Spectrum of Somatic Hypermutations and Implication on Antibody Function: Case of the anti HIV-1 antibody, b12

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2015

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Program Authorized to Offer Degree:

Molecular and Cellular Biology

University of Washington

Abstract

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Sequence diversity, ability to evade immune detection and establishment of human immunodeficiency virus type 1 (HIV-1) latent reservoirs present a formidable challenge to the development of an HIV-1 vaccine. Structure based vaccine design stenciled on infection elicited broadly neutralizing antibodies (bNAbs) is a promising approach, in some measure to circumvent existing challenges. Understanding the antibody maturation process and importance of the high frequency mutations observed in anti-HIV-1 broadly neutralizing antibodies are imperative to the success of structure based vaccine immunogen design. Here we report a biochemical and structural characterization for the affinity maturation of infection elicited neutralizing antibodies IgG1 b12 (b12).

We investigated the importance of affinity maturation and mutations accumulated therein in overall antibody function and their potential implications to vaccine development. Using a panel of point

reversions, we examined relevance of individual amino acid mutations acquired during the affinity maturation process to deduce the role of somatic hypermutation in antibody function. Biophysical characterizations of b12 point mutant interactions with gp120 monomers from two Clade B viruses (SF162 and QH0692) indicates importance of cooperative contributions by individual mutations accumulated due to the extensive maturation processes in attaining the observed broadly neutralizing properties of b12. However, effects of individual mutations on epitope binding do not correlate with their effect on virus neutralization potency.

Establishment of viral latent reservoirs, rendering antibody-based immune responses ineffective as preventive treatments, precedes serum detections of infection elicited highly potent bNAbs. Bearing the goal of structure based vaccine immunogen design that recapitulate b12-like immune response, we also investigated the minimum mutations on germline antibody sequences required to garner b12 comparable epitope binding affinity and virus neutralization potency. Our progressive increase in mutations on germline b12 precursor antibody approach reveled the importance of mutations on both antibody complementarity determining (CDR) and framework regions (FWR) in epitope binding and virus neutralization. Here we report extensive mutations and prolonged affinity maturation are vital to the development of highly potent bNAbs.

One potential mechanism in attaining increased affinity maturation following the antibody maturation process is conformational antibody rearrangement and rigidification of antibody variable domains. To determine the role of extensive antibody mutations in the overall antibody structural flexibility and conformational rearrangements, we examined the structures of germline precursor b12 light chain variable domains. Accordingly, our structural analyses reveal absence of apparent effect on global structure of antibody light chain variable domains following affinity maturation. However, local structural affects are observed where germline precursor antibody CDRs exhibited conformational samplings distinct from b12 light chain CDRs. While germline precursor antibody CDRs sample different conformations, the process of antibody maturation focused the epitope binding region to specific conformation enabling high affinity epitope binding.

These data suggest that structure based vaccine development that utilize b12-like bNAbs as templet need to develop a scheme that enables extensive affinity maturation observed in bNAbs. The bodies of work presented here, representing work on multiple aspects of bNAb development, potentially highlight key basic science research on HIV vaccinology that inform HIV vaccine development.

Dedication

To my wife Genet Tadesse and daughter Tinsae Mesfin for enduring the headwinds and to my parents Mulugeta Gewe and Fetlework Bekele for believing in me.

Table of Content

List of Table

List of Figures

Abbreviations used

HIV	Human Immune deficiency Virus
AIDS	Acquired Immune Deficiency Syndrome
Env	HIV Envelope glycoprotein
bNAb	Broad Neutralizing Antibody
CD4BS	CD4 Binding Site
MPER	Membrane Proximal External Region
BCR	B-Cell Receptor
Fv	Antibody Fragment Variable
VH	antibody Variable region Heavy chain
VL	antibody Variable region Light chain
CDR	Complementarity Determining Region
CDRH	Complementary Determining Region Heavy chain
CDRL	Complementary Determining Region Heavy chain
SHM	Somatic HyperMutation
c/scFv	Cleavable Single Chain Fv
SPR	Surface Plasmon Resonance
SA	Streptavidin
k_a	Rate of association
k_d	Rate of dissociation
K_D	Equilibrium binding constant
mSR	Mature single reversion to germline sequence
gRB	Germline block reversion to mature sequence
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide gel electrophoresis
SEC	Size Exclusion chromatography
CD	Circular Dichroism
T_m	Denaturation temperature
NKG2D	Natural Killer cell receptor group 2D
NKG2DL	NKG2D ligand
MHC	Major Histocmpatability Complex
HLA	Human Leukocyte Antigen
MIC	MHC class I like Complex

Antibody maturation and affinity maturation exchangeable usage

Chapter 1:

1 The virus – immune system interface: the case of HIV-1 and vaccine development

1.1 Introduction

Since the time of Edward Jenner's successful vaccination against smallpox in 1798 (1), science has uncovered the remarkable ability of the mammalian immune system to identify and eradicate pathogenic particles and toxic compounds. In addition, the system is also robust in recognizing previously encountered pathogens upon re-infection and hence capable of establishing immunological memory. The advent of vaccination coupled with immunological memory enabled the worldwide eradication of notorious infection causing agents like smallpox and those like polio, rubella and measles eradicated in regions where diligent vaccination protocols were implemented. Vaccines have proven to be effective prophylactic medical interventions against various pathogenic agents, yet despite decades of research and various attempts to develop protective immunity, vaccines against viruses like HIV-1 remained elusive.

HIV-1 is the causative agent of AIDS, a disease which, according to WHO, affceted the lives of over 35 million people worldwide by 2013 (2). Although prevalence of HIV-1 infection remains high, because of research in HIV-1 epidemiology, antiretroviral therapies and preventive measures, virus transmission is drastically reduced giving way to diminished rate of mortality and progression to AIDS (2-5). Given the current state of HIV-1, one ponders the need for a vaccine against HIV-1. The gained strides against HIV-1 are quite tenuous due to factors such as establishment of latent viral reservoirs early in infection, the need for reliance on lifelong adherence to drug regimens, unforeseen long-term side effects of drugs especially following aging, and the tremendous challenge due to emergence of drug resistant viruses. These could potentially reverse the current gains against HIV-1 (5-9). Ensuring continued progress in containing the virus and even ultimately ending the HIV epidemic requires vaccine based prophylactic immunity.

Vaccines against HIV-1 could be therapeutic where the goal is boosting the immune system of HIV-1 seropositive individuals or could be protective (10). Therapeutic vaccines act by depressing initial rates of HIV-1 replication thereby maintaining low viral load in HIV-1 seropositive individuals. Decreased viremia correlates with reduced probability of virus transmission (11, 12). In contrast, the goal in developing vaccines that create protective immunity is to provide sterilizing immunity through neutralization and hindering virus entry into naive cells (13, 14). To this effect, since the discovery of HIV-1 as the causative agent of AIDS in 1983, the HIV scientific research community has been relentless in its quest for a safe vaccine with efficacy that predicts protection.

Initial vaccine discovery predictions failed to understand the complexity in HIV biology and virus-immune system interface. Conventional and empirical vaccinology, using either live-attenuated or inactivated viruses, proved cumbersome and ineffective with no or minimal efficacy (15-18). Vaccine development challenges reflect unique features at host - virus interfaces. HIV-1 route of entry, transmission and lifecycle makes proper immune response against the virus difficult. The virus mainly infects and establishes a latent reservoir in immune cells (CD4+ T cells and macrophages) impeding proper immune response against opportunistic pathogens. These immune cells serve as hubs and coordinate immune response against invading microbes. Furthermore, effective vaccine based protection is also coordinated through activation of these immune cells. Hence, infection of these sub-classes of immune cells gravely impairs proper immune protection and vaccine development. Propensity of the virus to establish a latent reservoir in immune cells at early stage of infection enables virus reactivation and reemergence following immune activation.(19-21).

HIV-1 biology, therefore, poses new challenges not encountered in previous vaccine development endeavors. HIV-1 immune evasion properties that enable the virus to escape immune detection are prominent hurdles in vaccine development. A well-documented immune evasion mechanism of HIV-1 is the low-fidelity reverse transcription of its RNA genome resulting in a high degree of sequence variability between generations (22-25). Inherent higher sequence variation in HIV-1 strains and the elicitation of highly autologous virus neutralizing antibodies that exert a natural selection pressure and reshape

immunogenicity of potential immune targets in subsequent generations further increase the viral strain diversity (26-29). These immune evasion mechanisms result in an arms race against the immune system where increased rate of viral genome divergence produce high enough diversity in the viral population leading to the emergence of resistant variants. The potential for virus immune escape results in a continuation of an immune race between the immune system and the virus (30-34).

The virus also disguises or shields highly immunogenic and immune-accessible regions using conformational rearrangements and/or host derived high glycan coverages. The viral genome expressed surface glycoprotein, envelope (Env), is highly glycan decorated with about 50 % of its weight composed of host-derived sugars (35, 36). The presence of host-derived glycans at or near potentially immunogenic regions on Env effectively masks important sites from immune surveillance. Immune resistant variants also arise through mutations that add or remove potential glycosylation sites on Env (37-39). Conformational rearrangements of Env subunits also mask conserved functional sites from immune detection (40-42). Sites like the co-receptor binding region, the fusion peptide and Membrane Proximal External Region (MPER) on gp41 are well buried in the trimeric assembly of Env in pre-CD4 binding conformation. However, conformational reorientations expose these regions post gp120 engagement by CD4 and co-receptors in a sequential manner (41, 43).

Env, although highly immunogenic, is decorated with cryptic and immuno-dominant epitopes that are antigenic but elicit only non-neutralizing antibody responses. Non-neutralizing B-cell responses against immuno-dominant regions result in overload of the humoral immune system. This focuses the antibody-based immune response away from potentially virus neutralizing antibodies (44-46). A defocused and diluted neutralizing response is an effective immune evading mechanism. Other factors like the non-covalent gp120-gp41 link make the gp120 monomers labile for shedding. This also results in gp41 trimer stumps and numerous incomplete Env structures that act as immunological decoys and elicit non-neutralizing antibodies (38, 47-49). Accompanying its role in populating non-native trimers, shedding results in low Env molecules per virion, which reduce availability of immunogenic epitopes.

In addition to virus specific scientific challenges, lack of detailed understanding in the host-virus interface also undermines the vaccine development research. Although possible immune activation post HIV-1 infection has been recorded, attributes of the immune response that predict protective immunity against viral infection are not well understood (50, 51). Clinically observed antibody based immune response post HIV-1 infection chronically yield virus neutralization potency in late phase of infection. The narrow time window for vaccine based immune activation prior to establishment of latent virus reservoirs hampers harnessing such immune responses for vaccine-elicited immunity. Lack of detailed understanding in attributes of virus derived immunogens that trigger the observed immune response and corresponding immuno-biological processes and mechanisms that enable HIV-1 recognition and yield reactive immune effector functions greatly challenge development of HIV vaccine. Furthermore, due to lack of appropriate and practical model organisms that mimic HIV-1 infection and corresponding immune response in humans, the limited designed potential immunogens for experimental vaccine studies have been tested in sub optimal conditions (52-54).

Although the challenges in HIV-1 vaccine development remain monumental and vivid, the past two decades have seen a shift in approach from empirical vaccine development techniques to structure based rational design of immunogens (13, 55). Building on the successful approach of bacterial vaccine development using 'Reverse vaccinology' where genome analysis enabled reverse design of bacterial vaccines (56, 57), structure based vaccine design attempts to recapitulate immune attributes observed in clinically isolated anti-HIV-1 antibodies that target parts of HIV-1 and neutralize diverse virus subtypes. The goal is to understand the biophysical and biochemical attributes of clinically isolated anti-HIV-1 broadly neutralizing antibodies (bNAbs) and their interaction with virus-derived antigens. The information enables bio-molecular engineers to design immunogens based on template bNAb – antigen interaction with the assumption that such immunogens could potentially elicit immune response with comparable potency as template antibodies.

Because the nature of antibody-based immune response against HIV-1 is the central foundation of design based vaccine development, herein I discuss characteristics of potential template antibodies clinically isolated post-chronic HIV-1 infection.

1.2 HIV-1 and antibody based immunity

HIV-1 infection triggers a broad antibody response in almost all seropositive individuals (26, 58-63). Characteristically, clinically isolated antibody pools are mainly non-neutralizing where the antibody response is due to antigenic but non-immunogenic antigens (64, 65) or strain specific narrow neutralizing conventional antibodies with the potential of neutralizing only limited virus strains (27, 66-68). However antibodies with greater potency and the potential to neutralize diverse HIV-1 strains are also elicited in ~20 % of chronically HIV-1 infected individuals with antibodies exhibiting exceptionally broad and high neutralization potency isolated from about 1 % of patients dubbed as elite neutralizers (26, 61, 62, 69).

The vast majority of infection elicited anti-HIV-1 antibodies are directed against Env, the trimeric viral envelope protein that forms spikes and decorates outer surface of the virus (32, 70-72). Env is encoded as a single precursor polypeptide (gp160) and enzymatically processed in the Golgi by cellular proteases like furin. Protease reactions yield mature glycoproteins; the surface exposed glycoprotein, gp120, and transmembrane anchored glycoprotein, gp41. Virion surface displayed Env spikes are heterotrimeric glycoproteins composed of heterodimers of gp120-gp41 associated by noncovalent interactions (36, 72).

Env plays a critical role in virus infectivity and life cycle. Env determines HIV-1 cell tropism. The host cell surface receptor, CD4 (a cell surface receptor on the surfaces of CD4+ T-cells and macrophages) engagement on a region on Env (CD4 binding site (CD4BS)) initiates entry of the virus into target host cell. This CD4-CD4BS interaction triggers conformational rearrangements on Env that expose co-receptor (CCR5 or CXCR4) binding surfaces. The sequential receptor and co-receptor engagement mediates further conformational changes on the surface that mediate viral and target cell

membrane fusions and hence entry into host cell (36, 73, 74). This important role constrains Env and specific regions within it to maintain relatively conserved sequence and structural features.

Various regions on Env elicit an antibody based humoral response. Conserved and functionally indispensable regions like the CD4BS, co-receptor binding sites, and epitopes at or near MPER are major targets of antibody-based humoral immunity and elicit bNAbs. However, infection triggered bNAbs targeting variable regions and host derived glycans are also isolated (Figure 1-1). Isolation of bNAbs that mainly focus on these regions suggests the potential to harness an immune response against these regions via vaccination. Dependence of the virus on specific interaction between virus-derived elements, host receptors for host cell identification, and entry makes the CD4BS immunologically a point of vulnerability on the viral Env architecture.

Clinically isolated bNAbs that target the CD4BS (antibodies like b12 (75, 76), VRC01 (77, 78), NIH 45-46 (78)) recognize mostly discontinuous epitopes directly competing for CD4 binding and hence provide a mechanism of virus neutralization potential through blocking virus entry into host cell. Biophysical analysis of such bNAbs indicates unusual interactions with the CD4BS that is mainly mediated through elements on their VH chain. Structural mechanisms of antibody-mediated abrogation of Env CD4 binding are relatively different among various CD4BS targeting bNAbs. While bNAbs like VRC01 and NIH 45-46 recapitulate the CD4-CD4BS interaction using antibody VH regions in conformationally analogous ways to gp120-CD4 interaction (33, 77-79), antibodies like b12 (76, 80), CH103 (81) and HJ16 (82) use extended CDRH3 that protrude to reach the depressed region on gp120 involved in CD4 binding (78). Biophysical and biochemical analysis illustrate the importance of antibody heavy chain and more specifically HCDR3 in antibodies like b12.

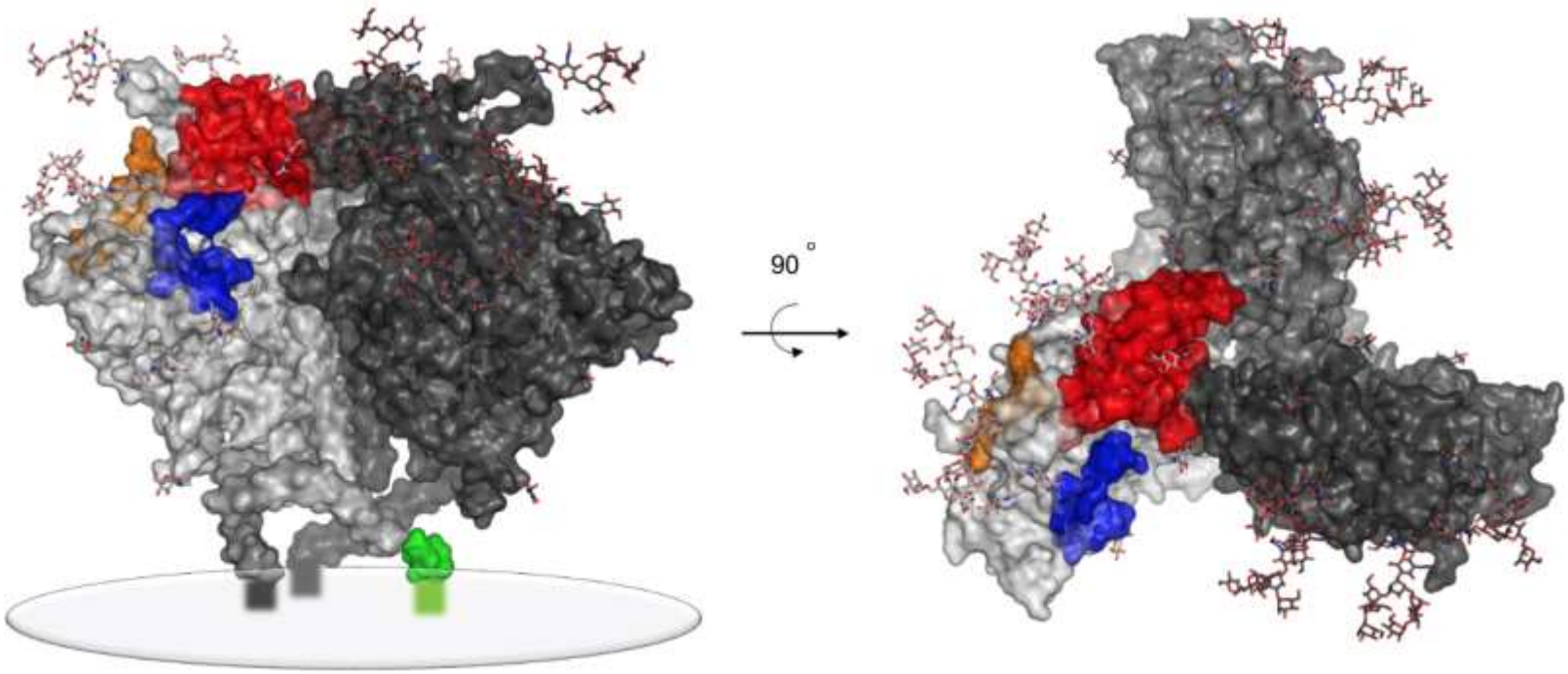

Figure 1-1. HIV-1 Env regions eliciting bNAbs
Side and top view of virion surface. Env model and bNAb targeted regions mapped on to the crystal structure of trimeric SOSIP Env structure PDB (83) accession code 4NCO (47)) based on presentation on West et al., (2014) (84). Trimeric HIV-1 SOSIP envelope structure presented as surface fill model with individual protomers colored with various shades of gray. Glycan molecules are presented in stick representations with individual atoms colored as gray (C), red (O) and blue (N). Regions identified as immunological hot-spots are indicated by surface representations and colored CD4BS targeted by bNAbs like b12, VRC01 (blue), Membrane Proximal External Region (MPER) eliciting bNAbs like 4E10, 2F5 (green), V3 region eliciting bNAbs including CD4 induced antibodies (orange) and V1V2 region and glycan that elicit bNAbs that recognize peptidoglycan epitopes (red).

Table 1-1. Antibodies that target CD4BS are both neutralizing and non-neutralizing

CD4BS Antibodies	Clonal Family	Neutralization breadth	Neutralization potency (μg/mL)	Key Reference
		bNAbs		
b12		70	2.82	(75, 80)
12A12	12A21, 12A30	100	0.06	(85)
VRC01	VRC02, VRC03, NIH-45-46	93	0.32	(33, 78)
VRC-PG04	VRC-PG04b, VRC-PG20	76	0.14	(33)
VRC-CH31	VRC-CH30, VRC-CH32, VRC-CH33, VRC-CH34	84	0.14	(33)
3BNC117	3BNC60, BNC62, 3BNC55	100	0.06	(33, 78)
8ANC131	8ANC37, 8ANC134, 8ANC195		0.18	(78)
PGV04				(86)
CH103		55	0.18	(81)
1B2530	1NC9, 1NC7, 1NC3		0.21	
HJ16		36	0.77	(82)
		non neutralizing antibodies		
F105				(87)
b3				(75)
b6				(75)
F91				(88)
m18				(89)
1F7				(90)
		Vaccine elicited non neutralizing antibodies		
GE136	GE148			(91)

The various clinically isolated and characterized bNAbs and their interactions with their respective epitopes pose additional challenges for structure based rational vaccine design. In addition to the previously discussed virus immune evasion mechanisms, the unusual nature of bNAbs isolated from HIV-1 natural infection greatly contribute to the hurdle of developing immunogens that trigger vaccine induced immunity. HIV-1 targeting bNAbs are rare and exhibit unconventional antibody development. These unique attributes of bNAbs include: (I) Germline B cell repertoires, targeted by virus-derived antigens subsequently giving rise to mature bNAbs, are not readily found in circulating naïve B-cells. Such precursor antibodies arise from germline gene recombination of rare heavy chain variable (VH) genes characterized by long complementary determining region 3 (CDRH3) (76, 84, 98-101). (II) Virus elicited bNAbs are solely isolated from chronically infected individuals with high viral load. In conjunction with prolonged infection and rarity of naturally elicited bNAbs used as template for vaccine development, (III) bNAbs exhibit increased mutation frequency in their variable regions when compared to non-neutralizing HIV-1antibodies (92, 93) and antibodies against non-chronic viruses like Influenza (94, 95). Anti HIV-1 bNAbs markedly exhibit extensive somatic hyper mutation (SHM) with 30-50 % amino acid mutation frequency over their respective germline precursor sequences (55, 82, 96, 97). The increased frequency of mutations following antibody maturation directly contributes to neutralization potency. (IV) As a result of prolonged affinity maturation, bNAbs exhibit high auto-reactivity and ploys pecificity (101-104). Autoreactive and polyspecific propensity could potentially be important in garnering antibody breadth (105, 106). (V) Even though affinity matured bNAbs bind their respective antigens with greater affinity, biochemical studies revealed germline encoded precursor antibodies do not interact with potential antigens on Env or bind weaker than the micro molar threshold required to initiate affinity maturation (107-111). Taken together with increased frequency of mutations, it is possible to hypothesize diminished cognate epitope recognition by germline precursors is potentially due to the frequent evolution of virus derived antigens dictating subsequent modifications in paratope sequences and structures. These underlining common attributes make developing vaccines that can elicit clinically isolated bNAbs-like response a tall order.

1.3 The antibody development paradigm

Harnessing the natural capability of host humoral immunity and its antibody-based immune response against HIV-1 by way of vaccination necessitates a brief look at antibodies and their development. Humoral response through recognition, eradication and establishment of immunological memory against pathogens and their toxic products is the primary function of B-cells in adaptive immunity (112, 113). Establishment of antigen-specific memory response is a key aspect in adaptive immunity and is the basis for vaccine-based immunity. This response, mainly mediated by antibodies, is exceedingly complex and specific.

Part of the adaptive immune system, antibodies are antigen binding effector glycoproteins expressed as surfaces receptors on B-cells (BCRs) or circulating immunoglobulins secreted by plasma B-cells. Antibody effector function involves diverse immune mechanisms. These include facilitating phagocytosis of pathogens through opsonization (where pathogenic particles are coated by antibodies), activation of the complement system that targets pathogens and infected cells, modulating the expression of other effector molecules, ability to bind and eliminate toxins, activation of other immune cells through antibody dependent cell mediated cytotoxicity and antibody mediated cytosolic proteolytic degradation of pathogenic particles (114-118). Such functional versatility enables antibodies to bridge between the adaptive and innate arms of the immune system.

Characteristic antibody diversity and functional versatility are inherent to antibody gene rearrangement and recombination of genes encoding antibody V (variable), D (diversity), J (joining), and C (constant) regions. Recombination is responsible for combinatorial diversity inter and intra VH, CH, VL and CL coding genes and junctional diversity in assembly of D segments. Whereas recombination results in diversification of the primary germline antibody repertoire, further antigen mediated antibody maturation, (aka affinity maturation) results in robust secondary antibody repertoire (119, 120). Recombination based initial BCR diversifications are mediated by initial foreign antigen encounter through wide affinity ranges. Secondary diversification following antigen dependent B-cell development

through iterative SHM and selection of small proportion of cells expressing high affinity BCRs has an enormous impact on the secondary BCR repertoire wherein humoral immune response is focused and specific. Antibody Ig molecules that initially define B-cells initiate this antigen dependent affinity maturation and become effector molecules following the differentiation of B-cells into plasma cells (118, 120-122).

The process of antigen dependent affinity maturation occurs in a specialized region within secondary lymphoid organs (123, 124). Foreign particle derived antigens are drained, filtered and accumulated in secondary lymphoid organs displayed on antigen presenting cells like follicular dendritic cells. In the follicles of these organs, circulating B-cells sample and interact with antigen specific T follicular helper cells and potential antigens. Initial antigen binding by antigen inexperienced B-cells trigger exit from the lymphatic circulation with subsequent migration and homing to form germinal center reactions. In this specialized organ B-cells undergo the affinity maturation process (123).

Hallmarks of affinity maturation are iterative generational mutations, competition for limiting antigens presented on the surfaces of follicular dendritic cells and rapid proliferation of B-cells. B cells expressing mutated BCRs with improved foreign antigen binding outcompete and preferentially survive in the antigen limiting germinal center. Hence, the process of antigen dependent affinity maturation can be assessed through ability to competitively and selectively bind limited antigen pool, improve antigen-dependent signaling and improve recruitment of follicular T-helper cells through efficient antigen presentation. B-cells expressing receptors with improved binding affinity to a specific antigen expand and ultimately produce the high-affinity antibodies that confer immunity and immunological memory (123, 125).

Somatic hypermutation and affinity based clonal expansion

Part of the antibody maturation process, SHM result in high mutation frequency in gene segments encoding the antibody hypervariable regions compared to random mutation in other parts of the genome. Rate of mutations in SHM are approximately 10^{-3} mutations per base pair per generation (126-128). The increased rates of mutations during B cell maturation are mainly mediated by Activation-induced cytidine

deaminase (AID) (129-131). AID mediated deamination of Cytosine generates Uracil which triggers the recruitment of various DNA repair machineries. Error during DNA repair introduces mutations in the germline antibody heavy and light chain coding sequence. SHM introduces random mutations mainly in the variable region of respective antibody genes (130, 132). Mutations are more favored in the hypervariable regions or complement determining regions (CDRs). Many of the mutations incorporated have a spectrum of effects on antigen recognition and many have deleterious effects on proper folding of the antibody protein. However some mutations, by either establishing new direct antigen contacts, stabilizing the antigen combining site in a preformed bound conformation, and/or modulating the overall protein stability and dynamics, result in antibodies with increased affinity for their cogent pathogenic epitopes (133-135). Although CDRs (both CDRs of heavy (CDRHs) and light (CDRL) chains) are preferentially targeted for mutation, mutations are also introduced in the antibody framework regions (FWRs) that flank CDRs (119, 132). Studies have shown the importance of these regions in effective epitope binding and virus neutralization (93).

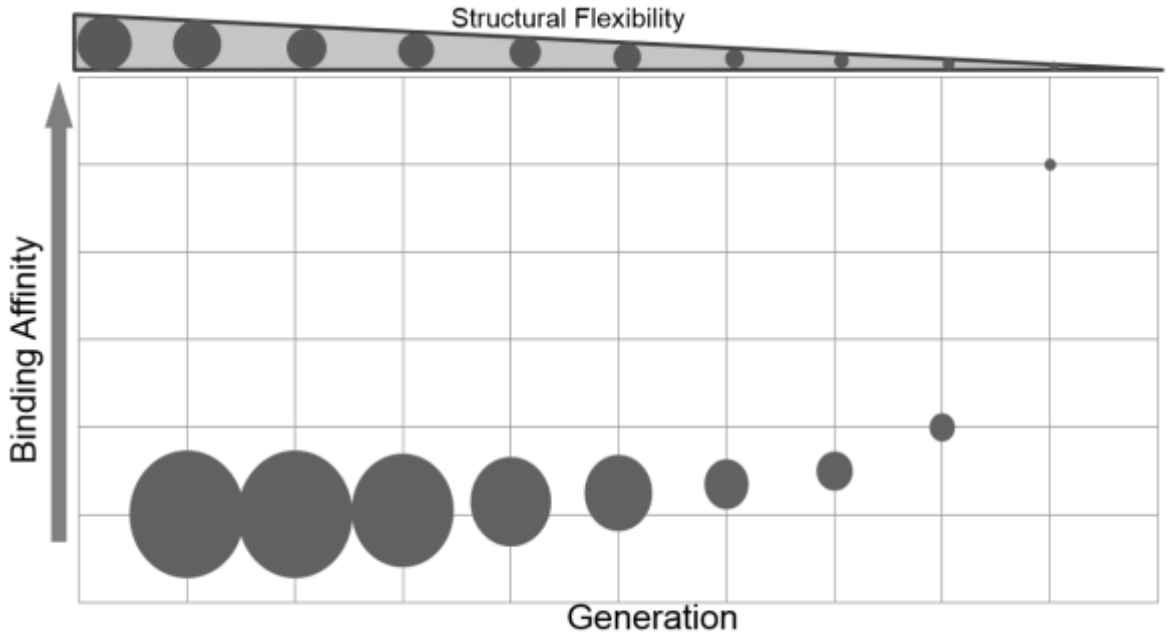

Figure 1-2. Role of affinity maturation in structural flexibility

Schematic representation of the role of antibody maturation in structural flexibility and binding affinity. Increases in binding affinity following iterative generational selection are plotted on Y and X axis respectively. Postulated decrease in structural flexibility are presented as decreased diameter of spheres

In addition to increased binding affinity, the maturation process focuses antibody specificity from the polyspecific nature of antigen recognition by germline encoded precursor antibodies (136, 137). Polyspecificity in germline encoded progenitor antibodies broaden the scope of antigen recognition by naive BCRs. The antibody maturation process fine-tunes antigen interaction mechanisms to yield highly specific antigen recognition. One mechanism of focusing antibody specificity during the maturation process is through rigidification of antigen binding site formed by antibody CDRs. Thus, SHM provides the foundations for positive selection resulting in high affinity antigen binding that is characteristic of a mature antibody based immune response. Therefore, a structural and biophysical change due to SHM in the antigen-combining region underpins evolution of mature antibodies from their respective germline precursors (Figure 1-2) (94, 119, 131, 132, 136-139).

The immune system's ability to develop antibody based immunological memory is the basis for vaccine based prophylactic treatments. Vaccines have proven their importance in public health and medicine especially in preventing virus caused diseases. One virus where research efforts to develop protective vaccine is currently a pressing priority is HIV-1.

1.4 The HIV vaccine design paradox

There are numerous unknowns in HIV-1 vaccine development, hence identification of virus derived immunogenic antigens, mechanisms of antigen presentations, expected immune response and determination of how protection is measured (what correlates with protection) are pressing priorities for global HIV vaccine scientific research. In addition to the inherent characteristics of HIV-1 infection, defining these parameters greatly affects vaccine development. These challenges necessitate effective benchmarks to delineate vaccine development. The gold standard for success in viral vaccines is elicitation of neutralizing antibodies that target viral particles and simultaneously neutralize viruses from different HIV-1 clades and hence block infection of naive cells (140).

In structure based rational vaccine design, the fundamental principle relies on understanding biochemical and biophysical characteristics of bNAbs that target virus and their interaction with virus

derived immunogenic antigens (13, 141). This knowledge is used in designing immunogens that recapitulate or mimic antibody based humoral response with the ability to neutralize viral infection. Hence, clinically isolated bNAbs and their interacting virus derived partners, particularly epitopes derived from conserved regions on Env, are used as vaccine design templates. The technology proved effective in preliminary Respiratory Syncytial Virus vaccine design (142).

As noted above, the potential elicitation of naturally circulating, infection-induced bNAbs in HIV-1 seropositive individuals predicts potential for vaccine induced immune response against HIV-1. Furthermore, the high frequency of bNAbs that target the HIV-1 surface protein, coupled with its surface accessibility and high immunogenicity renders Env an obvious potential candidate immunogen. Strategies that aim at developing vaccine immunogens that exploit susceptibilities on Env include the use of trimeric Envelope-like-particles (47, 49, 65, 110, 143, 144), monomeric gp160/gp120/gp41 subunit particles (145-147), minimized forms of gp120 (148-150), and bNAb interacting epitopes presented using scaffolds (48, 151, 152).

Immunogen design using envelope-like-particles and full length monomeric gp120 failed to elicit neutralizing antibodies in immunization trials despite observed comparable *in vitro* biochemical interaction with clinically isolated bNAbs. Attempts at vaccinating chimpanzees using soluble forms of monomeric gp120 resulted in protective immunity following subsequent intra-venous virus challenge (153, 154). However, such immunogens failed to elicit protective immunity in clinical trials suggesting neutralization epitopes are not optimally presented in monomeric preparation on Env subunits (13, 50, 140, 155-157). Existence of non-immunogenic but highly antigenic epitopes on Env subunits could burden the immune system and hence affect antibody titer and profile.

Antigen focusing with an aim to disguise or eliminate non-immunogenic epitopes has potential to increase neutralizing antibody titer following immunization. Structure based rational immunogen design approaches building on bNAb and their interactions with respective epitopes aims at focusing the immune response towards specific epitopes leading to elicitation of comparable antibody based humoral response. Attempts in immune focusing utilize fragment-based designs and scaffold based epitope presentation.

Conserved and functionally indispensable regions on Env like the CD4BS, co-receptor binding, regions on V1/V2 and V3 loops and epitopes at or near MPER are targeted by humoral immunity and hence are potential targets for rational vaccine design. The approach obliges extensive biochemical and biophysical characterization of the bNAb-epitope interface and interactions. Information gathered resulted in immense success in designing peptide and epitope scaffolding immunogens that can recapitulate comparable *in vitro* biophysical interaction with trimeric Env and/or monomeric gp120 (48, 158-160).

Progress in immunogen design that mimics interaction between bNAbs and epitopes on Env resulted in potential immunogens. However, extending designed immunogens to vaccines that garner prophylactic immune response in humans has faced challenges. Current vaccine development attempts show dismal success and failed to elicit protective bNAb based immune responses (15, 16) (140, 161). Designed immunogens bound to bNAbs used as design templates with comparable affinity to cognate antigen, elicited neutralizing antibodies in model organisms and successful preventive response against viruses challenges in non-human primates following passive immunization (69, 88, 91, 147, 155, 162). Some factors contributing to the bottleneck include a lack of understanding of B-cell development following HIV-1 infection and the process of antibody maturation in chronic HIV infections.

Given the extensive maturation process and extent of mutations acquired in HIV-1 targeting bNAbs, the relative contribution and importance of the various positions to epitope binding and virus neutralization are not well defined. Therefore, successful vaccine design benefits from in-depth analysis of mutations to parse the minimum antibody-antigen interaction that can initiate antibody maturation process and recapitulate clinically isolated anti-HIV bNAb-like immune response.

As stated above, bNAbs against HIV exhibit significant rates of SHM compared to their germline precursors however, it is unclear how the high frequency of SHM in bNAbs impact binding affinity. The fundamental antibody maturation paradigm assumes antibody maturation resulting in increased binding affinity due to SHM. AID mediated SHM in antibody development is mainly stochastic. Therefore, it is plausible to posit the potential of mutations with no apparent contribution to binding affinity be maintained through the maturation process due to random chance. In addition, the prolonged affinity

maturation and the high frequency of mutations in infection-induced bNAbs could imply mutations accumulate without initial increases in affinity, but such mutations are ultimately required to achieve broadly neutralizing function.

Our study attempts to understand the role of individual SHM accumulated through the process of affinity maturation. The role of SHM on antibody diversity, antigen presentation and survival of B-cells is well documented (163). However, its importance in elicitation of bNAbs and relation to neutralization potency is still elusive. Questions we addressed in this study include; can prolonged SHM dictate the emergence of mutations required for increased binding affinity and neutralization potency? Could there be bystander mutations that might not alter antibody function? Does increased binding affinity predict neutralization potency? Does prolonged affinity maturation in anti-HIV bNAbs result in increased structural rigidity? To ponder points raised above, we used the CD4BS targeting bNAb, b12, as a model antibody.

1.5 Documents presented in this Thesis

Understanding antibody based virus neutralization observed in chronically HIV-1 infected individuals is core to structure based rational vaccine approach, a technique hailed as promising approach for HIV-1 vaccine development. Working towards such a goal, our group, in collaboration with others, attempted to determine the importance of antibody maturation related mutations in antigen binding and subsequent virus neutralization. This Thesis presents work undertaken during my six-year Doctoral research in the Cellular and Molecular Biology program. Study results presented include:

Chapter 1 Introductory overview. In this chapter, I introduce the concepts of HIV biology, vaccine development, affinity maturation and the vaccine design approaches. I also discuss current challenges faced in the vaccine development process. Chronic infection triggered broadly neutralizing antibodies and their advantages and challenges for use as templets in vaccine design are also presented. This chapter raises the major questions addressed in the subsequent chapters

Chapter 2: Role of SHM on epitope binding and virus neutralization potency. In this chapter, I discuss results of experiments designed to deconvolute effects of individual mutations on epitope binding and virus neutralization using the retro analysis where mature antibody SHM sequences are reverted to germline precursor.

Chapter 3: Minimal SHM that garner b12-like binding and neutralization response. In this chapter, we examined minimum mutations required on germline precursor antibody background to elicit b12-like immune response. The main goal is to define mutations observed in mature b12 and their relevance in epitope binding and neutralization.

Chapter 4: Antibody paratope rigidification is proposed as a potential mechanism of attaining increased epitope binding affinity and specificity. To assess role of SHM on b12 rigidification, we studied structure of germline b12 precursor antibody using X-ray crystallography techniques. Here we report b12 germline precursor light chain antibody variable domain structure and the unorthodox light chain variable domain dimer.

Chapter 5: In addition to the work done on vaccine development and vaccine based prophylactic reagent against viruses, I have worked on a project that aims at elucidating the intersection of humoral anti-viral immunity and innate immunity with particular interest in the role of Natural Killer (NK) cells and their role in controlling virus based infection. To understand innate immune evasion by viral particles, I researched structural mechanisms of viral immune evasion that target NK activating ligands using Adenovirus (Ad) immune evasion by the third Early gene product E3/19K protein.

Chapter 6: Experimental techniques, detailed experimental methods and reagents used in performing the various experiments are described in this chapter.

Chapter 7: Discussions, concluding remarks and overall implications of results presented in this Thesis and future directions for b12 based vaccine design are presented in this chapter.

Chapter 2

2 Role of SHM on epitope binding and virus neutralization

2.1 Overview

Structure based vaccine design is predicated on the fundamental assumption that binding to specific regions on HIV-1 Env in a manner that replicates biophysical attributes of clinically isolated bNAbs predict protection through elicitation of bNAbs. Corollary to the hypothesis, bNAb based response produces antibodies that directly block viral entry and subsequently drive downstream immune activation (13, 59, 108, 116, 142, 164). This implies designed vaccine immunogens capable of successfully mimicking biophysical interactions between the rare bNAbs and virus-derived epitopes are poised to trigger antibody-based immunity. Because the paradigm necessitates rigorous investigation of the antibody – antigen interaction, abundant resources are devoted to characterizing bNAbs and their interaction with respective epitopes. An aspect of bNAbs relatively overlooked in their characterization is the role of mutations accumulated during the antibody maturation process, in regards to virus neutralization and positional importance in the development of broad neutralization capability.

As detailed in Chapter 1, the immune system is remarkable in recognizing foreign particles by generating high affinity antibodies with greater specificity through assembly of the limited diversity in germline gene repertoire into germline antibody pool (165, 166). Germline precursor antibodies are further diversified through the antigen dependent affinity maturation process. This intricate process is dictated in part by SHM and greater competition for antigen leading to stringent affinity based selection (99, 123, 129-131, 134, 166-168). The process is initiated when antigen inexperienced BCRs interact with foreign antigens with minimum binding affinity threshold (micro molar range rate of association) (119, 130, 133, 134, 138, 169, 170). Mutations, mainly in antibody regions that are determinants of antigen binding affinity and specificity, improve antigen binding either by; (1) establishing new direct antigen contacts, (2) stabilizing the antigen-combining site in preformed antigen bound conformation, and/or (3)

modulating the overall protein stability and solubility. Hence, structural and biophysical changes due to SHM in the antigen-combining region underpin evolution of mature antibodies from germline precursors (119, 122, 133, 136, 138, 170).

Biochemical research on bNAbs, predominantly on antibodies targeting host receptor (CD4) binding site (CD4BS), revealed the presence of high frequency of SHM. Correlation between rate of mutation and prolonged affinity maturation imply role of SHM in bNAb development (32, 70, 77, 95, 100, 139, 171). Although this phenomenon is observed in antibodies elicited following chronic infection (172, 173), little is known with regards to the role of individual amino acid positions altered by SHM on antigen binding and subsequent antibody functions. To define the minimum interaction that can initiate the maturation processes and yield antibodies recapitulating clinically isolated anti-HIV-1 bNAbs and associated immune responses, successful vaccine design benefits from in-depth analysis of mutations acquired through SHM. To determine effects of mutations in epitope binding and neutralization of anti-HIV-1 bNAbs, we set out to examine the effect of altering individual positions that differ from the germline precursor on the HIV-1 bNAb, b12.

Anti-HIV-1 bNAb, b12 was isolated from the bone marrow of asymptomatic 31-year-old male HIV-1 seropositive individual using combinatorial phage display (75, 174). Phage display in conjunction with combinatorial library approach enables capture of antibody repertoires and pan for antigen binding combinations of heavy and light chains (175). Versatility of the technique is attained by cloning antibody heavy- and light-chains independently into libraries using bacteriophage scaffolds, assemble and generate random antigen binding Fab fragments. Association of random antibody heavy and light chain domains in library assemblies, a caveat of the system, makes it impossible to definitively assert the natural existence of intact antibodies with given heavy and light chain combinations in the donor's antibody repertoire (175). Therefore, we acknowledge the particular b12 heavy and light chain expressing genes could be an artifact of the experimental process. However, sequence comparison with the predicted germline precursor reveal comparable mutation frequency on both light and heavy antibody variable domains

arguing for natural occurrence of the anti-HIV-1 antibody b12 and its germline precursors in the donor antibody gene repertoire (107).

B12, one of the first identified in the CD4Bs anti-HIV-1 broad neutralization spectrum, is biochemically and biophysically well characterized. Most of the work done in understanding b12 was aimed at reverse engineering potential vaccine immunogens that mimic b12-antigen interactions. Structural and epitope mapping experiments confirmed that b12 interacts with a conformational epitope near the CD4BS on HIV-1 Env protein (76, 92, 107, 176-181). Similar to other CD4BS interacting antibodies, b12 recognizes its epitope, a depressed region on CD4BS, mainly through its CDRH3 region. *In vitro* neutralization studies also deduced that b12 neutralizes 40 – 70 % (100) of HIV-1 strains with great potency (IC_{50} ~2 µg/mL) (77). Such in-depth analysis climaxed with designed antigens that recapitulate b12 biophysical attributes (151, 159, 160). However, none were elevated to a point of immunogenicity eliciting b12-like immune response.

Our goal is to determine relevant mutations that directly affect epitope binding and virus neutralization. We postulate paratopes that are involved in direct antigen interaction will have greater importance while the stochastic nature of AID induced mutation could result in accumulation of mutations on antibody regions that have no apparent importance to epitope binding or neutralization. The analysis will enable us define minimum mutations on germline b12 precursor antibody to elicit b12 like response. It also will enable us to decipher potential correlation between positional effect of SHM on antigen binding and neutralization potency.

2.2 Results

2.2.1 Amino Acid Sequence Analysis of b12 Mature and Germline Precursor

The gene sequence encoding the germline precursor of the anti-HIV-1 bNAb b12 was sequenced from peripheral blood mononuclear cells (PBMCs) isolated from the original donor. Heavy chain gene usage was determined to be VH1-03*01, D2-21*02 and J6*03 while genes encoding light chain antibody segments were deduced to be KV3-20*01 and KJ2*0141 (107). According to amino acid sequence comparisons of germline encoded b12 precursor and mature b12 antibodies, the antibody maturation process resulted in high degree of SHM with ~20% amino acid mutations in the antibody variable regions. Mutations are relatively more favored in the VH domain where amino acids are altered at 24 positions as compared to VL domain where mutations are defined in 21 positions (Figure 2-1 and Figure 2-2 B). In line with the paradigm of antibody maturation where SHM and subsequent selection are dictated mainly due to increased affinity in the interaction with cognate antigen, mutations in b12 are more favored on CDRs that form the antigen binding groove with 13% and 11% of positions are mutated on VH and VL domains respectively. In contrast, the FWRs that form scaffolding for the antigen-binding platform are less targeted during the affinity maturation process with 6% and 8% of positions of b12 FWRs on VH and VL domains respectively altered during the affinity maturation process (Figure 2-1).

Mutation of solvent exposed amino acids are more tolerated during the affinity maturation process while regions buried in the antibody Ig domain interfaces are less frequently mutated. Interface analysis using PISA (83) established that in the b12 variable domain VL - VH interface solvent inaccessible residues are less targeted during the affinity maturation process. Amino acid sequence comparison of matured b12 and its germline encoded precursor deduced, ~14% of positions in the interface region are altered versus 20% in the variable domain (Figure 2-2).

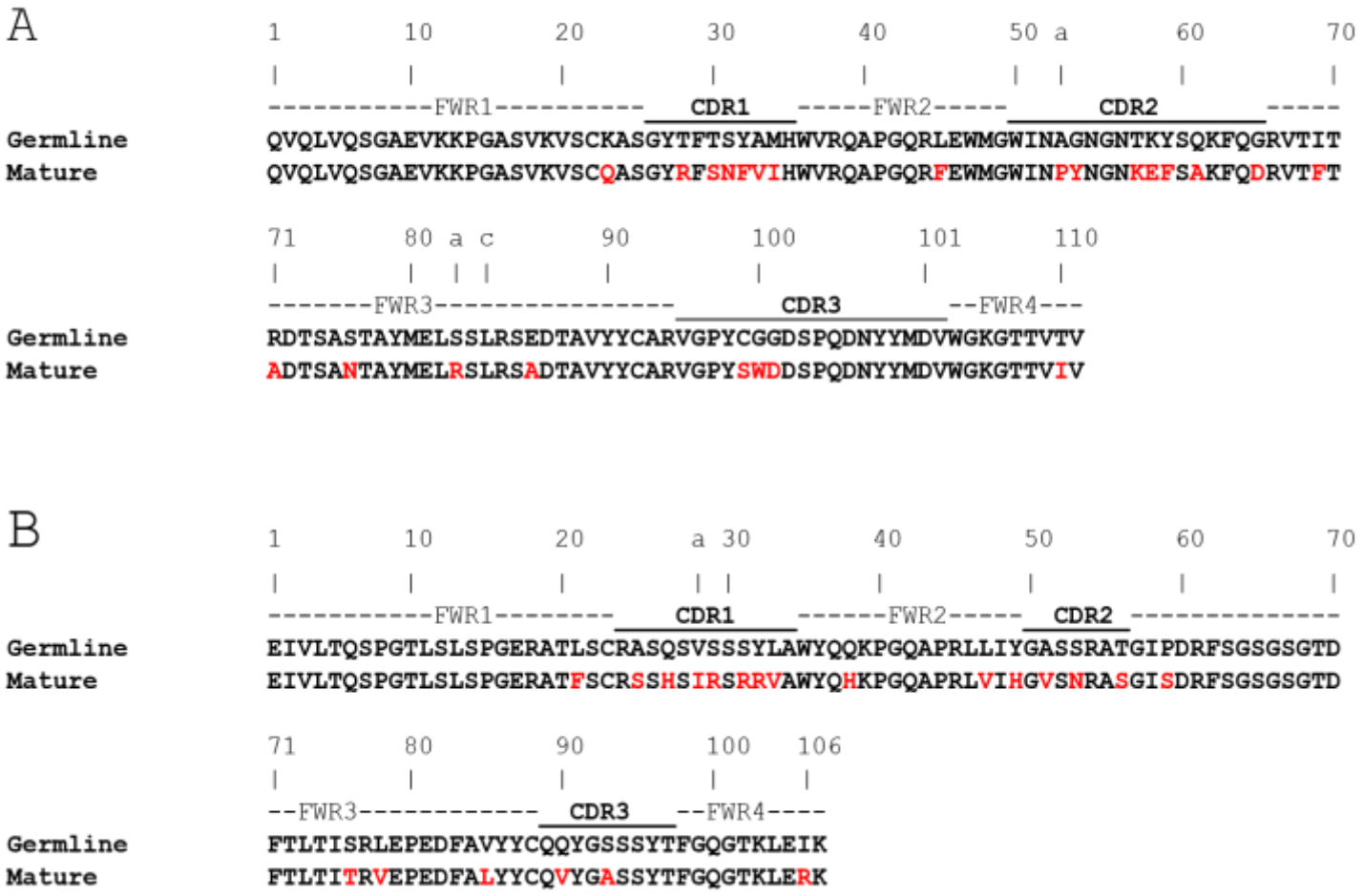

Figure 2-1 Somatic mutations during the maturation of b12
Amino acid sequence alignment for germline and mature b12 of the variable regions VH (A) and VL (B). The sets of somatic mutations introduced in the heavy and light chains are colored red. CDRs are indicated with line above the sequence and FWRs are marked with dashed lines. Residue numbering and CDRs defined according to Kabat antibody numbering system (182).

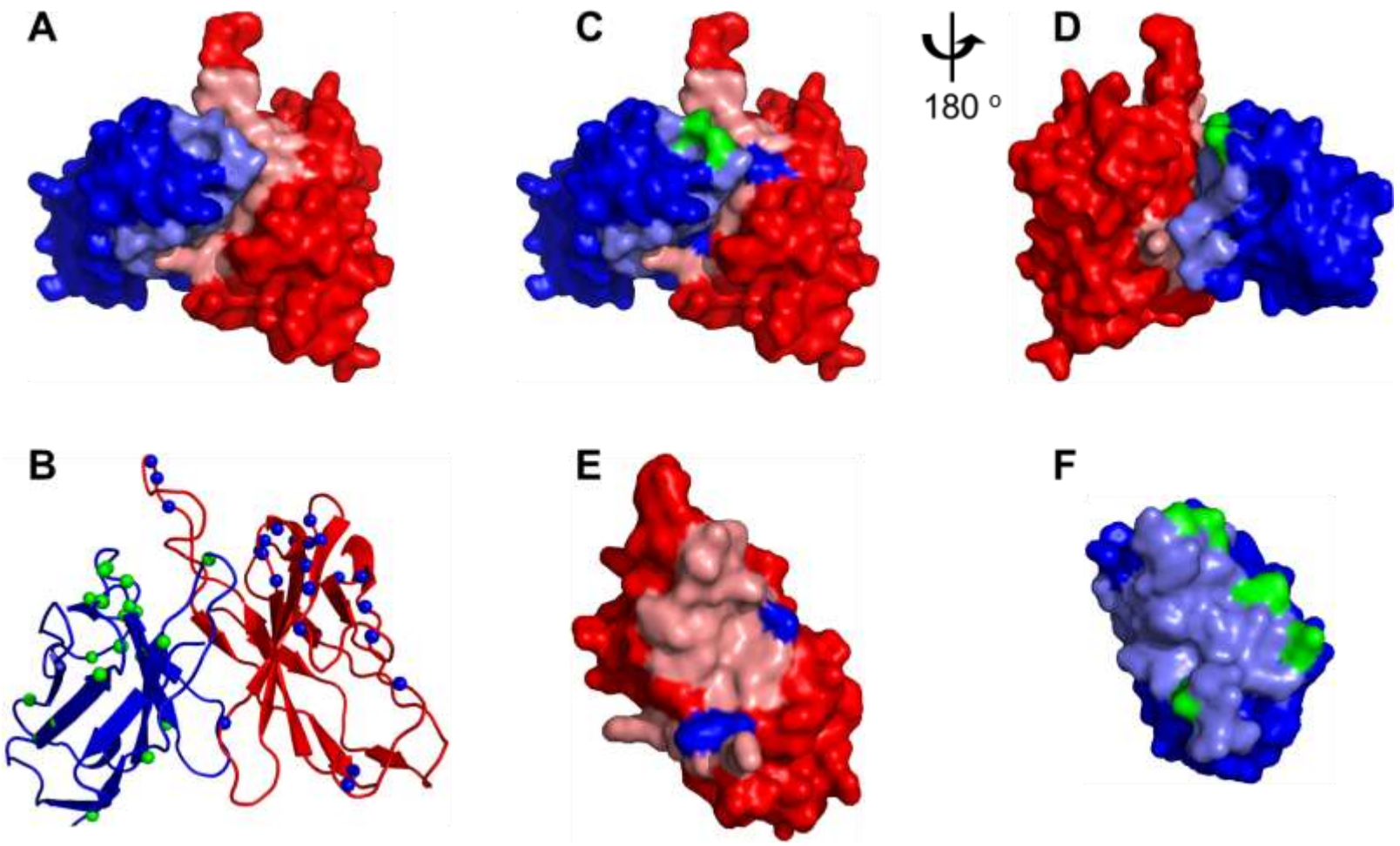

Figure 2-2. SHM derived positional mutations on the anti-HIV-1 bNAb, b12
Fv domain of b12 as presented in gp120 bond conformation (PDB accession code 2NY7). The variable region of b12 is presented in molecular surface (A) or cartoon (B) representations with VH domain colored red and VL domain colored blue. Positional mutations accumulated are represented in spheres with mutations on VH domain colored blue while mutations on VL domain colored green. B-F) Variable domain interface analysis using PISA(59). B - D, surface representation of antibody Fv domains with VH domain colored red and VL domain colored blue. Interface region formed by the two variable domains are indicated with salmon red and slate blue shade for VH and VL domains respectively. (C-F) VH-VL interface as in A with respective positions altered by SHM are colored blue (VH) and green (VL). C and D are 180^{o} rotational look at interfaces. E and F, surface representations of individual variable domains with VH (E) and VL (F). Molecular representations are done using Pymol graphics tool (183)

2.2.2 Characterization of b12 c/scFvs proteins

In previous biochemical and biophysical characterizations of the anti-HIV-1 bNAb b12, most binding, neutralization and crystallization studies were done using divalent full length Immunoglobulin G (IgG) antibodies or monovalent antigen binding fragments (Fabs) derived from proteolytic cleavage of full length IgGs (92, 151, 159, 176, 184, 185). Although there are examples that experiment using b12 Fv as reagents (181), we determined b12 cleavable single chain Fv (c/scFv) recombinant protein stability and ability to preserve b12 binding affinity and neutralization potency. C/scFv constructs expressed as single polypeptide VL-VH and Thrombin treated to resolve monodispersed monobody Fv domains (186) were examined for proper folding and thermal stability using Circular Dichroism (CD). Fv constructs showed wavelength scan consistent with proper Ig fold (Figure 2-3) (187, 188) and thermal denaturation at melting temperature (T_m) range of 62 ^oC – 67 ^oC indicating stable and properly folded protein.

To demonstrate c/scFvs recapitulating b12 Fab epitope interaction, antigen-binding activities were compared among the various c/scFv constructs. C/scFv samples used were mature b12, germline encoded b12 precursor c/scFv or Fab and chimeric b12 c/scFvs (Chimera 1 with germline heavy chain

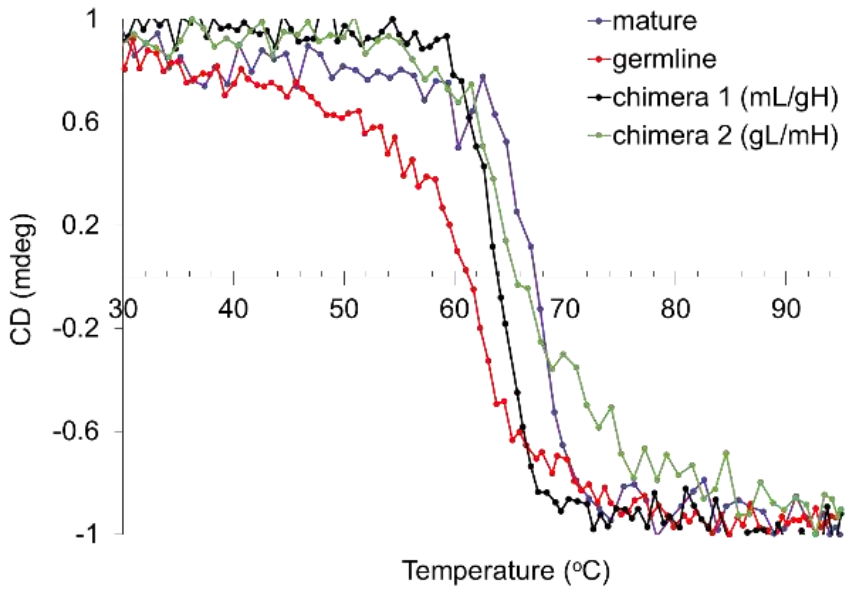

Figure 2-3. Thermal denaturation of b12 c/scFvs:
CD thermal denaturation analysis at 215 nm wavelengths. Mature b12 c/scFv colored blue, germline c/scFv red, Chimera 1 (mL/gH) black and Chimera 2 (gL/mH) green.

coupled with mature light chain (mL/gH) and Chimera 2 with mature heavy chain coupled with germline light chain (gL/mH). Binding properties of the different b12 c/scFvs were measured using Surface Plasmon Resonance (SPR) based binding assay. Monomeric clade B Tier 1 (SF162) and Tier 2 (QH0692) gp120s (189) were expressed with biotin acceptor sequence for *in vivo* biotinylation using co-expressed bacterial biotin ligase (BirA) (190). Biotinylated gp120 monomer ligands were immobilized on SA sensor chip (GE Healthcare). Antibody c/scFv reagents were used as analytes and injected over immobilized monomeric gp120 in duplicate concentrations ranging from 0.2 nM to 2 μM. Binding kinetics and equilibrium binding affinity are detailed in Figure 2-4 and Table 2-1.

Echoing reported high affinity binding, mature b12 bound gp120 with equilibrium binding affinity constants (K_D) of 0.06 nM and 0.9 nM against QH0692 and SF162 respectively (Figure 2-4). Analysis of binding kinetics indicated slow rate of dissociation (k_d) with slower k_d observed in mature b12 interaction with QH0692 than SF162 (Figure 2-4, A and B). As reported previously, b12 germline c/scFv or Fab did not show detectable binding to both gp120 monomers tested at high concentrations (1 uM) (107, 110, 111, 159, 176, 184, 191, 192). Chimeric b12 samples also recapitulated previously reported binding affinity (107). Highlighting importance of VL chain in mature b12 and epitope interaction, Chimera 2 (gL/mH) showed reduced binding affinity to both gp120 monomers tested with K_D of 28.3 nM and 60.2 nM to SF162 and QH0692 respectively. The decreased equilibrium binding affinity measured for Chimera 2 (gL/mH) and gp120 monomer interactions are mainly due to slower rate of association and significantly faster rate of dissociation where both kinetic values are altered by at least ten fold compared to mature b12 - gp120 monomer interaction (Table 2-1). In contrast, while SF162 gp120 monomer - b12 Chimera 1 (mL/gH) interactions fail below the SPR limit of detection for both rates of association and dissociation, Chimera 1 (mL/gH) interacted with QH0692 gp120 monomers at 0.1 uM K_D. This implies, as reported in Hoot (2013), mutations on VL chain alone do not suffice for efficient gp120 interaction.

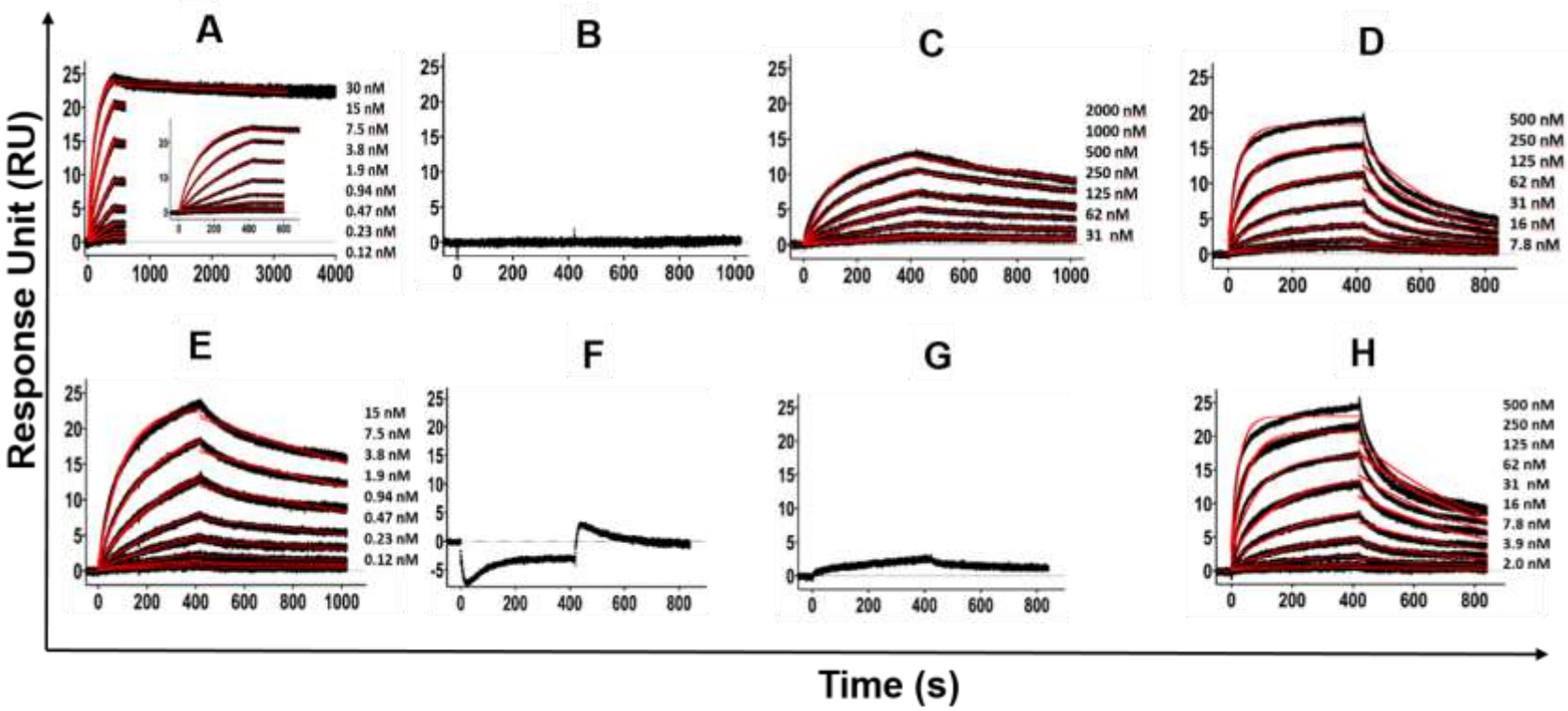

Figure 2-4. Biophysical analysis of b12 c/scFv interactions with monomeric gp120s:

SPR sensograms of the interaction between antibody c/scFv analytes b12 mature (A and B), germline precursor (C and D), Chimera 1 (gH/mL) (E and F), and Chimera 2 (mH/gL) (G and H) against in vivo biotinylated and SA captured gp120 monomeric ligands from Tier 2 clade B virus, QH0692 (A, B, C, and D) and Tier 1 clade B virus, SF162 (E, F, G, and H). Analytes were injected in duplicate and at random order. Matured b12 were injected in concentration serial dilutions from 15 nM to 0.12 nM. Triplicate 30 nM mature b12 were injected to derive rate of dissociation against QH0692. Sensograms for concentration series 15 nM to 0.12 nM are indicated in the inset. B12 germline precursor and Chimera 1 (mH/gL) analytes were injected at a single concentration of 1 µM against SF162 while injecting analytes of 1 µM germline precursor b12 assessed interactions against QH0692 and concentration series ranging from 2 µM to 31 nM of Chimera 2 (gH/mL). Experimental results are shown as black traces and kinetic 1:1 model fits are shown as red lines. Detailed experimental setup are listed in Table 6-1

To examine if b12 c/scFv constructs attain literature reported neutralization IC_{50}, neutralization potency were measured using standard TZM-bl luciferase reporter assay using cells pseudotyped with envelope sequences from SF162 and QH0692 as previously described (102, 186). Mirroring the observed lack of interaction between germline b12 and gp120 monomers, germline b12 Fab did not show measurable virus neutralization. Similarly, Chimera 1 (mL/gH) and Chimera 2 (gL/mH) c/scFv did not neutralize against both viruses. Recapitulating the high neutralization potency by mature b12, mature b12 c/scFv samples demonstrated literature comparable neutralization potency with IC_{50} of 0.13 and 2.92 ug/mL against SF162 and QH0692 respectively (Figure 2-5 and Table 2-1). The result suggests mutations on both VL and VH chains are necessary for effective virus neutralization.

Given the experimental results of protein stability, binding and neutralization potency presented above, we deduce b12 c/scFv constructs perform comparably to previously used b12 Fab reagents. Hence to study role of individual SHM positions on epitope binding and neutralization, our lab constructed b12 mature c/scFv with individual SHM positions reverted to their germline amino acid sequence (mSR) (193).

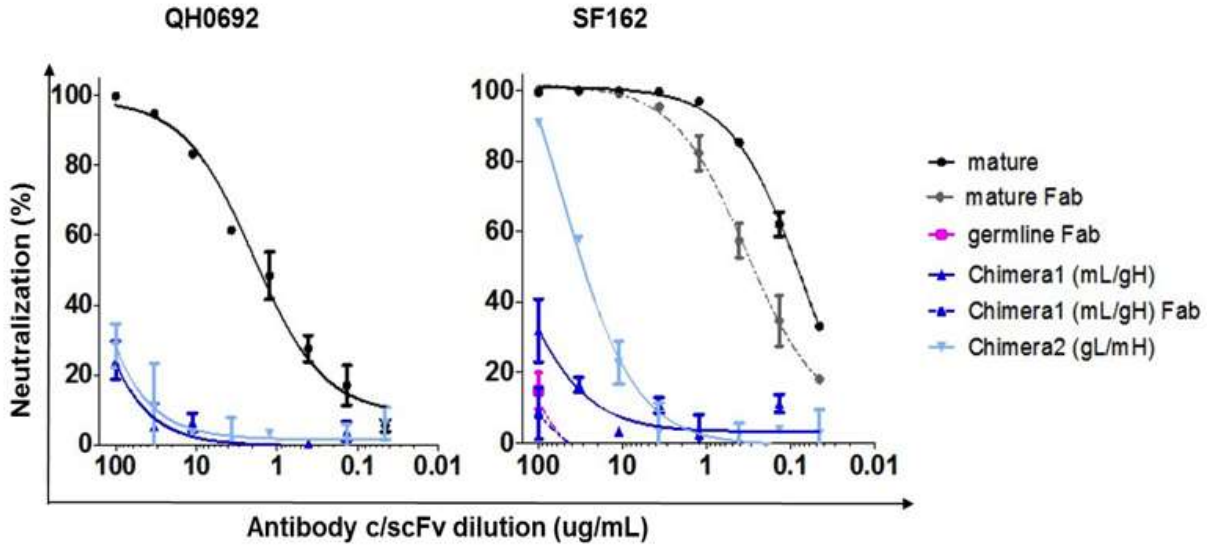

Figure 2-5. Neutralization potency of various b12 c/scFv constructs
Neutralizing activities of c/scFv against QH0692 and SF162 virions. Mature and germline precursor, Chimera 1 (mL/gH) samples were tested as c/scFv and Fab. Average data are indicated as; mature c/scFv ●, mature Fab ● germline Fab ■ Chimera 1 c/scFv (mL/gH) ▲ and Chimera 2 (gL/mH) ▼. Results are representative from two independent experiments

2.2.3 Epitope binding is affected by SHM on CDRs more than FWRs

Antibodies recognize their respective antigens mainly through their CDRs while FWRs play a role in scaffolding the antigen-binding groove (136, 194). During antibody maturation, mutations that increase binding affinity to specific epitopes are preferentially selected and drive the affinity maturation process. Although the antibody maturation paradigm suggest preferential targeting of positions that form the antigen binding interface during SHM, Klein, F. et al,(93) described the relative importance of mutations in FWRs in virus neutralization breadth for some anti-HIV-1 bNAbs. To explicitly define importance of individual SHM positions both on CDRs and FWRs in epitope binding and neutralization, b12 mature c/scFv with individual SHM positions reverted to their germline amino acid sequence (mSR) were constructed (193). Using mSRs, effect of single amino acid reversions on epitope binding and virus neutralization were examined.

Table 2-1. SPR binding kinetics for gp120 monomer interaction with b12 c/scFv constructs

SF162	k_a ***($M^{-1}s^{-1}$)***	k_d ***(s^{-1})***	K_D ***(M)***	***Neutralization IC_{50} (ug/mL)***
Mature	$6.32(1) \times 10^{5}$	$5.88(1) \times 10^{-4}$	$9.29(1) \times 10^{-10}$	0.13
germline b12			NB	>10
Chimera 1 (mL/gH)			BLD	>10
Chimera 2 (gL/mH)	$7.29(4) \times 10^{4}$	$2.06(1) \times 10^{-3}$	$2.83(2) \times 10^{-8}$	>10
QH0692	k_a ***($M^{-1}s^{-1}$)***	k_d ***(s^{-1})***	K_D ***(M)***	***Neutralization IC_{50} (ug/mL)***
Mature	$2.91(1) \times 10^{5}$	$1.85(1) \times 10^{-5}$	$6.35(1) \times 10^{-11}$	2.92
germline b12			NB	>10
Chimera 1 (mL/gH)	$4.62(1) \times 10^{3}$	$5.12\ (1) \times 10^{-4}$	$1.11(1) \times 10^{-7}$	>10
Chimera 2 (gL/mH)	$5.30(2) \times 10^{4}$	$3.19(1) \times 10^{-3}$	$6.02(3) \times 10^{-8}$	>10

NB = no binding
BLD = below limit of detection

As reported by Carrico (2013), individual mSRs did not greatly alter gp120 monomer binding affinity. Effect on rate of association contributed more to the observed difference on equilibrium binding affinity. Although minimal, irrespective of their position in relation to the antigen-binding groove, almost all mSRs exhibited some effect on epitope binding kinetics. Contrary to the initial hypothesis, mutations that were assumed to affect binding surface conformation did not exceptionally reduce binding affinity (Figure 2-6.) Given these, Carrico (2013) suggested gradual development of binding affinity mediated by not only direct epitope interaction but also allosteric effects and protein stability alterations due to mutations on positions distal from the antigen-binding surface.

Further analyzing the binding kinetics of mSR – gp120 interactions, in this section, I examined amino acid changes in b12 antibody CDRs and FWRs due to SHM and respective effect on epitope binding and kinetics. Consistent with the paradigm of affinity driven antibody maturation, relatively pronounced effects on K_D due to mutations on CDRs of both VL and VH domains than FWRs (Figure 2-7) are observed. Individual mSRs in CDRs lowered binding affinity to SF162 and QH0692 by 20 - 30 % compared to mature b12. Particularly mSRs in CDRL1, CDRH2 and CDRH3 affected antigen binding kinetics. In contrast, although mSRs on FWRs also affected binding kinetics, compared to CDR mutations, effects were negligible. Of the mSRs positioned at FWRs, position 47 on VL FWR2 demonstrated remarkable decrease in K_D matching only ~40% of mature b12 binding resulting in decreased binding affinity. As can be inferred from the mature b12 structure (76), VL position 47 is found buried in the VL β pleated sheet Ig fold interface.

Among mutations on CDRs, there are micro satellite regions particularly on CDRL1, CDRH2 and CDRH3 that exhibited pronounced effect on binding kinetics. Confirming previous reports, (76, 92, 176, 177, 184, 185) our results emphasizes the importance of CDRH3 in gp120 binding to elicit b12-like antibody response. Reversions at the apex of CDRH3 (S99C, W100G and D100aG) decreased K_D by ~40% compared to observed binding by mature b12. This further strengthens the significance of residues at CDRH3 apex where reversion of W100G abrogates important contact to a hydrophobic pocket on gp120 while S99C and D100aG could influence the overall environment of the binding pocket.

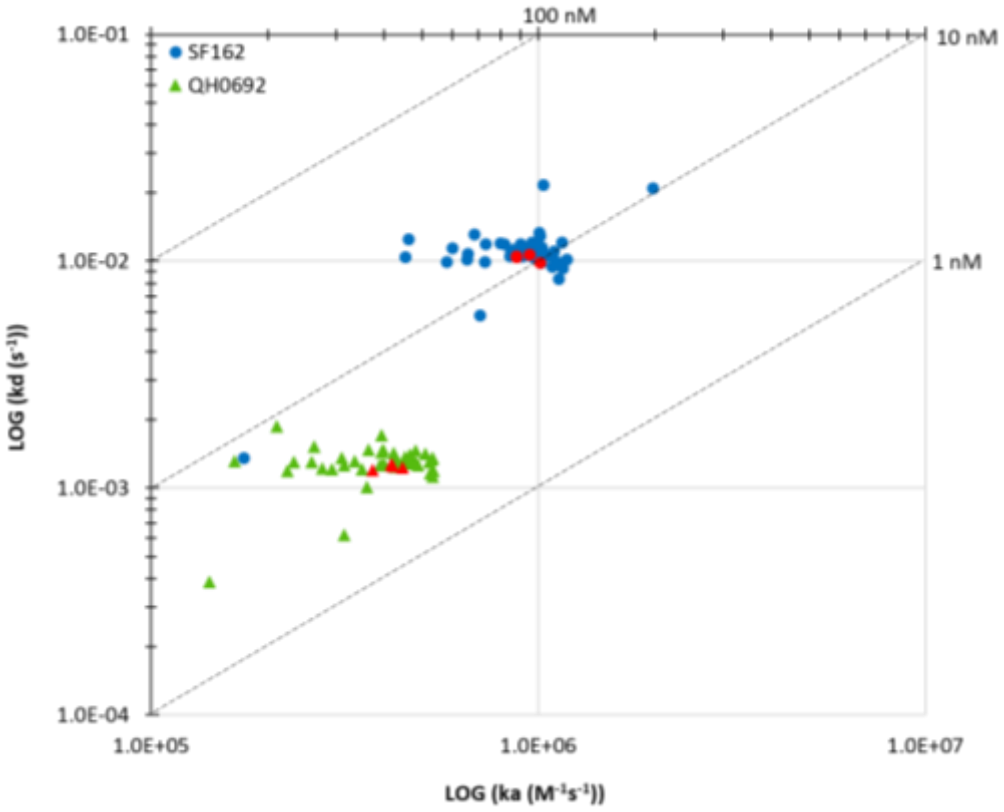

Figure 2-6. Binding kinetics of mSR - gp120 monomer interaction
SPR measured rate of association and dissociations of mSR –gp120 interactions where mSR - QH0692 are indicated by green triangles and mSR-SF162 interactions are indicated by blue circles. Data points for mature b12 are indicated with red circles and triangles. Log rate of associations are plotted on X-axis and log rate of dissociations are plotted on Y-axis. Diagonal lines indicate rate of equilibrium binding affinity in nM.

On the other hand, reversions on CDRH2 altered binding kinetics (k_a and k_d) with no measurable effect on K_D. Y53G on CDRH2 noticeably affected kinetics with marked decrease on k_a over both SF162 and QH0692 (~80% and 65% slower association compared to mature b12 respectively) accompanied by substantially decreased rate of dissociation. Given these, we posit residues on CDRH2 are important for initial complex formation but their impact on K_D, at least as observed on QH0692, is masked by other secondary contacts that help stabilize the bound conformation.

Reversions on VL CDRs, particularly positions on CDRL1, also singly affected binding kinetics. According to gp120 core bound mature b12 Fab structure (76), CDRL1 does not show any obvious

contact with gp120 and is positioned further distal from the binding site to influence residues at or near the binding pocket. Hence, mutations on CDRL1 were not expected to be significantly important in epitope binding. Contrary to the structural information, previous mutagenesis and alanine scanning studies observed (176) marked effect on binding mainly due to the SHM that result in a poly-Arginine motif on CDRL. Emphasizing this, our results argue greater effect on binding due to residues on CDRL1 where binding affinity decreased ~40% due to CDRL1 reversions of H27Q and R32Y compared to mature b12 binding.

Our reported b12 mature binding kinetics for monomeric gp120 interactions on either amine coupled or Streptavidin captured ligands show difference in binding K_D. We attribute these differences to the method of ligand presentation where in amine coupled gp120 monomers, potential binding interfaces could be occluded due to stochastic nature of amine coupling while the highly specific interaction between biotin and streptavidin presents potentially higher proportion of binding interfaces.

2.2.4 Individual mSRs exhibit minimal effect on virus neutralization potency

Although antibody maturation and hence SHM are geared to attain an increase in affinity to cognate antigens, it is important to delineate mutations that confer the observed virus neutralization potency in mature b12. To understand role of SHM on virus neutralization potency, percent neutralization potency of mSRs were assessed and compared against potency due to mature b12. Mature b12 c/scFv neutralized 80% of QH0692 and SF162 viruses at antibody dilutions of 10 ng/mL and 0.5 ng/mL respectively. Virus neutralization by antibody Fv fragments at set antibody dilutions (10 ng/mL and 0.5 ng/mL against QH0692 and SF162 viruses respectively) was compared to mature b12 neutralization against both viruses.

Virus neutralization potency was not altered considerably due to individual reversions when compared to mature b12. However, reversions that decreased antibody effect on viruses were localized mainly on CDRs while residues on FWR in general exhibited no effect. Similar to their effect on binding affinity, micro-satellite regions on light and heavy chain CDRs showed the most effect on neutralization

potency against both virus strains tested. Reversions of SHM positions on antibody CDRs demonstrated lower virus neutralization with individual reversions in CDRs on average accounting for ~20% reduction in neutralization potency. Reversion of W100G on CDRH3 exhibited ~90 % loss in neutralization potency against SF162 making it one of the most important single residue positions to affect neutralization potency. Highlighting the importance of positions on CDRH3, residues flanking Trp100 similarly showed substantial decrease in neutralization potency (~40 % reduction in neutralization due to S99C). A reversion at CDRH2 position 53 (Y53G) also greatly reduced neutralization potency against SF162 (~70 %). Although relatively small, similar effects were also observed due to reversions on CDRH1 (R28T and N31S with ~30% reduction in neutralization potency) (Figure 2-7). SHM positions on CDRL also lowered the effect of antibody on virus neutralization. CDRL1 mSR at position 27 demonstrated ~70% lower neutralization potency due to mature to germline H/Q reversion.

When compared to CDR positions, overall effects on neutralization potency due to mSRs on FWRs were negligible. However, two SHM positions on FWRL exhibited relatively lower neutralization potency. Reversions V47L and V78L, although both are buried in the immunoglobulin (Ig) fold β pleated sheet interface, affected neutralization against the Tier 1 virus strain SF162 by ~40% and 50% respectively. The effect due to V47L directly correlated with decreased binding, while V78L reversion discriminately affected neutralization without any measurable impact on binding kinetics.

2.2.5 SHM on CDR micro satellites exhibit correlated effect on epitope binding and virus neutralization

Uncoupling the role of SHM in epitope binding affinity from its role in virus neutralization, effect of mSRs on neutralization potency against both virus strains assayed show no correlation to observed effect on binding affinity. To assess correlations of epitope binding and virus neutralization potency, effects of individual mSRs on binding K_D and % neutralization were normalized against mature b12 binding affinity and neutralization potency. Normalized K_D and neutralization potency were compared for all mSRs and reversions on CDRs and FRWs (Figure 2-8). Although there exist micro-satellite positions that altered both epitope binding affinity and virus neutralization potency, effect of mutations on epitope

binding does not directly correlate with virus neutralization potency. This lack of correlation is consistently observed for mSRs on both CDRs and FWRs.

However coupling the role of individual mutations on antigen binding affinity and neutralization potency, some reversions on CDRs showed marked reduction in neutralization. As discussed above, reversions on CDRH2, CDRH3 and CDRL1 affected both binding affinity and neutralization potency. Reversion of W100G on CDRH3 reduced epitope-binding K_D by ~40% compared to mature b12 with ~90% loss in neutralization potency against SF162. This neutralization coupled effect of mSRs on K_D was also observed due to reversions at CDRL1. Of particular importance is position 27 on CDRL1, where ~70% lower neutralization potency with a comparable effect on K_D were observed due to Q27H reversion.

In addition to positions that show correlated effect on equilibrium binding and neutralization potency, amino acid reversions that exhibited no apparent effect on binding kinetics showed remarkable reduction in neutralization potency, uncoupling binding kinetics from neutralization potency. A reversion at CDRH2 position 53 (Y53G) that altered k_a and k_d with unremarkable effect on overall K_D also greatly reduced neutralization potency against SF162 (~70%). Similar effects were recorded due to reversions on CDRH1 (R28T and N31S with ~30% reduction in neutralization potency).

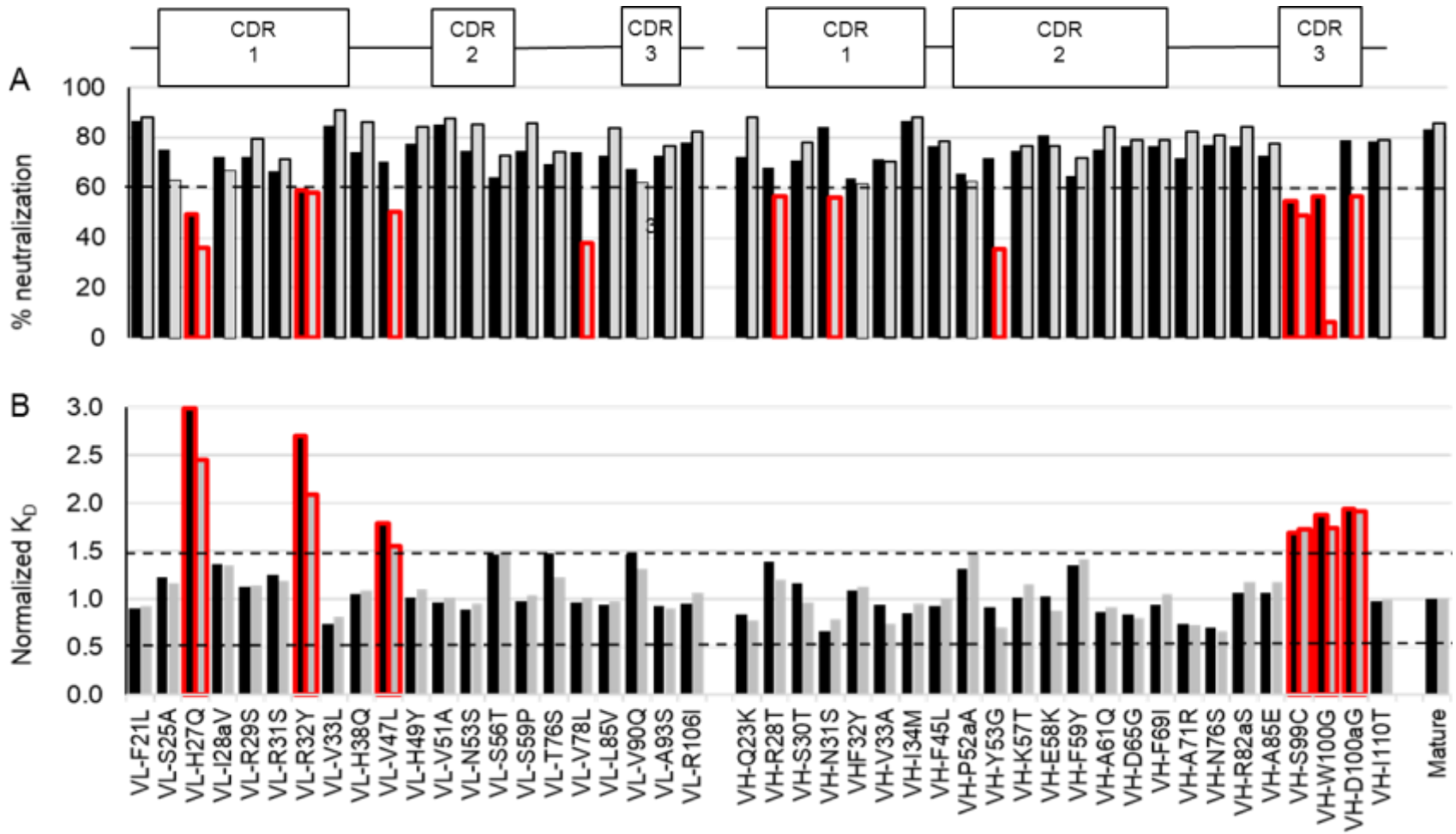

Figure 2-7. Effect of individual mutations on virus neutralization and binding
A) Effect of mSRs on neutralization potency are plotted as percent neutralization. (B) SPR binding analysis of mSRs to chemically surface coupled gp120 monomers. Single amino acid reversions were used as analytes injected at 30 nM concentration over chemically coupled gp120 (QH0692 and SF162) surfaces (Carrico, 2013). Equilibrium binding affinities (K_D) are normalized to mature b12 binding K_D. mSRs that resulted in fold K_D >1.5 fold over b12 mature are delineated with red borders. Black bars indicate effects against QH0692 monomeric gp120 and effect on SF162 monomeric gp120 are indicated by gray bars. Schematics representation of folded domains at the top and identity of individual mature to germline reversions corresponding to bars are indicated at the bottom.

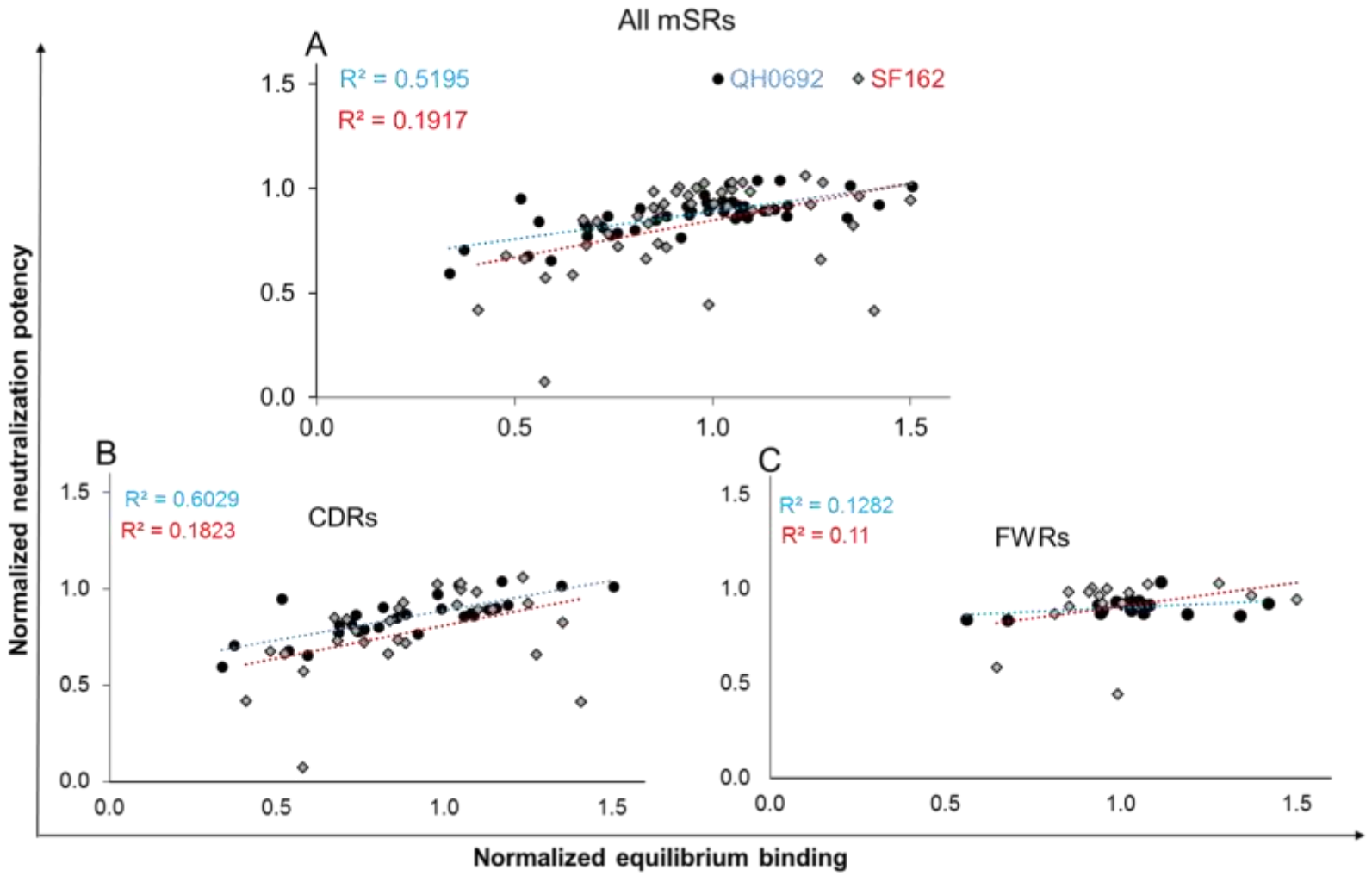

Figure 2-8. Correlation of mSRs effect on binding and neutralization

Equilibrium binding constants (K_D) and percent neutralizations of individual mSRs against gp120 were normalized to mature b12. Normalized K_Ds are plotted on X-axis and normalized neutralization potencies are plotted on Y-axis. Data points for individual mSRs are indicated with black circles (QH0692) and gray rhomboids (SF162). Linear correlation line and correlation coefficients (R^2) are colored blue (QH0692) and red (SF162).Correlation between neutralization and binding are plotted for all mSRs (A), mSRs of CDR (B) and mSRs of FWR (C).

2.3 Discussion

Antibody maturation process is a key component of an effective humoral immune response. SHM and selection for high affinity antigen binding antibody variants in part dictate this antigen dependent selection process (119, 195). Although, the process is facilitated due to the deaminase activity of AID (129, 130, 135), illustrating the highly stochastic nature of SHM, mapping AID hotspots on antibody germline repertoire genes is unable to predict potential sites prone for SHM (132). This lack of amenability to computational *in silico* determination of potential SHM routes necessitates empirical determination of sites of SHM and their importance in epitope binding and downstream antibody function. The importance of such empirical analysis of positional impacts of mutations in virus derived epitope binding and neutralization potency are pronounced in structure based vaccine design strategy (48, 59, 141, 142).

To complement the wealth of data gathered on biochemical and biophysical attributes of CD4BS targeting bNAbs, we examined the role of individual SHM positions in b12 epitope binding and neutralization. In agreement with previously reported importance of SHM on CDRs (119, 132, 163), mutations on b12 CDRs affected epitope binding and neutralization albeit moderately. Deciphering the importance of mutations on VL and VH chains, the observed reductions in binding affinity and neutralization potency due to CDRH3 are congruent with structural (76) and mutational analysis (176). CDRH3 makes important contacts and affects binding and neutralization potency on diverse virus strains. The ability of b12 to probe CD4BS using the long CDRH3 loop (96) highlights importance of mutations of C99S, G100W and G100aD in influencing the overall architecture of the binding groove.

While mutations on CDRH3 affected overall epitope binding and virus neutralization, our SPR based binding studies indicate relevance of CDRH2 in overall epitope binding and neutralization. As can be inferred from binding kinetics, mSRs at CDRH2 affected mainly rate of associations. Y53G, a mutation on CDRH2 did not affect equilibrium binding kinetics (K_D) yet greatly altered overall rate of association (k_a) and neutralization potency (% neutralization). Hence, other secondary contacts that help

stabilize the bound conformation mask overall impact of CDRH2 mutations on K_D, at least as observed on QH0692. Fast complex formation in neutralization tolerant strains implies the role of CDRH2 in facilitating antigen-binding rate of association could be an acquired feature during the affinity maturation process. The differential effect of mSRs on HIV-1 strains tested further informs role of CDRH2 mutations on epitope binding. Given these, we posit residues on CDRH2 are important for initial complex formation while other important contacts stabilize the bound conformation.

An interesting observation is the importance of CDRL1 in binding and neutralization. Despite lack of direct epitope contact on gp120 monomers in structural studies (76), the region exhibited CDRH3 comparable effect on binding and neutralization. Indeed, previous mutational analysis also suggested a potential role of CDRL1 in epitope binding (176) where the presence of a poly Arg motif was put forth as a conformational modulator. Other potential reasons for the observed effect of CDRL1 in binding and neutralization are its proximity to CDRH3. Antibody maturation through changes in germline precursor antibody amino acid sequences around the CDRL1 region could alter overall CDRH3 conformation; hence improve epitope recognition and binding. Similarly, the minimal gp120 core used in b12-gp120 structure encapsulates only minimal interactions of b12 and its epitopes. Hence, potential forces that maintain the bound b12 could be lost in structural analysis.

Current advancements in Env trimeric structures enabled us to examine the importance of b12 CDRL1 on epitope binding. In our modeling exercise where we superposed the gp120 core on Env trimeric structures (47), in addition to its importance in maintaining CDRH3 conformation, CDRL1 is deduced to make important contacts with variable loop regions on Env. We acknowledge the pitfalls in modeling structural analyses given the high structural flexibility of Env trimers, yet the results are in accordance with our binding and neutralization findings and therefore we conclude that b12 CDRL1 is essential for direct envelope binding and virus neutralization.

Even though affinity maturation related mutations are more prevalent on CDRs (119, 125, 132), while studies also indicated importance of FWR mutations in epitope binding and downstream antibody functions (93). Similarly, our analysis revealed the importance of mutations on VL chain that affected

epitope binding and neutralization. Mutations L47V and L78V on VL chain affected either binding affinity, neutralization potency or both compared to mature b12. According to structural analysis using the available gp120 core bound b12 structure, these positions are buried in the VL Ig fold interface. The conservative substitution of a hydrophobic residue (Leu) by Val is tolerable and not detrimental for protein stability. However, this substitution can potentially alter overall protein folding. In thermal shift based protein stability test Carrico (2013) recorded minimal thermal stability effect due to mutation on this position suggesting the subsequent decrease in binding and virus neutralization is not a mere contribution of Leu to Val mutation but rather an effect on overall prevalence of other important mutations. The position can also affect overall binding due to allosteric effects where CDRs that directly contact epitope alter their conformational and biophysical characteristics due to minor change like the reported conservative substitutions.

Similar to the observed differential effects of mutations on CDRs on the two virus strains tested in regards to binding and neutralization potency, FWR mutations altered neutralization potency against the neutralization sensitive strain (SF162). These discriminatory effects on binding kinetics and neutralization potency due to regional mutations elude to a potential mechanism of virus neutralization. We postulate that b12 neutralize Tier 1 viruses through direct binding and inhibition of target cell receptor contact while Tier 2 viruses require mechanisms that involve other routes in addition to direct blockage of receptor contact.

Affinity maturation and subsequent antibody function assumes correlative relations between antibody binding to virus derived epitopes and functional virus neutralization. An intriguing observation from our mSR analyses is the minimal overall effect of single reversions of mutations from mature to germline sequences on both epitope binding and virus neutralization potency. While CDRs endure the most of binding interaction to epitopes on HIV Env, mutations of positions distal from the binding site exhibited minor effects on overall binding and neutralization potency. However, as deduced from regression analysis observed effects do not show correlation. The differential effects of mutations on epitope binding and antibody function are specific to neither FWR nor CDR mutations. Although

mutations on CDRL1 and CDRH3 altered both epitope binding and neutralization potency, in overall normalized analysis the correlation is localized to mutations on specific positions and is not generalizable to all CDR mutations. The lack of correlation implies antibody maturation is not a mere process of affinity maturation but mutations important to achieve neutralization potency also play important role during the selection process.

Although individual mutations are maintained through evolution for an array of potential roles including increased antibody repertoire, increased antigen presenting efficiency of B-cells and generation of memory B-cells (163); in its role of affinity maturation, SHM in bNAbs against chronically infectious agents like HIV-1 needs a deeper examination. Given the importance of mutations distal to epitope binding in binding strength and virus neutralization, we postulate that, in chronic infections like HIV-1 the exceptional high affinity and broadly neutralizing ability observed in elite neutralizers is a function of gradual molding of the antibody response. The lengthy antibody maturation process observed in anti-HIV-1 bNAbs enables the accumulation of mutations that result in broadly neutralizing antibody function. Hence, individual and one generational interactions do not suffice to yield potential mature b12-like antibody response.

2.3.1 Structural implication of antibody light chain

Identified from combinatorial phage display antibody library, the role of b12 VL in antigen binding and virus neutralization were questionable. Structural elucidation of b12 bound to gp120 core demonstrated epitope binding mediated mainly through antibody VH chain (76). These structural inferences contradicted previous mutational studies that claim significant effects on epitope binding and neutralization potency due to modifications on VL chain (176, 185). Biophysical analyses presented here and reported by Hoot et al (2013) using chimeric antibodies emphasize importance of VL in overall binding and virus neutralization. Our analyses utilizing individual germline sequences rather than arbitrary substitutions elucidate the role of CDRL1 in epitope binding and neutralization in addition to FWR positions on VL domain that altered epitope binding

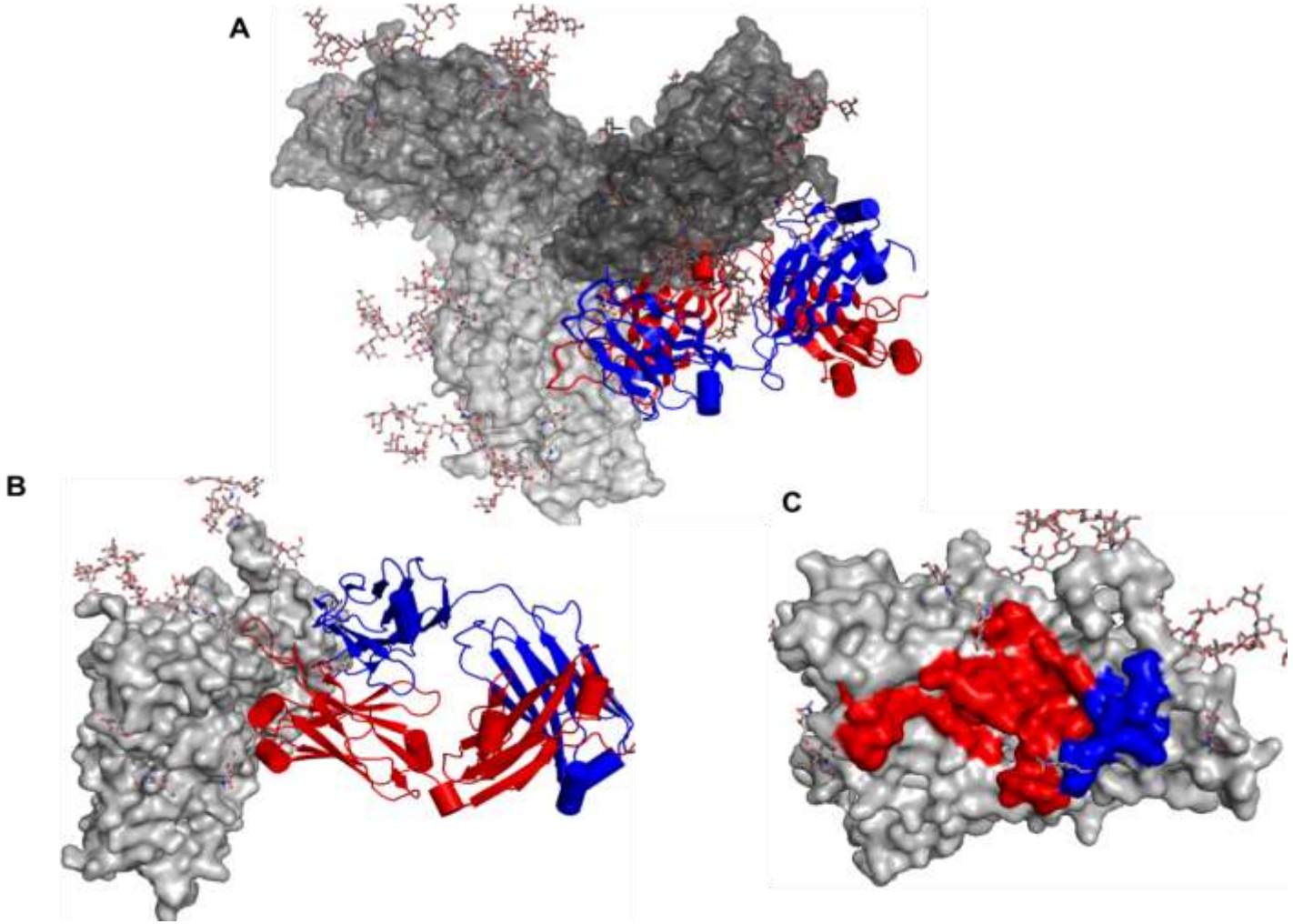

Figure 2-9. Mature b12 binding footprint on gp120 surfaces

A, Surface representation of Env trimer (PDB (83) accession code 4NCO (47)) in light gray shade with mature b12 binding as elucidated on core-gp120 structure (PDB accession code 2NY7 (76)) and superposed on a single protomer with VH chain colored red and VL chain colored blue. Glycan molecules are presented in stick representation where atoms are labeled as gray (C), red (O) and blue (N). B, Mature b12 binding as in A with a closer look at a single protomer. C, Surface representation of full length gp120 from 4NCO shaded light gray with b12 modeled footprint on gp120 colored as in A.

The recent revelation of native-like Env trimer SOSIP structures further points out the importance of VL domain (47, 49). Modeling b12- gp120 interaction on full-length gp120 from trimeric Env structures indicates the extensive contact footprint due to VL interactions (Figure 2-9C). Inferring molecular footprint from interface analysis using PISA (83), a higher proportion of gp120 interactions are mediated through VH chain with 1260 $Å^2$ surface area on gp120 monomers covered by VH chain during b12 interactions. VL-gp120 interaction also covers 314 $Å^2$ surface area and footprint analyses indicate substantial coverage by b12 VL domain. The modeling experiment supports results presented in this document that demonstrate pronounced effect on epitope binding and neutralization due to VL chain interactions with Env, especially CDRL1.

The observed differential epitope binding in Chimera 1 (mL/gH) and Chimera 2 (gL/mH) interaction with gp120 monomers revealed importance of b12 VL chain. In contrast to Chimera 2 (gL/mH), Chimera 1 (mL/gH) showed micro molar binding to QH0692 but not SF162 implying diminished binding affinity due to lack of interactions with affinity matured and mutated VL residues. Examining the differences in binding due to mature b12 or Chimera 2 (gL/mH) interaction with the two gp120 monomers tested, the loss in binding affinity due to absence of mutations on VL (up to 100 fold reduction) indicate substantial alteration on epitope binding due to b12 VL.

2.4 Acknowledgment

Results presented in this section were done in collaboration with Christopher Carrico as part of a larger project that assesses biophysical and structural attributes of b12 antibody maturation with the goal of epitope design for potential vaccine immunogens. Results from the joint effort are currently being compiled into a research article. Carrico generated individual mSRs and analyzed SPR based binding on amine coupled gp120 monomers with help from Della Friend. His insight in expression construct design and discussions during data interpretation were invaluable. I am also grateful to Della Friend who analyzed SPR based biophysical interaction of mSRs and gp120 monomers. Her insight and helpful discussions regarding SPR data analysis are paramount to the completion and depth of work presented here. Sara Carbonetti in Leo Stamatatos Lab at SeattleBiomed did neutralization studies. The tremendous work done by Sara to generate the complete set of neutralization data that complemented SPR based binding analysis was invaluable for my work. I am grateful for the intellectual help I amassed from her.

Chapter 3

3 Minimal SHM that garner b12-like binding and neutralization response

3.1 Overview

Understanding the molecular mechanisms that separate elicitation of infection induced HIV-1 neutralizing antibodies (especially bNAbs) from the majority of ineffective and narrowly neutralizing antibodies that swarm the immune system advances future attempts in design based HIV-1 vaccine development (13-15, 140, 141). Research in understanding the interplay between HIV-1 and host immune response uncovered a wealth of knowledge fuelling vaccine research. Vast biophysical and structural research aimed at characterization of HIV-1 Env and regions that elicit bNAb response (47, 49, 145, 196-199) unveiled important attributes that focus antibody based neutralization response. Research focused on defining what distinguishes bNAbs from the vast majority of non-neutralizing or narrowly neutralizing antibodies elicited during HIV-1 infection also elucidated characteristic features of highly cross reactive and bNAbs isolated from chronically HIV-1 infected individuals (28, 58, 61, 101, 102).

Env is the only virus encoded protein that is accessible for immune surveillance and antibody mediated immune response. It is indispensable and plays a vital role in viral infectivity and host targeting (36, 65, 72). Its functions necessitate preservation of regions involved in host receptor interaction implying greater susceptibility to antibody based immune response. Even though the vast majority of bNAbs isolated are targeted against regions on Env, an array of immune evasion strategies deployed by the virus (200-203) delay antibody based immune response rendering the response ineffective (204-206).

Biochemical and biophysical analysis on the growing list of bNAbs isolated from chronically HIV-1 infected individuals revealed interesting characteristics of such antibodies (detailed review in Chapters 1 and 2). In bNAbs that target the host receptor binding site (CD4BS), in addition to the high SHM frequency suggesting such antibodies require extensive virus derived antigen exposure for their development and maturity, numerous studies demonstrated the lack of measurable epitope binding by

germline precursor antibodies despite high affinity epitope binding by bNAbs (107-111). The exceptionally high mutations frequency and prolonged affinity maturation in bNAbs predicts evolution of mature antibodies away from their germline precursors. This divergence can contribute to the lack of measurable interaction between cognate epitopes and germline encoded antibodies. Given these features, it is possible to postulate that molecular mechanisms of bNAb development could be shaped by the high degree of variability in viral sequence post infection.

One possible hurdle in HIV-1 vaccine design is sifting through the vast number of mutations acquired through affinity maturation for mutations and amino acid positions relevant for the recorded bNAb response. Such analysis enables defining the minimum number of mutations important for immune response that correlate with protection and important antibody amino acid positions required to stimulate infection elicited bNAbs-like response. Immunogen design coupled with relevant information regarding importance of affinity maturation related mutations could potentially enable directing immunogens to relevant interactions for expedited elicitation of bNAb response.

A progressive approach that identifies amino acid positions that play important role in epitope recognition and progressively analyze their additive effect is a potential technique in fine-tuning the immunogen design process. The technique will streamline the design process to focus on those positions that potentially confer comparable mature antibody like epitope recognition. In the previous chapter (Chapter 2) we attempted to address the importance of individual positions on b12 altered through the process of SHM and their relative importance in overall antibody stability, virus derived epitope recognition and virus neutralization. Following results of positional importance in antigen binding and virus neutralization, in this section we attempted to address key positions on b12 germline precursor antibody that confer cognate antigen recognition. In addition, we attempted to assess the possibility for focused SHM towards limited number of mutations on germline precursor antibody that enable mature b12 comparable antigen binding and neutralization.

3.2 Results

3.2.1 gRBs for progressive SHM analysis

The anti-HIV-1 bNAb b12 accumulated 45 mutations in its Fv region. Although these mutations are mainly focused on CDRs, as discussed in Chapter 2 and Carrico 2013 (193), individual mutations did not alter antigen binding and virus neutralization dramatically other than the observed minor variations among positions. In order to define the minimum mutation required during antibody maturation to confer mature b12 comparable binding and neutralization, cleavable and STREP tagged b12 germline precursor c/scFvs that contain blocks of positions reverted to mature b12 sequence in germline background were synthesized and expressed.

Using the information presented in Chapter 2 of this document and Carrico 2013 (193), positional mutations were prioritized based on their effect on K_D, k_a and/or k_d (Figure 3-1). Four reversion blocks (gRB) incorporating groups of mutations that could drive matured b12 like binding kinetics were designed. The first block of reversions (gRB1) was composed of mutations (VH-C99S, VH-G100W, VH-G100aD, VL-Q27H, VL-Y32R and VL-V47L) that exhibited the highest reduction in binding affinity (K_D less than a standard deviations) compared to mature b12 against the two gp120 monomers tested. According to the mature to germline single reversions (mSRs) analysis, mutations on CDRH3 (VH-C99S, VH-G100W, VH-G100aD) individually reduced overall gp120 binding K_D. Similarly reversions of amino acid positions on CDRL1 (VL-Q27H, and VL-Y32R) individually reduced overall binding K_D. In structural elucidation of b12 interaction with gp120 core (76) CDRH3 provide extensive interaction with the gp120 core while CDRL1 is not presented as relevant in epitope binding. Given the decreased binding affinity to gp120 monomers in mSRs, we postulate that incorporation of these mutations in germline background will show measurable binding to b12 cognate epitope. In addition to positions on CDRs, remarkable reduction in epitope binding is observed due to reversion of a position on VL framework region (FWR). VL-position 47 is located within the Ig domain β-sandwich where it can potentially affects overall protein fold and stability. Reverting the mature sequence (Val) to the germline equivalent (Leu) in

our mSR analysis reduced overall antigen binding affinity (Chapter 2, Carrico, 2013 (193)). The mature antibody sequence at VL position 47 was incorporated in gRB1 with the assumption that at VL position 47, Val potentially facilitates protein solubility and folding and hence subsequently contributing to increased epitope-binding affinity.

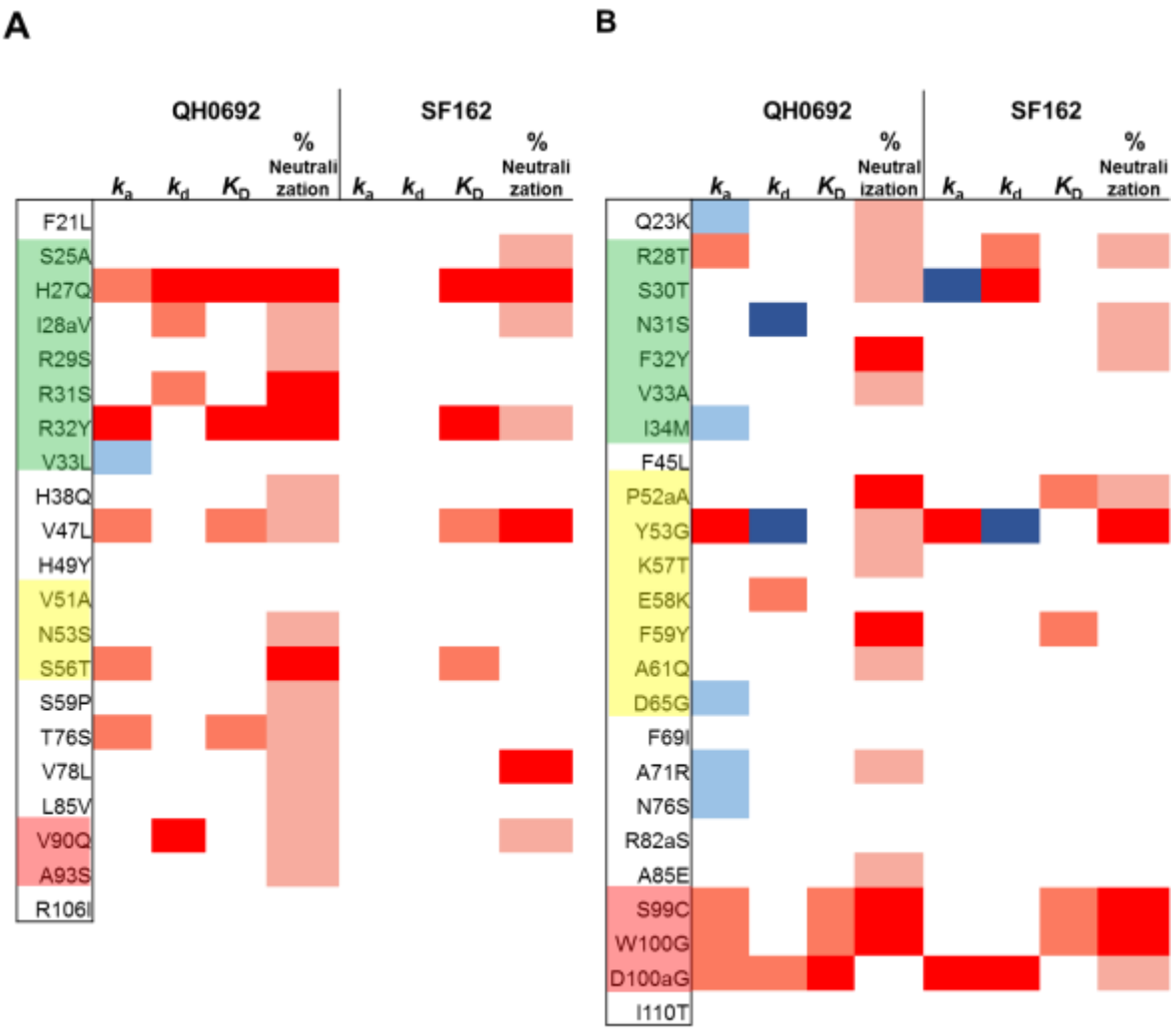

Figure 3-1. Analysis of single mature to germline sequence mutation effects on binding kinetics and neutralization
Standard deviation analysis of mSR effects on binding kinetics and neutralization potency A) of mutations on VL chain and B) of mutations on VH chain. Rows indicate individual mature to germline reversions, kinetics parameters and neutralization potency are given in columns. Mutations in CDR regions are indicated o both chains as green shades CDR1, yellow shades CDR2 and red CDR3. Effects are indicated as blue for improvement or red for reduction compared to mature b12. Standard deviations are presented using intensity of colors. Regular red or blue indicate 2 standard deviation away from mature b12 while light red or blue indicate 1 standard deviation from mature b12.

Likewise, amino acid positions with mature b12 sequence incorporated in gRB2 included mutations in gRB1 and additional four residues in CDRH1 and CDRH2. As inferred from the structural rendition of mature b12 and gp120 core interaction (76) and mutational analysis (176), positons on CDRH1 and CDRH2 contact gp120 core structure directly and alterations on these positions abrogated direct interactions and virus neutralization. In our biophysical analysis of individual mSRs and examination of SPR sensorgrams, positions of CDRH1 and CDRH2 demonstrated significantly slow k_a (VH-T28R, VH-S31N, VH-Y32F, and VH-G53Y) accompanied with no or minimal change in overall binding affinity. We hypothesize these amino acid positons affect initial antigen recognition while presences of other secondary contacts maintain overall stability of the antibody-antigen complex.

In addition to mutations on CDRs, FWR amino acids also altered b12 mature epitope binding. Previous work from the Bjorkman's lab showed mutations on FWR amino acids of anti-HIV-1 bNAbs measurably affect epitope binding and neutralization (93). Mutations that are not predicted to alter epitope binding through direct interaction are incorporated into germline b12 precursor sequence and engineered as gRB3 construct. The construct will enable assessment of the role of SHM on positions that do not affect binding and neutralization. Positions mutated to their respective mature b12 sequence in gRB3

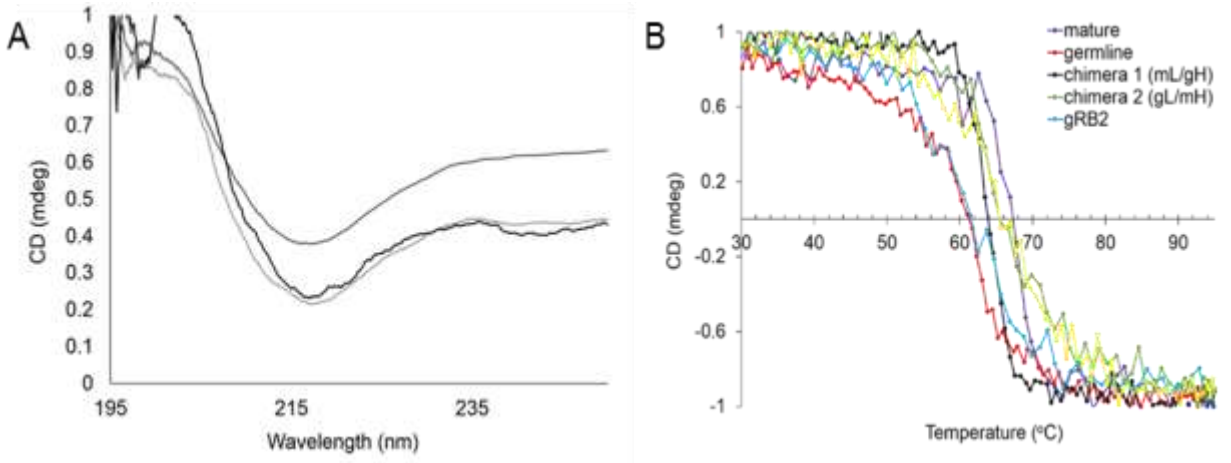

Figure 3-2. Protein stability analysis using Circular Dichroism (CD)

A) CD wavelength scans of various c/scFv proteins. Protein secondary structures were deduced from wavelength scans. CD spectra indicated protein secondary structure of mostly composed of beta-sheets. Representative scan spectra are given in gray. B) Thermal denaturation analysis at 215 nm wavelength. Mature b12 c/scFv colored blue, germline c/scFv red, Chimera 1 (mL/gH) black, Chimera 2 (gL/mH) green and gRB2 light blue

(VH-T30S, VH-A52aP, VH-Y59F, VH-S82aR, VH-E85A, VL-V28aI, VL-S29R, VL-S31R, VL-Q38H, VL-T56S, VL-S76T, and VL-Q90V) are mainly involved in the overall Ig fold of the Fv region and yet in mSR analysis exhibited measurable effect on binding kinetics.

In the above-described groups of mutations, mutations are subdivided according to their perceived importance in antigen binding. However, these positions (regardless of their respective importance in formation of antibody CDR and FWR) could affect overall binding and neutralization through either direct epitope contact or overall antibody conformational stability. Given these assumptions we engineered germline b12 precursor Fv constructs with positions described in gRB1-3 reverted to mature sequence as gRB4. The incremental increase in SHM sequence in the germline precursor background simulates progressive intermediates in the affinity maturation process. These positions were selected as described above, for their effect merely on binding kinetics with deviations greater than at least three standard deviation compared to mature b12.

All gRBs were synthesized as c/scFv constructs with VL and VH domains linked with poly Gly linker encompassed by double thrombin cleavage site. Samples were expressed in mammalian HEK 293F cells using lentivirus based transduction system. Of the four designed constructs, gRB1, gRB2, and gRB4 were expressed as soluble secreted protein while, gRB3 did not express in our mammalian expression system. Expressed protein samples were protease cleaved to generate mono-dispersed mono-body Fv recombinant protein. Fv samples were biochemically characterized using comparative SDS-PAGE under reducing and non-reducing conditions and SEC.

Protein stability analysis using CD wavelength scan and thermal denaturation at 215 nM wavelength confirmed proper protein folding. The three gRBs tested exhibited antibody c/scFv comparable wavelength scan spectrum with characteristic Ig-fold (Figure 3-2). Analysis of protein stability using thermal denaturation assay confirmed, compared to the other b12 c/scFv constructs, nearly all gRBs adopt stable protein conformation with Tm values in spectrum of b12 mature and germline precursor c/scFvs.

3.2.2 b12 comparable binding kinetics necessitate extensive mutation of germline encoded precursor antibodies

To assess if the incorporation of specified mutations in b12 germline antibody precursor affected binding kinetics to cognate epitope on Tier 1 and 2 gp120 sequences (SF162 and QH0692 respectively) (189), SPR based binding assay was performed. To measure binding kinetics of gRBs to monomeric gp120s, *in vivo* biotinylated and SA chip coupled gp120 monomer SF162 and QH0692 were utilized as ligands while gRBs were injected as analytes.

gRB1 shows weak binding below the instrument limit of detection for measurements (Figure 3-3 and Table 3-1) to either gp120 monomers tested. Although the interaction between gRB1 and gp120 monomers fell below the instrument's limit of detection to generate binding kinetic statistics, gRB1 c/scFv exhibited a minor increase in gp120 recognition when compared to germline b12 precursor c/scFv. According to b12 structural (76) and mutational analysis (176), the antibody CDRH3 particularly Trp100 and surrounding charged residues were postulated to play important roles in maintaining CD4BS interaction through the hydrophobic extended apex of CDRH3 in given conformation. SHM positions on CDRL1 and FWRL2 affected overall binding affinity; the expected gain in binding affinity was not observed when amino acid sequences at these positions in germline precursor antibody are mutated to mature b12 sequence. Even though mutations incorporated in gRB1 make direct contact with gp120 and individually show the highest effect on binding affinity, confined mutations to these positions potentially are not solely sufficient to exert mature b12 comparable binding and virus neutralization. However, given the observed sub molar interaction between gRB1 and gp120 monomers, we speculate pre-alterations at these positions due to either pre-initiated B-cell development or initial virus derived epitope stimulation could engage naïve BCRs and stimulate B-cell proliferation and affinity maturation.

Additional mutations in gRB2, mainly of positions on CDRH1 and CDRH2, conferred higher yet micro molar binding to monomeric gp120s as compared to germline precursor b12 antibody Fv or gRB1 while greatly diminished binding compared to mature b12 (Figure 3-3 and Table 3-1). Mutations accumulated due to SHM on CDRH1 and CDRH2 that potentially make direct epitope contact improved

binding kinetics parameters against both Tier 1 and Tier 2 virus derived gp120 monomers. Increased binding affinity is attained mainly due to a faster rate of association (k_a,) with minimal effect on the rate of dissociation (k_d). Compared to gRB1, additional mutations in gRB2 bring ~35 fold faster antigen recognition and association. Complex formation by gRB2 was slower only by ~ 2 - 7 fold (against SF162 and QH0692 respectively) compared to mature b12. The faster k_a could indicate positions on CDRH2 further improve the kinetic favorability of the paratope facilitating initial antigen recognition and binding events while further stabilization of the bound conformation are achieved through subsequent mutations. Evidently, mutations on CDRH1 and CDRH2 play important role in stabilizing the paratope in initial Env interaction conformation.

Given the gradual improvement in antigen binding observed due to mutations incorporated in germline precursor when compared to germline precursor Fv constructs, we hypothesized that incorporation of all antibody maturation related mutations that show measurable effect on binding in mSR

Table 3-1. SPR binding kinetics for gRB interaction with gp120 monomers

SF162	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)	Neutralization IC_{50} (µg/mL)
Mature	6.32(1) x 10^5	5.88(1) x 10^{-4}	9.29(1) x 10^{-10}	0.09
germline b12			NB	>10
gRB1	BLD	BLD	BLD	>10
gRB2	3.44(3) x 10^5	1.40(1) x 10^{-2}	4.07(5) x 10^{-8}	>10
gRB4	7.67(1) x 10^5	7.93(1) x 10^{-4}	1.03(1) x 10^{-9}	3.86
QH0692	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)	Neutralization IC_{50} (µg/mL)
Mature	2.91(1) x 10^5	1.85(1) x 10^{-5}	6.35(1) x 10^{-11}	1.61
germline b12			NB	>10
gRB1	1.17(1) x 10^3	1.69(1) x 10^{-3}	1.44(1) x 10^{-6}	>10
gRB2	4.24(1) x 10^4	1.99(1) x 10^{-3}	4.71(1) x 10^{-8}	>10
gRB4	3.74(1) x 10^5	4.16(1) x 10^{-5}	1.11(1) x 10^{-10}	3.76

NB = no binding
BLD = below limit of detection

studies (Chapter 2, and Carrico, 2013) will enable mature b12 comparable binding affinity. Accordingly, incorporation of extensive mature b12 sequences in germline precursor background in gRB4 enabled mature b12 comparable binding affinity and kinetics (Figure 3-3 and Table 3-1). Combination of germline to mature mutations added in gRB4 altered overall binding mainly through increasing the stability of the bound complex. These mutations (mainly located on FWRs) stabilized the complex by ~ 20-50 fold (against SF162 and QH0692 respectively).

The data indicate, while mutations on CDRs are important for initial complex formation, mutations on FWRs stabilize the complex and hence maintain the antibody – antigen complex in bound conformation. Some mutations, as can be inferred from our gRB analysis, might not directly contribute to epitope binding and hence potentially are dispensable in the development of vaccine-induced b12 like antibody response. We were able to attain b12 comparable binding kinetics through the mutation of half the positions altered by SHM during the maturation of b12 from its progenitor. However, the number of mutations required to mimic b12 antigen interaction is yet greater than average mutation observed in conventional antibodies elicited post-vaccination (130-132, 134, 166, 169).

3.2.3 Mature b12 comparable neutralization potency does not correlate with binding affinity.

A prominent assumption in vaccine design entails correlation of cognate epitope binding affinity and neutralization potency where immunogens that elicit higher affinity binding antibodies potentially neutralize viruses more efficiently. Particularly bNAbs that target host receptor binding regions on Env, like CD4BS, are expected to bind to their target epitope with higher affinity and hence block key host-virus interactions. Given the lack of measurable correlation in antigen binding and neutralization potency in b12 mSRs studies (Chapter 2), we hypothesized that assembling mutations that exhibited some degree of effect on binding affinity and kinetics could accentuate the correlation between epitope binding and virus neutralization.

To assess correlation between epitope binding and neutralization, gRBs were assayed for their neutralization potency against Tier 1 and 2 viruses. Given the gradual increase in binding affinity from

the observed lack of binding by the un-mutated germline precursor to gRB1, gRB2 and mature comparable binding by gRB4, we hypothesize gradual increase in neutralization potency culminating in mature b12 comparable neutralization IC_{50}. As observed in mSRs analysis (Chapter 2), positions incorporated in gRB1, particularly mSRs on CDRH3 exhibited the largest reduction in neutralization potency. Accordingly, the mere incorporation of mature CDRH3 sequences in germline precursor background could greatly increase virus neutralization potency.

To define the minimum mutations on germline antibody that confer mature b12 comparable neutralization potency, gRBs were assayed for ability to block infection using standard TZM-bl assay against viruses pseudotyped with SF162 and QH0692 envelope sequences. Mirroring the low binding affinity observed in SPR based binding analysis, gRB1 and gRB2 exhibited weak or no virus neutralization (Table 3-1). Given the effect of single amino acid reversions on neutralization potency presented in Chapter 2, the lack of measurable neutralization at concentrations tested by gRB1 and gRB2 is intriguing.

Individual SHM positions on CDRL1 and CDRH3 recorded remarkable reduction in neutralization potency compared to mature b12 when germline sequences are incorporated at V_L-Q27H, V_L-Y32R and V_L-V47L of CDRL1 and V_H-C99S, V_H-G100W, and V_H-G100aD of CDRH3 positions. Greater percent neutralization reductions were observed due to CDRH3 SHM positions when reverted to germline sequence in mSR analysis with average of 50% neutralization observed as mature b12. Similarly, additional mature b12 sequences in CDRH1 and CDRH2 incorporated in germline precursor antibody background were unable to rescue neutralization potency. In mSRs analysis, while these positions did not reduce virus neutralization potency pronouncedly compared to mature b12, they altered epitope binding kinetics. The lack of gain in neutralization by gRB4 with incorporation of extensive mutations on CDRs that affected epitope binding suggested mutations could affect antibody function without altering direct epitope binding (Figure 3-4).

Measurable neutralization potency IC_{50} was observed only when ~half the mutations gained during affinity maturation were incorporated in germline b12 precursor background. Consistent with

increased binding observed by gRB4, an antibody that incorporates extensive mutations neutralized both viruses. Demonstrating the importance of virus neutralization potency and uncoupling from effect of mutations on epitope binding, gRB4 neutralized SF162 and QH0692 with diminished neutralization potency compared to mature b12 (Figure 3-4 and Table 3-1). This is despite the mature b12 comparable gain in binding affinity and kinetics against gp120 monomers.

In addition to the diminished neutralization potency, the antibody neutralization profiles greatly differ between the Tier 1 and Tier 2 viruses. The moderately neutralization resistant (189) Tier 2 virus strain QH0692 is neutralized at ~2.5 fold IC_{50} compared to mature b12 (Figure 3-4 and Table 3-1). In contrast, gRB4 antibody poorly neutralized the neutralization sensitive Tier 1 virus strain SF162 with ~ 40 fold higher IC_{50}. The result argues for uncoupled epitope binding and virus neutralization. Positions important in epitope interactions play role in virus neutralization; additional positions targeted by SHM are also required to achieve b12 comparable neutralization potency. Similarly, the striking difference between neutralization of SF162 and QH0692 imply, in Tier 1 viruses in addition to direct epitope binding other attributes of mature antibodies are necessary to garner greater neutralization potency.

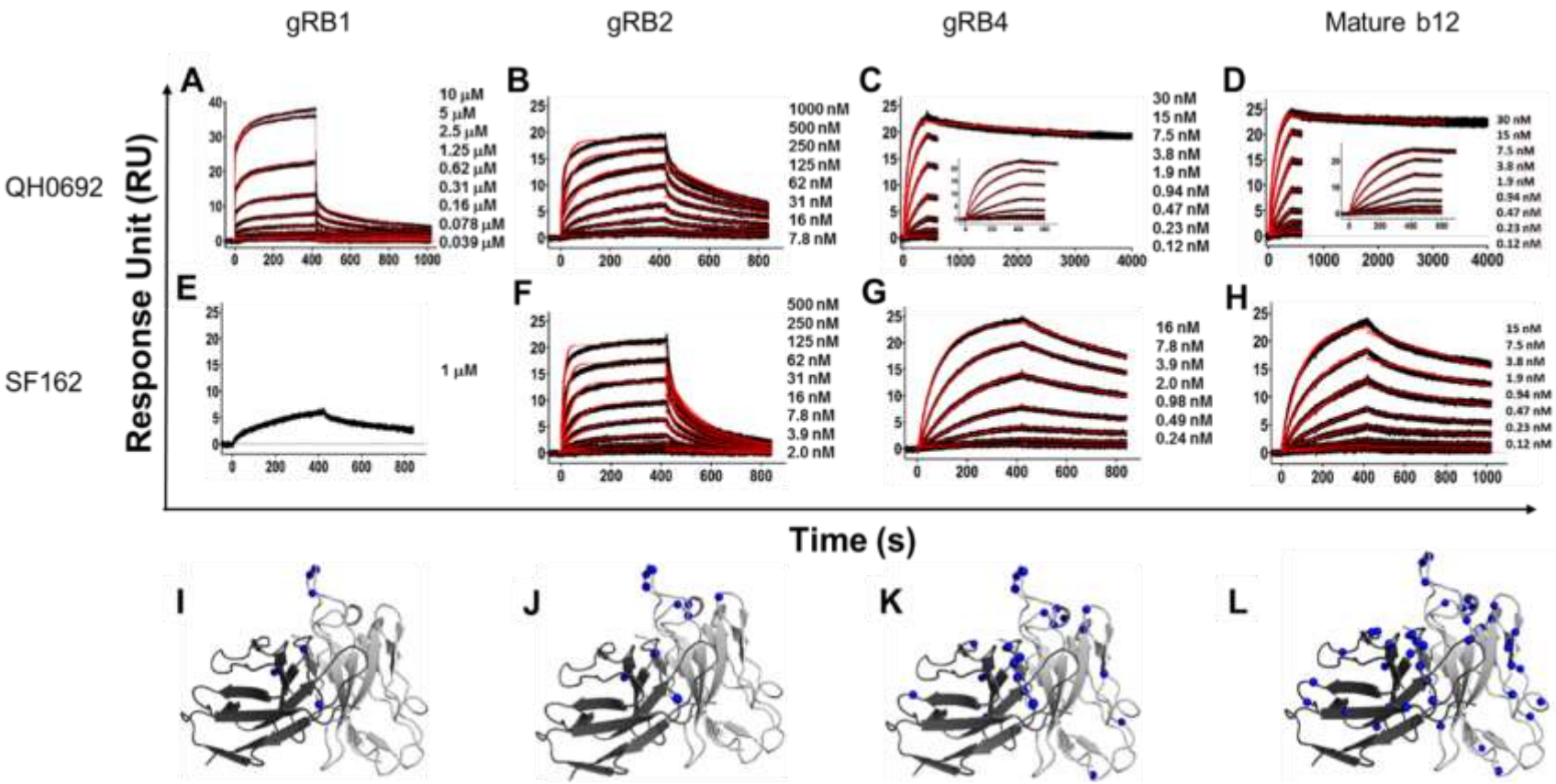

Figure 3-3. SPR binding analysis of gRBs

SPR sensograms of the interaction between antibody c/scFv analytes gRB1 (A and E), gRB2 (B and F), gRB4 (C and G) and mature b12 (D and H) against *in vivo* biotinylated and SA captured gp120 monomeric ligands from Tier 2 clade B virus, QH0692 (A, B, C and D) and Tier 1 clade B virus, SF162 (E, F, G and H). Analytes were injected in duplicate and at random order. Concentration of samples injected and respective serial dilutions are indicated next to respective sensograms. Injecting triplicate 30 nM gRB4/mature b12 samples derived rate of dissociations for the gRB4/ mature b12-QH0692 gp120 monomer interaction. Sensograms for concentration series of gRB4/mature b12 15 nM to 0.12 nM are indicated in the inset (C and D). For gRB1 and SF162 interactions analytes were injected at a single concentration of 1 µM Experimental results are shown as black traces and kinetic 1:1 model fits are shown as red lines. Detailed experimental setups are listed in Table 6-1. Cartoon representation of the antibody variable regions with VH in light gray and VL domain with dark gray shade from b12 structure as in PDB (83) accession code 2NY7 (76) with mutations introduced in gRB1 (I), gRB2 (J), gRB4 (K) and mature b12 (L) indicated with spheres colored blue

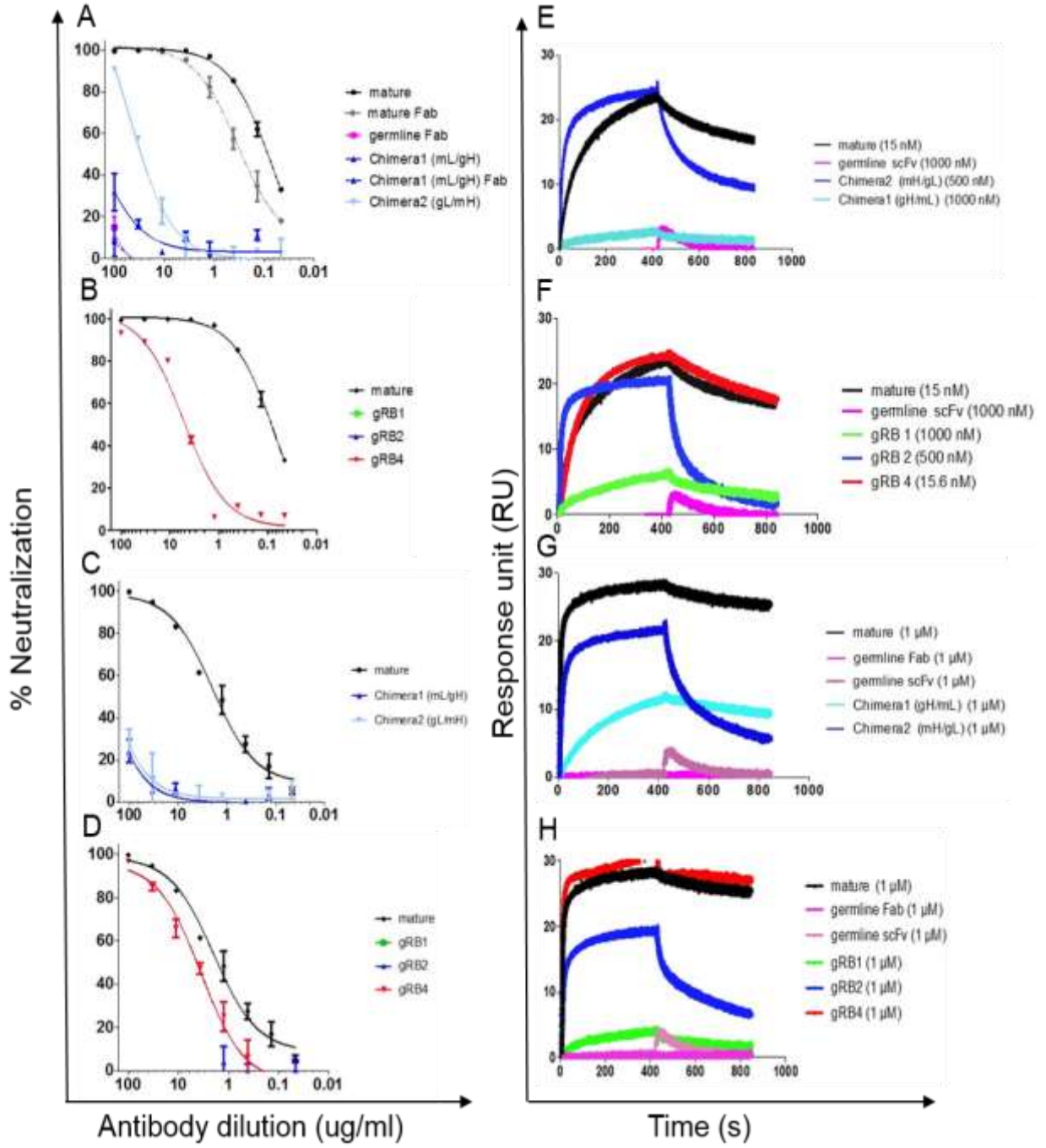

Figure 3-4. Binding and neutralization characterization of germline reversion groups
(A-D) Neutralization potency of b12 mature, Chimera 1(mL/gH) and 2(gL/mH), germline precursor c/scFv (A, C) and gRBs c/scFv (B, D) against SF162 (A, B) and QH0692 (C, D) pseudovirus strains. Neutralization potency and IC50 are determined based on antibody dilutions and corresponding percent neutralization. SPR binding interaction between mature b12, Chimera 1(mL/gH) and 2(gL/mH), germline precursor c/scFv (E, G) and gRBs c/scFv (F, H) against SA captured in vivo biotinylated monomeric gp120 SF162 (F) and QH0692 (H). Curves are colored as mature b12 (black), germline b12 precursor (magenta), gRB1 (green), gRB2 (blue) and gRB4 (red). For interaction with SF162 curves obtained by injecting analytes at concentrations of 15 nM b12 mature, 1 µM b12 germline precursor, 1 µM of gRB1, 0.5 µM of gRB2 and 15.6 nM of gRB4. SPR analyses against QH0692 were performed with all analytes injected at 1µM concentration.

3.3 Discussion

Our results support the theory of SHM based affinity maturation whereby gradual and incremental amino acid changes shape the overall epitope-paratope interactions and subsequent antibody function. As shown using gRB analysis, in addition to mutations implicated in direct epitope contact, reversions to mature sequence at positions distal from the direct epitope binding are required for mature b12 comparable binding. Results presented in this section supporting conclusions presented in Chapter 2, indicate an additive and cooperative effect of mutations on epitope binding.

An important observation from our gRB analysis is the diminished neutralization potency of mutations incorporated in germline precursor antibody against both viruses. Addition of mutations that directly or indirectly affected epitope binding increased binding affinity incrementally to achieve b12 mature comparable binding. However, the mutations were not sufficient for comparable gradual increase in neutralization potency. Mutations on CDRs mainly affect initial antigen recognition and FWR mutations stabilized formed complexes as indicated by slower rates of dissociation. In contrast, mutations dispensable for epitope binding play important role in virus neutralization. This finding argues differential importance of antibody maturation related mutations in antigen binding and neutralization potency.

The discrepancy in neutralization potency is more elaborate when analyzed against tested virus strains. Diminished neutralization potency against the neutralization sensitive SF162, when compared to the moderately neutralization resistant QH0692, implies differential targeting of viruses by such antibodies. Strengthening the argument that extensive mutation in bNAbs that mirrors increased virus sequence evolution is due to prolonged affinity maturation. Although mutations incorporated in gRB4 sufficed to attain b12 comparable binding, the observed differential effect on neutralization potency against the sensitive viruses suggests tailored virus targeting and neutralization mechanisms. Our results argue for the need to aim beyond binding attributes of bNAbs and incorporate importance of mutations in neutralization potency in the design function.

Our findings agree with coevolution of antibody variable domain sequences following persistence of infection accompanied by increased sequence divergence of the viral genome. HIV-1 targeting bNAbs are elicited following iterative and protracted activation and modification of BCRs. Initial stimulation of b12 progenitor naive BCRs is due to interactions (probably weak and avidity mediated interactions) with initial founder viruses that are prone for immune surveillance and targeting (81, 207, 208). While initial naïve BCR interactions results in minor paratope modifications, subsequent virus derived CD4BS sequences, continue the stimulation process. Persistence of bNAbs through the affinity maturation process with considerable sequence divergence from their germline sequence highlights relative conservation of the CD4BS. Metadata analysis of anti-Influenza antibodies (209), revealed the phenomenon of antibody response titer shift with initial diverse response focused overtime resulting in persistence of antibodies targeting conserved regions. Similar immune focusing following HIV vaccination using designed HIV immunogens could result in bNAbs-like response, provided proper sequence divergence and stimulation are outlined. Hence, we conclude that b12-like response following vaccination requires proper selection of initial progenitor BCR stimulating immunogen and subsequent sequential vaccination to simulate antibody evolution.

3.4 Acknowledgment

Results presented in this section are done in collaboration with Christopher Carrico as part of a larger project that assesses biophysical and structural attributes of b12 antibody maturation and epitope design for potential vaccine immunogens. Intellectual discussions with Carrico on gRB designs, neutralization data analysis and experimental planning were instrumental. Della Friend analyzed SPR based biophysical interaction of gRBs and gp120 monomers Friend's patience and attention to detail enabled the data presented herein. I am grateful for her input in the work presented here. Sara Carbonetti in Leo Stamatatos Lab at SeattleBiomed did neutralization studies. The tremendous work done by Sara to generate the complete set of neutralization data that complemented SPR based binding analysis was invaluable for my work.

Chapter 4

4 Antibody maturation and structural flexibility

4.1 Overview

The process of SHM and subsequent antigen dependent selection results in mature antibodies that bind their respective antigens with greater affinity in a lock and key mechanism evolved from the conformational sampling mode of binding suggested for their germline precursor antibodies (130, 131, 133, 136). This antibody maturation process, in addition to achieving greater gain in binding affinity, focuses antibody specificity from polyspecific progenitor germline antibody binding (121, 137, 138). Immune surveillance necessitates naïve B-cells to maintain the capability of recognizing diverse pathogenic particles and hence need only interact weakly. One potential mechanism to ascertain promiscuous interaction is through polyspecificity. In such polyspecific interactions antigen inexperienced BCRs can sacrifice binding affinity to achieve flexibility in their antigen-binding region. While polyspecificity in germline antibodies increases the antibody response against diverse pathogens and broadens the scope of antigen recognition, highly specific and strongly binding antibodies mainly compose the antibody based humoral immune response against foreign particles, particularly viruses. This specific pathogen recognition by mature antibodies is attained through the iterative affinity maturation process. One mechanism to attain increased affinity and specify in mature antibodies following the affinity maturation process is through stabilization of the antigen-combining region. Following SHM, the region is constrained in antigen bound preformed conformation (122, 170).

The role of SHM in paratope rigidification is well studied in antibodies that target small molecules and proteins (134, 169, 210, 211). These studies uncovered subsequent rigidification of CDRs in elicited mature antibodies following affinity maturation compared to their respective germline precursors or intermediate antibodies. Whereas germline precursor antibody CDRs exhibit high degree of flexibility, structural studies show minor conformational change in mature antibody CDRs. Similar

phenomenon is also observed in antibodies that target viral epitopes derived post-acute viral infection (212, 213). Although the body of structural information has grown, antibody structural evolution following chronic viral infections like HIV-1 is at its infancy. Studies that aim at deciphering the molecular architecture of bNAbs that target MPER on HIV Env indicated inherent flexibility in mature antibodies more so than germline precursors that characteristically enabled such antibodies to simultaneously recognize MPER and proximal lipid bilayer (214). Similarly, studies that aimed at structural evolution of CD4Bs antibodies indicated relatively minor change in flexibility due to affinity maturation (215). These observations beg for more research to parse out importance and correlation of antibody maturation and structural rigidification of bNAbs banked as templet for vaccine design.

In addition to the prolonged period from infection to elicitation of anti HIV-1 bNAbs, such antibodies exhibit extensive sequence divergence from their germline precursors. In part due to the high sequence divergence, while highly mutated mature antibodies target their respective antigens with exceptionally high affinity, their germline precursors do not show apparent cognate epitope binding (108, 109, 111). As documented in Chapter 3, sequence divergences not only affects binding strength but also alters neutralization potency and breadth. Given the correlation between sequence divergence and increased potency, we hypothesize that the increased binding affinity and specificity observed in bNAbs is mainly due to structural rigidification of the antigen-binding groove formed by CDRs. In an attempt to define the effect of SHM and extensive sequence divergence on antibody flexibility and specificity, we analyzed structural attributes of mature b12 and germline precursor antibodies. One of the first identified CD4BS targeting anti HIV-1 bNAb, b12, acquired 45 amino acid substitutions in its antigen-binding region following its extensive affinity maturation. Similar to other bNAbs that recognize the CD4BS on HIV-1 Env, the extensive affinity maturation resulted in high affinity despite a lack of epitope recognition by its germline precursor antibody (data presented in chapter 2 and (107)).

As postulated for a matured antibody, b12 exhibits characteristic features of structural rigidification. Structural comparison of unbound, affinity matured full length IgG b12 antibody (180) and gp120 core bound mature b12 Fab (76) bare the apparent rigidity in the mature antibody. Superposition of

the two structures indicate no or little structural flexibility (root mean square deviation (RMSD) Cα of 1.3Å) in the overall antigen binding fragment (Fv). Consistent with preformed bound conformation stabilization of the antigen binding groove, in three dimensional structural model comparison, CDRs exhibit no apparent flexibility irrespective of the crystallographic condition or binding partners (Figure 4-1B). Particularly, the extended arm of CDRH3 that plays significant role in probing the CD4 binding region on gp120 does not show noticeable flexibility. In accordance with affinity based antibody maturation paradigm, the apparent rigidification of overall Fv conformation in general and CDRs in particular imply greater specificity and increased binding affinity due to rigidification of b12 antibody during its long protracted affinity maturation.

The observed structural rigidification is with the presence of a hydrophobic Trp100 residue at the tip of CDRH3 which makes important contact with a hydrophobic patch on gp120 near the CD4 binding site (179). In the unbound b12 IgG structure (180), this residue is solvent exposed and maintains a side chain orientation resulting in entropically unfavorable conformation. Irrespective of the presence of such hydrophobic residue at the apex of CDRH3, the three dimensional crystallographic structures show extraordinary rigidification in the CDRH3 loop region (Figure 4-1A). Previous mutagenesis based studies attributed the rigidification in mature b12 CDRH3 to the presence of highly charged amino acids proximal to Trp100. Minor variations in rotamer conformation of Trp100 on CDRH3 are also observed dependent on binding partners associated with mature b12 (either gp120 or designed peptide). Comparisons of side chain rotomers near CDRH3 implicate relatively larger flexibility on Trp100 when compared to other amino acids positions nearby. In the unbound mature b12 structure, the tryptophan indole ring is swung outwards by ~90^{o} in comparison to bound structures.

To investigate the role of antibody variable region rigidification in increasing antigen specificity and binding affinity, we set forth to determine the structural model of b12 germline precursor antibody. Structural analysis of germline, proxy-intermediates and mature antibodies could elucidate the role of extensive affinity maturation in increased affinity, specificity and neutralization potency.

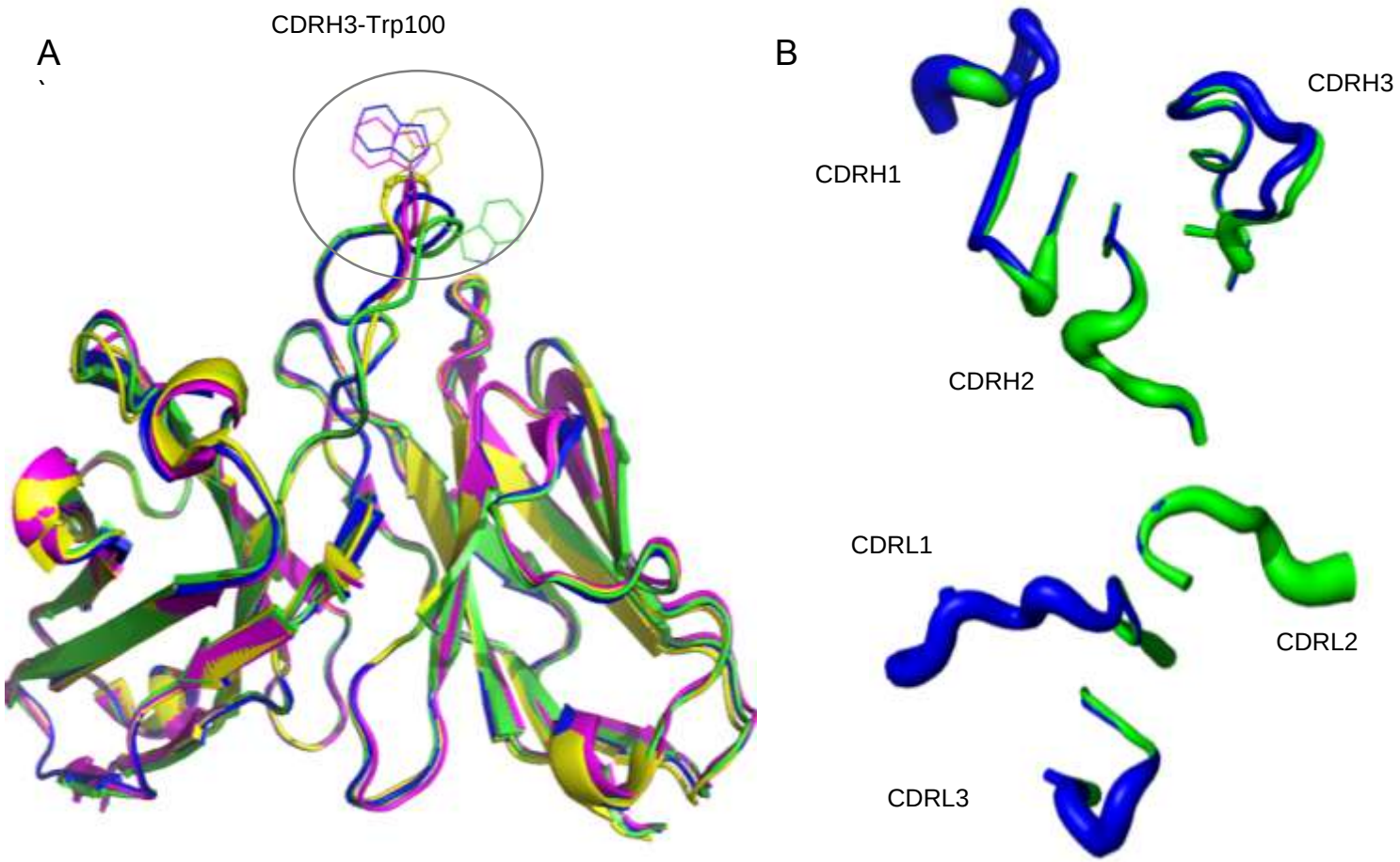

Figure 4-1. Structural analysis of b12 rigidification
A Mature antibody b12 VL domain superposition in ribbon representation. Core gp120 bound mature b12 (PDB (83) accession code 2NY7 (76),) are colored blue, unbound mature b12 (PDB accession code 1HZH(180)) represented in green colored ribbon representation while structure of peptide bound matured b12 (PDB accession code 1N0X(159)) and Epitoe scaffold bound b12 Fab (PDB accession code 3RU8 (160)) are represented with yellow and magenta colored ribbons. Regions of CDRH3 and Trp100 are indicated with dark circle. Trp100 side chain is presented with stick representation. B) CDR flexibility is assessed using B-factors as surrogate for flexibility. CDRs are presented using B-factor putty with CDRs from bound b12 mature are colored blue while unbound mature b12 CDRs colored green. Structural analysis are done using Pymol (183).

4.2 Results

4.2.1 C/scFvs as crystallization reagents

To examine the conformational attributes of germline VL and VH both as germline VL-VH pairs and irrespective of their corresponding germline VL or VH chains, different b12 constructs that address various aspects of the germline antibody were used as crystallization reagents. As the aim of our crystallographic endeavor is to examine relative structural flexibility in the antibody Fv domains, constructs were designed as c/scFvs. VL and VH domains were designed as intact germline precursor and chimeric assemblies paired with respective mature VL and VH domains. Constructs designed and expressed as c/scFv for crystallographic studies were germline b12, Chimera 1 (mL/gH), Chimera 2 (gL/mH). In additions germline b12 c/scFv constructs designed with blocks of mutations discussed in the earlier chapter were also used as crystallization reagents.

Constructs were engineered as cleavable polypeptides and expressed using mammalian lentiviral transduction system. Linker peptide that connects VL-VH domains resolved using Thrombin cleavages of c/scFvs. Proteolytic cleavage resolved mono- and di-body molecules. As discussed previously (186), expression of antibody VL and VH domains as single polypeptide result in potential mixture of mono- and di-body molecules, which, according to our previous crystallography experience, could hamper proper protein crystal formation. Hence engineering a cleavable short linker as described in the Methods section facilitates purification of monobody molecules suitable as crystallographic reagents.

One potential pitfall of using c/scFv constructs in protein crystallography is the relative weak interface between light and heavy chains domains. Compared to full antibody or Fab fragments, where the light and heavy chain dimer interface is stabilized by disulfide bridges in addition to hydrophobic interactions, one could argue c/scFvs are less stable due to a weaker dimerization interface (83). This weak dimerization interface, formed mainly through hydrophobic interactions and some electrostatic molecular bonds, can potentially be disrupted under non-physiological crystallization conditions. The

presence of ample antibody variable domain structures using c/scFvs from our group and others justify the use of these constructs as crystallization reagents.

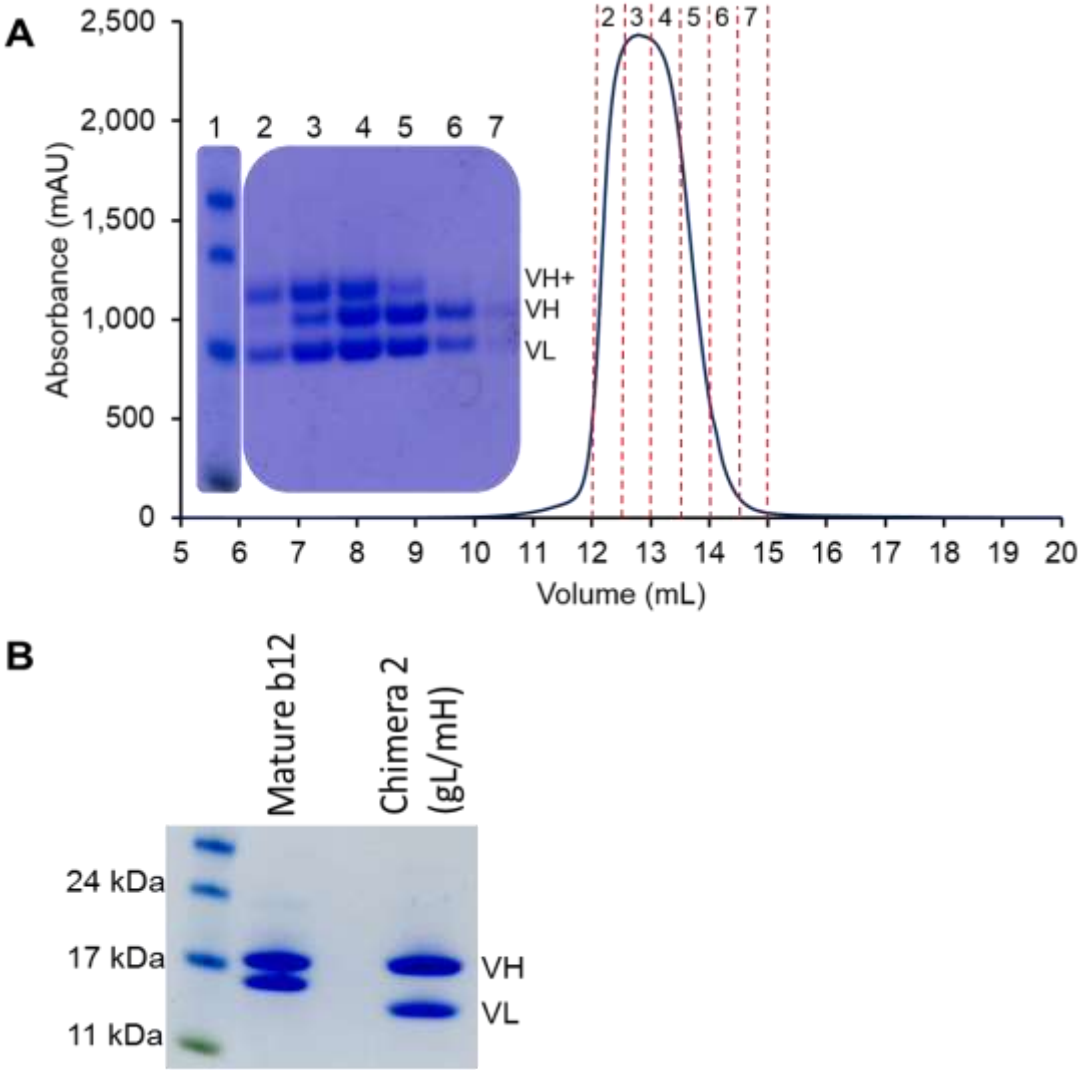

Figure 4-2. Purification of b12 c/scFv constructs
A) Mature b12 c/scFv run on size exclusion chromatography. SEC trace and peak fractions indicated with numbers 2-7. Peak fractions run on SDS_PAGE gel, where Lane 1 indicates molecular weight size marker while lanes 2-7 containing corresponding SEC fraction numbers. Higher molecular weight VH chain (VH+), VH and VL chains are indicated B) C/scFv constructs expressed in bacterial expression system as insoluble inclusion body and *in vitro* refolded. SDS-PAGE analyses of mature b12 and Chimera 2 (gL/mH) with respective VH and VL bands are indicated.

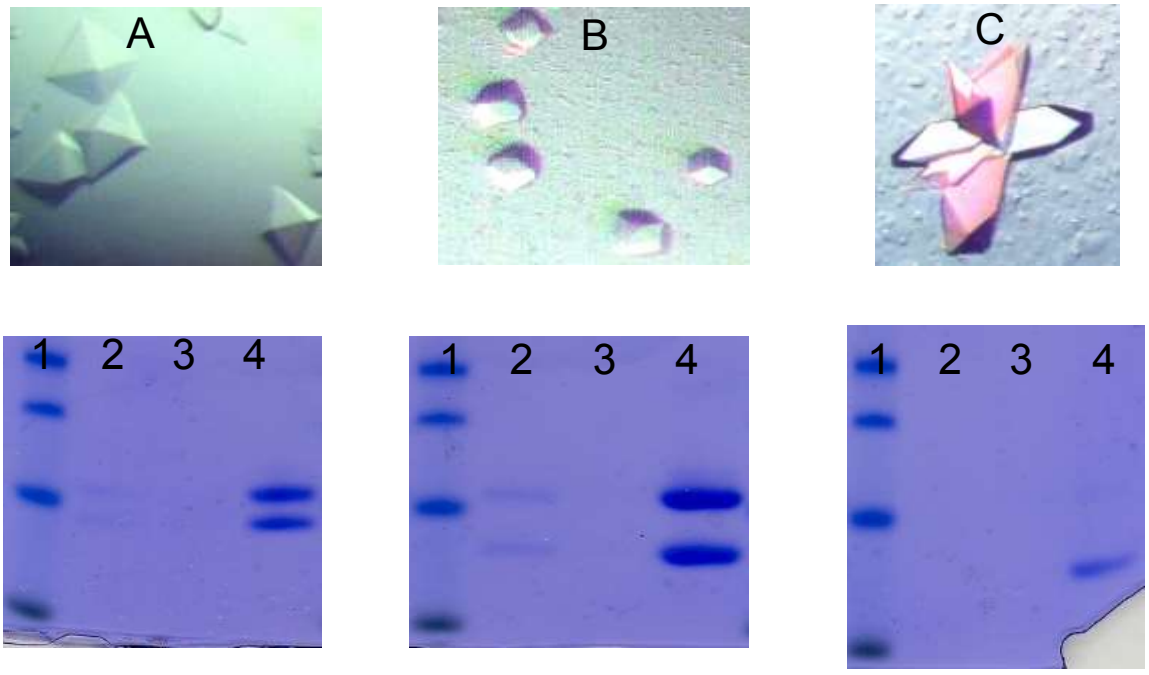

Figure 4-3. Characterization of various b12 c/scFv crystals
A) upper panel, image of representative mature b12 c/scFv crystals; lower panel, SDS-PAGE analysis of b12 mature c/scFv crystals where lane 1 contain protein molecular weight markers, lane 2 first crystal wash with mother liquor, lane 3 second crystal wash with mother liquor and lane 4 contain crystals dissolved in water. B) upper panel, image of representative Chimera 2 (gL/mH) c/scFv crystals; lower panel, SDS_PAGE analysis of Chimera 2 (gL/mH) c/scFv crystals with lane assignment as in A. C) upper panel, image of representative germline b12 c/scFv crystals; lower panel, SDS_PAGE analysis of germline b12) c/scFv crystals with lane assignment as in A

Initial expression of c/scFvs indicated the presence of a higher molecular weight 'contaminating' mixture. Given the architecture of our expression construct, we hypothesized the 'contaminating' fragment to be the result of either (1) incomplete thrombin cleavage, (2) aberrantly maintained secretion signal peptide or (3) Stochastic stop codon runoff during the translation process. To pinpoint the causative factor for the presence of a 'contaminating' protein fragment and decipher the three potential factors described above, we analyzed protein samples using comparative SDS-PAGE and SEC analysis.

Partial protease reaction resulting in cleavage at either thrombin sites designed to excise the VL-VH linker potentially results in fragments containing linker sequences on either VL or VH chains. If one of the two engineered thrombin cleavage sites are not readily protease accessible, one can assume the presence of VL C-terminus attached linker or VH N-terminus attached linker peptide resulting in apparent higher molecular weight fragment. However, protein characterization of pre- and post- thrombin cleavage

Table 4-1. Data collection and refinement statistics

	Germline b12 V_L	gRB2 V_L	Chimera 2 (gL/mH)
Data collection			
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_13$
Cell dimensions			
a, *b*, *c* (Å)	49.16, 98.71, 102.65	44.60, 71.62, 73.23	167.66, 167.66, 167.66
α, β, γ (°)	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00
Resolution (Å)	150.0-1.90 (1.93-1.90)	50.00-2.40 (2.44-2.40)	50.00-2.85 (2.93-2.85)
R_{sym} or R_{merge}	0.077 (0.327)	0.093 (0.505)	0.094 (0.477)
$I / \sigma I$	22.6 (3.6)	22.1 (4.6)	21.6 (7.66)
Completeness (%)	100.0 (100.0)	99.9 (100.0)	99 (100.0)
Redundancy	5.8 (5.9)	5.3 (5.5)	9.0 (11.1)
Refinement			
Resolution (Å)	102.6-1.90 (1.95-1.90)	44.6-1.90 (1.97-1.90)	40.55-2.86 (2.93-2.86)
No. reflections	37,898(2,670)	18,661 (1,119)	34,540 (2,518)
R_{work} / R_{free}	0.175/0.208	0.179/0.230	0.258/0.291
No. non-hydrogen atoms			
Protein	3,387	3,320	8,095
Ligand/ion	101	147	7
Water	237	118	98
R.m.s. deviations			
Bond lengths (Å)	0.015	0.011	0.009
Bond angles (°)	1.697	0.959	0.234
Ramachandran plot statistics (MolProbity)			
MolProbity score	1.57	1.16	2.89
Residues in most favored regions (%)	96.85	95.71	92.68
Residues in disallowed regions (%)	0.0	0	1.31
Est. coordinate error (max. likelihood ESUc) (Å)	0.083	0.199	0.515

using SDS-PAGE and Western blot showed presence of the 'contaminating' mixture pre cleavage (data not shown).

Expression construct engineering dictates improperly excised secretion signal increase the molecule weight of VL chain with no apparent effect on VH chain. However, in post cleavage samples, the 'contaminant' fragment is deduced to arise due to VH domain (Figure 4-2). Sequence analysis of the

expression vector used in cloning lentiviral expression constructs indicated the presence of encrypted stop codon a few nucleotides past the engineered stop codon. Given this and minimal protein expression checks in transformed mammalian expression systems, it is plausible to attribute the aberrant expression of 'contaminant' fragment due to stop codon runoff.

To establish the inherent instability in c/scFvs used, mature b12 and Chimera 2 (gL/mH) amino acid sequences used were codon optimized for bacterial expression system. We expressed c/scFvs as inclusion bodies (IB) and *in vitro* refolded following protocols described previously (216). Subsequent Thrombin digestion and SEC and SDS-PAGE analysis indicated the absence of 'contaminating' fragment form the VH chain (Figure 4-2B).

To assess the effect of adding additional sequence to the C-terminus in crystal formation and gp120 binding, I used mature b12 constructs as a surrogate. SEC fractionations of cleaved b12 mature c/scFv were performed to isolate scFv fractions with VH domain containing 'contaminating' sequence and scFv fractions without the 'contaminating' sequence (Figure 4-2). According to SEC based binding analysis, monomeric gp120 bound mature scFv irrespective of the additional fragment suggesting no apparent effect on epitope binding due to the additional fragment (data not shown). This is consistent with structural elucidation of binding interface of core gp120 and mature b12 (76) where N- and C- terminus of Fvs do not possess direct and significant role in the interaction. In contrast, the effects of 'contaminating' fragment in c/scFv crystallization were pronounced. While b12 mature c/scFv readily crystallized in an array of crystallization conditions, crystallization attempts using the higher molecular weight 'contaminant' fragment VH domain containing c/scFv did not result in noticeable crystal formation. This suggests the presence of some proportion of c/scFvs with the additional stop codon runoff fragment can poison crystal formation.

4.2.2 Crystal structure of b12 germline light chain domains

Crystal structure of b12 germline VL, both by itself and as chimeric c/scFv paired with mature VH, were determined from germline b12, gRB2 and Chimera 2 (gL/mH) c/scFvs at resolution ranges of

1.9 Å, 2.4 Å and 2.8 Å respectively (Table 4-1). Initial sitting-drop crystallization screening for crystals of germline b12 and Chimera 2 (gL/mH) c/scFv resulted in potential crystallization conditions while crystals of Chimera 1 (mL/gH) c/scFvs were not observed in the various conditions tested. Similarly, germline c/scFv constructs designed with various reversions to mature amino sequence at predetermined positions (detailed in chapter 3) were also used as crystallization reagents. Crystals observed in screening trays containing gRB2 c/scFvs. Initial screening conditions optimized in hanging-drop plates at room temperature.

Diffraction data collection and analysis described in the Methods section. Diffraction data for germline b12 merged and scaled to crystallographic space group p212121 in orthorhombic unit cell. Attempts to phase integrated and scaled b12 germline diffraction data using molecular replacement (MR) with variable domain of mature b12 (3RU8 (160)) as initial sub-structural search models yielded solutions with minimal figure of merit statistics indicating poor correlation between experimental data and models used for phase determination. Subsequent phasing efforts using MR with mature VL domain ensembles resulted in four VL molecules per asymmetric unit. Upon further crystal packing analysis, we determined that the asymmetric unit was composed of VL chain molecules and crystal forming precipitants, ions and solvent molecules. We confirmed presence of only VL chains in germline c/scFv crystals as compared to mature b12 and Chimera 2 c/scFv (Figure 4-3). Individual crystals were isolated from the mother liquor, washed with well solution and dissolved using water. SDS-Page analysis bare the presence of intact VH and VL chains in mature b12 and Chimera 2 (gL/mH) while only VL chain is observed in crystals from germline precursor crystals.

Similar crystallographic analysis done on diffraction data collected from gRB2 crystals. Although ten positions on gRB2, mainly on CDR, are reverted to mature b12 sequence (detailed in chapter 3), crystals of gRB2 exhibited features observed in germline b12. Diffraction data merged as p212121 crystallographic space group and the asymmetric unit is composed solely of germline VL molecules. Further model building and refinement confirmed the presence of four VL chains in the crystal asymmetric unit forming two dimers mediated by a PEG molecule.

Three-dimensional structures of Chimera 2 (gL/mH) were determined from crystals grown in crystallization condition containing 2.0 M NaCl, 0.1 M Citric acid pH 4.0. Unlike germline b12 c/scFv crystals, crystals of Chimera 2 (gL/mH) contain both VL and VH chains in unit cells. Data analysis and subsequent solvent content calculation using Matthews coefficient (217, 218) revealed the presence of five molecules per asymmetric unit in a cubic unit cell and space group of $p2_13$. Analysis using TRUNCATE (219) in ccp4i (220) indicated the diffraction data exhibit merohedral twining with twin fraction (α) of 0.35 and twining operator of -K, -H, -L. Model building was done using COOT (221) followed by iterative refinement using REFMAC 5 (222). Twin refinement was implemented on the final refined model. As can be inferred from refinement statistics, although four molecules of gL/mH were built with good model agreement to experimental electron density data, the fifth molecule was highly disordered which hampered proper placement of solvent molecules and loop regions at or near this molecule. Proper placement of molecules and refinement were assessed using PROCHECK (223) and Molprobity (224).

To investigate structural flexibility of b12 germline precursor prior to affinity maturation, structural analyses were done on the three dimensional structures of germline VL domains determined by X-ray crystallography. Superposition of various germline VL structures revealed the presence of remarkable three-dimensional similarity between the different germline VL structures. VL molecules showed high structural agreement with Cα RMSD of 0.48 Å (225). In agreement with antibody Ig folding, and contrary to posited flexible germline precursor antibodies that ensure polyspecificity, the overall antibody variable domain framework region is structurally rigid. The limited flexibility observed is likely due to crystal contacts and crystallization conditions.

In depth look at the CDRL regions show minor conformational differences among the various germline b12 VL domains. Conformational flexibility, although minor, are observed on CDRL1 with Cα deviation (RMSD) of 1.4 Å. Loop regions of CDRL1 sample a conformational distance span ~4 Å. Mutations in this region show effect on binding while the effect on overall structural flexibility is minor and unremarkable. Compared to the structural rigidity observed in mature CDRL1 bound and unbound

structures (Cα RMSD of 0.22 Å), the region is relatively flexible with Cα RMSD of 2 Å when compared to bound mature b12 CDRL1. The observed flexibility is maintained despite altering three SHM positions to mature sequence (Q27H, Y32R and V47L) suggesting, although individually the positions contribute in epitope binding (chapter 2 and (193)), they play minor role in CDRL1 conformational rigidity observed in mature b12 antibody.

Similar structural features were observed on other CDRs with CDRL2 exhibiting high structural rigidity with Cα RMSD of 0.29 Å and Cα RMSD of 0.34 Å between bound and unbound mature VL CDRL2 and mature unbound and germline CDRL2 respectively. This argues that CDRL2 is structurally rigid irrespective of antibody stage of maturation. From structural analysis based on the gp120 core bound b12 crystallographic structure (76), it is evident that the region plays an important role in antibody fold while contributing minimally to epitope binding. In contrast, CDRL3 shows the highest overall flexibility observed in germline VL with Cα RMSD of 1.6 Å, sampling conformations covering ~6 Å distances (Figure 4-4). Comparison with mature b12 CDRL3 revealed, similar to CDRL1 the region is highly rigid following antibody maturation (Cα RMSD of 0.16 Å) while is relatively flexible when compared to mature CDRL1 (Cα RMSD of 1.27 Å).

To analyze if affinity maturation resulted in rigidification of CDRs in antibodies elicited following chronic viral infection, we compared structures of affinity matured b12 VL domains with germline structures. Even through VL chain of b12 antibody accumulated 21 amino acid mutations over its germline precursor sequence through its extensive affinity maturation process, the overall structure of both mature and germline b12 antibody variable domains exhibit minor structural differences with Cα RMSD of 1.7 Å (Figure 4-4). Given the assumption that germline antibody polyspecificity is attained through structural flexibility of CDRs, we compared mature b12 CDRs with its respective germline antibody. In-depth look at the antigen binding regions particularly CDRL1 and CDRL3, regions that show no detectable flexibility between the bound and unbound mature b12 structures, show significant conformational flexibility when compared to the germline structure (Figure 4-5A). This observed flexibility is greater or comparable to previously reported structural flexibility in NIH 45-46, a family of

CD4BS bNAbs (215) (Figure 4-5B). Given the observed extraordinary rigidity in the mature b12 antibody, irrespective of epitope interaction, the observed minor flexibility in the germline Fv and particularly VL CDRs imply potential plasticity and hence polyspecificity in germline b12 precursor.

Using Chimera 2 (gL/mH) as a surrogate for full antibody variable domain, we investigated VL-VH interface and inter-domain flexibility. Structural analysis of Chimera 2 (gL/mH) Fv domains using superposition of mature VH domains demonstrated high structural agreement among not only mature VH domains but mature and germline precursor VL Ig domains (Figure 4-5). The dimerization interface in both mature and Chimera 2 (gL/mH) b12 c/scFv variable domains are mediated mainly through hydrophobic interactions between aromatic head groups and other hydrophobic side chains. VL residues that contributed mainly in the VL-VH interface formation are Tyr 36, His 39, Pro 44, His 49, Leu 46, Tyr 87, Tyr 88, Gln 89, Tyr 91, Tyr 96, Phe 98. Consistent with preferential exclusion of interface amino acid positions in SHM, VL interface forming residues are preserved in the affinity maturation process with only two substitutions from germline to matured antibody sequences (H39Q, H49Y).

In-depth analysis and further comparison of superposed Chimera 2 (gL/mH) structures and mature b12 using Pymol (183) reveal subtle difference between germline and mature VL domains. Aligning Chimera 2 (gL/mH) and mature b12 crystallographic models on their common mature VH domain expose the presence of slight structural differences at the base of light chain variable domains. The germline VL domain in Chimera 2 (gL/mH) is displaced outwards by ~2 Å as compared to its mature homolog that widens the VL-VH interface in Chimera 2. Given this minor change in the VL-VH interface, we hypothesize potential flexibility in the dimerization interface implying relative overall flexibility of the germline Fv domain. The lack of structural rigidity in the germline precursor is consistent with polyspecific germline antibody where subsequent affinity maturation result in Fv rigidification and hence mature antibody specificity and high affinity.

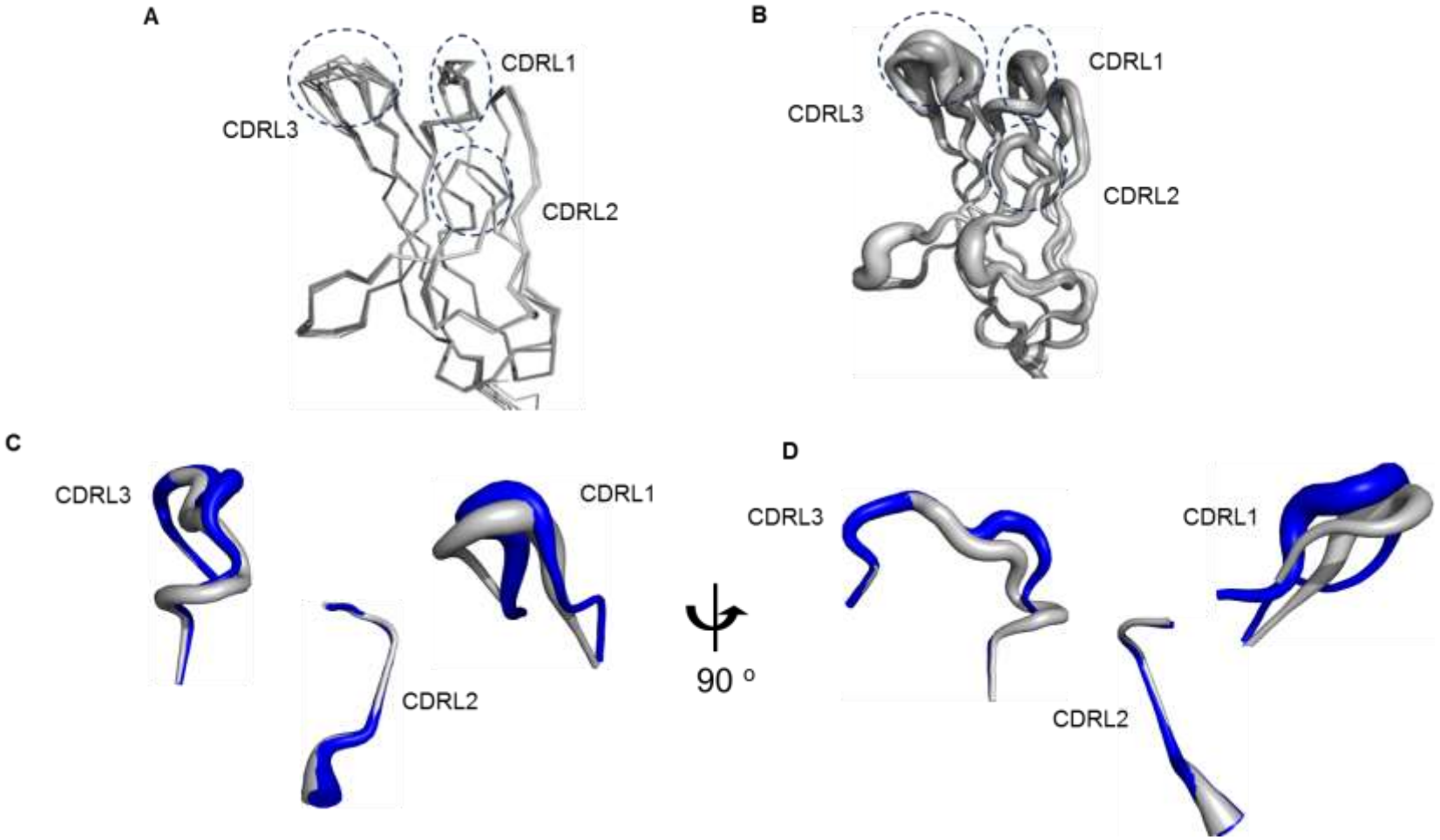

Figure 4-4. Structural analysis of germline b12 VL flexibility
A) Superposition of b12 germline precursor V_L domains from V_L dimer (PDB accession code 4QXN), gRB2 (PDB accession code 4QXY), and Chimera 2 (gL/mH) (not deposited), structures in gray shade ribbon representation B) Structural flexibility of chains presented in (A) are assessed using B-factor putty. CDRs are numbered and indicated with black dashed circles. A closer look at individual CDRs and comparison to bound mature antibody at 90 ° Y axis rotation are presented in C and D. Antigen bound mature b12 CDRS are colored blue.

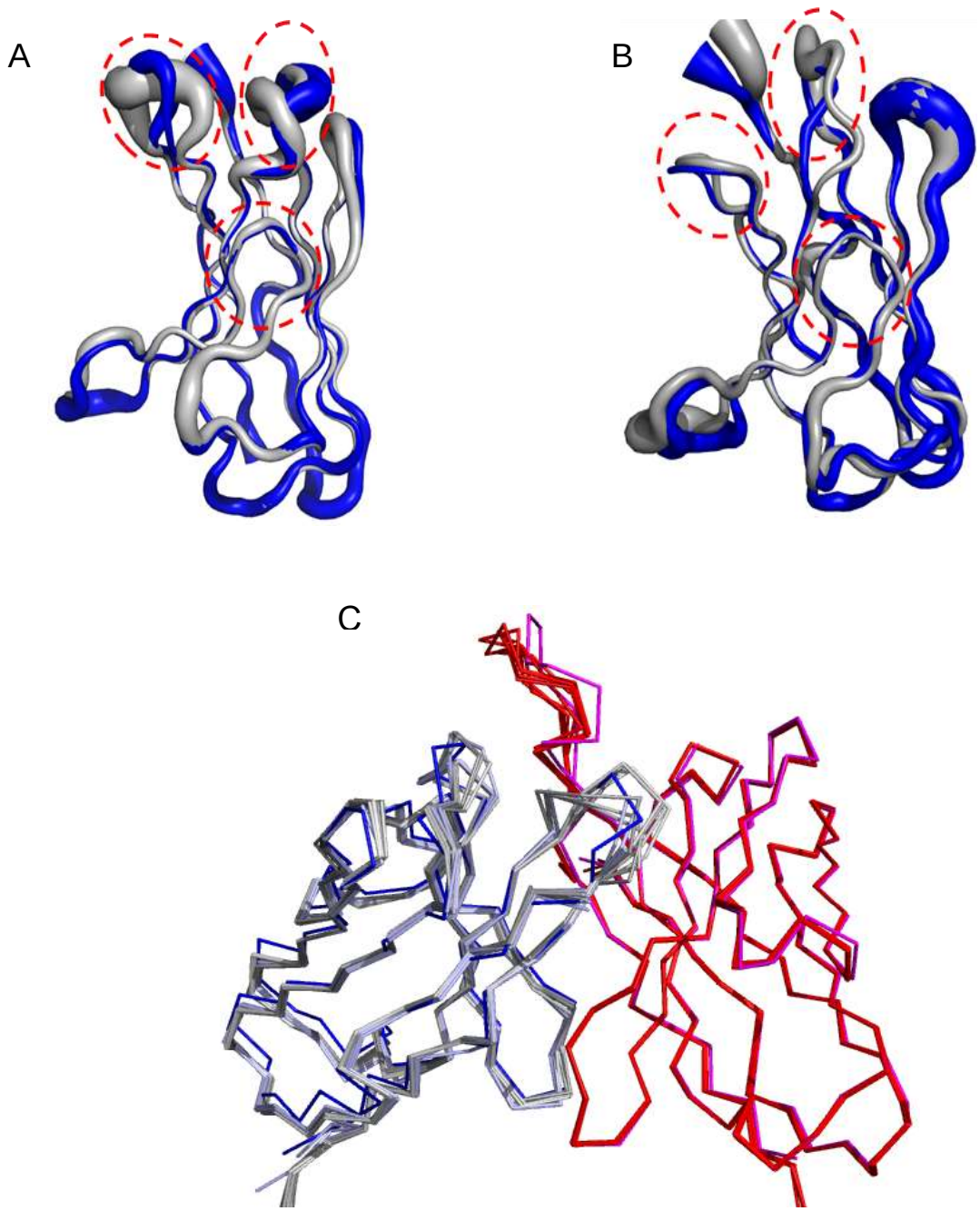

Figure 4-5. Structural comparison between germline and mature b12 VL
A) Analysis of structural flexibility in b12 VL domains between b12 mature (PDB accession code 2NY7 (76)) and its germline precursor (PDB accession code 4QXN and 4QXY). Germline precursor VL domains colored gray while mature VL domain colored blue. (B) Effect of maturation on structural rigidity of CD4 binding site VL domains from related bNAb structures. Structural superposition of mature NIH45-46 mature VL (PDB accession code 3U7W (226)) colored blue against germline precursor of NIH45-46 VL (PDB accession code 4JDV (215)) colored gray. VL chains in A and B are presented as B-factor puttys and CDRs are numbered and indicated with red dotted circles. C) Superposition of Chimera 2 (gL/mH) with mature b12 structure (PDB accession code 2NY7 (76)) aligned on VH domains. Mature b12 VH domain from PDB accession code 2NY7 colored magenta and VL domain colored blue, mature VH domain from Chimera 2 (gL/mH) colored red and germline precursor VL domains colored gray.

4.2.3 Conformational Sampling in germline b12 light chain CDR1 and CDR3

Studies have implicated SHM in evolution of germline antibodies from their plasticity due to conformational sampling mode of binding to the rigid antigen-binding region observed in mature antibodies that interact with specific antigens through lock-and-key mechanism of binding (121, 137, 138). To determine presence of conformational sampling in b12 germline antibody, we analyzed structural features of germline b12 VL domain CDR regions. This analysis revealed striking conformational differences among the various molecules. While all molecules maintain similar conformation in their CDR2 region, they sampled different conformations in their CDR1 and CDR3 regions (Figure 4-6). While continuums of conformations are observed in these regions, two predominant conformations are recognized. These conformational changes sample distances of ~3.5 Å in CDR1 and ~5.5 Å in CDR3.

To assess importance of SHM in antibody conformational stabilization, particularly CDR rigidification, we compared light chain CDR structures of germline and mature b12 structures. In mature and germline VL domain structures, CDR2 maintained the same conformation. In constrast, while under

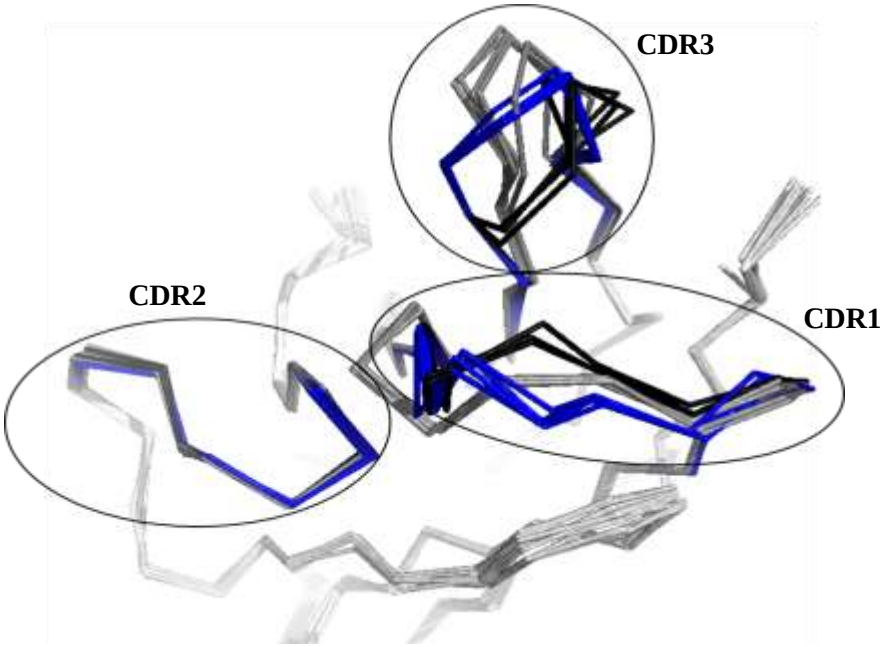

Figure 4-6. conformational sampling in b12 light chain CDRs
Close-up view of b12 light chain from the CDR regions down through Ribbon representation of σ-carbon atoms mature b12 (PDB accession code 2NY7 (76)) in blue and germline b12 (PDB accession code 4QXN and 4QNY) in gray and back. CDRs are indicated with black circles and numbered accordingly.

various crystallization and bound antigen environments mature b12 light chain CDRs maintain similar conformations, germline b12 light chain CDR1 and CDR3 exhibit conformational deviation from their respective mature regions. These conformational differences are predominantly due to deviations at or near positions altered due to SHM. These results implicate roe of SHM in regasification of the antigen-binding domain in b12.

4.2.4 Germline b12 light chain dimers

We characterized the unexpected b12 germline precursor VL dimer structures analyzing their crystallographic attributes. In crystallization experiments with b12 germline VH sequence containing samples, four VL molecules per asymmetric unit formed homo-dimeric crystal packing in both germline b12 and gRB2 crystal structures. Analyzing the VL homo-dimers and comparing to classical antibody Fv VL-VH structures indicate, unlike the mature b12 antibody VH-VL heterodimers, the presence of a Poly Ethylene Glycol (PEG) composed of 6-10 ethylene glycol repeats facilitated the dimerization in VL homo-dimers. VL dyads forming homo-dimer are related by pseudo two fold axis of 179^{o} non crystallographic rotation (Figure 4-7 A,C). This pseudo two-fold axis also passes through the dimerization interface. The dimer interface is wider by as much as 3Å when compared to canonical antibody FV domains and hence the two VL dimer dyads form an opening ‘canal’ (Figure 4-7 A,B) encompassed by the proximate CDRL3 regions and similar arrangement by loop regions of FWR2.

Even though a physiologically irrelevant alternate PEG molecule mediates dimerization, the homo-dimer orientation of VL molecules did not alter overall fold of b12 germline precursor variable domains. Individual VL molecules preserved overall Ig fold where the VL FWRs formed β- pleated sheets linked by two disulfide bridges between Cys 23 and Cys 88. Loop regions that comprise CDRs maintain predicted Ig variable domain conformations (227, 228). Other than observed minor structural flexibility in CDRLs, despite the close proximity between two CDRL3 regions and other crystal contacts, regions of the antigen combining sites on VL exhibit remarkable attributes transferable among structures solved from the three different conditions.

In a VL homo-dimer, dimer facilitating PEG molecule abuts two VL chains. The pseudo two fold rotational axis discussed above passes in-plane with PEG molecule that also occupy the 'canal' formed by the wider VL-VL interface. Hydrophobic interactions mainly facilitate protein-PEG interactions that enable VL dimer formation. The protein-protein dimerization component constitutes only ~7% of the individual VL chain surface area. Analysis of the molecular interface between PEG and VL molecules using surface electrostatics reveals the presence of highly hydrophobic patch of residues (Figure 4-7). The main contributors in this hydrophobic interaction are residues Ala 34, Tyr 36, Pro 44, Lys 46, Tyr 49, Tyr 87, Tyr 91, Tyr 96, and Phe 98 with aromatic rings of Tyr and Phe residues engulfing the PEG molecule (Figure 4-7C). As inferred earlier, these residues are fairly conserved during the affinity maturation process with the only substitution occurred at position 49 (Y49H). The residues also are conserved in other Ig variable domain structures where they serve in forming variable domain antibody interfaces. The interaction between PEG and VL domains is further stabilized by hydrogen bond formation between amide head groups of Q89 from both dimer partner VL chains and ether moieties on the PEG molecule (Figure 4-7).

Structural alignment and comparison of VL domains were used to investigate biophysical attributes of VL regions that potentially facilitate dimerization. In overall fold and dimer arrangement, the VL dimers resemble antibody variable regions from VL-VH heterodimers. However, comparisons of structural orientations of the dimer partners to mature b12 c/scFv (76) indicate wider dimerization interface with ~3 Å gap difference compared to mature VL-VH domains (Figure 4-7A). This increased gap enables the formation of 'canal' feature in b12 germline precursor VL-VL dimers, which accommodates the dimer mediating PEG molecule. Furthermore the dimeric angular orientation between VL-VL dimers in b12 germline precursor orient the molecules in pseudo two fold orientation at 179^{o} while this angular relation between VL-VH chains in mature b12 is 103^{o}. Despite the pseudo two-fold dimeric orientation, presence of wider gap in germline light chain dimers and dimerization facilitating PEG molecules, comparable amino acid positions are involved in hydrophobic interactions that facilitate dimerization interface formation in both VL-VL homo-dimers and VL-VH heterodimers.

In vivo expressed and secreted monoclonal free light chains have been previously reported in immunoproliferative disorders like Fanconi's syndrome (229). Structural elucidation of such free light chain protein (Bence Jones protein) elucidate the propensity of free VL domains to form pseudo-dimers, similar to what is observed in germline VL, through a pseudo two fold axis (229-231). Hence, exploring similarity and differences between the b12 germline VL dimers structure and Bence Jones protein dimers are used to further parse out features of VL-VL dimer formation.

There exist notable differences between the two forms form free light chain dimer structures signifying the unusual nature of b12 germline VL structures described here. In both free VL reported dimerization, the domains maintain overall Ig fold while unlike the b12 germline VL dimers, Bence Jones protein dimers resemble Ig VL-VH complexes with the dimerization interface mediated by residues from the protein chains only; not due to the presence of an artificial crystallization facilitating molecules. In addition, similar to mature b12 VL-VH interface, the Bence Jones protein dyads are related by a pseudo two fold axis of ~109^{o}. This natural Fv VL-VH resembling interface formation hinders formation of the wider 'canal' observed in b12 germline VL dimers. These main differences point out that, although free VL domains are more stable in pseudo-dimer form, the dimerization observed in germline b12 is non-physiological and born out of the vagaries of crystallization experimentations where the introduction of physiologically irrelevant molecules to facilities crystal formation potentially distorts physiologically relevant states of protein molecules. Even though the physiological relevance of the VL dimer is negligible, the individual VL domains still provide important structural information regarding b2 germline VL domains.

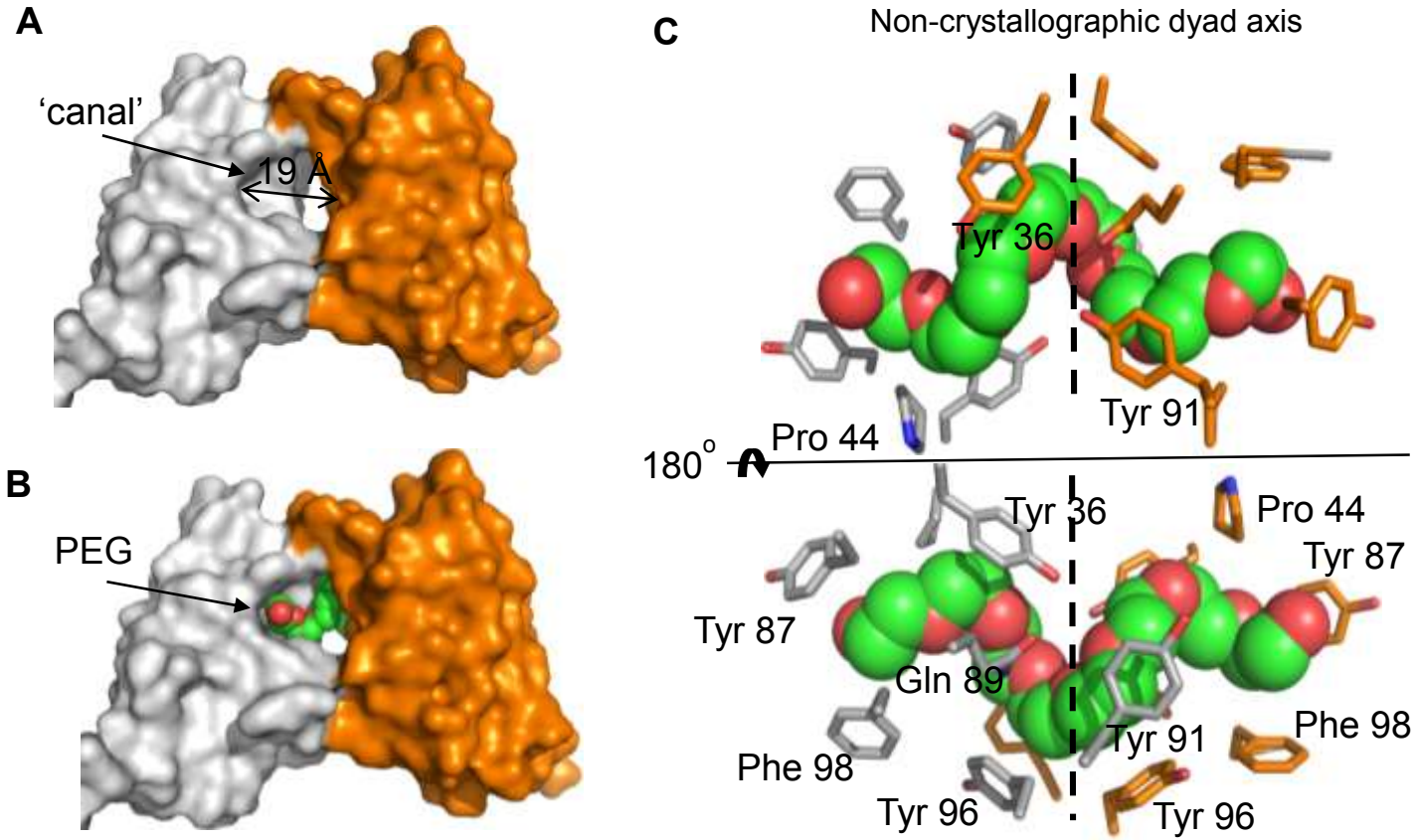

Figure 4-7. Germline b12 VL dimer interface analysis

A) Surface representation of germline b12 VL dimer with protomers colored gray and orange. B) Dimerization mediating PEG molecule presented in sphere representation with carbon and oxygen atoms colored green and red. C) Side chains that coordinate PEG molecule presented in stick representation. Carbon molecules from the protomers colored either orange or gray while oxygen and nitrogen molecules are colored red and blue respectively. 180 ° rotation of C along the Y-axis.

4.2.5 Protein stability and validation

To assert our claim that these dimer structures are artifacts of the process of crystallization but not physiologically important or symptomatic of protein degradation and stability problems, we assayed our germline b12 samples using analytical SEC and comparative SDS-PAGE gel. Protein samples were stored at 4 ^{o}C in sample running buffer for three months and 20 months. SEC analysis points out proper VL-VH complex formation in solutions. Prolonged storage of protein samples post purification mildly affected stability of VL-VH complex formations (Figure 4-8). Samples are stable after three-month storage and most of the protein remained stable VL-VH heterodimer after 20 months of storage. The observed minor degradation post 20 month storage resulted in aggregated protein but not free VL domain. This indicates the structurally observed VL dimers is not inherent phenomenon to the b12 germline FV precursors rather is mainly due to non-physiological crystallization conditions.

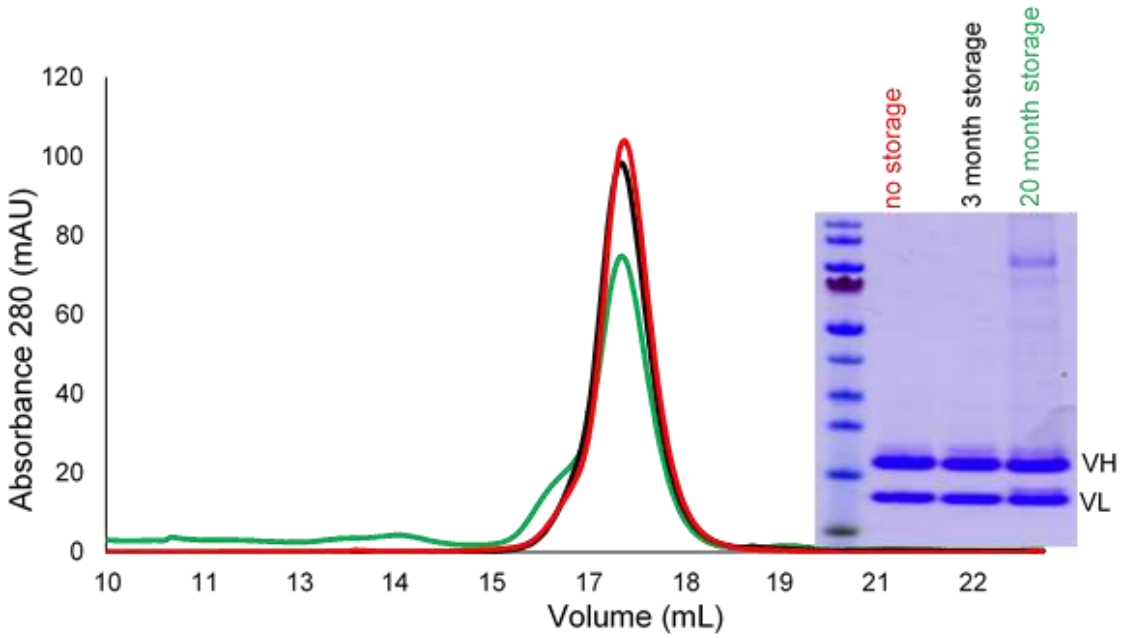

Figure 4-8. Germline c/scFv protein stability analysis
Germline c/scFv protein stored for different lengths of time analyzed using SEC and SDS-PAGE. SEC trace where red colored traces indicate fresh germline b12 c/scFv samples, black traces indicate samples stored for three months and green traces indicate samples stored for 20 months. SDS-PAGE gel runs are also labeled with similar colors. Gel bands corresponding to VL and VH chains are indicated

4.3 Discussion

One of the proposed structural mechanisms by which affinity maturation attains increased binding to antigens is through rigidification of the antigen combining region and maintaining a bound conformation whereby overcoming an energetic threshold to facilitate antigen recognition (94, 132, 137). Such structural rigidification implies optimization of binding interface for specific antigen binding and locking-in structural features of the bound conformation for increased binding following affinity maturation. The structurally observed rigidification of b12 Fv domains as deduced from the bound (76) and unbound (159, 160, 180) structures of mature b12, argue for such rigidity following affinity maturation. In our attempt to pinpoint regions of antibody Fv where structural rigidity observed due to SHM, we show that germline precursor b12 VL domains retain structural features observed in the VL regions of mature antibody.

Our structural analysis of pseudo VL dimer b12 germline precursor antibody, although deficient in deciphering features of the VH chain structures, provides sufficient representation of region on VL. Germline b12 VL shows some degree of flexibility, particularly in CDRL1 and CDRL3. Given the current trimeric Env structural knowledge, it is plausible to postulate b12 VL directly interact with Env and hence alter binding kinetics. In addition, proximity of CDRL1 to the protruding CDRH3 also enables VL structural flexibility to affect the binding conformation of the antibody. Hence, even the minor flexibility observed in VL CDRs could suffice to ablate high affinity binding observed in mature b12 antibody. This study (Chapter 2) and previous reports (107) illustrate the importance of the VL domain in antigen binding and virus neutralization potency as previously reported. This structural characteristics of the germline light chain Fv, although not unprecedented in CD4BS antibodies (96, 215), suggests the VL region of germline antibodies could be rigid in structure while rigidification and rearrangements in CDRs as a result of maturation attains exceptional specificity and binding affinity observed in affinity matured antibodies.

The lack of germline precursor b12 VH chain structural features in the different crystallographic reagents tested could signify a spectrum of underling reasons including experimental problem and structural features of the antibody. As presented in Figure 4-2, the presence of a 'contaminating' fragment on VH chain could interfere with crystallization experiments. While such protein impurities repress proper crystalline formation, the propensity of VL domains to dissociate and form pseudo VL dimers can promote crystal growth. While this experimental pitfall could contribute to the lack of VH structures in germline and Chimera 1 (mL/gH), our ability to grow crystals of b12 mature and Chimera 2 (gL/mH) with proper mature VH chain argue for possible structural feature of VH chain as culprit. Based on the results described above, we are now seeking better crystallographic germline VH containing reagents that enable proper crystalline formation of intact Fv domains.

Given the results inferred from b12 germline VL domain and described in this section, we conclude that germline b12 is minimally flexible. While overall Fv domains show remarkable rigidity, the observed minor flexibilities are localized near CDR regions. Hence, SHM on VL domain, while affecting antibody binding and neutralization, the structural mechanism involve rigidity of the antigen binding region minimally. Given previously observed minor conformational changes and structural flexibility of germline VL domains of CD4BS antibodies, it is apparent that we need to solve the structure of the germline VH domain. We posit that higher degree of flexibility could be observed on germline b12 VH chains.

4.4 Acknowledgments

The progress reported in this chapter was mainly accomplished with great help and assistance from Christopher Carrico, a former graduate student in the Strong lab. The work reported here was a joint effort between Carrico and myself. His input in experimental design and construct engineering were instrumental to the success reported in this chapter. I am also grateful for the input and intellectual discussion provided by Peter Rupert during crystallographic data collection, analysis and interpretation.

Chapter 5

5 Immune evasion by adenovirus E3/19K

5.1 Introduction

Natural Killer (NK) cells are part of the innate immune system involved in immune surveillance. Unlike their counterparts in the adaptive immune system (T and B cells), NK cell activation depends mainly on invariant receptors expressed on their surface. These cell-surface receptors do not show germline receptor rearrangement but recognize stress induced signals. Therefore, NK cells are poised to act when needed and their activation does not require prior sensitization or clonal expansion. The mechanism plays a key role in the recognition of stressed cells by the immune system (232, 233).

A balance of signals from stimulating and inhibitory receptors on NK cell surface mediates their effector functions. One of the best-characterized NK receptors is the activating receptor Natural Killer Group 2D (NKG2D). NKG2D is a type II transmembrane protein with an extracellular C-type lectin-like domain expressed on surfaces of NK cells, αβ T cells, γδ T cells and macrophages. Cellular signaling through NKG2D is mediated through the DAP10 and DAP12 adapter molecules that in turn recruit phosphatidylinositol 3-kinase, Grb2, and RAGE (234-236). Cascades of signaling due to NKG2D and its interactions with respective ligands activate cytotoxic NK and T cells and promote lysis of cells expressing stress-induced molecules (237-239). Even though a specific receptor-ligand interaction governs NK cell activation and function, NKG2D binds promiscuously to a plethora of ligands. This plasticity in NKG2D interactions with its ligands allow the receptor to recognize a large array of diverse molecules in the absence of an important conformational change upon ligand binding (240).

In humans, identified NKG2D interacting ligands (NKG2DL) include diverse molecules like the major histocompatibility complex (MHC) chain class I related sequences A/B (MIC A/B), and UL16 binding proteins 1-6 (ULBP1-6) (241-244). Predominantly, these molecules are expressed on gastrointestinal epithelium, endothelial cells and fibroblasts (245-247). They exhibit restricted expression

profile in normal tissue while overexpressed in cells enduring stress-causing factors like transformation, pathogen infection, hypoxia, inflammation and DNA damage (247-249). Overexpression of NKG2DL is also linked to poor outcome in organ transplantation and cancer prognosis (250, 251).

There exists minimal sequence similarity (~30% identity) among the various NKG2DL while they demonstrate structural similarities and comparable NKG2D mode of interaction. One structural similarity between NKG2DL is in the overall architecture of the receptor interacting interface. The platform domain, including α1 and α2 helixes, form the NKG2D interacting surface and is a common structural motif in all NKG2DL. NKG2D forms an obligate homodimer and binds monomeric NKG2DL with comparable surfaces on each NKG2D protomers interacting with distinctly different surfaces of α1 or α2 domains (252, 253). While the platform domain is conserved in all NKG2DL, the attached α3 domain is missing in some NKG2DL like ULBPs (254, 255). Similarly, NKG2DLs also exhibit remarkable differences in their cell surface anchoring mechanisms. Molecules like MIC A/B express a transmembrane domain that facilitates cell surface expression. In other cases, mo ULBP1-3 are anchored on the cell surface by glycosyl-phosphatidyl-inositol (GPI) linkers (241, 242). However, the relevance of differences in mode of cell surface adhesion in NK cell activation and subsequent immunological function is yet to be elucidated.

In addition to the structural similarity among the various families of NKG2DL, they are also distant structural homologs of MHC class I molecules (Figure 5-1). Both MHC class I molecules and MICs are functionally unrelated and exhibit low sequence homology (20-30% sequence homology per domain). Structurally, similar to MHC molecules, NKG2DL contain α1 and α2 domains (256, 257). This region forms a receptor binding interface in NKG2DL while it constitutes an antigen binding groove and T cell receptor (TCR) interacting surface in MHC class I molecules. Similar to MHC class I molecules, some NKG2DL like MIC A/B possess α3 domains, while the region is missing in others like ULBPs. There exist notable architectural differences between the MHC and MIC class of molecules.

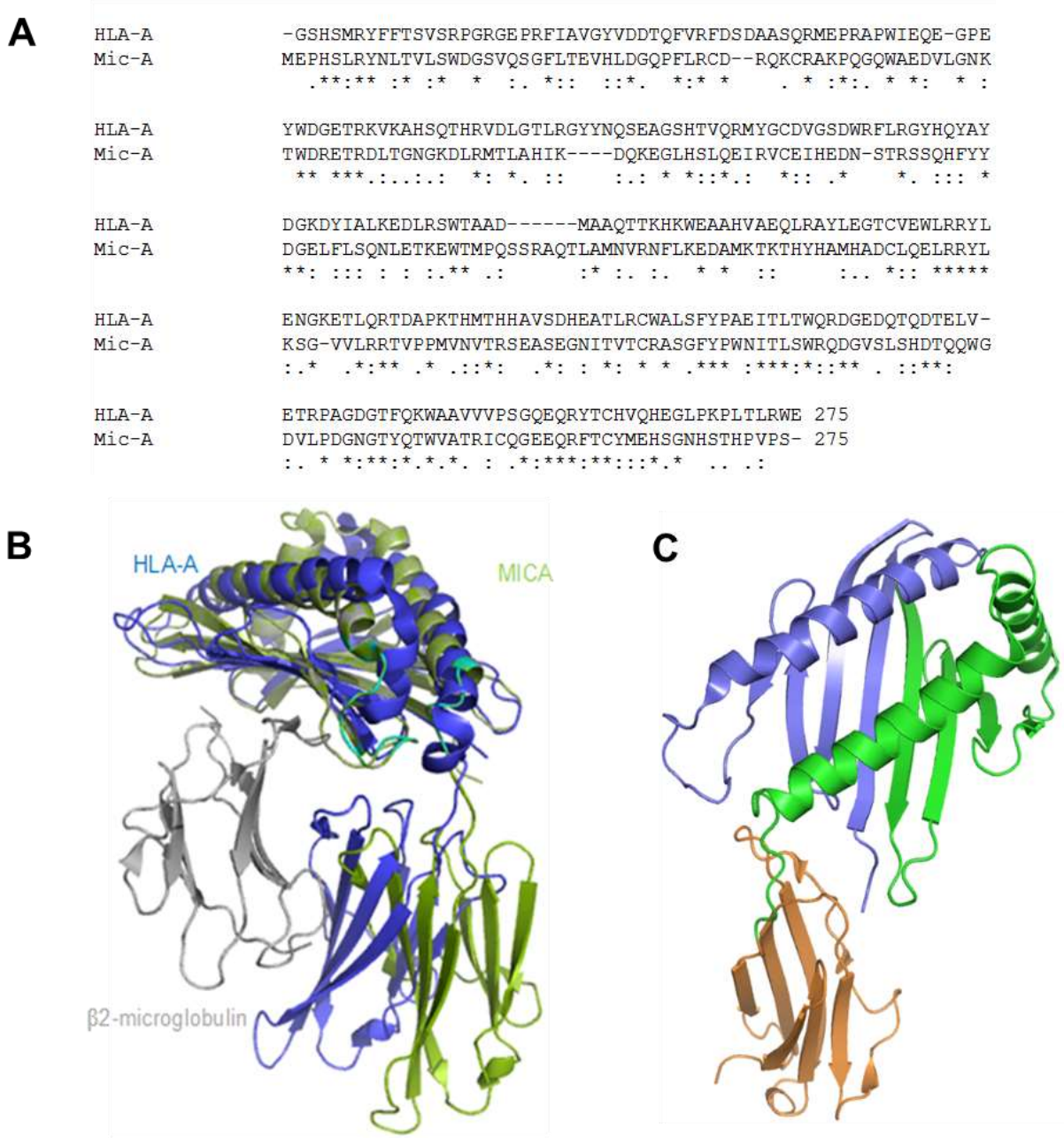

Figure 5-1: Sequence and structural differences between NKG2DL MICA and MHC class I HLA-A
A) Sequence comparison between HLA-A and MICA. B) Structural comparison between HLA-A and MICA performed using Pymol. Structures are presented as models in cartoon representations. σ1, σ2 and σ3 domains of HLA-A are colored blue while β2-microglobulin are colored gray. MICA σ1, σ2 and σ3 domains are presented in green color. C) MICA σ1, σ2 and σ3 domains are shown using green, blue and orange colors respectively.

While classical MHC class I molecules associate with β2-microglobulin, bind to host or non-host derived peptides and present antigens to cytotoxic T-cells through recognition by TCRs, MIC proteins neither associate with β2-microglobulin, nor bind and present antigens to T-cells. Crystallographic models of NKG2DL elucidated factors contributing to MIC proteins inability to bind antigens where the putative antigen binding groove formed by α1 and α2 helices is narrower in MIC proteins and one of the groove defining helices (α2) contains a disordered flexible region which is ordered when NKG2DL binds its receptor (240, 253, 257).

Similar to MHC class I molecules, MICs are highly polymorphic (258). Thus far, 65 different polymorphic MICA alleles and 17 MICB alleles have been identified (http://www.ebi.ac.uk/imgt/hla/). Most of the polymorphisms on the extracellular domain of MIC molecule arise from single amino acid substitutions resulting in non-conservative changes. Even though frequent substitutions are observed in the α2 domain (15 substitutions), polymorphisms are not restricted to a specific region of the gene or region on the protein structure. Most of the conserved residues across species and conservative substitutions occur on the underside of the α1 and α2 platform (opposite to the NKG2D binding site) (259). Polymorphic variations are also observed on the NKG2D binding platform where at least one polymorphic position directly interacts with NKG2D (216). Despite polymorphism in the NKG2D binding surface, NKG2D and its ligands interact in a similar manner across different MIC alleles and organisms albeit with varying affinity.

Even though functional relevance of polymorphism in MICs is not well defined, correlation of polymorphic alleles and their distribution among population groups suggest importance of MIC polymorphisms. In different populations, studies have unveiled differential distribution of MIC polymorphic alleles (260-262). This haplotype diversity correlates with susceptibility to certain autoimmune diseases (263-267). Some epithelial derived tumors also correlate with MIC polymorphisms and their expression (268, 269). Further exemplifying the relevance of polymorphisms in cancer biology, NKG2DL shedding plays important role in cancer immune evasion where NKG2DL shedding down regulates surface expression of NKG2D (270-272).

Despite the listed correlations in disease prevalence and MIC polymorphisms, there exists a knowledge gap in polymorphism-driving factors in these molecules. Given the invariant nature of their receptor interactions, their importance in immune modulation and targeting by viral and tumor immune evasion molecules, we hypothesize the need to bind cellular ligands while evolving away from viral immune evasion molecules is one polymorphism-driving factor.

The importance of NK cells and hence NKG2DL can be inferred from the various immune evasion mechanism used by viruses to elude NK based surveillance (273, 274). Viral immune modulatory mechanisms that manipulate NK receptors include targeting activating ligands for degradation (275), virus derived decoy ligands for inhibitory receptors (276), selective up regulation of inhibitory MHC class I antigens (277), and down-regulation of NKG2DL using viral protein (278, 279). Often, viruses down regulate expression of MHC class I molecules presenting viral antigens to CD8+ T cells, which activates immune response by the adaptive immune system. Hence, down regulation of MHC class I molecules simultaneously activates NK cell responses (280, 281). Viruses also encode proteins that sequester activating ligands resulting in decreased cell surface expression. Human Cytomegalovirus (HCMV) encodes a protein, UL16 that sequesters NKG2DL like MICB, ULBP1 and ULBP2 and down regulates their surface expression (282-286). Another HCMV encoded protein, UL142, uses a similar mechanism to down regulate MICA surface expression (286-288).

Various serotypes of Human Adenovirus also encode proteins that modulate surface expression of NK activating receptors. Wilkinson's and Burgert's groups showed that the Adenovirus E3/19K protein sequesters MICA/B proteins in the endoplasmic reticulum (ER) and down regulates their surface expression (289-291). E3/19K was initially identified because of its ability to abolish viral peptide presentation by MHC class I molecules and to evade cytotoxic T cells mediated immunity (292-294). It is a type I membrane glycoprotein, expressed by the E3 region of the virus genome (295). Phylogenic sequence analysis revealed conservation of E3/19K encoding gene in most adenovirus serotypes (296, 297). E3/19K is a glycoprotein that contains a large (about 110 aa long) N terminus domain that localizes in the ER lumen, a transmembrane domain (~20 aa), and a short cytoplasmic tail (~15 aa) equipped with a

dilysine ER anchoring motif (297-299). Structurally, E3/19K is stabilized by the presence of conserved Cys residues forming disulfide bridges (300). The N-terminal domain of E3/19K binds either peptide filled or peptide deficient MHC class I molecules passing through the ER due to cellular trafficking. Retention of immune modulatory molecules in the ER lumen is mainly due to the presence of a C-terminus ER anchoring motif (301-303). MHC molecule binding does not involve either cytoplasmic or transmembrane domains of E3/19K (304, 305). Recent structural examination of E3/19K and HLA-A interactions from the Bouvier group revealed that E3/19K makes contact with all σ1, σ2, σ3 and β2-microglobulin domains.

Based on analysis of interactions between NKG2DL and immune modulating virus-derived molecules described previously, E3/19K could bind to the various domains on MIC molecules (Figure 5-2). E3/19K could mimic NKG2D-MIC interactions and hence interact on the platform region that overlaps the receptor-binding interface. Such interactions potentially serve as selection pressure on NKG2DL and hence drive polymorphism. The mode of binding has been previously described for immune modulation by the Cytomegalovirus protein UL16 and other virus derived immune evasion molecules. Specific interactions with the MIC platform region is also used to discern interactions with distantly related NKG2DL (284, 306, 307).This hypothesis is also supported by the structural elucidation of E3/19K-HLA-A where the HLA-A antigen presenting domain makes extensive contact with adenovirus E3/19K . Understanding tumor immune evasion mechanisms where, in some tumors, direct interaction of metalloproteases and disulfide isomerases with MICA σ3 domain is deduced to drive MICA shedding (308, 309). We can also infer potential structural mechanisms of binding and sequestration of MICs by adenovirus E3/19K due to direct interaction with the σ3 domain. The structural elucidation of HLA-A and E3/19K interaction also suggest, although small, some degree of contact with the σ3 domain. Hence, biophysical analysis, using various domains of MICs could enable direct delineation of E3/19K binding partners.

We selected the MIC A/B – E3/19K interaction as a model to study the role of viral immune evasion protein interactions with NKG2DL in driving MIC polymorphisms. Most viral immune evasion proteins involved in the down regulation of MIC surface expression are selective to either MICA or MICB; the CMV UL16 can only bind MICB while UL142 binds only MICA (283, 284, 287). E3/19K, however, engages both MICA and B in addition to HLA proteins (297, 310, 311). According to binding

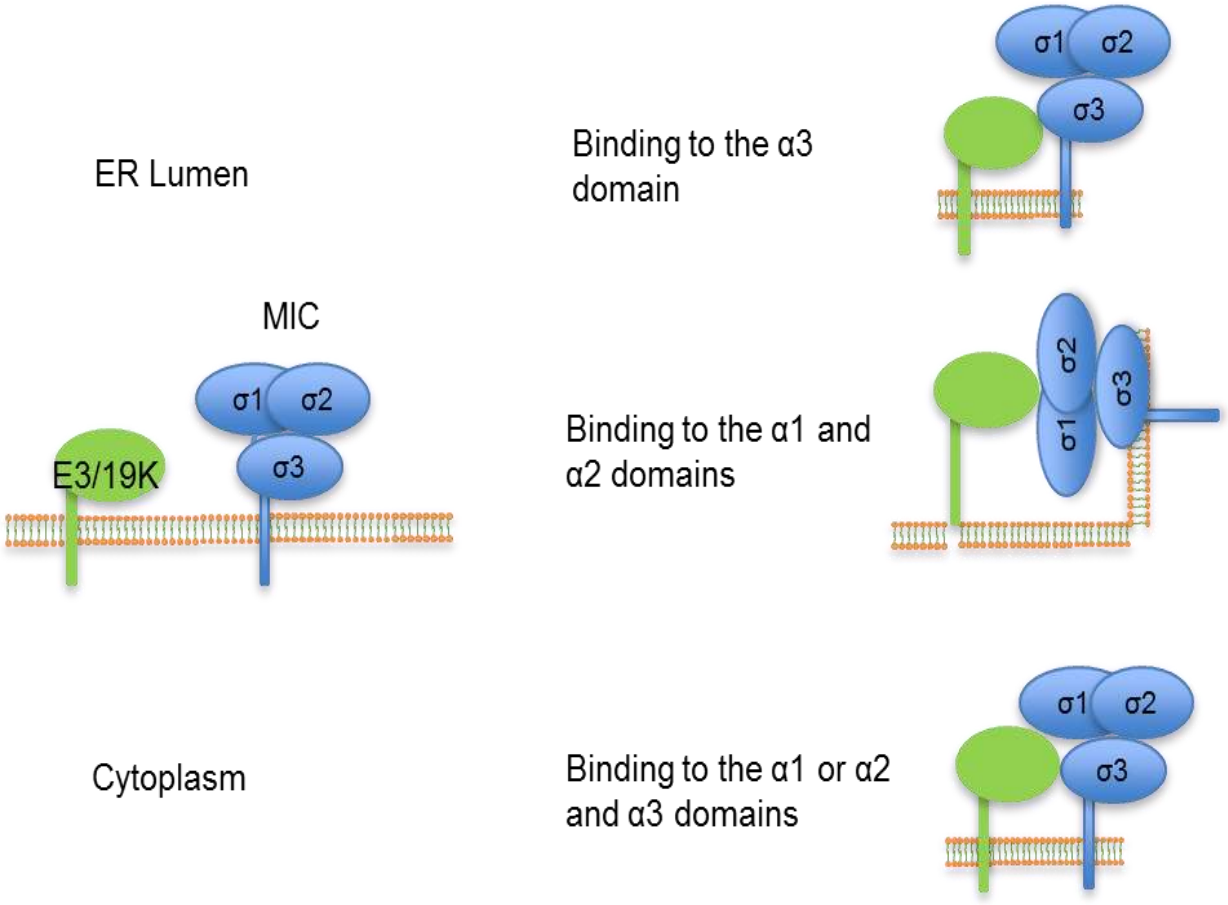

Figure 5-2: Model for E3/19K - MIC interactions

Different models of E3/19K and MIC modes of interactions are presented. Schematics on left panel indicate orientation of molecules with cytoplasm and ER lumen sections designated in relation to molecules placed on ER membrane. E3/19K luminal domain are represented using green ovals while MIC σ1, σ2, and σ3 domains are presented using blue ovals. Model for E3/19K – MIC σ3 domain interactions are given on right top panel. Right middle panel illustrate E3/19K mode of binding to MIC platform domains while right bottom panel illustrate possible model for E3/19K interaction σ3, σ1 and/or σ2 domains.

studies done on HLA alleles, it is not farfetched to hypothesize that MIC-E3/19K interactions could exhibit varying affinity to different MIC alleles.

Understanding how E3/19K binds to highly polymorphic MIC and the nature of MIC residues involved in such interactions could provide insight into the potential contribution of selection on NKG2DL. Our goal is to use structural attributes and biophysical characteristics of MICs and E3/19K interaction to define the binding interface. Constraints that drive polymorphism in MICs will be deduced from information gathered through these experiments. Although our attempts to use X-ray crystallography to determine a complex structure of MICA - E3/19K interaction are yet unsuccessful, biophysical analysis using Surface Plasmon Resonance (SPR) and Size Exclusion Chromatography (SEC) enabled us to define potential E3/19K contact domains on MICA.

5.2 Results

5.2.1 Structural analysis of MICA - E3/19K interaction

Structural definition of E3/19K interaction with HLA-A from the Bouvier group (312) elucidated the main structural features in its interaction with MHC Class I molecules. In an HLA-A –E3/19K interaction, the binding interfaces mainly encompass the antigen-binding groove formed by σ1 - σ2 domains and part of σ3 domain. E3/19K also makes reasonable contact with the β2-microglobulin. Given this structural information and apparent structural homology between MHC class I molecules and MIC proteins, we postulate that E3/19K binding interaction with NKG2DL does not overlap with the NKG2D binding surface. Modeling experiments, where crystallographic structure of MICA bound to NKG2D homodimer is docked on the E3/19K – HLA-A structure using Pymol (183), revealed that E3/19K could potentially sequester surface expression of NKG2DL by direct interaction with the σ1, σ2 and/or σ3 domains (Figure 5-2). However, structural docking experiments do not take into account structural flexibility, a caveat in docking experiments. Crystallographic studies of MICA and HLA-A infer distinct features that may not be obvious in modeling experiments. Crystallographic studies deduce structural rigidity between the various domains in HLA-A held together by the presence of β2-microglobulin (313, 314), and conformational flexibility between the platform and σ3 domains in MICA (216, 315). These observations suggest, although MHC class I molecules and NKG2DL like MICA exhibit remarkable structural similarity, E3/19K might not interact with both classes of molecules with similar modes of binding.

Sequence and structural comparison between HL-A and MICA to delineating the MICA interacting E3/19K interface revealed differences in amino acid sequences in the interface-forming region. A greater portion of the potential E3/19K binding interface on MICA involve amino acids that were different from cognate HLA-A sequences (Figure 5-3). Changes that alter local electrostatics of the interface potentially affect overall interaction. The negatively charged Glu in HLA-A position 54 that is

involved in an hydrogen bond forming interaction with E3/19K is carrying a hydrophobic amino acid (Val) in MICA. Presence of a hydrophobic residue at this position could abrogate potential interaction.

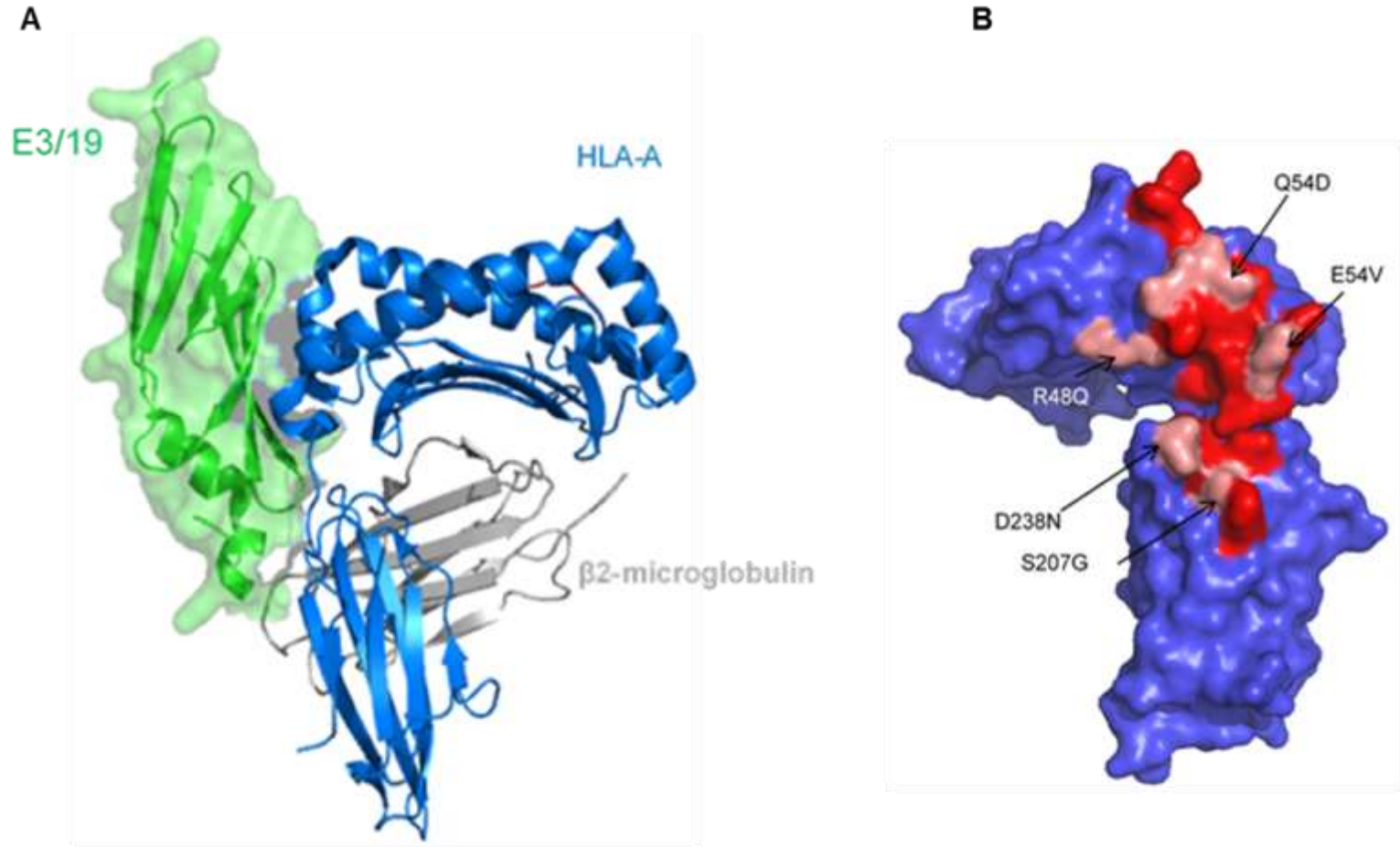

Figure 5-3: Structural comparison of E3/19K interaction with HLA-A and MICA
A) X-ray crystallographic model of E3/19K – HLA-A complex structures (PDB accession code 4E5X modeled in cartoon representation. HLA-A σ1, σ2, and σ3 domains are colored blue. HLA-A β2-microglobulin is colored gray while adenovirus E3/19K is presented in green ribbon surrounded by transparent green surface. B) MICA (PDB accession code 1HYR) presented in surface representation with Adenovirus E3/19K interacting interface deduced from modeling experiments indicated with red shading. Amino acid positions in the presumed binding interface that differ from HLA-A sequences are presented with light red shading. With each amino acid pair, the first residue is from HLA-A and the second from MICA. Structural models were analyzed and generated using Pymol.

Position 48, that displays the positively charged Arg is substituted with a non-polar Gln and the difference at position 238 where Asp residue on HLA-A is substituted with Asn on MICA, could also affect potential binding. Although structural analysis depict E3/19K potentially utilizing a comparable interface to interact both MHC class I and MIC molecules, observed sequence differences between the molecules imply different mode of binding.

5.2.2 E3/19K protein characterization

Various attempts at expression of E3/19K failed due to the unusual protein-folding pattern. However, Lentivirus based mammalian expression system using an expression chaperone lipocalin protein as fusion partner enabled expression of properly folded Adenovirus E3/19K protein. This was

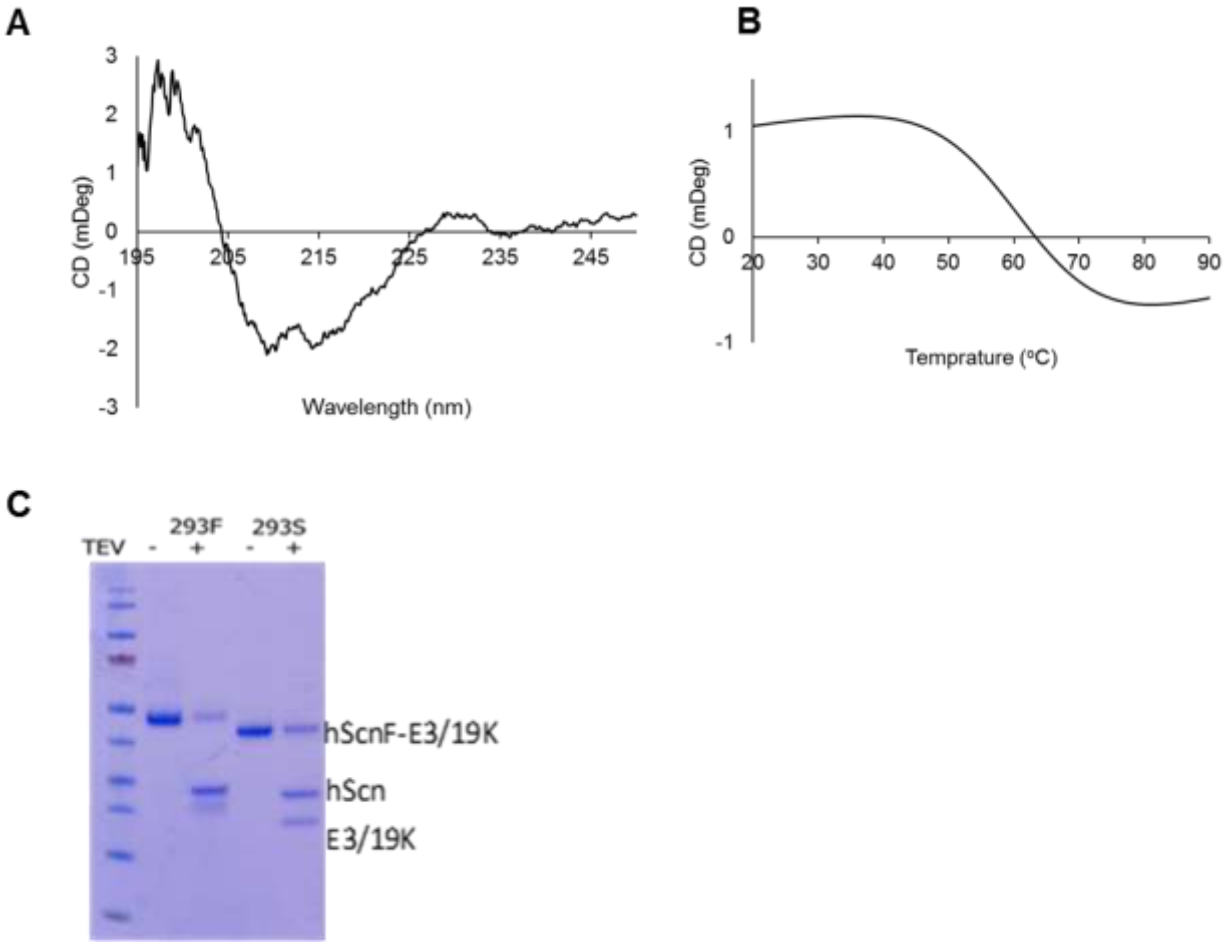

Figure 5-4. E3/19K Circular Dichroism and expression profile
A) Wavelength scan of E3/19K. B) Thermal denaturation analysis of E3/19K analyzed in the temperature range of 20 °C to 90°C at 214 nm wavelength. T_m was calculated to be 62 °C (+/- 0.55 °C) C) Comparative SDS-PAGE gel analysis of proper folding and TEV cleaved hScn-E3/19K fusion protein expressed in HEK 293F and HEK 293S cells. Difference in E3/19 K molecular weight is attributed to the lack of complex glycan in HEK 293S expressed protein.

deduced from CD analysis of protein sample generated characteristics spectra for σ helical and β sheet mix folding. Similarly thermal denaturation analysis indicated the high stability (T_m =62 ^oC) of the protein. The presence of two predicted glycosylation sites in the luminal domain of E3/19K barred the use of this preparation for crystallization experiments. However, expression in N-acetylglucosaminyltransferase I deficient 293S cell line resulted in homogeneous sample suitable for downstream experimentation (Figure 5-4).

5.2.3 MIC platform (σ1 - σ2) domain is sufficient to bind E3/19K

Given the differences between MICA and HLA-A, we assessed potential E3/19K binding to sub-domains on NKG2DL. Previously described sequestration of MICs due to E3/19K expression (290), preliminary SPR based binding assays and SEC based complex formation analysis reasoned that E3/19K luminal domain interacts with full length MICA (Figure 5-5, Figure 5-6). Accordingly, full length MICA interacts with E3/19K with a binding affinity slightly stronger or comparable to the NKG2D interaction (reviewed in (316)). The observed MICA-E3/19K interaction is weaker than reported HLA-A –E3/19K binding affinities (297). We hypothesize this difference is attributed to the diversity in NKG2DL and evolutionary adaptation of E3/19K to modulate NK cell based immune activation through recognition of diverse NKG2DLs.

To parse out the importance of various MIC domains in E3/19K binding, we assessed E3/19K complex formation with MIC platform (σ1 – σ2) domain and σ3 domain alone. The MIC platform region is sufficient to interact with E3/19K while σ3 domain alone does not show any interaction. In both SPR and SEC experiments, MICB platform domain readily formed E3/19K complexes with binding affinity comparable to full length MICA. The importance of the platform domain is consistent with the mode of interaction suggested in molecular docking exercises. Unlike the HLA-A and E3/19K interactions, σ3 domain might not add significantly to the overall binding strength. Given these results, we posit that the principal interacting partner and important biochemical interactions are mediated through the MIC platform region rather than than the σ3 domain. In-depth understanding of the structural factors that

govern NKG2DL and E3/19K interaction and mechanism of immune evasion necessitate structural further analysis using E3/19K and MICA complex.

To validate the relative importance of the different SPR and SEC determined MIC domains, we attempted X-ray crystallographic based structural model determination. Although various constructs of E3/19K from different Adenovirus serotypes were used, resulting crystals contained full length MICA alone. Crystallographic reagents of Adenovirus serotype 2 E3/19K were expressed in both bacterial and mammalian expression systems. Protein expressed in bacterial system as inclusion bodies and *in vitro* refolded resulted in soluble E3/19K, however, protein samples were not amenable for storage and crystallography. E3/19K expressed in mammalian expression system using both HEK 293F and HEK 293S systems with hScn fusion partner resulted in soluble and stable protein samples. Crystals were observed in screening conditions containing full length MICA and HEK 293F expressed E3/19K. Further analysis using diffraction data from these crystals revealed crystal lattices composed of only full length MICA (data not shown).

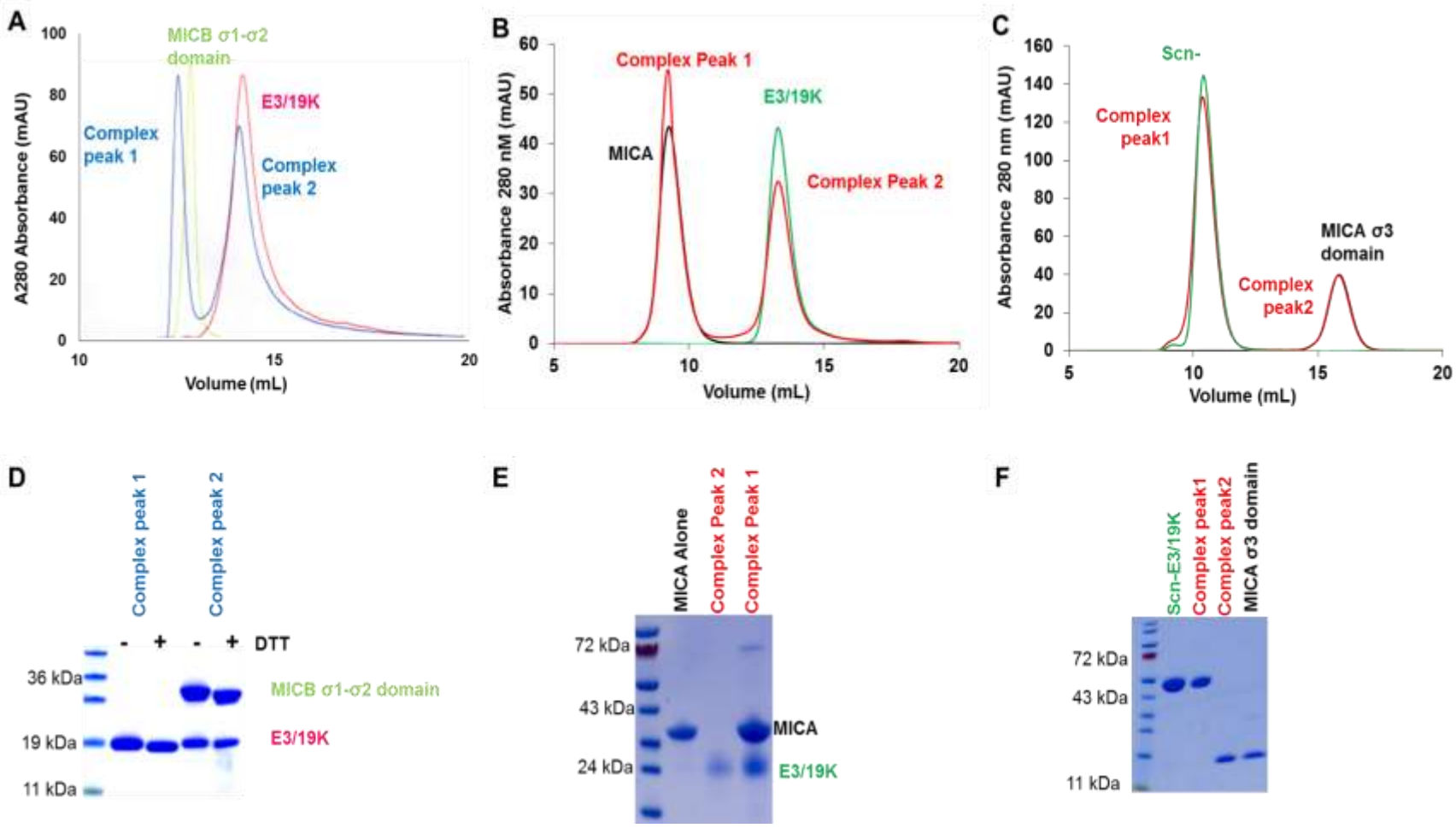

Figure 5-5: Binding Analysis of Adenovirus E3/19K interaction to MIC domains

Binding analysis of full length MICA to Adenovirus serotype 2 E3/19K analyzed using SEC (A-C) analyzed using analytical superdex 75 column. (A) Complex formation of E3/19K with Platform MICB. SEC curves for uncomplexed MICB domain presented as light green lines, uncomplexed E3/19K curves colored red while complex run are presented with blue lines (B) E3/19K complex formation with Full length MICA. SEC curves for uncomplexed Full length MICA presented as black lines, uncomplexed E3/19K green curves and complex run presented with red color. (C) E3/19K complex formation with MICA σ3 domain. SEC curves for uncomplexed MIC σ3 domains presented as black lines, uncomplexed E3/19K curves colored green and complex run presented with red color. SDS-PAGE gels of respective curves are presented with standard molecular weight markers for E3/19K complexes with platform domain MICB (D), Full-length MICB (E) and MICA σ3 domain (F).

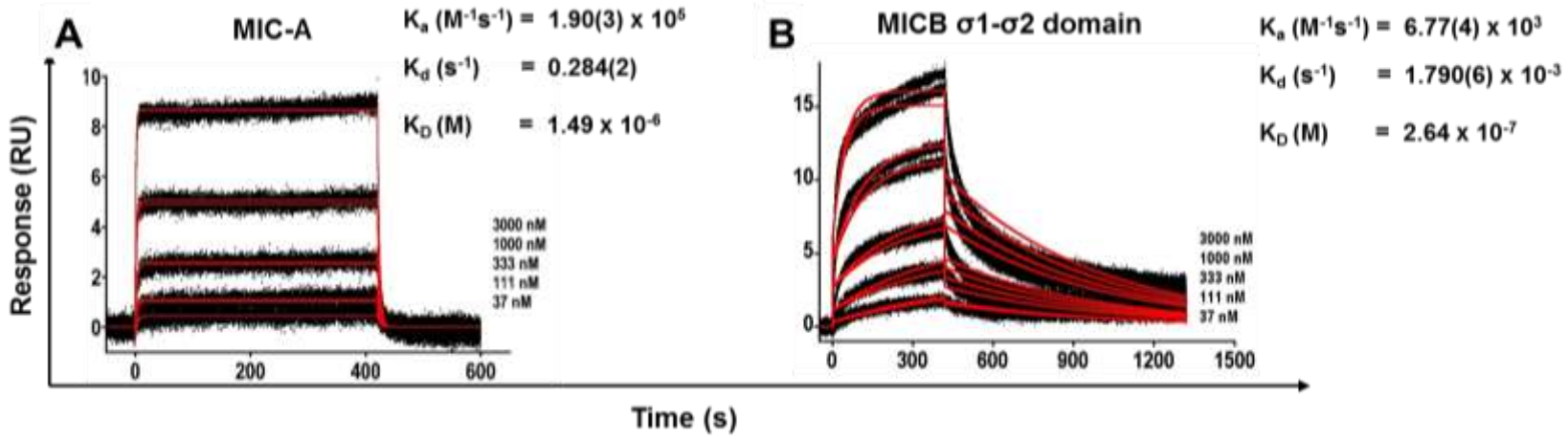

Figure 5-6. SPR based binding of Adenovirus E3/19K to MICA/B
Preliminary SPR based binding analysis of full length MICA and E3/19K interaction are presented with duplicate serial dilution sample runs given in black while model fits presented in red lines. SPR sensorgrams of the interaction between (A) Full length MICA analytes and E3/19K ligands and (B) MICB platform domain analytes and E3/19K ligands. Duplicate experimental run results are shown as black traces 1:1 model fits are shown as red lines

5.3 Discussion

Adenovirus infection is more prominent and detrimental in immunocompromised hosts. Reports have indicated increased infection post solid organ, bone marrow and hematopoietic stem cell transplantation (317, 318). Furthermore, presence of immune compromising viral infections like HIV further increased the advent of such opportunistic viral infections. In humans, opportunistic viruses like Adenovirus deploy various immune compromising and evading mechanisms. One arm of the immune evading mechanism involves eluding immune surveillance by NK cells. As discussed above E3/19K is a small protein expressed by various Adenovirus serotypes and residing in the ER lumen. E3/19K binds various HLA-A and MIC alleles and down regulates their surface expression.

Biophysical and structural analysis were used to delineate the E3/19K binding interface on MICs. According to our structural modeling experiment, E3/19K could make extensive contact with the MIC platform and σ3 domain. This suggests the conformationally flexible molecule MIC is maintained in the rigid HLA-A conformation to facilitate E3/19K binding. Conversely, the binding of E3/19K could induce the flexible MIC molecule to maintain an HLA-A conformation. Although MICs are structurally homologous to HLA-A, sequence comparison suggest potential sequence differences in the proposed E3/19K binding interface that suggest differing modes of E3/19K interaction.

Defining E3/19K binding partner domains by using biophysical characterization techniques, we showed the platform domain of MIC is sufficient to bind E3/19K. In contrast to the structurally observed HLA-A – E3/19K mode of binding where E3/19K makes reasonable contact with different domains on HLA-A, the main binding interface on MIC concentrates on the platform domain. Whereas absence of potential complex formation by the σ3 domain, could not exclude possible minor contacts, it suggests dispensability of the domain in E3/19K interactions. Given the high degree of flexibility observed in the MIC platform and σ3 domains, E3/19K binding geared towards the platform domain is also amenable to efficient sequestration of MICs. Furthermore, because NKG2DLs also include a family of molecules that lack σ3 domains and are anchored by GPI linkage, mere binding to the platform domain posits the

versatility of a single viral protein (E3/19K) to down regulate surface expression of distantly related NKG2DLs.

Binding analysis using SPR and SEC techniques provide immense biophysical information. However, sensitivity limitations and lack of atomic resolution detailed information hinders precise definition and comparison of various immune evasion techniques by E3/19K. Because X-ray crystallographic techniques enable us to parse the binding partners and atomic forces involved, we anticipate determining the molecular model for E3/19K bound MIC to provide a clearer understanding of the interactions. This will reveal the exact nature of the amino acids that make direct contacts and could define the molecular mechanisms for the interactions.

Immune modulating molecules like NKG2DL targeted by viral immune evasion proteins are highly polymorphic. Although correlation of polymorphic allele expression and prevalence of certain autoimmune diseases and cancers are well documented, as of yet factors that dictate fitness of polymorphic alleles are not well defined. Given that these molecules are highly targeted by immune modulating virus and cancer cell derived molecules, we posit that the need to evolve away from such interactions while maintaining their ability to invariantly bind receptors impose evolutionary pressure that result in high degree of polymorphism. Our binding analysis studies confirm that the Adenovirus immune evasion molecules E3/19K binds predominantly on the platform domain of MICs. This highly polymorphic domain is also involved in NKG2D interactions. Understanding the role of viral immune evasion in driving NKG2DL polymorphism necessitate an in-depth look at E3/19K binding patterns to various MIC alleles.

The versatility of E3/19K to sequester surface expression of various antigen presenting MHC class I molecules is well-documented (311). As described in this section, its interaction with NKG2DLs is established only for the MIC family of molecules. To determine if viruses like Adenovirus utilize these highly expressed immune evasion molecules like E3/19K in modulating surface expression of other NKG2DLs, we propose binding studies with the ULBP family of NKG2DLs.

5.4 Acknowledgment

We are grateful for the generosity of Dr. Burgert who gratefully donated Adenovirus serotype 2 EcoRID fragment that also contain the E3/19K coding region. The progress reported in this chapter was mainly accomplished with great help and assistance from Bin Xu, a former post-doctoral fellow in the Strong lab. His input in experimental outline design and construct engineering were instrumental. I am also forever beholden to Della Friend who analyzed SPR based biophysical interaction of MIC domains and E3/19K.

Chapter 6

6 Materials and Methods

6.1 Expression constructs

Antibody c/scFv constructs were engineered as VL-VH single polypeptides with double thrombin cleavage site (Leu-Val-Pro-Arg-Gly-Ser) separated by a short spacer (Gly-Gly-Gly) to facilitate protein expression and resolve monodispersed, monobody c/scFv elements (Figure 6-1 A and B). a STREP tag (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) was engineered to the C-terminus of the constructs to monitor protein expression levels.

Antibody Fab constructs were designed as independent light chain (VL-CL) and heavy chain (VH-CH1) constructs with polyhistidine affinity tags (Figure 6-1). Constructs were cloned into various lentivirus shuttle vectors that contain either GFP (VH-CH1) or mCherry (VL-CL) as transfection readout.

Full-length gp120 monomers were engineered as a C-terminus fusion to a human Lipocalin protein Siderocalin (hScn) with N-terminus polyhistidine and short FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) affinity tags to facilitate protein expression analysis and purification. To generate soluble gp120s, a Tobacco Etch Virus (TEV) protease cleavage site (Glu-Asp-Leu-Tyr-Phe-Gln) was designed between C-terminus of hScn and N-terminus of gp120 (Figure 6-1 C). C-terminus biotin acceptor sequences (Gly-Leu-Asn-Asp-Ile-Phe-Glu-Ala-Gln-Lys-Ile-Asp-Trp-His-Asp) were engineered for *in vivo* biotinylation when coexpressed with bacterial biotin ligase (BirA). The biotinylation site was preceded by a thrombin cleavage site to facilitate recovery of unbiotinylated full-length gp120 for other biophysical and biochemical experiments.

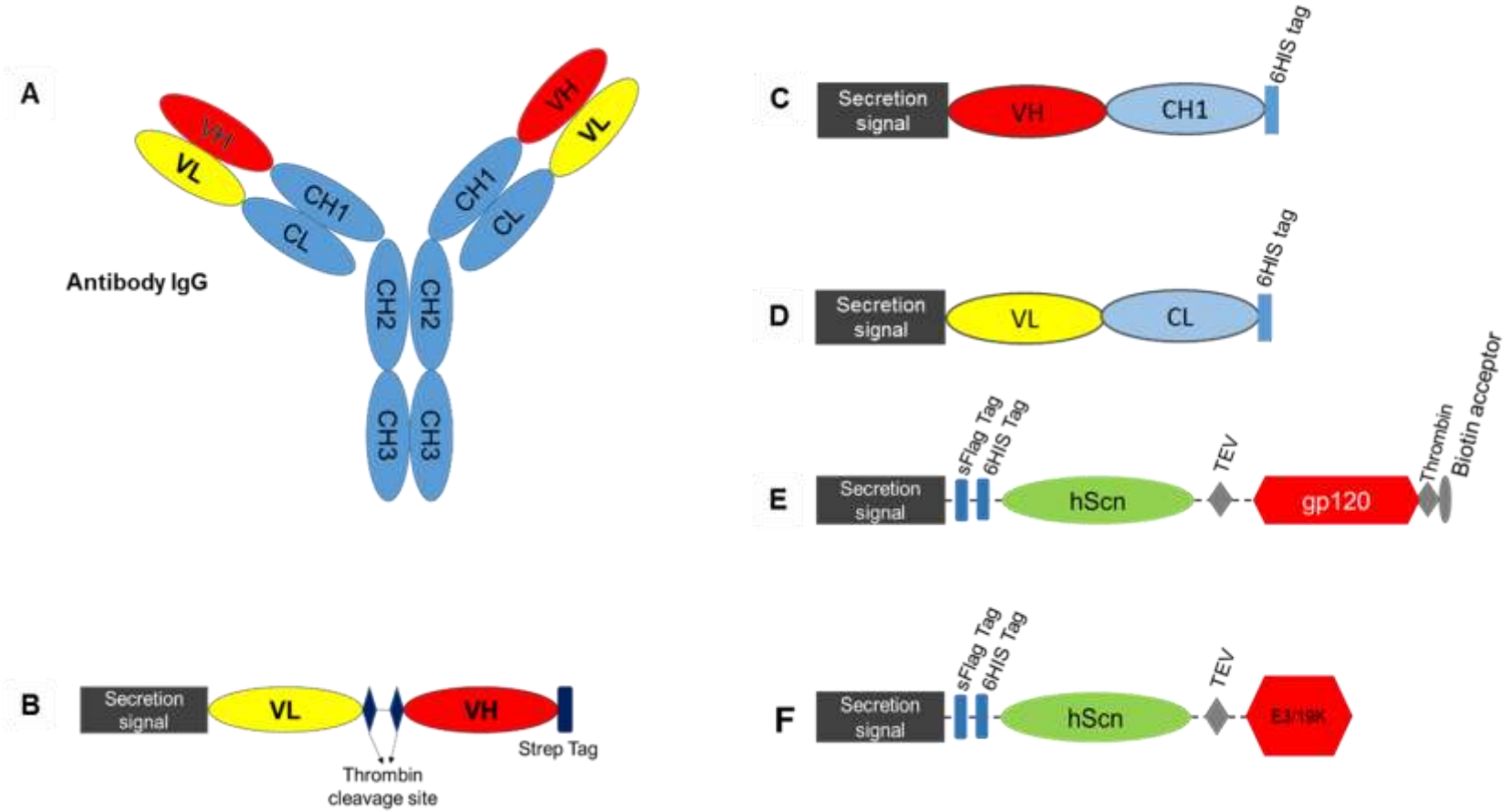

Figure 6-1. Schematics of expression constructs

A) Pictorial depiction of bivalent IgG antibody with variable and constant regions. Heavy chain variable domain colored red, light chain variable domain (VL) colored yellow and constant heavy (CH) and light (CL) domains colored blue. B) Schematic representation of c/scFv expression constructs. VL and VH domains, protease cleavage sites and affinity tag indicated. C,D) Fab expression constructs with VL-CL (C) and VH-CH1 (D) domains and affinity tags indicated. E) Schematic representation of monomeric gp120 expression construct. Monomeric gp120 represented with red polygon with a short linker connecting I with its fusion partner hscn (green oval). Gray diamonds indicates protease sites while affinity tags are presented as blue rectangles. Biotin acceptor sequence for endogenous BirA biotinylation is presented as gray oval. F) Schematic representation of Adenovirus serotype 2 mammalian expression construct with color coding as in E.

6.2 Optimized DNA sequences used for expression

Nucleotide sequences were optimized for mammalian expression using Genscript codon optimizer and synthesized at Genscript. Constructs were cloned into in-house mammalian expression vectors with 3' IRES dependent GFP or mCherry coding sequence for use in lentivirus based transduction or transient transfection (319). Antibody light chain (VL-CL) and heavy chain (VH-CH1) Fab constructs were cloned into vectors 5' to either mCherry or GFP IRES dependent florescent protein expression respectively.

Mature b12 c/scFv

catatggaaatcgtcctgacccaatcaccgggcaccctgagcctgagtccgggcgaacgcgcaacctttagctgtcgttcgtcccatagcatccgtagtcgtcgcgtggcatggtatcagcat
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cacggtcatcgtgagttcccatcaccatcaccatcactaatgactcgag

Chimera 1 (mL/gH) c/scFv

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Chimera 2 (gL/mH) c/scFv

ctcgagatggatatgcgggtcctggctcagctgctgggcctgctgctgctgtgtttccctggggcaagatgcgagattgtcctgactcagagtccaggcactctgagtctgtcaccaggagag
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Germline b12 precursor c/scFv

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Mature b12 VH-CH

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Germline precursor b12 VH-CH

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Germline precursor b12 VL-CL

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QH0692 gp120_birA

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caagggctt aac gat atc ttt gag gcc cag aaa ata gag tgg cac gaa taatgaagtcgaccgagcatc

SF162 gp120_birA

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gRB1

ctcgagatggatatgcgggtcctggctcagctgctgggcctgctgctgctgtgcttccctggggcaaggtgtgaaatcgtcctgactcagagcccagggaccctgagtctgtcaccaggaga
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gRB2

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gRB3

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gRB4

ctcgagatggatatgcgggtcctggctcagctgctgggcctgctgctgctgtgcttccctggcgctagatgtgaaatcgtcctgactcagtccccaggaactctgagtctgtcaccaggagag
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gaaaaataatgaggatcc

Human Adenovirus Serotype 2 E3/19K cDNA

atgaggtacatgattttaggcttgctcgcccttgcggcagtctgcagcgctgccaaaaaggttgagtttacggaaccagcttgcaatgttacatttaaatcagaagctaatgaatgcactactctt
ataaaatgcaccacagaacatgaaaagcttattattcgccacaaagacaaaattggcaagtatgctgtatatgctatttggcagccaggtgacactaacgactataatgtcacagtcttccaagg
tgaaaatcgtaaaactttatgtataaatttccattttatgaaatgtgcgatattaccatgtacatgagcaaacagtacaagttgtggccccccacaaaagtgtttagagaacactggcacctttttgttc
caccgctctgcttattacagcgcttgctttggtatgtaccttactttatctcaaatacaaaagcagacgcagttttattgatgaaaagaaaatgccttga

HScn - E3/19K fusion mammalian expression construct

Atggagacagacacactcctgctatgggtactgctgctctgggttccaggttccactggtgactacaaggacgagcatcaccatcatcaccatggtggaagccaggactccacctcagacct
gatcccagccccacctctgagcaaggtccctctgcagcagaacttccaggacaaccaattccaggggaagtggtatgtggtaggcctggcagggaatgcaattctcagagaagacaaaga
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gggagtacttcaagatcaccctctacgggagaaccaaggagctgacttcggaactaaaggagaacttcatccgcttctccaaatctctgggcctccctgaaaaccacatcgtcttccctgtccc
aatcgaccagtgtatcgacggcggangtagcgaaaacctgtattttcagggaggcggccgcgccaagaaggtggagttcaaggagcccgcttgcaacgtgaccttcaagagcgaggcca
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agtgatagcgggccgctaaggatcc

6.3 Protein expression and purification

Human Embryonic Kidney (HEK) suspension adapted freestyle 293F cells (Invitrogen) were used for protein expression following lentiviral based stable transduction or transient transfection with polyethylenimine (PEI) (sigma) transfection reagent respectively following previously outlined protocols (214, 319). Stable lentiviral based transductions were performed as follow, in brief, plasmids containing appropriate constructs were incorporated into replication incompetent lentiviral particles as described previously. HEK-293F cells plated in fresh media at one million per mL in 10 mL of fresh freestyle media were transduced with pseudoviral particles. Cells were incubated at 37 ^{o}C with 8% CO_2 and 80 % humidity. 8-16 hours post transduction; cells were supplemented with 25 mL of fresh freestyle media. Transduction efficiency and protein expression were verified qualitatively by assessing GFP/mCherry expression using EVOS fluorescence imaging systems (Life technologies). Three days post transduction, cells were expanded at a cell density of 0.5 million per mL.

Transient transfections were done in short as follows, HEK freestyle (293F) cells were plated at one million per mL in 100 mL of fresh freestyle media. A DNA:PEI complex was formed at 1:3 mass ratio and applied directly onto cells suspended in flasks. Cells were incubated at 37 ^{o}C with 8% CO_2 and 80 % humidity. 8-16 hours post transfection, cells were pelleted by centrifugation at 1000 rpm for 5 min and resuspended in fresh freestyle media and incubated at 37 ^{o}C. Four days post transfection, supernatant were harvested by centrifugation at 8000 rpm for 15 min and clarified by passing through 0.22-micron Centricon ultrafilter (Millipore).

Cleavable c/scFvs for mature b12, germline encoded precursor, b12 chimeric forms, gRBs, b12 Fab constructs (mature, germline, Chimera 1 (mL/gH) and Chimera 2 (gL/mH)) and hScn fused full-length gp120 monomers were expressed using stable transduction of HEK 293F cells as described above. Pseudoviral particles containing VH-CH1 and VL-CL were used to co-transduce 293F cells plated as described above. Qualitative estimation of co-transduction efficiency was done using EVOS fluorescence imaging systems (Life technologies).

Full-length biotinylated gp120 sequences were expressed in HEK 293F cells using lentiviral based stable transduction as described above. Cells transduced with viral particles that contain cDNA for hScn full-length gp120 fusion and confirmed for protein expression using SDS-PAGE and western blot were retransduced with pseudoviral particles containing the BirA biotin ligase enzyme. Efficiency of double transfection was assessed using EVOS (life technologies). Biotinylation efficiency was assayed using streptavidin agarose beads (Thermo Scientific).

Large-scale expression and purification of c/scFvs, Fabs and hScn-gp120 fusions were performed in HEK 293F cells. Supernatant clarification and protein purification were done as described previously with minor modification. Briefly, supernatants were harvested by centrifugation at 8000 rpm to remove cells and clarified by passing through 0.22-micron filter (Thermo scientific). Clarified supernatants were supplemented with Sodium Chloride and concentrated using 10,000 molecular weight cutoff Amicon Stirred Cell concentrator (Millipore). Recombinant proteins were further purified by size exclusion chromatography (SEC) using a Superdex 75 and/or Superdex 200 column (GE Healthcare) running in 25 mM piperazine-N,N'-bis(2-ethanesylfonic acid) pH 7.0, 150 mM NaCl, 1 mM EDTA, 0.02% NaN_3 (PNEA) or phosphate-buffered saline (PBS) running buffers. Eluted fractions were analyzed using SDS-PAGE. Protein concentrations were determined using calculated protein extinction coefficients and absorbance at 280 nm as surrogate measure.

Protein expression and purification protocols for samples used as crystallization reagents obtained using protocols outlined above with modifications described below. Because of the presence of a contaminating fragments due to aberrant stop codon overall in our mammalian expression construct, c/scFv constructs expressed for crystallization were further purified using SEC fractionation to yield crystallization quality protein samples. C/scFv samples were run and fractionated into 250 uL fractions on Superdex 200 column (GE Healthcare) with 25 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), 150 mM NaCl, 1 mM EDTA, 0.02% NaN_3 (PNEA) or phosphate-buffered saline (PBS) running buffers. Fractions with no or minimal contaminating fractions were collected following comparative SDS-PAGE gel

analysis under reducing and non-reducing conditions. Samples were concentrated to protein concentrations suitable for crystallographic experimentation.

MICA full length, MICB platform domain and MICA σ3 domain were cloned into bacterial expression vector pET22b(+)(Clontech, Mountain View, CA), transformed into BL21(DE3) (Life Technologies, Grand Island, NY) cells, and subsequently produced insoluble inclusion bodies. Inclusion bodies were resolubilized and *in vitro* refolded as described previously (186).

Both bacterial and mammalian expression systems were used to generate soluble E3/19K protein suitable for biochemical characterization and structure determination. Bacterial expression constructs were generated by PCR amplification of Adenovirus serotype 2 E3/19K region from the EcoRID fragment generously provided by Dr. Burgert (University of Warwick, Coventry, UK) and cloning into pET22b(+) expression vector (Clontech, Mountain View, CA). Insoluble inclusion body were expressed in BL21 DE3 (Life Technologies, Grand Island, NY), *in vitro* refolded, and purified as previously described (186). For mammalian expression, Adenovirus serotype 2 E3/29K region was engineered as fusion protein to fusion partner human Siderocalin (hScn) as described in previous chapters, fusion constructs were cloned into lentivirus transduction vectors and expressed in HEK293F (Invitrogen, Grand Island, NY) or HEK293S (CRL-3022) (ATCC, Manassas, VA) using protocols discussed in chapter 2. Soluble secreted fusion proteins were purified using affinity chromatography. Purified hScn-E3/19K fusion protein samples were treated with TEV protease (Invitrogen) to liberate E3/19K soluble protein from its fusion partner.

Adenovirus E3/19K protein, expressed in HEK 293 expression system was properly folded. HScn fused E3/19K expression exhibited extensive glycosylation when expressed in HEK 293F cells. To make the protein amenable for crystallography the protein was also expressed in HEK293S GnTI^{-} cells that do not express N-acetylglucosaminyltransferase I and hence produce protein with reduced N-linked glycans. Absence of higher order glycans on protein expressed in HEK 293S cells was deduced using comparative SDS-Page analysis where E3/19K from HEK 293S is lower in molecular weight than protein sample from 293F (320).

6.4 Protein validation and thermal denaturation

Protein expression and proper folding were analyzed using Western Blot, SDS-PAGE gel under reducing and non-reducing conditions and size exclusion chromatography (SEC). Initial c/scFv protein expressions were monitored by probing for STREP tagged c/scFvs using nti-streptavidin HRP conjugate (Thermo Scientific). FLAG tagged hScn-gp120 fusions were probed with mouse monoclonal anti-FLAG primary and AP conjugated goat-anti-mouse secondary antibody (BioRad) in Western Blots using standard protocols. From initial crude supernatant to purified protein, proper protein folding and disulfide formation were monitored using SDS-PAGE (life technologies) under reducing and non-reducing conditions.

Thermal stabilities of *in vitro* expressed proteins were assayed using Circular Dichroism (CD) with J-815 spectrometer (Jasco) instrumentation at protein concentration of 10 – 20 µM in 10mM phosphate buffer pH = 7.2. Based on wavelength scanning analysis, protein-melting temperatures (T_m) were analyzed at 210 nM wavelength and temperature range from 20 ^oC to 90 ^oC at temperature ramp of 2 ^oC/minute slope.

6.5 Neutralization assay

Neutralization potency of b12 c/scFvs and Fabs were assayed as previously described (102) using replication incompetent virus psuedotyped with QH0692 and SF162 envelope sequences. Assays were performed using a luciferase reporter TZM-bl cell line as target. Percent neutralization and IC_{50} were calculated from two independent runs for b12 c/scFv and Fab variants as described and outlined previously. Percent neutralization by single amino acid reverted b12 c/scFvs were assayed at antibody dilutions where ~80% neutralization potency against QH0692 and SF162 were observed by mature b12 c/scFv (10 ng/mL and 0.5 ng/mL Ab dilutions respectively). All neutralization assays were done in replicate.

6.6 Binding Kinetics using Surface Plasmon Resonance (SPR)

SPR experiments were carried out on a Biacore T-100 instrument with the T200 sensitivity enhancement (GE Healthcare) at room temperature (25 ºC) in HBS-EP+ (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant p20, pH 7.4) running buffer supplemented with 0.1 mg/mL bovine serum albumin.

Binding kinetics of b12 mature, b12 germline, b12 Chimeras (1 and 2), gRB b12 c/scFvs and Fabs to gp120 surfaces were done using SPR. Cleaved and uncleaved biotinylated hScn-gp120s were captured on SA sensor chip (GE Healthcare). Chips were preconditioned with 1 M NaCl and 50 mM NaOH following manufacturer's recommendations. Both hScn-gp120 fusion and cleaved gp120 were used as binding ligands. Cleaved gp120 (SF162 and QH0692) monomers were captured on flow cell four while uncleaved hScn-gp120 fusions (SF162 and QH0692) monomers were captured on flow cell two. Flow cell one and three were left blank for double referenced data analysis. To capture on SA chips, biotinylated binding ligands were injected at 0.2 µg/mL in running buffer at a flow rate of 10 µL/min and immobilized up to ~120 RU. Analytes (mature, germline, and Chimeric c/scFvs or Fab) and blank buffer injections were randomized and injected in duplicate at a flow rate 50 µL/min. Detailed individual concentration series, association time and dissociation time are presented in Table 6-1.

SPR based binding analysis of E3/19K and MICA/B were carried out on a Series S CM5 sensor chip (GE Healthcare). 73.9 response units (RU) of bacterial expressed and refolded E3/19K in 10 mM sodium acetate (pH 5.5) were immobilized on CM5 sensor chips using standard amine-coupling chemistry. Activated and conditioned empty flow cell was used as a reference surface. To assess binding of E3/19K to full length MICA and MICB platform, refolded full length MICA, and MICB platform domain were used as analytes. Samples and buffer blanks for double referencing were injected as duplicates in random order. Analytes were injected as serial three-fold dilutions with concentrations ranging from 3000 nM to 37 nM, injected at a flow rate of 50 µL/min for 7 min followed by a 15 (MICA full length) or 3 min (MICB platform) dissociation phase.

Surface regenerations were done by injecting 10 mM glycine, pH 1.5, at 50 μL/min for 5s. Kinetic analyses on double referenced data were analyzed using BiaEvaluation software. Data were fitted globally using a 1:1 binding model. Sensorgrams were analyzed using a 1:1 binding model with Biacore T100 Evaluation Software (version 2.0.4; GE Healthcare).

Table 6-1. SPR binding analysis protocol

Analyte	**Captured SF162 gp120 monomer (RU)**	**Concentration Range**	**Inject Time(s)**	**Dissociation Time (s)**
Mature	111	15→ 0.12 nM	420	600
germline b12	111	1 μM	420	420
Chimera 1 (mL/gH)	111	1 μM	420	420
Chimera 2 (gL/mH)	111	500 → 2 nM	420	420
gRB1	111	1 μM	420	420
gRB2	111	500 → 2 nM	420	420
gRB4	111	15.6 → 0.24 nM	420	420
Analyte	**Captured QH0692 gp120 monomer (RU)**	**Concentration Range**	**Inject Time(s)**	**Dissociation Time (s)**
Mature	120	30→ 0.12 nM	420	3600 or 180
germline b12	120	1 μM	420	420
Chimera 1 (mL/gH)	120	2 μM → 31 nM	420	600
Chimera 2 (gL/mH)	120	500 → 7.8 nM	420	420
gRB1	120	10 μM → 39 nM	420	600
gRB2	120	1 μM → 7.8 nM	420	420
gRB4	120	30→ 0.12 nM	420	3600 or 180

6.7 Crystallization

Crystals of b12 germline, mutant germline b12 (gRB2) and Chimera 2 (gL/mH) were obtained from commercially available standard crystallization screens in a sitting drop setup. Initial crystal hits were recorded for b12 germline at ~10 mg/mL from crystallization condition containing 0.1 M MES buffer pH 6.5, 2.0 M Ammonium sulfate, and 5% polyethylene glycol 400 after 20 days. Similarly, gRB2 crystals were observed in conditions containing 1.2 M Lithium sulfate, 0.1M HEPES pH 7.5 and 20% PEG 1000. Initial screening trays for crystals of b12 Chimera 2 (gL/mH) were set at ~10 mg/mL and initial crystal hits were recorded after three days in crystallization condition containing 0.1 M citric acid pH 4.0 and 1 M NaCl. Further optimizations of crystallization conditions were performed using hanging-drop vapor diffusion method. Diffraction quality crystals were grown at room temperature. Crystals of germline b12 and gRB2 were cryopreserved in mother liquor containing 15 % v/v Glycerol while Chimera 2 (gL/mH) were cryopreserved in mother liquor supplemented with 35 % v/v Ethylene glycol.

6.8 Diffraction data analysis and model building

Diffraction data were collected at the Advanced Light Source beamline 5.0.1 (Lawrence Berkeley National Laboratory, Berkeley, CA). Data reduction and indexing of germline b12, gRB2 and Chimera 2 (gL/mH) were done using d*TREK (321) and HKL-2000 (322) respectively. Initial phase information were determined by Molecular Replacement (MR) using PHASER (323) from the CCP4i program suite (220). The molecular replacement sub-structural search models were generated using b12 Fab structure (PDB accession code 3RU8 (160)) with variable fragment portions and CDRs trimmed using Chainsaw as implemented in CCP4i program suite (220). Subsequent refinement using REFMAC 5 (222) and model building using COOT (221) were performed to improve phase information. Structure validation was carried out using MolProbity (224). Using L-and H-tests in CCP4i (220) program suite detected significant twinning statistics on Chimera 2 (gL/mH) data set. Twinning was taken into account during the final round of refinement using REFMAC 5 (222). Twinning fractions were determined using twinning analysis in REFMAC 5 (222).

Chapter 7

7 Discussion and Future Directions

As a vaccine development approach, structure based vaccine design utilizes antibody-antigen structural, biophysical and biochemical knowledge to rationally design immunogens that recapitulate bNAbs and their interaction with respective antigenic epitopes (13, 55, 162, 180, 324). Approaches sought in the field include utilization of native Env subunits as potential immunogens, resurfacing Env subunits to focus immune response and epitope grafting into scaffolds. While individual subunits display important immunogenic epitopes, the plethora of virus immune evasion mechanism hindered successive use of such immunogens as vaccines (87, 149, 155, 161). Yet, to date the modest success in HIV vaccine development is recorded from subunit based vaccine studies (161, 162, 325).

Techniques of immune-focusing (326) attempted to design Env subunits that display highly immunogenic epitopes while shielding or removing immunodominant antigenic epitopes that are potential competitors during antibody elicitation (44, 77, 158). Research in this area resulted in resurfaced gp120 monomers that remove hypervariable loop regions, manipulate glycan coverage and subunit stabilization which remarkably reduced elicitation of non-neutralizing antibodies without compromising native gp120 comparable bNAb recognition(155) (77, 92). However, such gp120-based immunogens were unable to elicit antibody based immunity response comparable to bNAbs, that is, superior to wild type gp120 (88, 155, 323, 327-330).

A promising approach that aims at grafting specific hyper immunogenic epitopes on scaffolding proteins enabled successful elicitation of antibodies in RSV (142). The technology also resulted in various potential immunogens that recapitulate Env comparable bNAb binding (151, 159, 160, 192, 331-335). Despite extensive characterization of designed immunogens, none elevated to vaccine status (55, 156, 336). Disconnect in designed immunogens' ability to bind template bNAbs and their failure to elicit relevant antibody responses in model organisms is indicative of a knowledge gap in attributes assessed

and their biological relevance. A potential shortfall is the lack of proper understanding of extensive mutations observed in bNAbs and their importance in antibody function.

As part of an overarching aim of designing vaccine immunogens using CD4BS bNAbs as design templates, our research into the biogenesis of b12 development and role of SHM uncovered important attributes of the antibody described in previous studies while revealing novel characteristics that impact structure based vaccine design (82, 107, 111, 184). Although briefly described in previous work (107), we have addressed the relevance of VL chain in epitope binding and neutralization. As detailed in chapter 2, antibody light chain domain greatly affected both epitope binding and neutralization. Further illustrating the importance of VL domain, our data indicates mutations at and near CDRL1 as instrumental in antibody function. Modeling analysis demonstrated presence of VL chain footprint and hence interaction with loop regions on trimeric Env (Figure 7-1). The results described in Chapter 2 emphasize the importance of VL chain (CDRL1 in particular) and hence strategies of structure based immunogen designs that utilize b12 as potential design template must consider the role of VL interaction in their algorithms.

Modeling experiments also revealed extensive interactions of b12 on neighboring protomers in addition to binding to CD4BS on its cognate protomer (Figure 7-1). The observed extensive contact on adjacent protomers and potential atomic clashes might affect use of b12 and its interaction as a design template. The antibody-antigen orientations in a b12-like interaction also suggest b12 cognate epitope is not readily accessible and shielded due to quaternary structure through trimerization (42). However, given the reported flexibility in native Env trimers (46, 337), it is plausible to achieve b12 comparable antibody based immune response following vaccination with immunogens that mimic b12-Env interaction. The observed extensive contacts with neighboring protomers need further investigation. Even considering Env flexibility, the current analysis suggests potential obligate contacts between b12 and neighboring paratopes. Contact through antibody constant regions might alter specificity and binding affinity. Hence we recommend these interactions need further in-depth investigation to delineate their importance in overall antibody binding and downstream function.

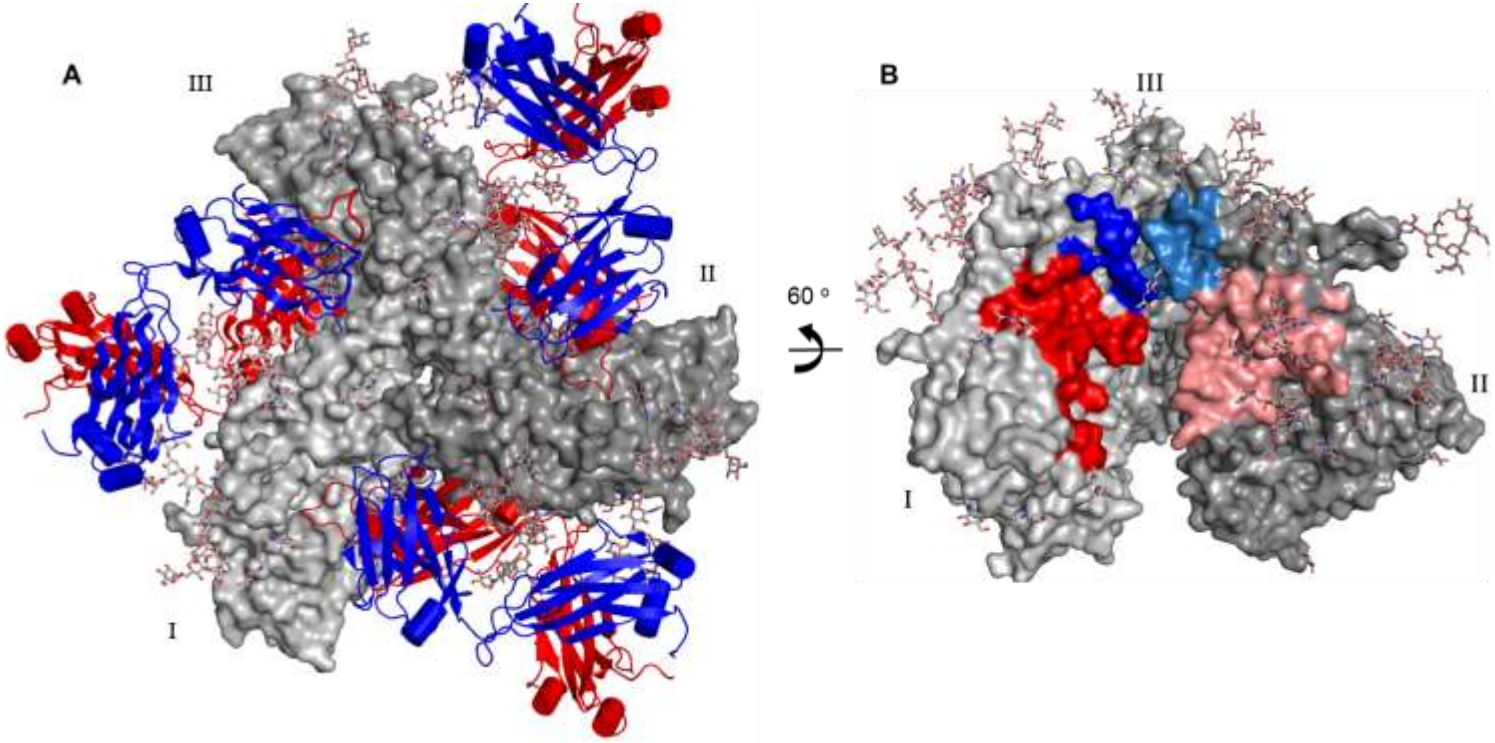

Figure 7-1. Mature b12 binding on trimeric Env protomers

A, Surface representation of Env trimer (PDB (83) accession code 4NCO (47)) in light gray shade with mature b12 binding as elucidated on core-gp120 structure (PDB accession code 2NY7 (76)) and superposed on a single protomer with VH chain colored red and VL chain colored blue. Glycan molecules are presented in stick representation where atoms are labeled as gray (C), red (O) and blue (N). Individual trimeric Env protomers are numbered I, II, and III respectively. B, 60^0 X axis rotation of Env trimer with similar representation and mature b12 binding as in A with footprints due to b12 VH on protomer I colored red and on protomer II colored salmon red. Footprints of VL on protomer I colored blue and on protomer II colored light blue.

Mutation based selection process is a key component of an effective humoral immune response. A novel discovery, in both single reversion of matured amino acids to their germline progenitor sequence and progressive incorporation of SHM in germline precursor antibody resulted in incremental and cooperative increases in binding affinity. No single position or groups of mutations are found to be deterministic of antibody function. Mutations on CDR regions improved initial epitope recognition mainly due to faster rate of association and modest CDR rigidification (as seen on CDRLs). Antibody maturation related mutations on FWR increased stability of antigen - antibody complexes. While this incremental and cooperative role of SHM in epitope recognition and binding affinity is consistent with the theory of affinity maturation, the effects on neutralization potency is complex and necessitates extensive SHM. Our observations further strengthen the hypothesis that the observed high frequency of mutations in bNAbs is predominantly due to gradual molding and shaping of the antibody Fv domain through the antibody maturation process. The process requires many iterations of incremental incremental increases in binding and neutralization potency.

Initial antibody responses dominated by diverse antibody profile, mainly non-neutralizing response, subside in antibody titer overtime. While non-neutralizing antibodies that target variable regions gradually fade, those targeting conserved regions are maintained past the decline phase and over prolonged affinity maturation (209, 212). High sequence evolution through HIV generations, while sustaining the diversity in antibody titer, also enables focusing antibodies that target relatively conserved regions for prolonged period. The extensive exposure to the evolving virus derived antigen results in an evolutionary arms race of Env and antibody paratope and hence constant molding and reshaping of the antigen-combining region.

The antibody maturation process and the observed extensive SHM on b12 like bNAbs, as described in this document, indicate the need for antibody diversity following initial immunization where potential antibodies that target bNAb-triggering regions are maintained through the diminished antibody titer phase. This implies that vaccination regimens need to maintain immunogen diversity to guide antibodies through the extensive antibody maturation process. Indeed, the ability of the immune system to

elicit bNAbs following vaccination is well established albeit for HIV vaccinations it may require prolonged and diversified immunization using either cocktail based or sequential/booster based immunizations to maintain the level of diversity observed following natural infection (338, 339). Such regimens can potentially lead to elicitation of multiple classes of bNAbs that can synergistically ameliorate the problem of immune escape (338, 340).

An extensive and protracted affinity maturation process coupled with immunogen sequence diversity can burden effective antibody elicitation following vaccination. Biogenesis of b12 and its antibody maturation does not promote confidence in structure based immunogen designs that utilizes rare bNAbs like b12. Our results and conclusions argue for reconsideration of the current reductive approach of structure based vaccine design and epitope grafting in particular, that attempt to simplify antibody elicitation following infection or vaccination to mere protein-protein interactions (108, 324). Tailor made vaccine development, where beginning with infection-elicited bNAbs and their cogent epitope, working backwards using design-based epitope grafting is a conceptual marvel. However, given the current state of the art necessitates recalibration and refocus in HIV design based vaccinology research. Elucidations of three-dimensional native envelope spikes reveal occlusions and orientationally limiting accesses of conserved bNAb triggering epitopes on Env (35, 47, 196, 197, 341). In addition, continuous revelations in the underlying properties of clinically isolate bNAbs (214, 342, 343) including the role of mutations in epitope binding and neutralization potency reported here indicates the potential hurdles in the use of bNAbs as template for vaccine design. Therefore, it is important to buttress the design based epitope grafting approach with other conventional and empirical vaccine development techniques.

One approach currently under investigation is the use of native-like trimeric or multimeric Env reagents as immunogens coupled with technologies aimed at stabilizing trimeric Env structures and focusing immunogenic regions (25, 48, 65, 109, 143, 146). Extensive research unveiled the superiority of multimeric native Env-like immunogens in eliciting antibodies compared to monomeric subunits. Closed Env structures are maybe required to trigger progenitor B cells and subsequently lead to elicitation of bNAbs than open and CD4 bound conformations or designed and grafted epitopes (157),(344, 345).

Hence, our current knowledge, both in virus biology and the immune response against HIV, suggests approaches that maintain the overall quaternary structure of Env simultaneously using modifications to harness the natural immunological sensitivity of regions on Env should also be given due consideration.

While the hallmark of success in vaccination is elicitation of bNAbs (212, 346, 347), the unconventional properties observed in the virus and immune system interface during HIV infection call for vaccine immunogen development that manipulates diverse immune responses. Initial infection results in abundant antibody based responses that include narrowly neutralizing antibodies. Given the narrow sequence divergence in founder viruses, vaccination tools that blend faster elicitation of such antibodies with other humoral and innate immune based protection could serve as potential solutions. Previous vaccination trials emphasized the importance of T-cell based immunity (108, 330, 345, 348) and antibody dependent cellular cytotoxicity (150, 349). Immunogens that target conventional and narrowly neutralizing antibodies and vaccination elicited T-cell based immunity needs to also be a focus for HIV vaccine development.

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