock-treated groups from the humanized NSG mouse challenge study in Figure 3.9B-C. Samples were taken 1-3 days post eCD4-Ig each week. Dashed line indicates the limit of detection of the assay at 1 ng/mL.

The *in vivo* properties of eCD4-Ig variants were further investigated in rhesus macaques using a rhesus macaque form of eCD4-Ig<sup>mim2</sup> (rh-eCD4-Ig<sup>mim2</sup>). To minimize potential adverse reactions *in vivo*, the Fc domain of the rh-eCD4-Ig<sup>mim2</sup> was generated from rhesus macaque IgG2, which binds Fc receptors and complement components less efficiently than IgG1. A gene expressing this rh-eCD4-Ig<sup>mim2</sup> was inserted into a self-complementary AAV (scAAV) vector, and  $2 \times 10^{13}$  AAV1 particles delivering this vector were administered into the quadriceps of three 2-year old, Indian-origin, male rhesus macaques. To promote efficient sulfation of the CCR5<sup>mim2</sup> component, a separate scAAV vector expressing rhesus tyrosine-protein sulfotransferase 2<sup>228</sup> (TPST-2) was also administered in the same injections at a 1:10 ratio with scAAV-rh-eCD4-Ig<sup>mim2</sup>. Note that, although this TPST-2 vector improves eCD4-Ig sulfation in *in vitro* studies (not shown), it is not yet clear whether it does so *in vivo*. Blood was collected from AAV-inoculated macaques at -4, -2, 0, 2, 4, 8 and 10 weeks after administration and sera were analyzed for rh-eCD4-Ig<sup>mim2</sup> concentrations and anti-rh-eCD4-Ig<sup>mim2</sup> antibody responses. rh-eCD4-Ig<sup>mim2</sup> titers stabilized to 6-12  $\mu\text{g/ml}$  by week 10 (Figure 3.10A), indicating the transgene expressed efficiently, but at somewhat lower levels than has been reported with antibody-like immunoadhesins delivered with a similar vector<sup>129</sup>. No adverse reactions were observed for any macaque throughout the experiment.



**Fig 3.10. Characterization of rh-eCD4-Ig<sup>mim2</sup> in rhesus macaques.** (A) Sera titers of rh-eCD4-Ig<sup>mim2</sup> from animals 255-12, 258-12, and 260-12 at the indicated weeks post AAV inoculations were measured by ELISA with immobilized SIV gp120. (B) Anti-transgene response measured by ELISA with immobilized rh-eCD4-Ig<sup>mim2</sup> using sera samples from the indicated weeks post scAAV inoculation. Dotted line indicates limit of detection as determined by an anti-rhesus CD4 antibody. All titers were below this limit equivalent to 0.0014 µg/ml of an anti-rhesus CD4 antibody. (C) A standard curve used to determine limit of detection of the anti-rhesus CD4-Ig antibody in panel b. Limit of detection is again represented with a dotted line. (D) HIV-1 pseudotyped with 89.6 or SF162 envelope glycoproteins was pre-incubated with sera samples obtained 2 weeks before ('pre') or 10 weeks ('wk 10') after scAAV inoculation, or with pre-inoculation sera spiked *in vitro* with purified rh-CD4-Ig or rh-eCD4-Ig<sup>mim2</sup> to a concentration of 10 µg/mL, based on the ELISA measurements of panel A.

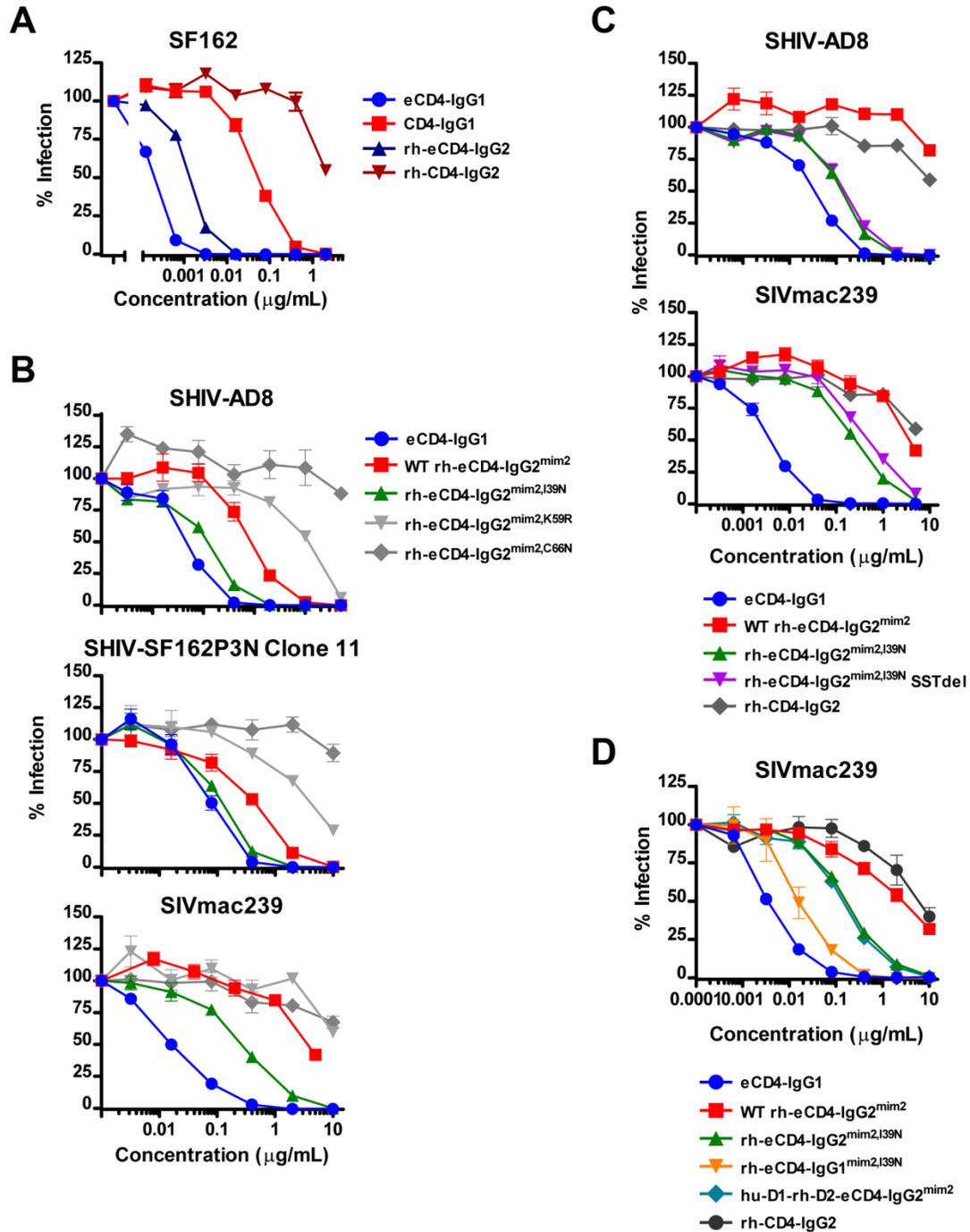
Studies in rhesus macaques using adeno-associated virus (AAV) vectors to express neutralizing antibodies or CD4-Ig have shown that anti-transgene antibody responses can emerge, and that these responses can interfere with the activity of AAV-delivered inhibitors<sup>129</sup>. For example, the majority of 12 macaques expressing the anti-SIV antibodies 5L7 or 4L6 produced antibodies recognizing these AAV-expressed antibodies detectable four to six weeks after administration of the AAV vector (S.F., R.C., in preparation). Similarly, Johnson *et al.* reported that two of three macaques expressing a single-chain immunoadhesin form of 5L7 also generated anti-immunoadhesin responses, although no such response was observed with the 4L6

immunoadhesin<sup>129</sup>. The same study observed that one of three macaques expressing approximately 5 µg/ml of a rhesus form of CD4-Ig (N4-Ig) developed an anti-N4-Ig response in the same time frame. In contrast, anti-rh-eCD4-Ig<sup>mim2</sup> antibody responses could not be detected through week 10 in any of the three macaques expressing this transgene (Figure 3.10B-C). These data suggest that rh-eCD4-Ig<sup>mim2</sup> is not more immunogenic than N4-Ig, 5L7, or 4L6 in rhesus macaques.

To determine if AAV-expressed rh-eCD4-Ig<sup>mim2</sup> retained its activity *in vivo*, we compared sera from the three inoculated macaques with pre-inoculation sera mixed with rh-CD4-Ig or rh-eCD4-Ig<sup>mim2</sup> at the same concentrations. Serum from all three inoculated macaques neutralized two HIV-1 isolates with 40-50% of the activity of sera mixed with the same concentrations of rh-eCD4-Ig<sup>mim2</sup> indicating that they expressed approximately 3-5 µg/ml of functional rh-eCD4-Ig<sup>mim2</sup> (Figure 3.10D). No neutralization was observed with rh-CD4-Ig mixed with pre-inoculation sera. Note that the IC<sub>50</sub>s observed in Figure 4d are higher than those in Figs. 1 and 3, because the rhesus CD4 ortholog binds the 89.6 and SF162 envelope glycoprotein much less efficiently than does human CD4 (ref. <sup>229</sup> and Figure 3.11A). Thus rh-eCD4-Ig<sup>mim2</sup> can be expressed efficiently as an AAV transgene, and it retains much but not all of its activity *in vivo*.

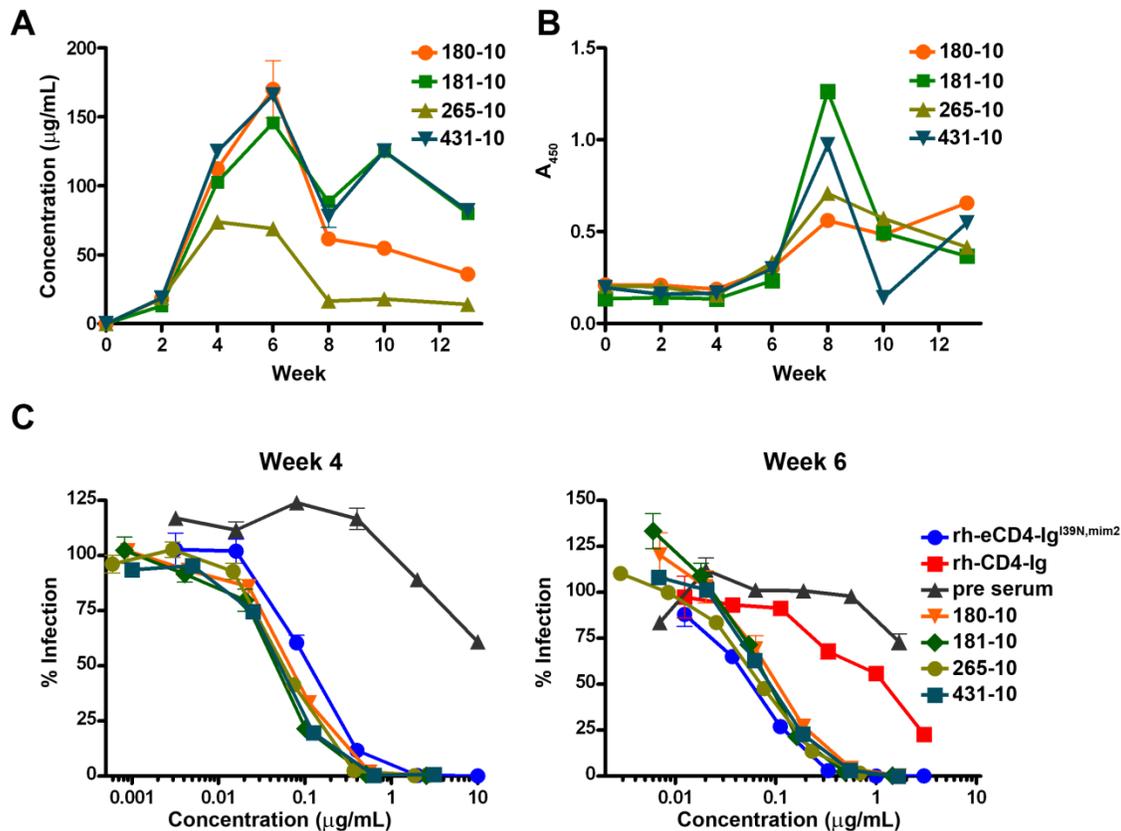
Next, we sought to evaluate the potency of rhesus-eCD4-Ig<sup>mim2</sup> as this will be used in future non-human protection and therapy experiments. We first observed that rh-eCD4-Ig<sup>mim2</sup> was less potent as the human-eCD4-Ig (Figure 3.11A). We tested this construct in neutralization assays on three isolates: SF162P3N Clone 11, SHIV-AD8, and SIVmac239. In all three cases, rhesus-eCD4-Ig<sup>mim2</sup> performed less efficiently than human eCD4-Ig, including - surprisingly - on SIVmac239 (Figure 3.11B). In an attempt

to restore potency, we made three human CD4 residues into the rhesus CD4 portion of rhesus-eCD4-Ig<sup>mim2</sup>: I39N, K59R, and C66N. The I39N mutation increased neutralization of SHIV-AD8 and SHIV-SF162P3N Clone 11 but not SIVmac239 (Figure 3.11B). Because of the presumable “claw-like” binding mechanism by which eCD4-Ig binds Env, we speculated the IgG hinge could modulate neutralization. Thus we deleted three residues preceding the hinge (SST) that predicted to be O-glycosylated, which might impact the hinge flexibility. This deletion had no effect on neutralization on either SHIV-AD8 or SIVmac239 (Figure 3.11C). Interestingly, changing from rhesus-IgG2 to rhesus-IgG1 had the greatest effect on SIVmac239 (Figure 3.11D). Finally, we then swapped out domain 1 of rhesus-eCD4-Ig<sup>mim2</sup> with human CD4 domain 1. The eCD4-Ig variant composed of human CD4 domain 1 with rhesus IgG2 and CCR5mim2 did not have an impact on potency as it neutralized at a similar level as the I39N rhesus variant (Figure 3.11D). Through this effort, we determined that the I39N variant will be suitable for SHIV challenge and therapy experiments while the rhesus IgG1 variant will be used in future SIV challenge and therapy experiments.



**Figure 3.11. Characterizing rh-eCD4-Ig<sup>mim2</sup> variants to gain sensitivity on SIV and SHIV isolates.** (A) TZM-bl neutralization assay comparing human eCD4-Ig, human CD4-Ig, rhesus eCD4-Ig<sup>mim2</sup>, and rhesus CD4-Ig. (B) rh-eCD4-Ig<sup>mim2</sup> variants with either the I39N, K59R, or C66N mutations were tested against SIVmac239, SHIV-AD8, and SHIV-SF162P3N Clone 11 isolates by TZM-bl neutralization assay. (C) TZM-bl neutralization assay comparing rh-eCD4-Ig<sup>mim2</sup> variant with deletion of possible O-glycosylated residues by the hinge region (SST del) with eCD4-Ig on SIVmac239 and SHIV-AD8 isolates. (D) TZM-bl assay comparing rh-eCD4-IgG1<sup>mim2</sup> and a variant that swapped rhesus CD4 domain with human CD4 domain 1 (hu-D1-rh-D2-eCD4-IgG2<sup>mim2</sup>) with eCD4-Ig on SIVmac239.

Lastly, we wanted to test whether using single-stranded AAV (ssAAV) vectors could improve rh-eCD4-Igmim2 transgene expression in macaques. The ssAAV vectors contain components shown to increase transgene expression such as a wood chuck element (WPRE) and a full CMV promoter. For this study, we used the I39N variant described in Figure 3.11. When using the ssAAV vectors containing a full CMV promoter and WPRE, we observed greater expression of rh-eCD4-Ig<sup>I39N,mim2</sup> in macaques with titers peaking at greater than 100 µg/mL in three of the four macaques we tested (Figure 3.12A). However, the greater expression did not come without consequence as we were able to detect antibodies against the rh-eCD4-Ig<sup>I39N,mim2</sup> construct in all four animals (Figure 3.12B). Expectedly, immunogenic response we detected correlates with the drop in rh-eCD4-Ig<sup>I39N,mim2</sup> titers seen after week 6. However, in this study, we also increased the amount of TPST-2 AAV inoculation to 20%. At this level, we observed the expressed rh-eCD4-Ig<sup>I39N,mim2</sup> retained 100% of its activity at weeks 4 and 6 post AAV inoculation (Figure 3.12C-D).



**Figure 3.12. rh-eCD4-Ig<sup>I39N,mim2</sup> expression with ssAAV vectors.** Similar experiments as performed in Figure 3.10. (A) Sera titers of rh-eCD4-Ig<sup>I39N,mim2</sup> from animals 180-10, 181-10, 265-10, and 431-10 the indicated weeks post ssAAV inoculations were measured by ELISA with immobilized SIV gp120. (B) Anti-transgene response measured by ELISA with immobilized rh-eCD4-Ig<sup>mim2</sup> using sera samples from the indicated weeks post AAV inoculation. (C) HIV-1 pseudotyped with 89.6 envelope glycoproteins was pre-incubated with sera samples obtained 2 weeks before ('pre'), 4 weeks ('Week 4'), or 6 weeks ('Week 6') after ssAAV inoculation, or with pre-inoculation sera spiked *in vitro* with purified rh-CD4-Ig or rh-eCD4-Ig<sup>I39N,mim2</sup> to a concentration of 10 μg/mL, based on the ELISA measurements of panel A.

### 3.D. Discussion

Taken together, our data show that eCD4-Ig and its derivatives are at least as potent as most well-characterized bNAbs and broader than any bNAb. The reason for their unusual breadth is clear: eCD4-Ig binds solely the CD4- and coreceptor-binding sites of gp120. These sites are functionally important and highly conserved<sup>230,231</sup>, consistent with eCD4-Ig's ability to potently neutralize 100% of the HIV-1 isolates

assayed and its unique ability to efficiently neutralize SIV and HIV-2 isolates. In contrast, the gp120 epitopes of bNAbs are larger than these sites, and in every case include variable residues that allow viruses to evade neutralization without loss of fitness<sup>91,188</sup>.

The reasons for eCD4-Ig's potency, however, are more complicated. It is clear that the CCR5-mimetic sulfopeptide in eCD4-Ig blunts the ability of CD4-Ig to promote infection. It is also apparent that the sulfopeptide and CD4 components of eCD4-Ig can simultaneously engage one or more HIV-1 envelope glycoprotein, because eCD4-Ig<sup>mim2</sup> is markedly more potent than mixtures of CCR5mim2-Ig and CD4-Ig<sup>166</sup>. Further, the CD4 domain of eCD4-Ig cooperatively enhances the affinity of its two sulfopeptides, which, in contrast to the CD4 domains, can each bind one monomer of the envelope glycoprotein trimer<sup>109,165</sup>. Thus, although CD4-Ig and most bNAbs can bind only one trimer epitope, eCD4-Ig has the potential to bind three with high affinity – one CD4-binding site and two coreceptor-binding sites (Figure 3.3). The ability of eCD4-Ig to generate more potent ADCC than CD4-Ig or an HIV-1 neutralizing antibody is also of interest, especially since, at higher concentrations, more CD4-Ig molecules bind cell-expressed envelope glycoproteins than eCD4-Ig molecules (Figure 3.2A). This observation may suggest that some Fc domains are more favorably oriented toward effector-cell CD16<sup>227</sup>, or that natural killer cells more efficiently recognize a localized array of Fc domains.

Highly potent anti-HIV-1 bNAbs have been a recent focus of intense interest for several reasons. First, they provide insight into the properties of antibodies that an HIV-1 vaccine should elicit, and perhaps provide guideposts in step-wise efforts to raise these

antibodies<sup>226</sup>. The example of eCD4-Ig provides similar insight. Its potency relative to CD4-Ig underscores one challenge to eliciting potent CD4bs bNAbs: to be broad, such an antibody must bind similarly to CD4, but to be potent, an antibody must suppress its ability to promote infection. Indeed, the best CD4bs neutralizing antibodies are much less efficient than sCD4 at inducing the CCR5-binding epitope of CD4i antibodies<sup>137</sup>. The potency of eCD4-Ig also highlights the ability of CCR5mim1 and CD4i antibodies to improve access to the CD4-binding sites of the envelope glycoprotein trimer<sup>22,109</sup>. Thus broad CD4i antibodies might enhance the potency of the many CD4bs antibodies in sera that are less potent than for example 3BNC117 or NIH45-46.

A second reason that bNAbs are of interest is their potential to prevent or treat an HIV-1 infection<sup>188-190</sup>. We have shown that eCD4-Ig when passively administered retains its activity *in vivo*, protecting humanized mice from a high-dose HIV-1 challenge. Although passive administration remains difficult and expensive, gene therapy technologies, including those using AAV vectors, make possible stable, long-term expression of bNAbs or inhibitors like eCD4-Ig<sup>129,232-234</sup>. Antibody-like immunoadhesins and bNAbs delivered as AAV transgenes have been demonstrated to protect rhesus macaques and humanized mice, respectively, from SIV or HIV-1 challenges<sup>129,232</sup>. In this context, eCD4-Ig has several advantages over any bNAb or related CD4i antibody/CD4 fusion construct<sup>168,173</sup>. It has fewer non-self antibody and T-cell epitopes than heavily hypermutated bNAbs, and unlike antibodies, it is small enough to be delivered through more efficient self-complementary AAV vectors<sup>233</sup>. The conservation of the eCD4-Ig binding sites on gp120 suggests that emergence of escape variants is less likely than with a bNab, and viruses that do bypass prophylaxis may be less replicative in the continued

presence of eCD4-Ig, and thus less likely to be retransmitted. If so, an AAV-delivered eCD4-Ig might also be useful as a supplement to or replacement for current antiretroviral regimens, especially when compliance with these regimens is a concern.

A key hurdle to the use of AAV vectors is the possibility that the expressed transgene will be cleared or inactivated by an anti-transgene antibody response. However, in contrast to previous and concurrent primate studies of antibody-like immunoadhesins<sup>129</sup> or full-length antibodies expressed from AAV-delivered transgenes, none of three rhesus macaques treated with scAAV-rh-eCD4-Ig<sup>mim2</sup> raised an anti-transgene antibody response. The relatively low titers of eCD4-Ig in these animals (<15 µg/mL) may account for the absence of an endogenous antibody response. We did, however, observe an anti-transgene response when using the I39N variant in the ssAAV vectors. The anti-transgene response in this case might have been caused from the I39N mutation or from higher titers (>100 µg/mL) of the transgene. Higher-powered *in vivo* studies will be necessary in order to determine which eCD4-Ig variants are more immunogenic and what levels of transgene can be tolerated without yielding an anti-transgene response. Encouragingly, rh-eCD4-Ig<sup>mim2</sup> retained much, or all, of its efficacy in macaque sera, especially when raising the amount of TPST-2 to 20% of the inoculated AAV. The observed levels of eCD4-Ig expression for both scAAV and ssAAV vectors, if sustained in humans, would likely prevent most HIV-1 transmission events. These properties of eCD4-Ig and its variants, combined with their exceptional potency and unmatched breadth, suggest that this class of inhibitors can be useful as microbicides, as supplements to antiretroviral therapies, or as expressed transgenes in an alternative vaccine.

## **CHAPTER 4: DISCUSSION**

### **Implications for the Development of an HIV-1 Vaccine**

The current work has highlighted the advantages of targeting the CD4- and coreceptor-binding sites on Env. Without a conventional HIV-1 vaccine, we must look to alternative approaches for targeting the several vulnerable sites of Env. In this chapter, we will discuss the state of HIV-1 vaccine efforts and the antibody responses observed in the current vaccine trials. We then focus on antigen design and the important factors of HIV-1 antibody development. The use of bNAb cocktail therapies and the possible role of CD4i antibodies in these cocktails is considered. Finally, the prophylactic and therapeutic uses of AAV-delivered eCD4-Ig is examined.

#### **4.A. The state of HIV-1 vaccine efforts**

There have been numerous efforts to make an effective HIV-1 vaccine. In the early 1990's, rhesus macaques given a live attenuated virus (SIVmac239/nef-deletion) had 100-fold lower viral loads than macaques receiving the wild-type strain<sup>235,236</sup>. This nef-deletion attenuated virus elicited a high antibody response, although it was unknown whether the resulting antibodies were bNAbs. Trials using an engineered nef-deleted attenuated virus were halted when a later report showed that this virus was infectious in infant macaques<sup>237</sup>. Since then, the vaccine field has moved to subunit antigens and non-replicating viral vectors in place of an attenuated virus vaccine strategy. However, it is worth noting that live attenuated vaccines can generate T-cell responses which control infection. Indeed, accumulated data suggest that vaccines that use both arms of the immune response will be more effective compared to those focusing on cellular or humoral responses alone.

One step in this direction was the RV144 Thai trial. In this trial, the cohort was primed with an inert canarypox viral vector delivering recombinant forms of HIV-1 gag, pol, and env. The vaccine recipients were then given a boost of a recombinant gp120 from clades B and E. In this trial, vaccine recipients were 31% less likely of becoming infected compared to the placebo group<sup>238</sup>. Since the report was published, the antibody response from this cohort has been analyzed extensively. One of the studies found that IgG antibodies targeting the V1/V2 loop were detected in individuals who received the vaccine<sup>239</sup>. However, no neutralizing antibodies were detected in any individual. Thus continuous stimulation of the humoral response and ongoing evolution of Env may be necessary to drive the numerous rounds of somatic hypermutation apparently necessary to generate bNAbs. A recent vaccine study in rhesus macaques identified V1/V2 loop antibodies as a correlate of protection<sup>240</sup>. Some studies have suggested a role protection for the ADCC, which requires less potent antibodies such as those found in the RV144 Thai trial participants. However, it is still unclear if V1/V2 loop antibodies and ADCC-mediating antibodies are sufficient for an HIV-1 vaccine, or whether they are a byproduct of the immediate immune response following Env exposure.

Other vaccine studies attempt to induce a T-cell response that would be able to protect from a wide array of HIV-1 isolates. To achieve an effective T-cell response, mosaic vaccines have been developed that combine elements from all known HIV-1 isolates for various viral proteins including Gag, Pol, and Env<sup>241,242</sup>. Delivered after priming with an adenoviral vector, these vaccines are thought to boost specific immune responses that prevent infection against a broad range of HIV-1 isolates. Challenge studies have shown that this vaccine strategy (Ad prime followed by mosaic vaccine

boost) has some effectiveness against both SIVmac251 and SHIV-SF162P3 infection<sup>243,244</sup>. Although anti-Env antibodies were observed, these antibodies could only efficiently neutralize tier 1 isolates, moderately neutralize some tier 2 isolates, but not tier 3 isolates.

A recent vaccine failure was the HVTN 505 trial which uses a similar approach with DNA vectors carrying Gag, Pol, Nef, and Env followed by a recombinant Ad5 boost<sup>245</sup>. Results demonstrated a CD8+ T-cell response against Env. However, previous studies have pointed out that this kind of response does not yield protection. An initial animal study showed that no bNAbs were produced by this vaccine and that it was only successful in animals challenged with SIVsmE660 but not with SIVmac251<sup>246</sup>. These limitations underscore the difficulty of protecting against a heterologous challenge.

There have been other promising approaches to HIV-1 vaccine design. One study used a rhesus CMV viral vector to deliver Gag, Rev/Nef/Tat, Env, and Pol in order to establish a persistent T-cell response<sup>247</sup>. Although these vectors were unable to protect the animals from challenge, about half of the vaccine-treated animals were able to clear the viral infection, and virus was not detected in any reservoir tissues as measured by the most sensitive PCR techniques. However, despite the inclusion of Env in the viral vector, there were little to no Env-directed antibodies produced. If such a vaccine were able to produce Env antibodies, it might more efficiently prevent initial infection.

#### **4.B. Developing antigens that will generate HIV-1 bNAbs**

It will take major advances in antigen design in order to generate a bNAb response. HIV-1 bNAbs have a large amount of necessary somatic hypermutation in

their framework region and usually have long CDR-H3s. This degree of somatic hypermutation may hinder the therapeutic usefulness of these antibodies. Reverting these antibodies back to their germline sequences might limit this immunogenic response. However, many such germline mutations impact the neutralization potency of these antibodies<sup>248</sup>. New antigens designed to elicit bNAbs from their germline precursor will need to take these factors into account.

To develop a useful antigen, we need a better understanding of the coevolution of the virus and antibody maturation process by which bNAbs emerge. Recently, one group tracked the mutations of a founder virus as it changed the glycosylation on N332, a supersite of vulnerability to current glycosylation bNAbs<sup>249</sup>. Over the course of the infection, a glycosylation site emerged after six months but it later reverted. Both non-glycosylated variants were resistant to PGT128. This type of study provides insight to the interplay between the evolving virus and generation of bNAbs that may prove useful in vaccine design.

An effective subunit vaccine may require rational structure-based antigen design. For example, McLellan et al designed variants of RSV F in the pre-fusion state that maintained the antigenic site Ø<sup>250</sup>. Immunization of these stable RSV F variants in mice and macaques elicited a neutralizing antibody response that was able to protect the animals from RSV infection. Although RSV is considerably easier to neutralize than HIV-1, it is conceivable that this idea can be extended to HIV-1 Env or gp120. Variants of Env that expose vulnerable sites, like the CD4-binding site, may accelerate the maturation of neutralizing antibodies that specifically target that site. In any case, an

antigen(s) eliciting neutralizing antibodies will be a critical component of a successful HIV-1 vaccine.

#### **4.C. Soluble trimeric Env as an antigen**

The recently published crystal structure of the Env trimer may be useful for vaccine design<sup>82,251</sup>. A soluble Env trimer antigen can elicit antibodies that recognize quaternary epitopes. Unfortunately, a soluble trimer still has the same fatal flaw that a gp120 antigen has: it is only one Env. An evolving Env may be necessary to drive high levels of somatic hypermutations found on bNAbs. Thus, a stepwise approach using antigens that continually target and mature a specific germline precursor might be necessary. A recent study that evaluated the antibody development pathway after HIV-1 infection showed that V1/V2 loop antibodies could be generated within months of infection<sup>240</sup>. Interestingly, these antibodies had modest somatic hypermutation and long CDR-H3s. These results suggest that HIV-1 bNAbs, under the optimal conditions, might only take weeks to develop rather than years.

#### **4.D. Antibody cocktails as a new HIV-1 therapy**

Until a conventional vaccine is available, we must consider alternate approaches. Our data imply that targeting multiple epitopes on Env can generate greater potency than targeting one epitope from one inhibitor or bNAb. Specifically, we show that the CD4- and coreceptor-binding sites can be targeted simultaneously for potent neutralization, either by two antibodies or a fusion construct. Although both the receptor epitopes are highly conserved, they are a challenge to target. CD4bs antibodies are rare and difficult to elicit with a single antigen. In general, CD4i antibodies are not potent and only

neutralize tier 1 HIV-1 isolates. CD4i antibodies also have difficulty accessing the coreceptor-binding site because of the V1/V2 and V3 loops surrounding the epitope.

The field has already recognized the value of using bNAbs in a cocktail therapy for treating HIV-1 infection<sup>188-190</sup>. A cocktail of 3 to 5 bNAbs was effective at suppressing infection in a humanized mouse model while combinations of 2 to 3 bNAbs could suppress SHIV infection in rhesus macaques. Interestingly in these latter studies, PGT121 by itself could suppress SHIV-SF162P3 infection in 3 out of 4 macaques<sup>190</sup>. Considering the potency of PGT121 and breadth of 10E8, using antibody cocktails as a therapy that combines a variety of epitopes like the CD4-binding site, V1/V2 loop glycosylation, V3 loop glycosylation, and MPER, may be effective at suppressing a wide range of HIV-1 infection.

There are a few obstacles that currently face the antibody cocktail therapy. First is the possibility of escape mutants from the antibodies used in the cocktail. If the antibodies target multiple epitopes, there is a greater chance of success at suppressing a wide range of HIV-1 isolates. However, an isolate may already be resistant to all but one of the antibodies in the cocktail. Should this be the case, escaping the last remaining antibody is less difficult than escaping multiple antibodies. This scenario raises the possibility of using variants of an antibody to cover escape mutation pathways. NIH45-46<sup>G54W</sup> has been engineered with this idea in mind. NIH45-46m2 and NIH45-46m7 variants were just as potent as the predecessor antibody and combining all three antibodies did not allow for any escape mutants to occur in a humanized mouse model<sup>145</sup>. Even with this success, it is still unclear whether a cocktail should target a single epitope, anticipating multiple escape pathways, or multiple distinct epitopes.

Because no known antigen can elicit a response that is similar to a bNAb cocktail, passive transfer or gene therapy delivery of the antibody cocktail remain the only viable approaches. Passive transfer studies have shown most of these bNAbs to have a half-life of 2 days post injection in humanized mice<sup>188</sup>. In rhesus macaques injected with an inoculum of 20 mg/kg of antibody, titers decreased to low concentration levels by day 7 post injection. In order to make passive transfer a viable therapy, new technologies need to be developed in order to increase the time the antibody lasts in the host. Gene therapy viral vectors bypass the need for continuous passive administration. Numerous studies, including our own, have shown that AAV vectors can be effectively used for the constant production of immunoadhesins or antibodies by muscle cells<sup>129,232,252,253</sup>. Questions still remain of the long term production of such inhibitors but data suggest that their production can continue for two years or more. However, the consequence for long-term, high-level antibody production in the absence of infection must be better understood. Going forward, studies would need to address potential problems such as host responses against the antibodies and how to stop antibody expression should problems arise.

#### **4.E. Translating bNAb *in vitro* properties**

There are general concerns about the non-neutralizing functions of bNAbs and whether *in vitro* potency translates into an *in vivo* model. The TZM-bl neutralization assay determines the potency of an antibody against a large panel of HIV-1 isolates. Although this assay can determine potency and breadth of an antibody, it only accounts for neutralizing cell-free virus, which might not determine activity in an *in vivo* model. It is not clear that this assay predicts the efficacy of antibodies and Env inhibitors against T-cell associated viral reservoirs. Newly developed *in vitro* assays determine whether these

antibodies can neutralize cell-to-cell transmission of virus. Flow cytometry analysis has been utilized to show that these antibodies can limit cell-to-cell spread<sup>254</sup>. It is clear that if antibodies will be used therapeutically, more physiological systems may be necessary to assess *in vivo* potency of a bNAb. The field is now closely evaluating the non-neutralizing effector functions of antibodies and how they influence protection. This was apparent in the RV144 Thai trial where there were apparent ADCC antibodies. These antibodies may be one subset of antibodies for a vaccine to elicit should they have a role in protection.

#### **4.F. The role of CD4i antibodies in an HIV-1 vaccine**

Both studies presented here highlight the importance of blocking coreceptor binding. First, we showed that E51, an antibody whose epitope overlaps the coreceptor binding site, can increase the potency of the CD4bs antibodies by increasing the association for Env through quaternary changes. Second, fusing a CCR5mim peptide to CD4-Ig increased the binding of CD4-Ig to Env, limited enhancement of infection by CD4-Ig, and increased avidity presumably by binding multiple epitopes on a single Env. Currently, the available CD4i antibodies are at the state most bNAbs were at 5 years ago: poorly neutralizing and lacking breadth. Is it worth undertaking a search for better CD4i antibodies? Identifying additional CD4i antibodies may not be difficult. An antigen could be engineered such that a gp120 monomer would be held in the CD4-bound state by fusing CD4 to gp120. This would create an accessible CD4i epitope for B cells to target. Identifying antibodies of this class from a library is straightforward. Key features, such as VH1-69 lineage or a tyrosine-sulfation motif in the CDR-H3, would be helpful in quickly identifying those antibodies that target the CD4i epitope.

Although we can generate and identify new CD4i antibodies, it remains unclear whether new CD4i antibodies will be more potent than current antibodies in this class. As we and others have shown, CD4i antibody binding requires quaternary changes in Env and incur an entropy cost as they induce the open conformation on Env. The potent HIV-1 bNAbs – VRC01, PGT121, etc. – have low Env perturbation factors that allow for higher affinity binding and faster on-rates<sup>140</sup>. Any CD4i antibody may necessarily incur a free energy cost as it binds because its epitope is occluded or differently structured in the absence of CD4. The energy required to induce the open state of Env could limit the potency and breadth of any CD4i antibody. Further, the coreceptor epitope is smaller than that of the CD4-binding site. Antibodies directed to the CD4i epitope will have numerous contact residues that are highly variable, again limiting their breadth and enabling viral escape.

CD4i antibodies may therefore have limited application. However, our data suggests a role for CD4i antibodies in a possible HIV vaccine. The potent CD4bs antibodies can be thought as having two key properties: they block access to CD4 and they avoid promoting necessary coreceptor binding. However, less potent CD4bs bNAbs, like F105, have been shown to have higher perturbation factors compared to more potent ones and may induce the CD4i epitope<sup>140</sup>. The presence of CD4i antibodies might enhance the neutralization of these high perturbation factor CD4bs antibodies

#### **4.G. Using eCD4-Ig as a vaccine alternative**

The absence of a silver bullet antigen to elicit HIV-1 bNAbs raises the question whether we can develop better therapies for treating HIV-1 infection. Use of a single entry inhibitor almost inevitably leads to viral escape. However, we have shown that

eCD4-Ig, through its mechanistically distinct mode of neutralization that targets the CD4- and coreceptor-binding sites simultaneously, is a highly potent entry inhibitor and has breadth that is unmatched by any HIV-1 antibody. Its exceptional breadth likely derives from the fact that, unlike antibodies, eCD4-Ig specifically targets only the most conserved regions on gp120. As escape from eCD4-Ig is characterized, it will be interesting to see what kind of fitness cost the virus pays in order to gain resistance to the inhibitor. Nonetheless, it appears that eCD4-Ig has the unique properties useful as a vaccine alternative or therapy.

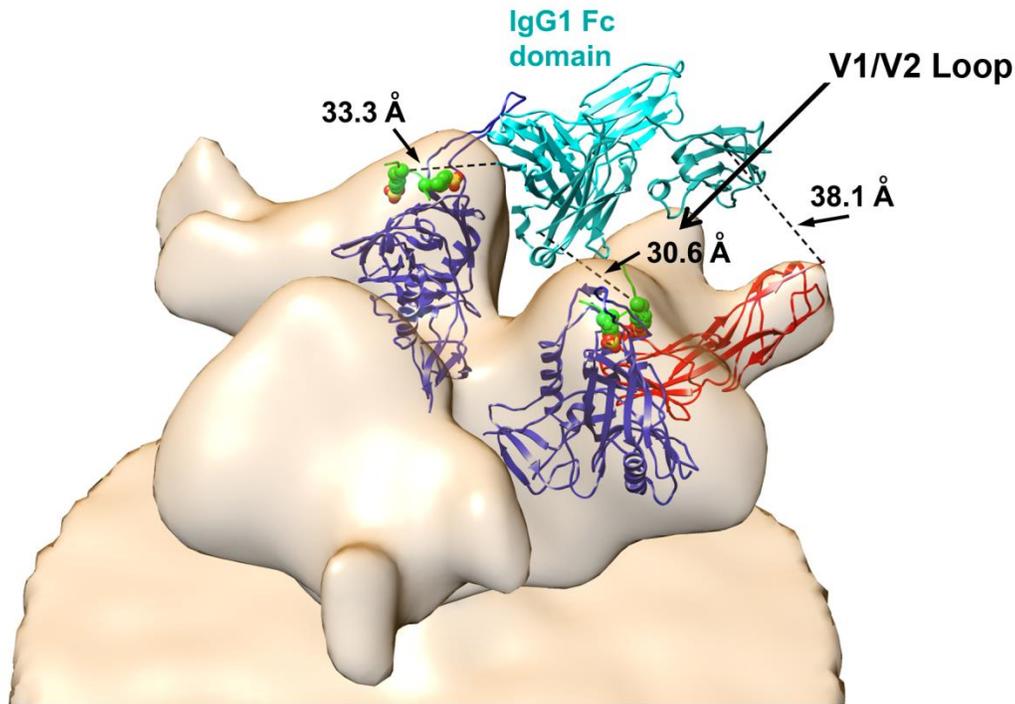
Passive administration of any new therapy, including bNAbs, is not currently feasible, but, as we demonstrate, ssAAV vectors can produce large amounts of functional eCD4-Ig in rhesus macaques. To determine the efficacy as eCD4-Ig as a vaccine alternative or therapy, we are developing higher-powered rhesus macaque studies to determine if these levels of eCD4-Ig (>100 µg/mL in serum) can protect from both SHIV and SIV infection. Given that AAV vectors take 4-6 weeks to produce these levels of eCD4-Ig, it will be necessary to suppress viral load with antiretroviral drugs so the virus does not escape as the transgene expression ramps up. Although it has been suggested that one antiviral drug might limit AAV transduction, an easy alternative to drugs is a bridge of a high-dose passive transfer of eCD4-Ig during AAV inoculation. In either case, given enough time for transgene expression, anti-retroviral drug and/or passive administration therapy could be ended while AAV therapy continues production of eCD4-Ig to maintain viral suppression.

#### 4.H. Predicting escape pathways of eCD4-Ig

As mentioned, we are developing an *in vitro* model of eCD4-Ig escape. We have currently shown that eCD4-Ig neutralizes 100% of the HIV-1, HIV-2, and SIV isolates assayed. However, isolates resistant to eCD4-Ig may still exist. By consistently passaging virus in the presence of eCD4-Ig *in vitro* and in macaques, we expect to gain insight on pathways by which the virus can mutate in order to become resistant to eCD4-Ig. There are several possibilities. First, the virus could adapt and become less dependent on CD4 binding, thus relying more on coreceptor usage. CD4-independent viruses tend to be X4 viruses and eCD4-Ig can efficiently neutralize this subset of viruses. However, subtle mutations around the CD4-binding site may occur. These mutations could alter the structure of binding region such that the virus decreases its affinity for CD4, decreasing affinity for the CD4 portion of eCD4-Ig. Similarly, subtle mutations might emerge in sulfotyrosine-binding pocketing of the coreceptor-binding site. Should escape occur because of changes to either receptor-binding site, we anticipate a corresponding cost to viral fitness.

A more likely escape pathway may include changes in the variable loop regions. If we assume the “claw-like” binding by eCD4-Ig on Env, the repositioning of the V1/V2 loop could sterically exclude eCD4-Ig. As modeled in Figure 4.1 (based on the mechanism we assume eCD4-Ig binds Env from our biochemical and neutralization studies), the V1/V2 loop in the cryo-EM of Env is in close proximity to the Fc portion of eCD4-Ig. CD4 binding relocates the V1/V2 loop to create the open form of Env and a longer V1/V2 loop has a greater chance of interfering with the eCD4-Ig Fc domain, preventing its peptide portion from binding. SIV neutralization studies with the rh-

eCD4-Ig<sup>mim2</sup> suggest this possibility. The original rh-eCD4-Ig<sup>mim2</sup> construct included rhesus IgG2 as the Fc. When we changed the Fc to rhesus IgG1, we observed a marked increase in the potency of rh-eCD4-Ig<sup>mim2</sup>. Specifically, the hinge of rhesus IgG1 is six amino acids longer than rhesus IgG2. We hypothesize that the longer hinge allows for greater flexibility for eCD4-Ig to reach around the V1/V2 domain and bind Env with its sulfopeptides. Consistent with this, SIVmac239 Env has a V1/V2 loop of 100 amino acids, much longer than the average 70 amino acids of HIV-1. The longer IgG1 hinge likely limits steric interference from the V1/V2 loop of SIVmac239.



**Figure 4.1. Modeling Env escape from eCD4-Ig.** Here we show the cryo-EM structure of Env with two gp120 subunits modeled into their respective locations. In red is CD4 and the sulfotyrosines atoms are modeled in green. The Fc receptor has been modeled in approximately the location for where CD4 would reach around to have the sulfopeptides bind gp120. Distances are included for determining how far the Fc would need to reach in order to get the sulfopeptides to bind in a “claw-like” state. Labeled is the location of the V1/V2 loop in the CD4-bound state. The V1/V2 loop is repositioned following CD4 binding. eCD4-Ig puts Env in the CD4-bound state, thus the V1/V2 loop would be repositioned outward. A longer V1/V2 would sterically interfere with the Fc domain of eCD4-Ig as it tries to reach around to bind the sulfopeptides at the C-terminus to their respective epitopes on Env. Thus, using the IgG1 Fc is beneficial for eCD4-Ig binding.

Lastly, escape mutations might emerge in the V3 loop. The sulfotyrosine-binding pocket is at the base of the V3 loop which is repositioned after CD4 binding to enable access for the coreceptor. Mutations or insertions in the V3 loop could prevent access for the sulfopeptide to bind in two ways. Mutations might lead to changes in the structure of the V3 loop that make it less flexible, thus making it harder to access the sulfotyrosine-binding pockets. Alternatively, larger V3 loops might also make the sulfotyrosine-binding pockets more inaccessible by occluding this site. In either case, the virus may exploit subtle differences in between the CCR5 N-terminus and the CCR5mim1 region of eCD4-Ig to allow the former but not the latter to bind.

#### **4.I. Effectively using AAV vectors for HIV-1 therapy**

eCD4-Ig is an antibody-like inhibitor with the potential to outperform even the broadest and most potent HIV-1 bNAbs. However, in contrast to bNAbs, there will never be an antigen developed to elicit eCD4-Ig *in vivo*. Thus, AAV vectors are necessary for use of eCD4-Ig in an *in vivo* model. Although earlier studies showed that scAAV vectors can produce large amounts of transgene<sup>129</sup>, we were only able to observe about 5 to 12 µg/mL of eCD4-Ig when using scAAV vectors. However, ssAAV vectors allowed us to incorporate a larger CMV promoter and the WPRE to stabilize RNA expression, which increased eCD4-Ig expression to levels >100 µg/mL. These levels should be sufficient to prevent SHIV and SIV infection in rhesus macaques and may be useful as a therapeutic.

Use of AAV vectors for gene therapy is not novel to the HIV field. Although it is not the traditional sense of a vaccine, such an approach is closer to realization than a conventional vaccine. Protection has been observed in mouse and macaque studies using AAV vectors, yet human studies present several hurdles. A major concern is the lack of

an off-switch – a mechanism that stops the expression of the transgene – in the event of a poor reaction to the AAV expressed transgene. The idea of an off-switch has already been realized in other gene therapy approaches through the uses of inducible caspase 9<sup>255,256</sup>. For similar strategy to be applied to AAV delivery of HIV-1 antibodies, the off-switch will have to be smaller in size. Another question is whether it is healthy for a patient to maintain high levels of a single, unchanging antibody. Without an off-switch, the only viable method for eliminating transgene expression would be a muscle biopsy, which has not been evaluated.

Another concern resides with the bNAbs themselves. These antibodies are fully matured, most with a large CDR-H3 and extensive somatic hypermutation. At high concentrations, these antibodies will likely elicit an endogenous antibody or a cytotoxic T cell response. As stated above, attempting to mutate some of these residues back to germline sequences limits the potency of these antibody so a certain number of mutations will have to be retained. Barouch *et al.* observed a faster viral rebound after a second dose of PGT121 which may be a result of a secondary anti-PGT121 response<sup>190</sup>. More data on the safety of these antibodies will be required before they are considered as a viable therapy.

Finally, AAV-delivery presents additional problems. Maximum eCD4-Ig expression was observed at 6 to 10 weeks post AAV-innoculation, providing a window for viral escape. The lower concentrations of an inhibitor found in the early stages might allow and drive escape before AAV transgene production reaches therapeutic concentrations. Horowitz *et al.* examined the use of anti-retroviral drugs with AAV expressed bNAbs 3BNC117 and 10-1074. It was reported that only half the mice that

received the AAV injection had efficient expression of antibody<sup>257</sup>. To circumvent potential problems combining the anti-retrovirals with AAV, a bridge of passively administered antibody was delivered while AAV inoculation was being performed. The majority of the mice receiving this treatment were able to maintain suppression of viral loads even after the bridge of passive antibody administration was stopped. Overall, some form of initial treatment to suppress the viral load must be undertaken for an AAV-based therapy to be effective.

Use of AAV to deliver antibodies is not limited to HIV-1 therapy. Broadly neutralizing anti-influenza antibodies are as difficult to elicit as HIV-1 antibodies. Current data suggests antibodies targeting the stalk of HA are the broadest and most potent because the stalk is a highly conserved, yet highly protected, area of HA. These antibodies are rare and come from the VH1-69 germline. Without a proper antigen to target this germline precursor, AAV-based therapy can be again applied to prevent and treat influenza A virus. Balasz *et al.* showed that their AAV vector efficiently produced an anti-HA influenza antibody, F10, in mice and that these treated mice were protected from subsequent influenza challenges<sup>253</sup>. As experience with delivery of AAV vectors for antibodies deepens, this class of therapeutics could be applied towards other pathogens lacking vaccines like dengue virus and malaria parasites, at least until conventional vaccines are developed.

#### **4.J. Other gene therapy ideas to be considered**

Using AAV vectors to deliver HIV-1 inhibitors that target Env is one possibility. Alternatively, one target the HIV-1 receptors themselves. As mentioned in the introduction, an antibody fused with the PG9 or PG16 scFv domains efficiently blocks

HIV-1 entry through the localization of the scFv domains to Env. Like eCD4-Ig, PG9/PG16-iMab could be expressed using AAV vectors. Another target for antibodies would be CCR5. One study showed that an anti-CCR5 vaccine protected a subset of macaques for SIV infection<sup>258</sup>. PRO140 is an anti-CCR5 antibody that blocks HIV-1 entry *in vitro* that was already evaluated in Phase 2 clinical trials. Like bNAbs, we could use AAV vectors to express PRO140 *in vivo* and be used as an alternate to an HIV-1 vaccine. However, in both cases, the safety of such approaches will be an even greater concern.

Interestingly, one gene therapy study targeted the CCR5 gene, excising it from T cells. Some populations, mainly in Scandinavia, have inherited a partially deleted CCR5 gene in chromosome 3 termed CCR5- $\Delta$ 32. This CCR5 deletion renders these people resistant to R5 strains of HIV-1 without apparent consequences. Using this insight, Tebas *et al.* modified CD4+ T cells of HIV-1(+) patients using zinc-finger endonucleases to delete the CCR5 gene<sup>259</sup>. The modified T cells were then infused into their donors and no side effects were noted in any of the 12 patients in this phase I clinical trial. Data also showed that viral copies decreased and the modified T cells continued to proliferate. Moving forward, there are many gene therapy approaches that can be applied to limit replication in HIV-1 infected individuals.

#### **4.K. Closing remarks**

Our data indicate that it is useful and effective for an HIV-1 entry inhibitor to target multiple epitopes of Env. Specifically, targeting the CD4- and coreceptor-binding sites of Env by a combination of CD4bs and CD4i antibodies is more efficient than targeting either site alone. Viral escape is less likely when multiple epitopes are targeted

so a vaccine that can generate neutralizing antibodies against several sites on Env, including the coreceptor binding site, should be considered. However, we are still many steps away from having a conventional vaccine that can elicit bNAbs. Numerous studies have shown that when using gp120 monomers or trimers to elicit Env antibodies, at best these antibodies neutralize a subset of tier 2 isolates. Although there is a role for non-neutralizing antibody effector functions, like ADCC, bNAbs are likely necessary for a vaccine to effectively protect from infection. However, bNAbs are probably not sufficient. An effective vaccine should make use of both arms of the immune system as these responses are tightly integrated and in combination is what protects us from disease.

Until an effective HIV-1 vaccine is developed, the field continues to pursue alternative prophylactic and therapeutic approaches. Cocktails of current bNAbs can be effective, but without an efficient mechanism of delivery, this therapy will likely not be practical. Even with more efficient means of passive administration, in which an antibody cocktail is administered every four months or so, this approach cannot be readily applied in developing nations. Although there are a number of concerns with using AAV vectors, they could transform bNAbs into a realistic therapy. Our data suggest that eCD4-Ig can be just as effective as the current bNAbs and should be considered as a possible vaccine alternative alone or in combination with bNAbs should AAV vectors prove to be safe and efficient as therapies. Our future studies will focus on the efficacy of eCD4-Ig in animal models as we attempt to develop eCD4-Ig into a new HIV-1 therapy.

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