FC Receptor-Mediated Activities of Env-Specific Monoclonal Antibodies Generated from Human Volunteers Receiving a DNA Prime-Protein Boost HIV Vaccine: A Dissertation

Matthew R. Costa
University of Massachusetts Medical School

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FC RECEPTOR-MEDIATED ACTIVITIES OF ENV-SPECIFIC MONOCLONAL ANTIBODIES GENERATED FROM HUMAN VOLUNTEERS RECEIVING A DNA PRIME-PROTEIN BOOST HIV VACCINE

A Dissertation Presented

By

MATTHEW ROBERT COSTA

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

OCTOBER 12th, 2016

IMMUNOLOGY AND MICROBIOLOGY
FC RECEPTOR MEDIATED ACTIVITIES OF ENV-SPECIFIC MONOCLONAL ANTIBODIES GENERATED FROM HUMAN VOLUNTEERS RECEIVING A DNA PRIME-PROTEIN BOOST HIV VACCINE

A Dissertation Presented
By
MATTHEW ROBERT COSTA

This work was undertaken in the Graduate School of Biomedical Sciences
Immunology and Microbiology Program

The signature of the Thesis Advisor signifies validation of Dissertation content

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The signatures of the Dissertation Defense Committee signify completion and approval as to style and content of the Dissertation

Madelyn Schmidt, Ph.D., Member of Committee

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Abraham Louis Brass, M.D. Ph.D., Member of Committee

Xiangpeng Kong, Ph.D., External Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

Paul Clapham, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the School.

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

October 12th, 2016
Acknowledgments

I would first like to thank my mentor, Dr. Shan Lu. He has provided the necessary structure that allowed me to continue my pursuit to become a scientist. I would also like to thank Dr. Shixia Wang for her support and mentorship in lab that was always a valuable resource. I would also like to thank the members of the Lu Lab that expertise helped me work through questions with my project, particularly Dr. Aaron Wallace and Dr. Diego Farfan. Also, I would like to thank Cindi Callaghan and Dr. Jill Serrano for all the assistance they were able to give over the years.

I would also like to thank the members of my TRAC committee: Dr. Ronald Iorio, Dr. Paul Clapham, Dr. Madelyn Schmidt, and Dr. Mohan Somasundaran. Their experience and helpful advice allowed me to develop and implement a significant research project. I would also like to thank Dr. Guido Ferrari for collaborating on one of the projects presented in this dissertation. The advice from all was vital during my time here at the University of Massachusetts Medical School.

Finally, I would like to thank my family and friends that have endured this journey with me. This accomplishment means so much more with the love and support that I had with me from you all. Thank you.
Abstract

Human immunodeficiency type 1 (HIV-1) is able to elicit broadly potent neutralizing antibodies in a very small subset of individuals only after several years’ infection and as a result, vaccines that elicit these types of antibodies have been difficult to design. The RV144 trial showed that a moderate protection is possible, which may correlate with antibody dependent cellular cytotoxicity (ADCC) activity. Previous studies in the Lu lab demonstrated that in an HIV-1 vaccine phase I trial, DP6-001, a polyvalent Env DNA prime-protein boost formulation, could elicit potent and broadly reactive, gp120-specific antibodies with positive neutralization activities along with multiple Fc mediated effector functions. I developed a protocol for the production and analysis of HIV-1 Env-specific human monoclonal antibodies (mAbs) isolated from these DP6-001 vaccinees. By utilizing a labeled gp120 bait to isolate Env specific B cells, paired heavy and light chain immunoglobulin (Ig) genes were cloned and allowed for the production of monoclonal antibodies with specificity for gp120. By using this protocol, 13 isolated mAbs from four DP6-001 vaccinees showed broad binding activities to gp120 proteins of diverse subtypes, both autologous and heterologous to vaccine immunogens, with mostly conformational epitopes and a few V3 and C5 specific mAbs. Equally cross-reactive Fc-mediated functional activities, including ADCC and antibody dependent cellular phagocytosis (ADCP), were present with both immune sera and isolated mAbs, confirming the induction of non-neutralizing functional antibodies by the DNA prime-
protein boost vaccination. Elicitation of broadly reactive mAbs by vaccination in healthy human volunteers confirms the value of the polyvalent formulation in this HIV-1 vaccine design.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADCC</td>
<td>Antibody Dependent Cell mediated Cytotoxicity</td>
</tr>
<tr>
<td>ADCP</td>
<td>Antibody Dependent Cellular Phagocytosis</td>
</tr>
<tr>
<td>ADCVI</td>
<td>Antibody Dependent Cellular Virus Inhibition</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>Ad5</td>
<td>Adenovirus 5</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral Drug Therapy</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>bNAb</td>
<td>Broadly neutralizing antibody</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CCR5</td>
<td>C-C chemokine coreceptor 5</td>
</tr>
<tr>
<td>CD4bs</td>
<td>CD4 binding site</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining region</td>
</tr>
<tr>
<td>CDRH3</td>
<td>Complementary Determining Region 3 Heavy Chain</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of Differentiation 4</td>
</tr>
<tr>
<td>CD4i</td>
<td>CD4 inducible</td>
</tr>
<tr>
<td>CSR</td>
<td>Class Switch Recombination</td>
</tr>
<tr>
<td>CXCR4</td>
<td>C-X-C chemokine coreceptor 4</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>Env</td>
<td>Human Immunodeficiency Virus type-I Envelope</td>
</tr>
<tr>
<td>Fab</td>
<td>Antibody fragment antigen binding region</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>Fc</td>
<td>Antibody fragment crystallizable region</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc Receptor</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal center</td>
</tr>
<tr>
<td>Gp41</td>
<td>Human Immunodeficiency Virus Type-1 glycoprotein 41</td>
</tr>
<tr>
<td>Gp120</td>
<td>Human Immunodeficiency Virus Type-1 glycoprotein 120</td>
</tr>
<tr>
<td>Gp160</td>
<td>Human Immunodeficiency Virus Type-1 envelope polyprotein</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human Immunodeficiency Virus Type-I</td>
</tr>
<tr>
<td>hmAb</td>
<td>Human monoclonal antibody</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MLV</td>
<td>Murine Leukemia Virus</td>
</tr>
<tr>
<td>MPER</td>
<td>Membrane Proximal External Region</td>
</tr>
<tr>
<td>NAb</td>
<td>Neutralizing antibody</td>
</tr>
<tr>
<td>NoNAb</td>
<td>Non-neutralizing antibody</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PIC</td>
<td>Pre-initiation complex</td>
</tr>
<tr>
<td>PMPA</td>
<td>(R)-9-(2-Phosphonylmethoxypropyl)adenine</td>
</tr>
<tr>
<td>SHM</td>
<td>Somatic Hyper-Mutation</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian Immunodeficiency Virus</td>
</tr>
<tr>
<td>UCA</td>
<td>Unmutated common ancestor</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
</tr>
</tbody>
</table>
Preface

The neutralization assay from Chapter IV in this dissertation (Table 4.1) was partially performed in the laboratory of Dr. Michael Seaman.

The ADCC assay from Chapter II and Chapter IV in this dissertation (Figure 2.2 and Figure 4.1, 4.2, 4.5) was performed in the laboratory of Dr. Guido Ferrari.

Figure 1.1 was adapted from the following publication:


Figure 2.9 was adapted from the follow publication:


Portions of this dissertation will appear in the following publication:

CHAPTER I

Introduction

1. HIV-1 Replication and Life Cycle

Human immunodeficiency virus type 1 (HIV-1) is a virus that uses the same cells that are a major component of the immune system as targets for infection and replication, such as CD4 T cells and macrophages. Disease is caused when late in the life cycle of HIV-1, production of HIV-1 virions causes death to infected immune cells including T helper cells and macrophages, and to a lesser extent dendritic cells and microglial cells. All these cells share the receptors necessary for binding and fusion of HIV-1 with the plasma membrane, CD4 and co-receptors CCR5 and CXCR4 (1). Fusion with the CD4 receptor and co-receptor is mediated by the envelope trimer on the surface of HIV-1 that consists of a trimer of the gp120/gp41 complex (2). After entry into the cytoplasm, host and viral proteins cooperate to reverse transcribe the viral RNA genome into proviral DNA. This viral DNA associates with viral and host proteins to form the pre-initiation complex and gain access into the nucleus, where integrase initiates integration into the cell genome (3, 4). From this point, HIV-1’s accessory proteins, Tat and Rev, along with additional viral and cellular factors allow the production of viral RNA, both genomic RNA and spliced mRNA. Spliced mRNA is transcribed into viral proteins, which allows for the final stages of virus production. Viral genome and assembly proteins are packed into a viral particle, after which the particle makes its way to the plasma membrane to
bud and acquire its envelope. HIV-1 Gag and its proteolytic products are necessary for packaging, budding, and virion maturation, which result in the creation of an infectious particle (5).

To develop an effective vaccine, the immune system has to be primed to recognize and respond to the HIV-1 viral glycoprotein on the surface of the virion. One of the main areas of research into HIV-1 is the envelope glycoprotein gp160, which is the basis for entry into cells, and consequently the main target of the humoral immune response. The viral glycoprotein is derived from the env gene which produces a protein gp160 which is cleaved into two parts, gp120 and gp41. A heterodimer is formed between gp120 and gp41, and three heterodimers form the trimeric spike on the surface of HIV-1. Like a type 1 membrane fusion protein, the gp41 protein mediates fusion after the gp120 protein engages with surface receptors on the target cell. With the advent of multiple monoclonal antibodies (mAbs) to epitopes on the trimer, combined with highly stabilized trimers, researchers have been able to determine high fidelity structures of the trimer. One of the main advances has been the BG505.SOSIP.664 trimer that mimics the antigenicity of native HIV-1 envelope protein (Env) by maintaining neutralizing epitopes and restricting non-neutralizing epitopes, giving rise to multiple structures describing in detail the full structure of the HIV-1 trimer (6–9). With these structures, the current model of the trimer involves gp41 forming a 4 helix bundle that is held in conformation by the carboxyl and amino end of gp120. When gp120 engages CD4 and CCR5, the conformational change in gp120 allows gp41 to form a helix that penetrates the cell membrane, followed by the three gp41 domains forming a 6 helix bundle that brings the
cell and viral membrane together for fusion (10). With this knowledge, we can see that while some neutralizing epitopes are accessible on the trimer, conformational changes during viral entry may expose further epitopes, while not being neutralizing, could have other anti-viral functions besides neutralization.

2. HIV-1 monoclonal antibodies

2.1 Neutralization versus Fc-mediated effector function

Historically, neutralization has been the sought after antibody function for protection from HIV-1 infection, but studies have also shown that Fc functions are also important. The Burton group utilized the SHIV macaque infection model and b12 antibody variants to study their effects. Two variants of b12 were utilized, the K322A variant that prevents binding to complement, and the L234AL235A (LALA) variant that prevents binding to FcγRI, FcγRIIb, FcγRIII and FcγRIV. Neither of these mutations affects Fab function and neutralization, so the effect is Fc specific. Following passive transfer of the three versions of b12 and challenge with SHIV, plasma viral loads were controlled by the wild type b12 and KA mutant, but the LALA mutant could not control viral load, and was also impaired when assayed for ADVCI, indicating that FcR function, but not complement function, is necessary for protection (11). A follow up study by the group using a low dose challenge model confirmed the necessity of Fc mediated function for full protection (12). Another group engineered the Fc domain of neutralizing antibody 3BNC117 to either have increased or decreased binding of FcRs compared to wild type. Using a humanized mouse model, the group confirmed the findings above, and also
showed that increasing FcR binding can improve the protective abilities of the antibody (13). One study has added confusion to this story. A non-fucosylated variant of b12 has increased binding to FcRgIII but does not improve infection in a macaque model (14). In general, anti-viral activities mediated through the Fc portion of antibodies seems to be a complementary and necessary component of the protective capability of antibodies in addition to neutralization.

2.2 Historical broadly neutralizing monoclonal antibodies

In the early days of HIV research, a few broadly neutralizing antibodies were isolated from HIV-1 infected patients that gave hope that the human body was capable of mounting a humoral defense against HIV-1. The trick was being able to create a vaccine to elicit a similar response. A certain number of caveats came with these early monoclonal antibodies. The CD4 binding site (CD4bs) broadly neutralizing antibody (bnAb) b12 was created from a phage display library, so it is not possible to determine if the antibody was generated endogenously in the body, which meant it was unknown if this type of antibody was a possible vaccine target (15). Another improbable target antibody was the glycan binding bnAb 2G12 that binds to a conformational epitope involving carbohydrates, but has the unusual feature of a domain swapped Fab region, which is not common in nature (16). This antibody was intriguing because it was able to protect animals at a much lower passive transfer titer as compared to b12 (17). The last two bnAbs of interest at this early time in HIV-1 research were both gp41 binding mAbs. The bnAbs 2F5 and 4E10 bind distinct linear but adjacent epitopes, along with another less well known bnAb Z13 that has an epitope that overlaps with 4E10 (18–21). While
interesting at the time of discovery, many more potent and broadly neutralizing antibodies have been isolated in the past 10 years with the advances in single cell molecular cloning of mAbs, with a lot of research going into how to elicit them by vaccination.

2.3 Contemporary broadly neutralizing antibodies

Over the last decade, numerous studies have been done with samples from HIV-1 infected patients. This has led to the development and study of a wide range of mAbs with varying degrees of activity and epitopes. The most potent and broadly neutralizing of these mAbs are classified bnAbs, which have taken advantage of a few regions on Env to bind and mediate neutralization. As shown in Figure 1.1, these mAbs include ones to the CD4bs, VRC01 (22), HJ16 (23), 3BNC60/117 (24, 25), and NIH45/46 (26); the quaternary glycan V1/V2 region, PG9/PG16 (27), and PGT145 (28); the V3 glycan region, HGN194 (23), PGT121-23, PGT25-26, and PGT28 (28); the outer domain glycan region, PGT135-36 (28); and the gp41 region, 10E8 (29), and 3BC315 (25). While potent and broadly reactive, these antibodies have been selected from patients that were screened for the best activity and developed in these patients over a long period of time. While interesting for insights into the epitopes accessible to antibodies, they may not be as easily targeted by vaccination.
Table 1.1: List of broadly neutralizing antibodies.

<table>
<thead>
<tr>
<th>hmAb</th>
<th>Targeted epitope</th>
<th>% virus neutralized</th>
<th>Reference</th>
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<tbody>
<tr>
<td>PG9</td>
<td>V1/V2, QNE</td>
<td>79%</td>
<td>Walker Science 2009, 326:285</td>
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<tr>
<td>PG16</td>
<td></td>
<td>74%</td>
<td></td>
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<tr>
<td>PGT141-145</td>
<td></td>
<td>38-78%</td>
<td>Walker L, Nature 2011, 477:466</td>
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<tr>
<td>VRC26</td>
<td></td>
<td>47%</td>
<td>Doria-Rose N, Nature 2014, 509:55</td>
</tr>
<tr>
<td>PGDM1400</td>
<td></td>
<td>83%</td>
<td>Sok D, PNAS 2014, 111:17624</td>
</tr>
<tr>
<td>CH01-04</td>
<td>V1/V2</td>
<td>38-49%</td>
<td>Bonsignori M, JVI 2011, 85:9998</td>
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<tr>
<td>PGT121-123</td>
<td></td>
<td>65-70%</td>
<td>Walker L, Nature 2011, 477:466</td>
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<td>PGT125-128</td>
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<td>50-72%</td>
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<tr>
<td>PGT130-131</td>
<td></td>
<td>40-52%</td>
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<td>PGT135-137</td>
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<td>16-33%</td>
<td></td>
</tr>
<tr>
<td>35O22</td>
<td></td>
<td>66%</td>
<td>Falkowska E, Immun. 2014, 40, 657</td>
</tr>
<tr>
<td>PGT151</td>
<td>CD4bs</td>
<td>66%</td>
<td>Scharf L, Cell 2014, 7, 785</td>
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<td>8ANC195</td>
<td></td>
<td>70%</td>
<td>Burton D, Science 1994, 266:1024</td>
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<tr>
<td>B12</td>
<td></td>
<td>&gt;90%</td>
<td>Wu X, Science 2010, 329:856</td>
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<tr>
<td>VRC01</td>
<td></td>
<td>57%</td>
<td></td>
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<tr>
<td>VRC03</td>
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<td>90%</td>
<td>Gao F, Cell 2014, 158:481</td>
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<tr>
<td>CH235</td>
<td>CD4bs</td>
<td>40%</td>
<td>Corti D, PLoS One 2010, 5, 8805</td>
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<tr>
<td>HJ16</td>
<td></td>
<td>84%</td>
<td>Wu X, Science 2011, 333:1593</td>
</tr>
<tr>
<td>VRC-CH31</td>
<td></td>
<td>76%</td>
<td></td>
</tr>
<tr>
<td>VRC-PG04</td>
<td></td>
<td>63%</td>
<td>Bonsignori M, JCI 2014, 124:1835</td>
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<td>CH98</td>
<td>MPER</td>
<td>55%</td>
<td>Liao H, Nature 2013, 496:469</td>
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<tr>
<td>CH103</td>
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<td>&gt;98%</td>
<td>Scheid J, Science 2011, 333:1633</td>
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<tr>
<td>3BNCC117</td>
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<tr>
<td>12A12</td>
<td></td>
<td>&gt;98%</td>
<td></td>
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<td>NIH45-46</td>
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<td>&gt;98%</td>
<td>Diskin R, Science 2011</td>
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<tr>
<td>10E8</td>
<td></td>
<td>~98%</td>
<td>Huang J, Nature 2012, 491:406</td>
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</table>
Figure 1.1: Structure of the HIV-1 Envelope on the virion surface. Key neutralizing epitopes labeled on the surface with relevant broadly neutralizing antibodies indicated.
2.4 ADCC Epitope mAbs

To better design vaccines that elicit protective responses, we must start by understanding the types of epitopes bound by non-neutralizing antibodies. Antibodies that mediate ADCC bind to epitopes that span a wide range of areas on both the gp41 and gp120 region of envelope. These epitopes can be both linear or conformational, and either neutralizing or non-neutralizing. Antibodies that mediate ADCC and target gp41 are mostly non-neutralizing, while the opposite is true for epitopes on gp120 (30). The prototypical ADCC mediating mAb is A32. The reason for A32 having this label is that the Fab fragment of A32 is able to abrogate the majority of ADCC activity in serum from chronically infected patients (31). This A32 blocking was also present in vaccine recipients in the RV144 trial, with >90% of recipients having ADCC activity that could be blocked by an A32 Fab fragment (32). A32 belongs to a category of antibodies that recognize CD4 inducible (CD4i) epitopes. This group can be broken down into three clusters; A, B and C. The epitope for cluster A is on the gp120 face masked by gp41 in the trimer. Cluster B binds proximal to the coreceptor binding site, involving the V1V2 domain. The coreceptor binding site is the epitope bound by cluster C antibodies. Cluster A antibodies had the strongest ADCC activity and could target ADCC activity during viral entry (33). Targeting a portion of the vaccine response to cluster A epitopes should provide useful functional support in protection.

Another area that harbors epitopes for ADCC-mediating antibodies is the V2 region in gp120. As mentioned above, the RV144 trial implicated V1V2 and ADCC activity in a correlates of risk analysis. The epitope recognized by two mAbs isolated
from RV144, CH58 and CH59, and the broadly neutralizing mAb PG9 overlap, though
the mode of binding was different, indicating that the epitope is structurally flexible. All
three antibodies can mediate ADCC, but CH58 and CH59 do not require trimeric gp120
or glycan for binding. CH58 binds an alpha helix coil structure, CH59 a 3-10 helix, while
PG9 recognizes a quaternary structure involving glycan (34). Study of the un-mutated
ancestor of CH58 has revealed that a large percentage of the binding, specifically the
glutamic acid/aspartic acid (ED) motif responsible for making salt-bridges with the V2
peptide backbone, are encoded in the germline sequence of only a very select group of
lambda chain germlines (3-10, 6-57). A few somatic mutations to the light chain
complementary determining region 2 (LCDR2) region increase the number of salt bridges
from 3 in the CH58 un-mutated ancestor (CH58UA) to 5 in CH58, and consequently
increase binding 2000 fold (35). While not neutralizing, these mAbs have shown ADCC
activity and the ability to improve antiviral activities with other RV144 mAbs through
synergy (32, 36). The CH58 and CH59 are important proofs that antibodies can be raised
with minimal mutation to biologically relevant regions of HIV-1 implicated in protection.
The germline encoded binding present in these mAbs provides evidence that other
vaccines may be able to induce similar responses.

Three other regions contain ADCC epitopes, the CD4bs, V3 and gp41. Gp41
epitope antibodies include the well-known MPER mAbs 2F5 and 4E10, along with mAbs
246-D, 4B3, 98-43, 50-69, 98-6, 126-50, 31710B and 120-16 (37–39). The CD4 binding
site also serves as an ADCC epitope and includes all neutralizing antibodies, including
VRC01, b12, 15e, 448-D, F105, 1125H and 5145A (31, 39–43). The last major epitope
for ADCC is V3, which while usually being strain specific because of the variability of the V3 loop, includes mAbs 694/98D, 4117C, 41148D, CH22 and CH23 (32, 39, 40). While these epitopes are not exclusive to non-neutralizing antibodies, they do indicate that a broad range of epitopes allow for Fc mediated effector functions.

2.5 Antibody Development

It is reasoned that knowledge of how broadly neutralizing antibodies are induced in infected individuals could be used to improve vaccines. This type of focused vaccine development is starting to develop results, as is the case with multiple collaborating groups designing immunogens to guide antibody development from precursor to matured neutralizing antibody by sequential immunization (44–47). There are now three distinct epitopes that have been studied from initial virus infection through development of neutralizing breadth and potency, which is defined as being able to neutralize a diverse panel of HIV-1 viruses and with only a limited amount of antibody necessary. CAP256-VRC26 is a V1/V2 antibody that coevolved with viral escape mutants to develop breadth from an initial recombination event that introduced a long CDRH3. As the immune system developed antibodies to the virus, escape mutations at the V1/V2 epitope would cause the immune system to raise further matured antibodies that had better binding to the new viral stock (48). The CD4bs was another epitope where co-evolution of antibody and virus diversification was observed. The unmutated common ancestor (UCA) of CH103 was able to strongly bind to the founder virus in an infected individual. Antibody breadth was developed after the virus developed diversity around the CD4bs epitope. The UCA was developed by inferring the germline sequence of mAbs isolated from the same
clonal lineage and sequence derived from deep sequencing (49, 50). It was also
discovered in this individual that another clonal lineage was helping to drive escape
mutations around the CH103 epitope, which further developed its breadth. CH235 could
neutralize early autologous virus, but then selected for mutants that escaped
neutralization and helped CH103 develop breadth by forcing the virus to mutate a residue
in the CH103 epitope, thus allowing for increased binding and further maturation (51).
The last and most recent epitope to be explored in this way was the high mannose patch
on V3 that has been susceptible to bnAbs. A group of mAbs (PCDN mAbs) were isolated
that developed independently from an initial burst of activation and diversification of a
naïve B cell. Like the other studies, these antibodies coevolved with the changing viral
stock, first developing autologous neutralization before developing breadth and
heterologous neutralization capability. The interesting thing about this PCDN group of
mAbs is that they contain members with relatively good neutralization breadth (47%) and
somatic mutation of only 11% (52). This type of antibody would be a good target for
vaccine development because of a neutralization level that may be protective while
having an activation level could be elicited by repeated vaccination. All these studies give
insight into how antibody maturation and virus evolution interact and how vaccine
strategies may be modified to try and mimic or adopt these pathways for vaccine
protection.
3. HIV vaccine studies

3.1 Summary of animal studies

Early studies lent evidence that targeting the virus early in infection can have a lasting benefit and protect late in disease progression. In the simian immunodeficiency virus (SIV) model, continuous PMPA treatment can prevent SIV infection. PMPA is a nucleotide analogue that works by being incorporated into a growing DNA strand during reverse transcription and halting elongation because PMPA is unable to form a phosphodiester bond. Shortened treatment with PMPA had reduced protection, but still showed control of viremia even after treatment cessation, indicating that treatment at early stages had lasting effects (53). This was studied further with passive immunization studies in macaques. Using a mixture of neutralizing antibodies, macaques were treated with antibodies and then challenged the next day with virus. Some animals had sterilizing immunity, but those that did not still showed some benefits in terms of the long term effects on disease progression. Peak viral loads were lower, correlating with reduced depletion of CD4 cells. Long term viral loads were also lower, even though the mAb has a short half-life (54). This has implications for immunization, since early intervention can have long term effects.

Animal studies have also given insight into the immune correlates that associate with protection. Some studies point to neutralization and cellular responses, like the heterologous protection seen in macaques by using an immunogen consisting of multiple HIV-1 immunogens including Gag, Pol, Tat and Env. Immunization of rhesus macaques
with this multi-immunogen formulation protected a portion of animals against challenge, with protection being correlated with both neutralization and cellular IFNγ responses (55). Still other studies show factors besides circulating neutralization are important, such as the prevention of SHIV acquisition in macaques by a gp41 virosome that was not linked to circulating neutralization, but instead mucosal IgA and IgG with transcytosis inhibition, ADCC and neutralization (56). The specific properties of the antibodies in the humoral response are also important. A macaque vaccine with 70% protection was correlated with anti-Env antibody avidity (57). These animal studies and others support the conclusion that multiple factors contribute to protection and this knowledge should help in the development of an effective vaccine.

More recent advances in the study of bnAbs from infected humans have influenced the direction of vaccine studies. Immunogens designed to elicit antibodies to specific epitopes have started to be tested in animal models (Figure 1.1). A few studies have looked into eliciting VCR01 like antibodies. Two studies looked into trying to induce broad neutralizing antibodies by targeting specific naïve B cells expressing the VRC01 germline gene through targeted immunogen design (58, 59). Most immunogens contain glycan modifications around the CD4bs that prevent the engagement and activation of naïve B cells that could develop into VCR01 like antibodies. When these glycans are removed, Env binding to these naïve B cells is enabled (58). Another approach to circumvent the inability of Env to bind VRC01-like precursor cells is to computationally develop and test engineered immunogens designed to specifically bind these type of naive B cells (59). Studies in mice with knock-in versions of CD4bs
germline reverted B cells indicates that these engineered immunogens are able to activate and induce characteristics similar to bnAbs, but are unable to promote the full development of neutralizing antibodies (24, 60). Most recently, this targeted immunization has been moved into mouse models with a full complement of non-rearranged human Ig genes and multiple targeted antigens to elicit neutralizing antibodies. These studies have shown some success in directing antibody evolution to sequentially bind designed immunogens, ending up with neutralization aimed at the CD4bs and N332 supersite (44–47). These types of studies have been able to utilize the information derived from mAbs and their epitopes to develop experimental vaccines with the promise of protection.

Another area of vaccine study is whether non-neutralizing antibodies are able to mediate protection in vivo. Thus far, the effects of Fc mediated effector function in relation to blocking acquisition has been indirect. Passive immunization studies using a LALA mutant of b12 were used to study the effect of blocking Fc effector function in a mouse model. Protection is decreased when Fc function is blocked by modifying the Fc region of b12 to no longer bind to Fc receptors, inferring that Fc function is needed for full protection (11). In a humanized mouse model, Fc binding mutants were used to either decrease or increase binding to FcγRs for a variety of neutralizing antibodies to diverse epitopes. Infection was correlated with level of FcγR binding (13). When comparing the effects of different vaccination strategies, the improved reduction in both acute and chronic viral load in macaques was correlated with ADCC, antibody dependent cellular virus inhibition (ADCVI) and transcytosis inhibition (61). The problem is that when
testing passive immunization in a NHP model, non-neutralizing antibodies have not been effective (62–64). One theory for the discrepancy between correlative studies and passive immunization studies is that the prozone effect causes high levels of antibody to be less effective, necessitating the optimization of antibody amounts to replicate the levels that would be seen by induction with a vaccine (65).

### 3.2 Human studies

Although a lot of study has been done in animal models, the true test of a vaccine must be in a human model. This is because animal models are not able to fully recapitulate the way in which HIV-1 interacts with the human immune system. Multiple times, results seen in simian models did not translate to humans. Along with animal models, vaccine trials in humans need to be performed. Even if they are not done in at risk populations, valuable information can be collected. One way of determining the effect of vaccination would be to perform a sieve analysis on the antibodies and viruses collected from vaccine trials. This type of analysis can help determine the types of viruses that escape vaccination and those that are blocked, along with the types of antibodies elicited during this sorting of virus transmission. Along with neutralizing antibody, Fc mediated function may also be interrogated by this method, helping to determine the most important function in protection against HIV-1 by vaccination (66).
Table 1.2: Late phase HIV-1 vaccine trials in humans.

<table>
<thead>
<tr>
<th>Study</th>
<th>Phase</th>
<th>Date</th>
<th>Immunogen</th>
<th>Population</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vax003</td>
<td>Phase III</td>
<td>1999-2003</td>
<td>Protein (CRF01_AE/B Env)</td>
<td>IDU (Thailand)</td>
<td>None</td>
</tr>
<tr>
<td>Vax004</td>
<td>Phase III</td>
<td>1998-2003</td>
<td>Protein (B Env)</td>
<td>MSM/High Risk Women (USA)</td>
<td>None</td>
</tr>
<tr>
<td>HVTN502</td>
<td>Phase IIb</td>
<td>2004-2007</td>
<td>Ad5 Vector (B Gag/Pol/Nef)</td>
<td>MSM/High Risk (Americas, Australia)</td>
<td>None</td>
</tr>
<tr>
<td>HVTN503</td>
<td>Phase IIb</td>
<td>2003-2007</td>
<td>Ad5 Vector (B Gag/Pol/Nef)</td>
<td>High Risk (South Africa)</td>
<td>None</td>
</tr>
<tr>
<td>RV144</td>
<td>Phase III</td>
<td>2003-2009</td>
<td>ALVAC Vector (B Gag/Pro, CFR01_AE Env) + Protein (CFR01_AE/B Env)</td>
<td>High Risk (Thailand)</td>
<td>31.2%</td>
</tr>
<tr>
<td>HVTN505</td>
<td>Phase IIb</td>
<td>2009-2013</td>
<td>DNA + Ad5 Vector (B Gag/Pol, A/B/C Env)</td>
<td>MSM/TG (USA)</td>
<td>None</td>
</tr>
<tr>
<td>HVTN702</td>
<td>Phase III</td>
<td>2016-2020</td>
<td>ALVAC Vector (C Gag/Pro/Env) + Protein (C Env)</td>
<td>South Africa (Planned)</td>
<td>-</td>
</tr>
</tbody>
</table>
3.2.1 Early Studies

Two of the earliest large scale human vaccine trials were Vax003 and Vax004. Both used a bivalent recombinant gp120 immunogen adjuvanted with alum. A lab adapted viral strain (MN) was used in both, while the second gp120 for each trial was from a region specific clade, CRF01-AE for Vax003 done in Thailand, and B for Vax004 done in the USA. Both trials found no efficacy when comparing vaccine groups to placebo groups in these high risk populations (67, 68). After these gp120 based trials failed, focus was shifted to a cell immunity based approach using an adenovirus vector based system that encoded the viral proteins Gag, Pol and Nef. The Step study (HVTN502) was halted early because an interim analyses showed no efficacy in either preventing infection or reducing viral loads (69). A parallel study that used the same MRKAd5 HIV-1 vaccine as the Step study had enrollment halted when the Step results were known, and no efficacy could be determined (70). After the failure of an antibody exclusive vaccine and cell based exclusive vaccines, efforts went into combining the approaches. While HVTN505, a polyvalent DNA prime including gag, pol, nef and env from clades A, B and C and polyvalent adenovirus boost with matched immunogens, did not produce efficacy at its interim testing, RV144 was able to show modest efficacy at the end of a Phase III trial in humans (Table 1.2) (71, 72).

3.2.2 RV144

RV144 was the first human vaccine trial to show limited protection against HIV-1. The trail was performed in Thailand with a canarypox prime (ALVAC-HIV) and
gp120 protein boost (AIDSVAX B/E) and peaked with a 60.5% efficacy in the first year that dropped to a final 31.2% vaccine efficacy (72, 73). When the trial was repeated in macaques, the vaccine regimen was shown to protect in 50% of the animals, which was correlated to low level neutralization and CD4 T cell responses against Env (74). Analysis of the correlates of protection identified two variables, IgG antibodies to the V1V2 region, and IgA responses. An inverse correlation with risk of infection was identified for the IgG antibodies to V1V2, while a direct correlation with risk of infection was identified for the IgA response (75, 76). When the viruses from vaccinated patients were analyzed, it was shown that there was selective immune pressure on the V2 region, giving further evidence that antibodies to the V2 region had an effect in the vaccine arm of the trial (77). This region has been implicated to interact with the α4β7 integrin receptor on the surface of immune cells. Blocking this binding may impact the ability of HIV-1 virions to accumulate and infect immune cells. Recently, it was shown that blocking this receptor can help to reduce SIV viral loads to undetectable levels, even after the cessation of ART and antibody treatment (78).

Another result from the RV144 trail showed that ADCC was a correlate of reduced infection risk in the group with low IgA C1 binding titers, with IgG titers to the A32 epitope region in C1 being implicated in reduced infection risk (31, 32, 76). This A32 epitope region has been shown to be a target of ADCC-mediated viral escape (79). The epitope is usually buried in the trimer state, but may be exposed on an infected CD4 cell surface by interaction with CD4 (31). Further studies determined that these IgA antibodies specific for the C1 region were able to block ADCC activity from the RV144
samples, indicating a mechanism for the reverse correlation of IgA titer with infection risk (80).

The RV144 trial was partially composed of a similar immunogen to the VAX003 trial, yet the outcomes were different. The boost for RV144 was composed of the protein immunization given in VAX003. The different outcomes may have been due to the Fc mediated functions elicited by each vaccine. RV144 was able to elicit highly functional IgG3 antibodies, while the repeated immunization of a subunit vaccine only pushed the VAX003 repertoire into the less functional IgG4 subclass (81). Recent studies in macaques have provided evidence for this notion. Utilizing both SIV and HIV immunogens, poly-functional antibody responses and antibody titer were correlated with increased sterilizing immunity (82).

Because of the success of RV144, further vaccine trials were designed to build on this knowledge. One such study is HVTN100, which utilizes an optimized version of the RV144 immunogens to test in sub-Saharan Africa. Because this trial will be performed in Africa, consideration needs to be taken with regards to the gp41 microbiota cross-reactive antibodies that can be elicited as seen in HVTN505 (83). The prime consists of the ALVAC-HIV vector used in RV144 with a subtype C immunogen (vCP2438) replacing the original B and CRF01-AE, while the boost was changed to a bivalent subtype C gp120 protein adjuvanted with MF59. This vaccine strategy does not contain gp41, which should help circumvent some of the problems seen in HVTN505. Along with the modifications with the immunization to improve efficacy, the trial participants will get an additional boost to help maintain the immunity that in RV144 peaked at 60.5% efficacy.
before diminishing after a year to its final efficacy of 31.2% (73). It was recently reported that the trial met the four benchmarks that were set at the beginning of the trial, allowing for the go ahead for a larger trial (HVTN 702) to be done in South Africa (Linda Gail Bekker, 21st International AIDS Conference, 2016).

3.2.3 DP6-001

There was also an early phase clinical trial done using research from the lab of Dr. Shan Lu at the University of Massachusetts Medical School. Human volunteers were tested for dose and reactivity against a polyvalent DNA prime/protein boost regimen. Six plasmids expressing env from multiple clades and one gag were immunized followed by a protein boost with matching gp120 antigens. This polyvalent vaccine was composed on Env from clades A, B, C and AE, with the goal to elicit a response against a wide range of antigen and possibly a qualitatively better response. Groups A and B volunteers first received three low dose (1.2 mg each time) DNA prime immunizations by ID and IM routes, respectively, with a polyvalent formulation including five gp120-expressing and one gag-expressing DNA plasmids, followed by two protein immunizations with a polyvalent (five) gp120 proteins produced from CHO cells formulated with adjuvant QS-21 (84). Group C volunteers received the same formulation except 1) a higher dose (7.2 mg each time) DNA prime immunization by IM route and 2) only one time gp120 protein boost immunization due to the early stop of the clinical trial in this group of volunteers. Initial DNA priming immunization did not elicit a detectable gp120-specific IgG titer in these volunteers’ sera, with the exception of one from Group C who received the higher dose DNA prime. However, after the first protein boost, gp120-specific IgG responses
can be seen to rise quickly to high titers. Serum neutralization was tested against a diverse panel of pseudotyped viruses from multiple clades (A, B, C, D, AE). Samples collected during the trial revealed that the humoral response developed in these volunteers had a broad gp120 binding profile along with limited neutralization. Because of the polyvalent nature of the vaccination, the responses seen were against multiple clades of HIV-1, being maintained out to a year (85).

4. Fc mediated function

Since the results of the RV144 trial showed that ADCC was a correlate of reduced infection risk in the group with low IgA C1 binding titers, interest in Fc effector functions in regards to HIV-1 protection has been renewed (32, 76). The first analysis of immune correlates in RV144 indicated that V1V2 binding antibodies inversely correlated with infection risk, while IgA antibodies correlated with risk. Secondary analysis also revealed that IgA had a confounding affect with other variables. When low IgA responders were analyzed, ADCC and neutralization were inversely correlated for risk of infection (76). Using serum from vaccine and placebo groups in the RV144 trial, Bonsignori et al. showed that the vaccine recipients elicited increased ADCC levels. Using A32 Fab, they were able to knock out most of the ADCC activity, indicating that most of the activity is focused on the A32 epitope in the C1 region. Isolation of mAbs from 6 RV144 vaccine recipients also confirmed these observations (32). Another group isolated mAbs from vaccine recipients that were specific for the V1V2 epitope identified in the RV144 analysis above. These antibodies did not bind free virions or neutralize Tier 2 isolates, though they did mediate ADCC activity. One of the antibodies, CH58, bound to the
V1V2 scaffold used in the risk analysis, and the lysine that the correlates of risk analysis indicated as a site of immune pressure is an essential part of the CH58 epitope (34). This matches with evidence from the risk analysis, but does not confirm that ADCC mediated by this type of antibody was responsible for reduced risk of infection.

There has been a long line of studies in HIV-1 infected individuals that show correlations between ADCC levels and disease progression. The majority of the studies show a correlation between high ADCC levels and control of the virus. Some of the first studies on ADCC indicated higher ADCC titers for a variety of circumstances. ADCC titer was shown to be elevated in both healthy seropositive patients and HIV-1 infected controllers (86, 87). A pair of early studies also looked at ADCC levels along the progression of infection, and showed titers to be elevated early in infection (88, 89). In the 1990s, multiple studies emerged linking ADCC titers with disease progression. In studies of children, and later adults, ADCC titer is inversely correlated to progression (90–95). More recently, groups have looked at viral load and found an inverse correlation with ADCC levels. These studies have ranged from acute infection studies to HIV-1 controller groups, all showing an inverse correlation (96–99). Although the majority of studies indirectly implicate ADCC in control of infection, there are a few early papers that failed to show a correlation between ADCC titers and progression (100–102). These studies indicated that there is a large body of evidence over an extended period of time supporting the conclusion that ADCC activity has a significant correlation with control of HIV-1 infection.
Even though Fc-mediated functions have been shown to be important in controlling infection, showing protection with only non-neutralizing antibodies has been elusive, though multiple studies over the years have implicated the effect of Fc-mediated effector functions in the course of HIV infection \textit{in vivo}. With this breadth of data, George Lewis has postulated that Fc mediated functions have the greatest effect early in acquisition and control of chronic infection (103). Elite Controllers with highly active ADCC activity were used to test passive immunization of polyclonal antibody. Purified non-neutralizing antibodies were given to macaques in low dose, but no protection or correlation with reduced disease progression was observed (64). Conversely, even though non-neutralizing antibodies (NoNAbs) have not been shown to be protective, passive immunization of polyclonal NoNAbs in a macaque model has been shown to reduce plasma viral load. When testing the combination of two non-neutralizing mAbs, 246-D and 4B3, the treatment was able to reduce viral load after challenge with SHIV (37). Along with passive immunization, it has been shown that NoNAbs can mediate Fc effector functions that induce immune pressure. Using NK cell activation as a marker for Fc-mediated activity, an epitope in the C1 region, which has been shown to be a non-neutralizing target, develops escape mutations in the concurrent virus population that prevents NK cell activation (79). The RV144 trial also showed immune pressure in gp120 V2 at position 169, which was an implicated ADCC epitope (77). One of the reasons for continued study into this type of protection is that most, if not all, passive immunization studies may be using antibody levels that skew into the prozone level for Fc mediated functions, thereby reducing the effectiveness of the therapy. The prozone effect occurs
when there is an excess of antibody that prevents both arms of an antibody from being able to bind to antigen, preventing the antibody from being able to crosslink on the target cell surface (65). In-vivo evidence also presents a compelling case for Fc-mediated function in protection, such as the inverse correlation between ADCC titers in the milk of pregnant HIV-1 infected mothers and transmission to newborns. This protection was not correlated with neutralization (104). Because Fc mediated functions have been shown to be important in HIV-1 disease progression and some clinical trials, further study is warranted.

The epitopes that mediate ADCC do not have to be accessible on an intact trimer, so both neutralizing and non-neutralizing antibodies can target them. The two main areas that epitopes for ADCC are exposed are during entry into cells and during budding from the cell. This allows both neutralizing epitopes such as the CD4bs and variable loops and non-neutralizing epitopes like those on gp41 and in the C1 region of gp120 to mediate ADCC and other Fc mediated functions (30). These non-neutralizing epitopes have been shown to be important because they are also under immune selection like neutralizing antibodies (79). When designing vaccines, epitopes that are both accessible and inaccessible on the trimer must be considered to fully realize the potential of the humoral immune response.

5. Research Framework and Objectives

The information provided above has led to the development of a thesis that will explore the effects of immunization in humans and how this information can be used for
the development of future vaccines. Much information has been discovered on the
development of bnAbs in HIV-1 infected patients and the interaction with the virus. More
recently, studies such as RV144 and subsequent animal studies have led the field to
appreciate that along with neutralization, vaccines should also focus on a combination of
effector functions that could include ADCC activity. The target with this original work is
to develop mAbs from human vaccine volunteers with the purpose to characterize
antibody effector functions and explore the fine specifics of the humoral response elicited
by vaccination in humans.

The first section in this dissertation deals with the development of a system to
isolate Env specific mAbs from frozen peripheral blood mononuclear cells (PBMC)
samples collected during the DP6-001 vaccine trial in humans. This was accomplished by
modifying and optimizing several protocols that utilized gp120 as a target for isolation of
Env specific mAbs. The gp120 bait was labeled on the amino terminal end which should
preserve most epitopes on gp120. This bait was used successfully to sort out B cells for
cloning. The variable region from B cells specific for gp120 were then cloned into
expression vectors for large scale production and characterization. Because single
memory B cells were isolated, a nested RT-PCR was needed to amplify the limited
amount of mRNA. This allowed for natural heavy and light chain pairing and production
of functional antibodies. This protocol successfully produced a panel of 13 gp120
specific mAbs. The second section takes these purified mAbs for binding and epitope
characterization so that the location of binding can be determined and compared to
information from mAbs developed in HIV-1 infected individuals and other vaccine
regimens. The majority of the mAbs bound to a broad range of gp120 antigens and bound to both linear and conformational epitopes, with conformational epitopes dominating the response seen in this panel. The few linear epitopes were against the V3 loop and an epitope at the amino end of C5. The last section looks into the effector functions from this panel of mAbs elicited during vaccination in humans, from neutralization to Fc mediated functions such as ADCC and antibody dependent cellular phagocytosis (ADCP). While very limited neutralization was only seen in a single mAb, most other mAbs exhibited broad Fc mediated function that included both ADCC and ADCP. The combination of data presented in this dissertation should advance the field of HIV-1 vaccine development by bolstering the depth of functional data we have from human vaccine trials and to show that antibodies with breadth in binding and functional activity beyond neutralization can be induced in humans through vaccination.
CHAPTER II

Utilization of a gp120 bait in isolating HIV-1 specific monoclonal antibodies from vaccine volunteers.

Introduction

Recent innovations in molecular cloning have allowed researchers to interrogate large numbers of human B cells for those of most interest. Before, the humoral response was mainly studied by looking at the polyclonal antibody response in the serum of HIV-1 infected individuals. The recent use of molecular cloning techniques to isolate human monoclonal antibodies has allowed a deeper understanding of the interaction of antibody with Env (25, 105, 106). Early efforts with these techniques focused on isolating broadly neutralizing and potent monoclonal antibodies from HIV-1 infected patients with strong control of viremia and associated broadly neutralizing serum responses (107, 108). While these types of antibodies have been instrumental in the characterization of epitopes and antibody development during HIV infection, further work is need on the type of antibody developed during vaccination.

The humoral response has been a cornerstone of study in immunology. With its study, we learn about the adaptive immune response and the sophisticated and precise mechanism that the body has to fight pathogens. In human adults, the bone marrow is where hematopoietic stem cells start developing, which differentiate into multiple immune cell lineages and become the backbone of the humoral response. For B cells,
stem cells develop into pro-B cells which allows them to start rearrangement of the IgH locus. Successfully rearranged heavy chains are paired with a surrogate light chain and displayed on the surface of pre-B cells. This leads to the rearrangement of light chain and the successful pairing of heavy and light chain on the surface of an immature B cell. Immature B cells leave the bone marrow and enter the circulation, displaying IgM on its surface. Immature B cells develop into naïve B cells when they begin expressing both IgM and IgD on their cell surface. The naïve antibody repertoire is composed of both immature and naïve B cells, which have not been exposed to antigen. Antigen crosslinking of membrane bound immunoglobulin creates the first signal for proliferation and internalized antigen is expressed on the B cell surface in complex with MHC class II. T helper (Th) cells recognize MHCII on the surface of the B cell and are activated. CD40 on the surface of the B cell then binds to CD40 ligand on the surface of the Th cell, inducing the second signal. These two signals promote the expression of cytokines and activate the B cell. After B cells become activated, they go through somatic hyper mutation (SHM) and class switch recombination (CSR) in the germinal centers located in secondary lymph organs such as the lymph nodes and spleen. The germinal center contains follicular dendritic cells (FDCs) that present antigen to B cells, and along with signals from Th cells, promote survival. B cells with less affinity do not receive these signals and die. (109). From germinal centers, cells can differentiate into either memory cells or plasmablast cells. Memory cells do not secrete antibody, but maintain surface expression that can recognize antigen in the bone marrow, circulation, or lymphoid organs (110). Plasmablast cells are proliferating antibody secreting cells that differentiate
into plasma cells that exit the lymph and enter circulation and long term in the bone marrow, maintaining antibody secretion into circulation (111). The circulation contains naïve and memory B cells (112). Consideration must be taken when looking to clone B cells from PBMCs and account for the low expression level and consequent mRNA content of memory B cells.

Protocols for developing mAbs have been well established in the mouse and rat models, but transfer of hybridoma creation into use for human B cells has been more problematic. One of the main reasons that hybridomas are less useful in the human system is that PBMCs are the main source of memory B cells for fusion, and their relative low numbers compared to the spleen in mice, combined with the low efficiency of fusion, means the number of hybridomas created from human B cells is low (113). EBV transformation has also been used to generate long lived antibody secreting cells in culture, though this method also has the problem of low efficiency. Much work has gone into making these methods more practical for human antibody production, and early anti-HIV antibodies were developed with these methods, although in limited quantities. The most recent surge in antibody production was due to the development of molecular cloning techniques of immunoglobulin genes from single B cells (22, 27, 28, 114–118).

DP6-001 was an experimental HIV-1 vaccination formulation tested in humans that utilized a DNA Prime/Protein Boost immunization to elicit protective responses to HIV-1. This initial study was a phase 1 trial used to determine the immunogenicity along with safety and tolerability of the vaccine in human volunteers. The DNA prime consisted of 5 env plasmids with a gag plasmid, while the boost contained five matched
gp120 proteins. In the trial, Groups A and B volunteers first received three low dose (1.2 mg each time) DNA prime immunizations by ID and IM routes, respectively, followed by two protein immunizations formulated with adjuvant QS-21 (85). Group C volunteers received the same formulation except 1) a higher dose (7.2 mg each time) DNA prime immunization by IM route and 2) only a single gp120 protein boost immunization due to the early stop of clinical trial in this group of volunteers. The protocol was tolerated well in most volunteers with the exception of some delayed type hypersensitivity in the group C arm of the study (84). When the trial was completed, the level of immune responses was characterized. The results indicated very good levels of cross subtype T cell responses and antibody responses, along with broad levels of neutralization with pseudotyped viruses (85).

The recent surge in the amount of bnAbs in the field has been due to the development of cloning techniques that allow the isolation of immunoglobulin genes from B cells from human patients and volunteers. Multiple groups have developed primer sets that allow the amplification of the variable region of heavy and light chain immunoglobulin genes (105, 106, 119). The first groups to isolate bnAbs employed multiple methods of identifying which B cells to clone. The group that isolated VRC01 used RSC3, a modified core antigen with specific mutations that only maintain the CD4bs, to identify B cells that could bind to RSC3 and isolate them using fluorescence-activated cell sorting (FACS), while the PG9/PG16 group screened for neutralization from B cells cultured in plates and cloned B cells with broad and potent neutralization activity (22, 27). In this chapter, the development and application of a modified
procedure to produce mAbs from DP6-001 vaccine volunteers utilizing a gp120 bait are described.
Results

**DP6-001 vaccine volunteers and their serum antibody responses.**

For my current work, a total of 13 human Env-specific mAbs were produced by the single B cell cloning approach from four DP6-001 volunteers (ABL-001, ABL-003, ABL-034 and ABL040). These volunteers were selected because of their serum responses. Initial DNA priming does not elicit a gp120-specific IgG titer, with the exception of group C which received the higher dose DNA prime (85). However, after the first protein boost, gp120-specific IgG responses can be seen to rise quickly to high titers and maintain levels out to a year for some of the volunteers (Fig 2.1a). The gp120 protein used to coat enzyme linked immunosorbent assay (ELISA) plates for detection of IgG titer was a proportionally mixed solution of the 5 autologous gp120s used in the DP6-001 immunization. Serum neutralization was tested against a diverse panel of pseudotyped viruses from multiple clades (A, B, C, D, AE) by Monogram sciences using a Phenosense assay that utilized pseudotyped virus that contained a firefly luciferase gene infecting U87 cells, which express CD4, CCR5 and CXCR4. For the select volunteers, positive neutralizing activity can be seen in immune sera after one or two protein boost immunizations across this panel of pseudotyped viruses, ranging for an ID50 titer of 1:20 to 1:977 (Fig 2.1b). Along with neutralization activity, I studied the presence and levels of Fc-mediated antibody activities in immune sera from these DP6-001 volunteers. After multiple immunizations including protein boost, gp120-specific ADCC activities can be seen in all volunteers, and is sustained out to 36 weeks which was the last time point we included in the ADCC study. ADCC activity is seen against both subtypes B and AE
gp120 antigens (Fig 2.2). Most significantly, ADCC activities can be detected by using either the assay format with target cells coated with recombinant gp120 antigens, or the infection format with viruses expressing HIV-1 Env antigens. ADCC levels in cells with viruses expressing HIV-1 Bal Env showed lower ADCC activities than the viruses expressing HIV-1 CM235. The levels of ADCC activities from volunteer ABL040 sera (Group C of DP6-001 trial) were lower than other three volunteers, most likely due to the fact that Group C volunteers did not receive the second protein boost (Fig 2.2). This initial finding of broad and potent antibody functions and the availability of PBMCs from these volunteers provided a rich resource for mining and exploring the monoclonal antibody response from vaccinated human volunteers.
Figure 2.1: Antibody sera responses from phase 1 vaccine trial DP6-001 subjects. (A) Gp120 specific IgG titers for select volunteers. The gp120 protein used to coat ELISA plates for detection of IgG titer was a proportionally mixed solution of the 5 autologous gp120s used in the DP6-001 immunization. Solid arrows indicate immunization with DNA prime (weeks 0, 4 and 12), and open arrows indicate immunization with gp120 protein boosts (weeks 20 and 28). Group A DNA prime was done ID (1.2mg), while group B and C were both IM (1.2 and 7.2mg). Group C was a high dose DNA prime, and only received the first boost. Protein boosts done by IM (0.375mg) (B) IgG titer data was determined previously in lab. Serum neutralization titers determined by PhenoSense assay at Monogram Biosciences (ID50).
Figure 2.2: Serum ADCC titers for select volunteers against gp120 coated cells from a Gran-Toxi-Lux ADCC assay (panel A) and infected cells from a Luc based ADCC assay (panel B). ADCC with sera from select DP6-001 volunteers against autologous Bal and AE in (A), and both autologous Bal and heterologous CM235 in (B). ADCC titers determined by Dr. Guido Ferrari at Duke.
Figure 2.3: Overview of the mAb production workflow. DP6-001 PBMCs are selected for B cells and labelled with gp120 for sorting of Env specific memory B cells. Sorted B cells are screened and Ig variable regions cloned into expression vectors for antibody production.
Generation of HIV-1 Env-specific human mAb from vaccine volunteers.

Labeled gp120 binding characteristics.

One of the first steps in developing the system to isolate Env specific mAbs (Figure 2.3) is to create the antigen to be used in binding and sorting the B cells of interest. Comparison of ELISA mAb binding between gp120 proteins modified with an Avitag peptide sequence indicates similar binding with most mAbs. The five gp120s from the DP6-001 trial were modified to contain the Avitag peptide signal (LNDIFEAQKIEWHE) at the C terminal of the protein (120). The binding of mAbs against a variety of epitopes was tested to both gp120 proteins that contain the Avitag peptide signal, along with the parent gp120 proteins. Both versions of gp120s A2, B and Bal had comparable binding for all 6 mAbs tested (Figure 2.4). For gp120s CZM and E, binding was always present in the Avitag form, while binding to the parent gp120 form was absent or reduced for mAbs 2G12 (glycan), 447-5D (V3), and R15 (C1), possibly due to a slight conformational change that allows for better binding to the Avitag form. For mAbs GB1 (C2), R53 (C4), and R56 (V3), levels of ELISA binding were comparable between both versions of g120, similar to the pattern seen with the A2, B and Bal comparisons. This indicates that the Avitag peptide signal maintains or may even improve multiple epitopes for human mAbs (Figure 2.4A) and rabbit mAbs (Figure 2.4B).
Figure 2.4: Comparison of mAb binding to Avitag or native forms autologous gp120. (A) ELISA binding to three human mAbs 2G12, 447-5D, and GB1. (B) ELISA binding to three rabbit mAbs R15, R53 and R56.
The insertion of an Avitag signal to the end of gp120 allows for the addition of a single biotin and subsequent fluorophore in a position that does not interfere with possible epitopes on the gp120 protein. This allows the labeled gp120 to be used for sorting Env specific B cells by FACS. After enzymatic addition of biotin to the lysine residue in the Avitag peptide sequence, the relative biotinylation success was tested by ELISA. Equal amount of MBP protein and gp120 were coated on an ELISA plate, and streptavidin-HRP used to detect the amount of biotin present. The control biotinylated MBP protein showed similar levels of biotin compared to the 5 biotinylated gp120-Avitag proteins (Figure 2.5). Each gp120 was successfully biotinylated and was then ready for conjugation to a fluorophore, either SA-PE or SA-APC.
Figure 2.5: ELISA detection of biotin enzymatically conjugated with autologous gp120-Avitag proteins. ELISA plates coated with biotinylated protein, and then detected with SA-HRP.
Comparison of sorting and screening protocols.

The labeled gp120 was used in the isolation of B cells from frozen PBMC cells that were collected during the course of the DP6-001 vaccine trial. There were two different frameworks used in the generation of mAbs. One framework isolates single B cells by FACS sorting directly into PCR plates, while the second framework bulk sorted B cells and stimulated FACS sorted B cells with feeder cells in tissue culture plates. As can be seen in Figure 2.6, B cells were selected by the B cell marker CD19 and dead cells were excluded by 7AAD incorporation. Then the final selection was done by selecting double positive gp120 binding cells. This double positive selection was used for the first framework, while a more inclusive gate including both double and some single positive cells were used for the second framework. The reason for the double positive selection was that the chances of a non-specific signal would be reduced when bound by two separate gp120-conjugated fluorophores. This gating was relaxed in the second framework to access B cells that would be missed. This relaxed gating was possible because of the ELISA screening protocol (Figure 2,) which would remove any non-binding wells before cloning.
Figure 2.6: Sort plot for DP6-001 B cells isolated with APC and PE conjugated gp120. B cells isolated from ABL040 PBMC cells, and then stained with anti-CD19, 7AAD, and 5 valent autologous gp120 (A2 (A), B, Bal (B), CZM (C), AE (AE)) labeled with APC or PE. Numbers in gates represent percent of parent population captured in gate.
**B cell culturing.**

A secondary technique utilized B cell culturing and ELISA screening to identify B cells of interest for cloning. Multiple stimulation conditions were tested. Four media conditions were tested, along with two cell lines used as feeder cells when plating B cells at near clonal dilutions. Clonal dilutions are described by a Poisson distribution, so that diluting a mixture to one cell/well will in practice end up with 0 cells 37% of the time, 1 cell 37% of the time, 2 cells 18% and so on. For the EBV conditions, in addition to EBV supernatant, the TLR9 agonist CpG (InvivoGen) was used to facilitate stimulation, while a checkpoint kinase 2 (CHK2) inhibitor (Calbiochem) was used to inhibit apoptosis and help with cell survival. The R848 conditions adapted the stimulation portion of an ELISpot protocol for the stimulation of B cells, using the TLR7/8 agonist R848 (InvivoGen) and recombinant human interleukin 2 to facilitate stimulation. The rabbit protocol used an activated rabbit splenocyte supernatant as the stimulation factor. And finally the human cytokine protocol used a combination of cytokines and CpG to stimulate B cell growth, including Blys, IL-2, IL-4, IL-6, and IL-10. Along with stimulating factors, the B cells were incubated with feeder cells, either EL4B5 cells or CD40L 293T cells. B cells were cultured for 7 days before screening for antibody production. The two successful conditions both used the EL4B5 feeder cells, Sort CC using the R848 protocol, and Sort HH using the rabbit stimulation protocol (Table 2.1). Three mAbs were isolated from each of these sorts; CC3C2, CC6B5 and CC6C11 from CC sort with ABL-003 volunteer B cells, and HH1G9, HH2D11, and HH4E4 from HH sort with ABL-034 volunteer B cells.
### Table 2.1: B Cell Culturing Conditions

<table>
<thead>
<tr>
<th>Sort</th>
<th>Patient</th>
<th>Bleed Day</th>
<th>Plates</th>
<th>Method</th>
<th>Feeder Cells</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>ABL010</td>
<td>252</td>
<td>4 (96)</td>
<td>EBV and R848</td>
<td>CD40L.293T</td>
<td>Wells tested, no binding mAb found.</td>
</tr>
<tr>
<td>BB</td>
<td>ABL022</td>
<td>252</td>
<td>6 (96)</td>
<td>EBV and R848</td>
<td>CD40L.293T</td>
<td>Wells tested, no binding mAb found.</td>
</tr>
<tr>
<td>CC</td>
<td>ABL003</td>
<td>252</td>
<td>6 (96)</td>
<td>EBV and R848</td>
<td>EL4B5</td>
<td>Wells tested, 3 binding mAb found, cloned, purified.</td>
</tr>
<tr>
<td>DD</td>
<td>ABL031</td>
<td>252</td>
<td>3 (96)</td>
<td>Rabbit Stimulation</td>
<td>EL4B5</td>
<td>Contamination.</td>
</tr>
<tr>
<td>EE</td>
<td>ABL031</td>
<td>252</td>
<td>3 (384)</td>
<td>R848</td>
<td>EL4B5</td>
<td>Sort problem, wells tested, no binding mAb found.</td>
</tr>
<tr>
<td>FF</td>
<td>ABL029</td>
<td>252</td>
<td>4 (96)</td>
<td>Rabbit Stimulation</td>
<td>EL4B5</td>
<td>Wells tested, low or insignificant binding, no VH clones found.</td>
</tr>
<tr>
<td>GG</td>
<td>ABL029</td>
<td>252</td>
<td>6 (384)</td>
<td>R848</td>
<td>EL4B5</td>
<td>Sort problem, same as EE, possible gp120 binding, no VH clones found.</td>
</tr>
<tr>
<td>HH</td>
<td>ABL034</td>
<td>252</td>
<td>4 (96)</td>
<td>Rabbit Stimulation</td>
<td>EL4B5</td>
<td>Wells tested, 4 binding mAb found, cloned 3 mAbs.</td>
</tr>
<tr>
<td>II</td>
<td>ABL010</td>
<td>224</td>
<td>½ (96)</td>
<td>Human Cytokines</td>
<td>EL4B5</td>
<td>40 cells plated, No ELISA results.</td>
</tr>
<tr>
<td>JJ</td>
<td>ABL033</td>
<td>224</td>
<td>4 (96)</td>
<td>Human Cytokines</td>
<td>EL4B5</td>
<td>Good percentage of IgG positive wells, but low gp120 percentage.</td>
</tr>
<tr>
<td>KK</td>
<td>ABL033</td>
<td>224</td>
<td>2 (96)</td>
<td>Human Cytokines</td>
<td>EL4B5</td>
<td>Low IgG and no gp120 positive wells found.</td>
</tr>
</tbody>
</table>
Cloning of immunoglobulin genes from B cells.

After cells were selected, nested RT-PCR was performed to isolate the variable region of each immunoglobulin gene. The RT was performed using a mixture of primers for the multiple heavy chain constant regions, along with a primer for each of the kappa and lambda constant region. As shown in figure 2.7, RT primers are oriented as reverse primers in the constant region, thereby coding from the constant region into the variable region. After RT, nested PCR was performed with a mixture of outer primers for heavy, kappa and lambda separately, followed by a PCR with inner primers specific for a single Ig subtype (Figure 2.7, Table 2.2). Primers were located in regions with relative sequence conservation. Even though there is a primer specific for each IgV subgroup, some primers contain degenerate sequence to account for allele differences within these subgroups. This degenerate sequence is most common in the forward inner primers that are positioned at the beginning of the variable region (Figure 2.7). The resulting product is a 400-500 bp band that contains the variable region of a rearranged and transcribed immunoglobulin gene, as shown in the example DNA gels in Figure 2.8.
Figure 2.7: Positions of representative RT and nested PCR primers on a recombined human IgG1 gene. RT primer is a reverse primer that converts variable region into cDNA. First nested PCR uses external primers to amplify this cDNA, followed by a second PCR with internal primers for final amplification.
Table 2.2: RT Nested PCR Primers

<table>
<thead>
<tr>
<th>RT Primers</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM-RT</td>
<td>ATGGAGTCGGGAAAAAGGAGTC</td>
</tr>
<tr>
<td>IgD-RT</td>
<td>TCACGGAGGTGGTGTTGTA</td>
</tr>
<tr>
<td>IgE-RT</td>
<td>TCACGGAGGTGGCATTTGGA</td>
</tr>
<tr>
<td>IgA1-RT</td>
<td>CAGGCGATGACACAGTTCC</td>
</tr>
<tr>
<td>IgA2-RT</td>
<td>CATGCGATGACCACAGTTCC</td>
</tr>
<tr>
<td>IgG-RT</td>
<td>AGGTGTGCACGCCAGTTGC</td>
</tr>
<tr>
<td>Cκ-new RT</td>
<td>GCAGGCACACACACAGAGGCA</td>
</tr>
<tr>
<td>Cλ-new-ext</td>
<td>AGGCCACTGTACAGCT</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Heavy Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer Forward</td>
</tr>
<tr>
<td>VH1-Ext</td>
</tr>
<tr>
<td>VH2-Ext</td>
</tr>
<tr>
<td>VH3-Ext</td>
</tr>
<tr>
<td>VH4-Ext</td>
</tr>
<tr>
<td>VH5-Ext</td>
</tr>
<tr>
<td>VH6-Ext</td>
</tr>
<tr>
<td>Outer Reverse</td>
</tr>
<tr>
<td>IgA-ext</td>
</tr>
<tr>
<td>IgD-ext</td>
</tr>
<tr>
<td>IgG-ext</td>
</tr>
<tr>
<td>IgM-ext</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inner Forward</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH1-Int tag</td>
</tr>
<tr>
<td>VH2-Int tag</td>
</tr>
<tr>
<td>VH3-Int tag</td>
</tr>
<tr>
<td>VH4-Int tag</td>
</tr>
<tr>
<td>VH5-Int tag</td>
</tr>
<tr>
<td>VH6-Int tag</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inner Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA1-Int tag</td>
</tr>
<tr>
<td>IgA2-Int tag</td>
</tr>
<tr>
<td>IgD-Int tag</td>
</tr>
<tr>
<td>IgG-Ext tag</td>
</tr>
<tr>
<td>IgM-Int tag</td>
</tr>
</tbody>
</table>
### Kappa Chain

#### Outer Forward
- Vκ1/2-Ext: GCTCAGCTCCTGGGGCT
- Vκ3-Ext: GGAARCCCAAGCDDAGC
- Vκ4/5-Ext: CTSITCTYTGGATCTCT
- Vκ6/7-Ext: CTSCTGCTCTGGGYTCC

#### Outer Reverse
- Cκ-ext: GAGGCAGTTCCAGATTTC

#### Inner Forward
- Vκ1-Int tag: CTGGGTTCCAGGTTCCACTGGTGACGACATCCAGWTGACCCAGTCT
- Vκ2-Int tag: CTGGGTTCCAGGTTCCACTGGTGACGATATTGTGATGACCCAGWCTCCAC
- Vκ3-Int tag: CTGGGTTCCAGGTTCCACTGGTGACGAAATTGTGTTGACRCAGTCTCCA
- Vκ4-Int tag: CTGGGTTCCAGGTTCCACTGGTGACGACATCGTGATGACCCAGTCT
- Vκ5-Int tag: CTGGGTTCCAGGTTCCACTGGTGACGAAACGACACTCACGCAGTCT
- Vκ6-Int tag: CTGGGTTCCAGGTTCCACTGGTGACGAAATTGTGCTGACWCAGTCTCCA
- Vκ7-Int tag: CTGGGTTCCAGGTTCCACTGGTGACGACATTGTGCTGACCCAGTCT

#### Inner Reverse
- Cκ-Int tag: GGGAGATGAACAGATGGT

### Lambda Chain

#### Outer Forward
- Vλ1-Ext: CCTGGGCCCAGTCTG
- Vλ2-Ext: CTCCTCASYCTCCTC
- Vλ3-Ext: GGCCTCCTATGWGCT
- Vλ3l-Ext: GTTCTGTGGTTTCTTCTGAGC
- Vλ4ab-Ext: ACAGGGTCTCTCC
- Vλ4c-Ext: ACAGGTCTCTGTGCTCTG
- Vλ5/9-Ext: CCCTCTCSCAGSCTG
- Vλ6-Ext: TCTTGGGCCAATTTTATG
- Vλ7/8-Ext: ATTCYCACTGTGCTG
- Vλ10-Ext: CAGTGGTGCCAGCG

#### Outer Reverse
- Cλ-new-ext: AGGCCACTGTCACTACGC

#### Inner Forward
- Vλ1-Int tag: CTGGGTTCCAGGTTCCA
- Vλ2-Int tag: CTGGGTTCCAGGTTCCA
- Vλ3-Int tag: CTGGGTTCCAGGTTCCA
- Vλ3l-Int tag: CTGGGTTCCAGGTTCCA
- Vλ4ab-Int tag: CTGGGTTCCAGGTTCCA
- Vλ4c-Int tag: CTGGGTTCCAGGTTCCA
- Vλ5/9-Int tag: CTGGGTTCCAGGTTCCA
- Vλ6-Int tag: CTGGGTTCCAGGTTCCA
- Vλ7/8-Int tag: CTGGGTTCCAGGTTCCA
- Vλ10-Int tag: CTGGGTTCCAGGTTCCA

#### Inner Reverse
- Cλ-Int tag: GGGYGGGAACAGATGGCAG
Figure 2.8: Representative example of immunoglobulin variable region PCR product. Final nested PCR products from ABL-001 B cells sorted into PCR plates by gp120 selection by either VH1 and CH internal primers, or VL3 and CL internal primers.
After isolation of the variable regions, fragments were inserted into a PCR cassette containing a promoter and immunoglobulin constant region to produce a full length IgG1, IgK or IgL gene, which was used to transfect 293T cells for antibody production. The promoter fragment contains a CMV promoter followed by an Ig leader sequence that has 25 bp sequence at the 3’ end that is also encoded in the 5’ end of the forward variable region primer. The constant region fragment contains either the IgG1, IgK, or IgL constant region followed by a polyA signal. The reverse primers for each of the variable region fragments has a corresponding sequence at the 5’ end of each constant region fragment. The overlapping PCR combination of a promoter fragment, variable region fragment, and constant region fragment creates a functional full length immunoglobulin protein that can be co-expressed in 293T cells to create mAbs (Figure 2.9).
Figure 2.9: Representation of overlapping PCR cassette for IgG1, IgK and IgL. Overlapping PCR is done with three fragments and primers flanking the outer fragments. Each fragment has overlapping sequence with the neighboring fragment, allowing priming and chain extension between fragments.
**DP6-001 HIV-1 Env-specific human mAbs.**

Frozen PBMCs from the DP6-001 vaccine trial performed with human volunteers were used for antigen specific cloning of human monoclonal antibodies (hmAbs). MAbs were isolated from 4 different volunteers (ABL-001, ABL-003, ABL-034 and ABL040) and three different vaccine strategy groups. The group A volunteers received a DNA prime by intradermal (ID) route, while group B and C received intramuscular (IM) DNA primes with group C receiving a high DNA dose. All groups received the protein boost by IM (85). This panel of 13 hmAbs were from single B cells isolated from frozen PBMCs collected at 30-36 weeks post initial DNA immunization (i.e. after one or two protein immunizations), with one hmAb (TA7) found in PBMC samples from 2, 10 and 32 weeks post protein immunization.

Immunogenetics analysis showed that IGHV of these mAbs are mainly from germline subtype 1 except two (CC3C2, subtype 3 and EA8 subtype 4) while IGLV is more diverse with a variety of variable gene subgroups utilized from both kappa and lambda germlines. The mutation rates of IGHV from germline are low, usually at 3-6% level with one at 9%. The CDR3 length varies ranging from 12 to 23 for the heavy chain, and 6 to 11 for light chain (Table 2.3, Figure 2.10). Taken together, this points to a generally typical B cell repertoire, with the exception of a disproportionate VH1 usage.
Table 2.3: Characteristics of the variable region of heavy chains and light chains of the DP6-001 vaccine elicited human mAbs

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>HmAb</th>
<th>Week</th>
<th>Heavy Chain</th>
<th>Light Chain</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ABL-034</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Group A)</td>
<td>HH1G9</td>
<td>36</td>
<td>1-2*02</td>
<td>2-28*01 (K)</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>HH2D11</td>
<td>36</td>
<td>1-2*02</td>
<td>3-11*01 (K)</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>HH4E4</td>
<td>36</td>
<td>1-46*01</td>
<td>7-43*01 (L)</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>ABL-001</td>
<td>GB1</td>
<td>30</td>
<td>1-69*9</td>
<td>1-5*03 (K)</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>(Group B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EA1</td>
<td>32</td>
<td>1-69*01</td>
<td>3-10*01 (L)</td>
<td>&lt;1</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>EA7</td>
<td>32</td>
<td>1-69*01</td>
<td>3-19*01 (L)</td>
<td>4</td>
<td>11</td>
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<tr>
<td></td>
<td>EA8</td>
<td>32</td>
<td>4-39*01</td>
<td>1-40*01 (L)</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>ABL-003</td>
<td>CC3C2</td>
<td>36</td>
<td>3-30*03</td>
<td>1-44*01 (L)</td>
<td>2</td>
<td>11</td>
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<tr>
<td>(Group B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC6B5</td>
<td>36</td>
<td>1-46*01</td>
<td>1-26*01 (K)</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>CC6C11</td>
<td>36</td>
<td>1-46*01</td>
<td>1D-17*02 (K)</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>ABL-040</td>
<td>TA6</td>
<td>30</td>
<td>1-3*01</td>
<td>3-10*01 (L)</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>(Group C)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>TA7</td>
<td>30</td>
<td>1-3*01</td>
<td>3-10*01 (L)</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>TB7</td>
<td>30</td>
<td>1-69*06</td>
<td>2-14*01 (L)</td>
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<td>10</td>
</tr>
</tbody>
</table>
**Sequence Analysis.**

Comparison of CDR3 amino acid length shows the bnAb heavy chain CDR3 length is on average longer than is typical in a normal human B cell repertoire, while the DP6-001 mAbs have a distribution of amino acid lengths around the average. When comparing light chains on the other hand, both DP6-001 and bnAbs have very consistent lengths, from 6-9 for kappa chain, and 10-11 for lambda chain (Figure 2.10, Table 1.1). These results are consistent with the understanding that vaccination is under normal repertoire selection pressures, while the constant stimulation during HIV-1 infection may drive up CDR3 lengths.

Figure 2.10: Comparison of CDR3 amino acid length between mAbs isolated from DP6-001 and a large selection of broadly neutralizing antibodies.
Two mAbs (TA6 and TA7) utilized the same germline for both heavy and light chain, and since they are from the same individual, indicate they could be related and from the same precursor B cell (Table 2.3). Protein alignment of the variable region highlights a high degree of similarity throughout, with the exception of the CDR3 region (Figure 2.11). This sequence similarity within the same individual sets up a few possible conclusions. One being that they are part of the same clonal lineage and diverged through SHM; and the other being that they arose separately and by chance utilized the same germline genes.

TA6  6  GYTFTNYAIHWVRQAPGRLEWMGWINGGNTKYSQKLQGRVTIRDTSASTAYMELSS  65  
GYTFT+YA+HWVRQAPG RLEWMGWINGGNTKYSQKLQGRVTIRDTSA+TA1MESS
TA7  1  GYTFTSYAMHWVRQAPQRLEWMGWINGGNTKYSQKLQGRVTIRDTSAATTAYMELSS  60

TA6  66  LRSEDSAVVYCYRRAYYGSRLDALLDVQGTLVTVSSASTKPSVFPPLAPSS  120
LRSED+AVYYC+R YY + D +D WQG LVTVSSASTKPSVFPPLAP S
TA7  61  LRSEDTAVVYCVRG-YYNTSGSTSYVDSWQGALVTVSSASTKPSVFPPLAPCS  114

Figure 2.11: Antibody heavy chain protein alignment of V3 specific ABL040 mAbs TA6 and TA7.
Discussion

The purpose of this study was to develop a system that would be able to produce mAbs from samples gathered from human vaccine trials. This will allow for the interrogation of different vaccine strategies and how they perform in a human system. The majority of human mAb studies have been with samples from infected volunteers. These types of antibodies could differ greatly from those vaccines elicit, which makes the development of mAbs from vaccine studies of great importance.

The samples that were available from the vaccine recipient volunteers consisted of sera and PBMCs. Since PBMCs were available, it was possible to try and isolate B cells from the PBMC samples. The main target for B cells in circulation are memory B cells, which display BCRs on their surface. But because memory B cells do not secrete antibody, the mRNA levels of immunoglobulin genes are low. This led to the development of an RT-PCR system that used a nested PCR step to amplify the PCR product to usable levels. The limited B cell supply in PBMCs also makes other cloning systems less ideal, like the well characterized hybridoma development system that is used in mouse and rat mAb development.

The initial impetus was to utilize frozen PBMCs from a human vaccine trial and develop a way to isolate gp120 specific mAbs. The first part was to select for gp120 binding antibodies. This was achieved by modifying gp120 to contain a fluorophore that would be used in FACS to sort out B cells that displayed surface immunoglobulin with the ability to bind to gp120. This was achieved by modifying the gp120 to contain an Avitag signal that could be biotinylated and then conjugated to a fluorophore. This minor
modification at the terminus of gp120 preserved most antibody epitopes on the gp120. The modified immunogens were tested against multiple mAbs against a variety of epitopes. The labeled gp120 allowed for the isolation of B cells with gp120 binding capabilities. With the number of PBMCs tested and the estimated frequency of gp120 specific B cells in the test population, the results indicate that FACS isolation was able to increase the frequency of B cells with gp120 specific BCRs. After cloning, the gp120 binding capability was not always present or strong, but a subset of broad gp120 binding mAbs was developed. One of the reasons that could account for this lack of gp120 binding after antibody isolation is that the B cells are not producing Env specific antibodies. It is possible that the sorting procedure has allowed non-specific B cells to be sorted. Because of limited vaccine PBMC samples, the approach taken was conservative so that Env specific B cells were not lost, which led to a greater number of non-specific B cells being included. Another possibility is that the ELISA screening protocol was more stringent than the sorting protocol, which led to B cells binding to the gp120 bait during FACS, but not binding to gp120 in a ELISA format after cloning. One final consideration is the antibody format. B cells expressing native BCR on their surface may not display an analogous binding profile to the cloned secreted version of the antibody, which places just the variable region into an expression vector with a replaced constant region. The mAbs that were Env specific and cloned exhibited low somatic mutation rates with average CDR3 lengths, which contrasts with that seen in most bnAbs developed from HIV-1 infected individuals. This is most likely due to the fact that vaccination is
performed on a shorter time frame with less antigen stimulation, while HIV-1 infection is a more consistent, long term stimulation.

A second system to develop monoclonal antibodies was also established. This system built off of the previous one by utilizing RT-PCR amplification of the variable region and subsequent cloning into an antibody expression system. The change is that instead of using FACS to isolate gp120 binding cells for direct RT-PCR cloning, B cells are cultured in plates and screened before the immunoglobulin gene cloning process is performed. This type of system, while more time consuming, allows for a diverse and modifiable selection criterion. Instead of being only selected by gp120 binding, culture supernatant can be screened for neutralization, Fc mediated effector function, or a specific epitope. Because gp120 binding may miss some antibody specificities, these other screening assays could fill in the gaps. This is specifically true for neutralization, which could help fill a hole in the current set of mAbs.

Multiple culture techniques were tested to determine a robust system that would be used in maintaining B cells in culture. While groups have been stimulating B cells for a while because of the need to expand populations of human memory B cells from PBMCs for antibody studies, such as hybridoma creation, our group wanted to do this stimulation on a smaller scale so that we could screen near clonal populations of cells for specific binding characteristics. The testing and modification of previous protocols converged on a protocol that uses a few simple stimulation factors with a feeder cell line to provide an environment that a few B cells can successfully be stimulated to produce a detectable amount of secreted immunoglobulin in a micro well environment.
With these cloning techniques, I was able to produce a small panel of mAbs from DP6-001 volunteer samples. While bnAbs in general have longer CDR3s, which is most likely a mechanism to bypass the glycan shield on the trimer, the DP6-001 panel of mAbs did not display this type of unusual characteristic (118, 121). In general, this is to be expected in a vaccination context because long CDR3s have a tendency to be more self-reactive and removed from the B cell repertoire (122, 123). Another aspect of bnAbs is a short CDRL3, which is also not present in the DP6-001 mAbs. The closest mAb that comes to this is GB1 with a 6aa CDRL3, but since the short CDRL3 is associated with removing steric hindrance to CD4bs epitopes, it is not unusual for this panel to not contain this highly unusual structure (124). Even though the DP6-001 mAbs do not have similarities to bnAbs, they do conform to the pattern seen in RV144, which was able to afford some protection with conventional antibodies with mutation rates below 5%, like those seen in the current DP6-001 panel (34).

An interesting pair of antibodies is TA6 and TA7 from the same individual. These mAbs utilized the same heavy and light chain germlines, yet had a different junction region. Even with this difference in the CDR3, these two mAbs maintained the same epitope as will be discussed in the next chapter. There are two different pathways that could have led to the generation of TA6 and TA7. One is that they were two separate B cell lines that had convergent recombination with similar heavy and light chain rearrangements that developed antibodies with a similar epitope. This line of thought is supported by the fact that two other mAbs (311-11D and 1334) from two separate individuals developed V3 specific mAbs with the same germline usage for heavy and
light chain (125, 126). The second pathway would have TA6 and TA7 being clonally related, and having a divergent SHM that modified the junction region to be different between the two mAbs.

One of the original goals of the project was to develop a panel of mAbs with neutralization capabilities that matched those seen in the sera of the volunteers. As will be shown in the following chapters, the number of mAbs produced was small and the number of neutralizing antibodies was even smaller. There are a couple of reasons for this shortfall as compared to groups working with HIV-1 infected patient samples that isolated multiple bnAbs. One of the major factors was the number of patients screened and PBMCs interrogated for Env specific antibodies. These groups were able to select patients that had very high titers of neutralizing antibodies from thousands of patients screened (27), or screen patient sera for a specific epitope like the CD4bs (22). With this greater number of samples to probe and specific criteria for donor patients, the odds of isolating a bnAb was greater. Another factor is the isolation methods used. For mAbs like VRC01, a specific probe was used to identify CD4bs antibodies and negatively select all other B cells (22). For other bnAbs like PG9/PG16, neutralization screens were run with culture supernatants from a large number of cells (27). The gp120 monomer bait used in the isolation of DP6-001 mAbs may miss some trimer specific antibodies. A significant portion of broadly neutralizing antibodies have trimer specific epitopes (25, 28, 127–129). Even though we were not looking for a bnAb, the decreased number of samples and low numbers of mAbs isolated contributed to this lack of neutralizing mAbs isolated from DP6-001. One note to make is that even with a low number of mAbs isolated,
another group in the Lu lab was able to isolate two mAbs with neutralization activity. These mAbs were developed using EBV transformation and screening limiting dilutions of cells for activity. One of the antibodies was a CD4bs antibody, while the other had an unknown epitope dependent on glycosylation. These binding specificities expand those seen in my panel.
CHAPTER III

Epitope specificity of vaccine elicited human monoclonal antibodies.

Introduction

Antibodies to HIV-1 have been studied for many years along with the epitopes on the HIV-1 surface receptor that these antibodies bind to. Some of these antibodies bind to the trimer, while others can bind to the monomer gp120. One of the main epitope considerations is whether the epitope is available on the surface of the virion and can mediate neutralization. While this neutralizing antibody and its corresponding epitope has been the focus of vaccine research for years, non-neutralizing antibodies that can mediate Fc dependent effector functions are of increased importance because of the results of the RV144 trial.

One important finding from the RV144 ALVAC-HIV (vCP1521) prime-AIDSVAX B/E boost vaccine efficacy trial is the identification of immune correlates of vaccine-induced protection from acquisition of human immunodeficiency virus type 1 (HIV-1). Antibodies targeting the Env gp120 V1/V2 region inversely correlated with infection risk, while IgA Env-binding antibodies to Env directly correlated with infection risk (76). Four antibodies (CH58, CH59, HG107, HG120) isolated from vaccine recipients have specific binding to this region and mediate ADCC activity (34). Another important epitope from RV144 are the Env-specific mAbs that preferentially use VH1
gene family and recognize multiple Env epitopes (32). The region implicated with the reduced risk of infection in this trial was the region overlapping with the A32 like epitopes in cluster A, which has been shown previously to be a target of ADCC-mediated viral escape (33, 79, 80).

Antibody responses to HIV-1 fall into two categories, neutralizing or non-neutralizing. The trimer on HIV-1 has developed multiple mechanisms to escape neutralization. A lot of the surface of the trimer is covered by glycans, which shield the underlying protein (130). When gp120 is in its monomer form, antibodies can develop against parts of the monomer that are hidden on the trimer, preventing the antibodies from being able to neutralize. The great diversity and high mutation rate of HIV-1 also prevents antibodies from being effective for incoming and current virus populations (131). One of the main areas that should be structurally consistent is the CD4bs, which must interact with the human immune system to infect cells. While some antibodies have been able to exploit this epitope, many are unable because of steric hindrances caused by the variable loops of HIV-1 and their interaction with the CD4bs on adjacent monomers in the trimer structure (132, 133).

Even though HIV-1 has developed systems to evade the immune system, there are a few well studied epitopes bound by antibodies. As discussed, the CD4bs is a prime candidate for neutralizing antibodies. This epitope is bound in both the monomer and trimer form, but only highly specialized antibodies can bind the trimer due to steric considerations. These antibodies are mainly derived from the VH-1-2 or VH1-46 germline due to the structural advantage encoded in these genes (124). Another major
epitope that elicits antibodies in infected individuals and from vaccination is the HIV-1 V3 loop (134–136). This group of antibodies can be divided into the three groups based on their specific binding modes and epitopes. In the two glycan-independent binding modes, the V3 crown either sits in the grove of the Fab fragment (cradle-like), or the crown sits in a bowl and a long CDRH3 interacts with the main chain of V3 (ladle-like) (137). The third binding mode is glycan dependent and been used by recently isolated antibodies to neutralize a broad range of HIV-1 isolates. The region at the base of V3 does not elicit many antibodies, but those few are very potent and usually depend on binding to glycans like the one at N332 (118). The RV144 trial highlighted another epitope that has multiple binding modes. The V1V2 loop is bound by three groups of antibodies. The V2i epitope is based around the α4β7-integrin binding site and conformation dependent. The V2q epitope is a quaternary epitope with a glycan component at N160. In contrast, the V2p epitope is present in V2 peptides and is glycan independent (138). The last major Env epitope is found in gp41. The membrane proximal external region (MPER) is highly conserved, but is poorly immunogenic (139).

In this Chapter, I have assessed the binding specificity of a panel of HIV-1 Env specific mAbs isolated from four volunteers who received a DNA prime-protein boost human vaccine in a previously reported phase I clinical trial DP6-001 (85). These mAbs could recognize a wide range of primary HIV-1 Env glycoproteins from multiple major subtypes. The characterization in this chapter highlights the multi-clade binding elicited by the polyvalent vaccine formulation, along with the diverse epitope elicitation that ranges from common linear V3 epitopes to probable conformational epitopes.
Results

DP6-001 vaccine volunteers and their serum antibody responses.

In the current study, a total of 13 human Env-specific mAbs were produced by the single B cell cloning approach from four DP6-001 volunteers (ABL-001, ABL-003, ABL-034 and ABL040) (Table 3.2). In DP6-001 trial, Groups A and B volunteers first received three low dose (1.2 mg each time) DNA prime immunizations by ID and IM routes, respectively, with a polyvalent formulation including five gp120-expressing and one gag-expressing DNA plasmids, followed by two protein immunizations with a polyvalent five gp120 proteins produced from CHO cells formulated with adjuvant QS-21 (85). Group C volunteers received the same formulation except 1) a higher dose (7.2 mg each time) DNA prime immunization by IM route and 2) only one time gp120 protein boost immunization due to the early stop of clinical trial in this group volunteers (84). In the current study, mAbs were generated from one Group A volunteer, two Group B volunteers and one Group C volunteer (Table 3.2).

Initial DNA priming immunization did not elicit a detectable gp120-specific IgG titer in these volunteers’ sera, with the exception of one from Group C who received the higher dose DNA prime. However, after the first protein boost, gp120-specific IgG responses can be seen to rise quickly to high titers (Figure 2.1a). Serum neutralization was tested against a diverse panel of pseudotyped viruses from multiple clades (A, B, C, D, AE). For volunteers included in the current study, positive neutralizing activity can be
seen in immune sera after one or two protein boost immunizations across this panel of pseudotyped viruses, ranging for an ID50 titer of 1:20 to 1:915 (Figure 2.1b).

**DP6-001 HIV-1 Env-specific ELISA Binding.**

Because the volunteers from whom the gp120-specific hmAbs were isolated were immunized with a polyvalent gp120 vaccine, a wide selection of gp120 antigens were used to test the breadth of binding for this panel of mAbs. Two patterns of binding are evident, with one group of hmAbs binding broadly to multiple clades of gp120 proteins, and the second group binding to gp120 antigens from B clade specifically. Nine mAbs (HH1G9, HH2D11 and HH4E4 from Volunteer 034 of Group A, GB1, EA7 and EA8 from Volunteer 001 of Group B, CC6B5 and CC6C11 from Volunteer 003 of Group B and TB7 from Volunteer 040 of Group C) belong to the first pattern although some of them have higher relative binding than the other hmAbs. Four hmAbs (EA1 from Volunteer 001 of Group B, CC3C2 from Volunteer 003 of Group B and TA6 and TA7 from Volunteer 040 of Group C) belong to the second pattern. This pattern was consistent between gp120 antigens used in ELISA autologous to vaccine components (Figure 3.1) and gp120 antigens used in ELISA heterologous to gp120 vaccine components (Figure 3.2). Those hmAbs with broad reactivity against autologous gp120 antigens are also able to recognize a wide range of heterologous gp120 antigens. One noticeable exception is gp120 antigen from subtype D. Given the fact that the gene sequences of subtype D viruses are more different from the consensus sequences than other major subtype viruses (such as A, B, C and AE), it is not surprising that the broadly reactive hmAbs cannot recognize subtype D gp120. In addition, no gp120 immunogen
from subtype D was included in the vaccine formulation. However, one broadly reactive hmAb HH1G9 (from Group A) was able to bind all gp120 antigens tested including the one from subtype D. Consistent with the notion that a polyvalent vaccination elicits an antibody repertoire that has binding breadth, most of the tested mAbs also display breadth that is not dependent on the combination of antibody paratopes, but is present in each individual antibody.
Figure 3.1: The reactivity of 13 hmAbs against a panel of five autologous gp120 proteins included in DP6-001 trail as measured by ELISA. HIV-1 ENV gp120 proteins were derived from the following isolates: 92UG037.8 (A2), 92US715.6 (B), Ba-L (Bal), 96ZM651 (CZM), 93TH976.17 (AE). (A) hmAbs from one Group A volunteer, ABL-034. (B-C) hmAbs from two Group B volunteers, (B) ABL-001, (C) ABL003. (D) hmAbs from one Group C volunteer, ABL-040. (E) Well characterized hmAb as controls.
Figure 3.2: The reactivity of 13 HmAbs against a panel of heterologous gp120 proteins revealed by ELISA. HIV-1 ENV gp120 proteins were derived from the following isolates: JR-FL, 93MW965 (C2), 92UG021 (D), consensus AE (AE Con). (A) hmAbs from one Group A volunteer, ABL-034. (B-C) hmAbs from two Group B volunteers, (B) ABL-001, (C) ABL-003. (D) hmAbs from one Group C volunteer, ABL-040. (E) Well characterized hmAb as controls.
Several well characterized human bnAbs in the HIV field were included in the binding antibody analysis as controls. VRC01 is able to bind to gp120 proteins from different subtypes but less well to the one from subtype D. PGT128 can only bind strongly to gp120 from subtype A but less well to gp120 proteins from other subtypes, reflecting its overall reduced binding to gp120 monomers. Not surprisingly, V3-specific hmAb 447-52D mainly binds to subtype B gp120 proteins, confirming its preferred subtype specificity. In contrast, A32 is able to bind to all gp120 proteins except the one from subtype D which may also explain its high ADCC activity against a wide range of HIV-1 viruses from different subtypes (Figures 3.1, 3.2).

Binding kinetics including affinities for this panel of DP6-001 hmAbs were also measured against the same autologous gp120 proteins as those used in the ELISA (Table 3.1, Figure 3.1). The levels of binding affinities are comparable to the binding profile seen in ELISA. The mAb HH1G9 reaches nM affinity ranges against B clade antigens, similar to bnAbs. There can also be seen a few hmAbs that are subtype B specific (EA1, CC3C2) (Table 3.1).
Table 3.1. Binding kinetics of DP6-001 hmAbs as measured by ForteBio system

<table>
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<th>Subject</th>
<th>hmAb</th>
<th>Binding affinities to gp120 proteins (KD)</th>
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* NB = No binding

- <1.0E-10
- 1.0E-9 ~ 1.0E-10
- 1.0E-8 ~ 1.0E-9
- >1.0E-8
Characterizing linear epitopes.

Using a peptide microarray system (JTP, Berlin, Germany), linear epitopes were determined for about half of the DP6-001 hmAbs included in this study (Table 3.2, Figure 3.3, Figure 6.1). The other half did not give clear dominant binding peaks, indicating that their epitopes may be conformational. The linear epitopes were mainly in the constant regions, including C2 (GB1 and CC6C11), C5 (EA1) or C2/C5 (HH1G9 and TB7) (Table 3.2). Representative JPT mapping results are shown in Figure 3.3. Two DP6-001 hmAbs (TA6 and TA7) had specific binding to peptides in the V3 region (Figure 3.3). For the V3-specific hmAbs, a peptide ELISA using cyclic V3 was used to confirm TA6 and TA7 binding, along with positive control V3-specific mAb 447-52D (Figure 3.4A). All other hmAbs did not display any binding to the cyclic V3 peptide (Figure 3.4B). For the C5 specific mAbs (EA1, HH1G9, TB7), ELISA binding was tested against a full length, linear C5 peptide derived from JR-FL. Binding is observed for EA1, but not HH1G9 or TB7 (Figure 3.5A), while no binding is observed for all other mAbs (Figure 3.5B).
Figure 3.3: Multiple epitopes elicited by vaccination in human volunteers revealed by peptide microarray. Purified HmAbs were tested on JPT slides containing overlapping peptides for a diverse set up gp120s from multiple clades, both autologous and heterologous, including Consensus sequences. Peptide microarray against multiple gp120 protein peptides, focused on epitopes in C2, C5 and V3.
Figure 3.4: The reactivity of 13 HmAbs against a consensus clade B V3 circular peptide revealed by ELISA. (A) V3 specific hmAbs. (B) Non-V3 specific hmAbs.

Figure 3.5: The reactivity of 13 HmAbs against a consensus clade B C5 linear peptide revealed by ELISA. (A) C5 specific hmAbs. (B) Non-C5 specific hmAbs.
Combining the known epitopes of EA1, TA6 and TA7 with the sequence data from gp120s tested in the ELISA format, a few possible amino acid differences may account for the binding specificity of these select mAbs. TA6 and TA7 bind to the V3 region of gp120 B, Bal and JR-FL (Figure 3.1, 3.2). These gp120s have an arginine in the GPGR/Q V3 motif, while all other gp120s tested by ELISA have a glutamine in place of the arginine (Figure 3.6A). EA1 binds to the C5 region of gp120 Bal and JR-FL, and weakly gp120 B (Figure 3.1, 3.2). According to JPT data, the probable epitope in C5 is KVVKIEPL. Gp120 Bal and JR-FL both have a lysine at the center of the epitope, while no other gp120s have a lysine in this position (Figure 3.6B). This sequence comparison data gives a plausible explanation for the differences seen in binding between gp120 proteins and clades for TA6, TA7 and EA1.
A.

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Possible Epitope

B.

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<td>RSELYKVVKIKIELPGLVAPTKAK</td>
<td>RSELYKVVKIKIELPGLVAPTKAK</td>
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Possible Epitope

Figure 3.6: Gp120 sequence comparison for epitopes of TA6, TA7 (A) V3, and EA1 (B) C5.
Testing for binding to conformational epitopes.

Linear and conformational epitopes were interrogated by testing against denatured antigen. Putative conformational epitope binding mAbs HH2D11, HH4E4, EA7, EA8, CC3C2, and CC6C11 all lost ELISA binding when gp120-Bal was denatured (Figure 3.7). The mAbs that have shown binding to linear peptides in microarray (TA6, TA7, EA1, 447-2D) also maintained full or reduced binding to denatured gp120-Bal (Figure 3.7). Four mAbs (HH1G9, GB1, CC6B5, TB7) that have shown binding to linear peptides in the microarray format were not able to bind to denatured gp120 (Figure 3.7). Also tested in this assay was the effect of deglycosylation on the binding of mAbs to antigen in the ELISA format. While the known glycan specific mAb PG9 lost binding to deglycosylated gp120-Bal, all other tested mAbs maintained binding (Figure 3.7). This data confirms the pattern seen in the peptide ELISA and supports the conclusion that some of the epitopes seen in the peptide microarray, like TB7, are false signals. Together, the data points to most DP6-001 mAbs having a conformational epitope.
Figure 3.7: ELISA binding to autologous gp120 Bal antigen deglycosylated and denatured. Native deglycosylated gp120 produced by PNGase without a denaturing step. Denatured gp120 produced by only denaturing. Denatured Deglycosylated gp120 produced by both denature followed by deglycosylation.
Since a large majority of the mAbs lost binding for denatured gp120, additional assays to test for conformational epitopes were performed. There was no evidence of binding of any mAb to a modified antigen that presents the V1V2 loop in a conformational form (Figure 3.8). Also, binding to multiple core specific antigens are absent in our set of mAbs (Figure 3.9). RSC3 is a very stringent CD4bs antigen, while the YU2 core contains the CD4bs epitope and any other epitope that may be present on a gp120 antigen with the variable loops removed. Testing against multiple conformational epitopes has not yielded any further information, leaving the majority of the mAbs with unknown conformational epitopes.
Figure 3.8: The reactivity of 13 HmAbs against a gp70V1V2-Bal or gp70 protein revealed by ELISA.

Figure 3.9: The reactivity of 13 HmAbs against a panel of gp120 core proteins revealed by ELISA. Proteins were derived from the following: RSC3, dRSC3, TU2 Core, YU2 Core D368R.
Some epitopes that are accessible on gp120 are hidden when in the context of a env on the surface of a virion. The V3 mAb TA6, and possibly TA7 to a small degree, was able to bind to JR-FL pseudo typed virus. The conformational epitope mAbs EA8, GB1 and HH1G9 are able to bind to the 816367.Co2 pseudotyped virus. No other mAbs were able to bind to pseudotyped virus in this assay. The V3 epitopes of TA6 and TA7 are known to be accessible on the trimer structure, which is confirmed in this assay. The amino terminal end of gp120 is most likely occluded by gp41 in the trimer structure, accounting for the lack of EA1 binding. The binding of conformational epitopes mAbs EA8, GB1 and HH1G9 indicate that the epitope on gp120 is also accessible on the trimer structure, or some form of the trimer structure present on pseudotyped virions.
Figure 3.10: Virion Binding. Pseudotyped virions tested for the ability to be captured by mAbs coated on the surface of an ELISA plate, with levels of virion capture assayed by ability to activate TZM-bl cells coated over the top of captured virions.
Table 3.2: Summary of Epitope information.

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<th>Peptide Array</th>
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<th>DG</th>
<th>Core</th>
<th>V1V2</th>
<th>Gp120 ELISA Binding</th>
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<tr>
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<td>HH4E4</td>
<td>Conf</td>
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<td>Y</td>
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<td>N</td>
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</tr>
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<td>C2</td>
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</tr>
<tr>
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<td>A2, B, Bal, JR-FL, D, AE, AE Con</td>
</tr>
<tr>
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<td>Bal, JR-FL, D</td>
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</tr>
</tbody>
</table>

- DNB: Denatured ELISA Binding
- DG: Deglycosylation
- Core: Binding to YU2 core and RSC3
- V1V2: Binding to gp70-V1V2
**HIV-1 V3 Specific hmAbs.**

There are a few mAbs that are of interest because of their epitope and similarity to other known mAbs isolated from HIV-1 infected patients. The V3 monoclonal antibodies TA6 and TA7 utilize the same heavy and light chain V gene as V3 specific mAbs 311-11D and 1334 (Figure 3.11). Alignment of the heavy chain for all four mAbs indicates strong similarity throughout the framework and CDR regions, with the exception of the CDR3. Most amino acid differences between the mAbs are conserved functionally. The CDR3 on the other hand is diverse between the 4 mAbs, with differing lengths and functional amino acid compositions. For the light chains, the entire variable region is highly conserved with only functionally similar amino acid substitutions present (Figure 3.11). The highly similar B cell rearrangement selection across multiple individuals, combined with binding to the same epitope, indicate that there could be an inherent binding capability for the V3 region of gp120 encoded in these germlines.
Figure 3.11: Protein alignment of 4 V3 specific monoclonal antibodies. TA6 and TA7 are DP6-001 mAbs. 311-3D and 1334 are previously isolated and characterized mAbs from the Gorny lab. (A) Heavy Chain. (B) Light Chain.
Discussion

The field of HIV research is now in the process of developing and analyzing the effects of vaccination in humans. One of the newly developed methods to interrogate vaccine efficacy is the isolation of mAbs in an effort to determine the specific binding characteristics and epitopes predominantly elicited during vaccination. This will allow for the comparison with antibodies elicited during natural infection. This type of information can help inform better vaccination strategies. In this chapter, I looked at the types of epitopes elicited by the DNA prime/protein boost vaccination strategy.

The current panel of mAbs from a polyvalent vaccine regimen was able to bind to a large variety of antigens. Along with binding to the antigens contained in the vaccine, most of the mAbs were also able to bind to heterologous antigens from multiple clades. There were a few mAbs that had restricted binding profiles, and mostly bound to only B clade antigens. Two of these B clade specific antibodies (TA6 and TA7) were V3 specific. The specific epitope of TA6 and TA7 contains an amino acid that has previously been shown to confer clade specificity (125).

When going to determine epitopes, multiple techniques were utilized. Because the vaccine regimen utilized plasmid derived env immunogens and protein gp120 immunogens, the epitopes could be varied. In this panel, a few linear epitopes along with multiple potentially conformational epitopes were discovered. To gain a general idea of the types of epitopes elicited by this vaccine regimen, a peptide microarray against multiple Envs produced an initial result. Most of the mAbs did not give a clear signal,
suggesting a preponderance of conformational epitopes. This reasoning is further supported by the ELISA done with denatured antigen. Like in the microarray, most mAbs were unable to bind to denatured antigen, with a few exceptions in both the microarray and denatured ELISA including TA6, TA7, and EA1. TA6 and TA7 both bound to V3 in the microarray, along with cyclic V3 peptides in an ELISA format. EA1 meanwhile bound to a C5 peptide in the peptide ELISA and microarray format, indicating that these three mAbs have linear epitopes. This C5 epitope may be adjacent to the epitope of cluster A antibodies such as C11. Antibodies from this region have been shown previously to be a target of ADCC mediated viral escape, along with a reduced risk of infection in RV144 (33, 80, 95). HH1G9, EA1, and TB7 all have an epitope that is adjacent with the cluster A epitope. Since most of the mAbs bound to conformational epitopes, additional assays are required to determine the remaining epitopes.

Unfortunately, these further assays were not able to clear up these epitopes. This could be explained by the antibodies not having strong enough binding in these assays to create a positive result. The more likely explanation is that these epitopes are conformational and not easily interrogated with the select assays performed. Deglycosylation of antigen did not have any significant effect on binding, in contrast to the known glycan-specific mAb PG9. The whole of the panel of mAbs were also unable to bind to YU2 core, RSC3, and gp70 V1V2. Together, this points to the conformational epitopes being glycan independent, and outside of core and V1V2 loops. Some competition assays with known epitopes would be a logical next step to elucidate these epitopes.
For many years, V3 mAbs have been studied widely because of their ubiquity in elicitation during an HIV-1 response. It used to be believed that V3 directed antibodies were the desired target for immunization because of studies in simians indicating that V3 specific mAbs could prevent infection (140–143). Shown here is that vaccination is able to produce V3 specific mAbs similar to those elicited during infection. Two of these V3 specific mAbs produced from DP6-001 had similar V gene usage to V3 specific mAbs 311-2D and 1334 isolated from HIV-1 infected individuals (125, 126). It is interesting that the CDRH3 regions are more diverse between these mAbs, while the other heavy chain regions and the entire light chain maintain significant similarity. This might indicate that the V gene itself confers an amount of binding to the V3 region, while the recombination region has less affect. This type of imbedded binding preference was also noted in the highly studied CD4bs bnAbs that use the VH1-2 and VH1-46 V-gene (26).

Some of the epitopes that are potentially linear may have a conformational component to them. The microarray data indicates a linear epitope, but this binding is not maintained when in the context of the entire denatured gp120 for HH1G9, GB1, CC6B5, and TB7. This will need to be further studied to determine the exact epitope, along with the make-up of the potentially conformational epitopes. A competition assay could be developed using other mAbs with known epitopes. For those few that have known linear epitopes, sequence analysis of the gp120s bound by ELISA shows that for EA1, TA6 and TA7, one amino acid may be implicated in the difference in binding in ELISA between gp120s. Structural information from V3 specific mAb 268-D indicates that a negatively charged pocket on 268-D forms salt bridges with the arginine in the GPGR motif, and
precludes the binding to other clades of gp120 that predominantly have a glutamine in this position (144). This gives 268-D a B clade restricted binding and neutralization profile, which could also be why TA6 and TA7 are B clade restricted (125).

A smaller panel of RV144 mAbs (CH58, CH59, HG107, HG120) were specifically isolated because of their V2 epitope that was correlated with protection in vaccine recipients and their ability to mediate ADCC against RV144 vaccine strains (34, 76). Like the DP6-001 mAbs, these RV144 mAbs developed with a low mutation rate from germline. In contrast, RV144 mAbs utilized multiple immunoglobulin gene families while DP6-001 mAbs have a VH1 focus. Another difference between the two panels of mAbs is that the RV144 mAbs were clade specific to AE and able to mediate neutralization against a couple tier 1 AE viruses (34). When antibodies against the two regions implicated in protection in RV144 (C1 and V2) were tested together, synergy was observed that increased the potency of CH58 to clinically relevant concentrations (36).

Since DP6-001 was an experimental study with limited volunteers and no virus challenge, we are unable to compare efficacy. With further isolation of mAbs from DP6-001, more comparisons of vaccine elicited epitopes will be possible. Because the epitope and activity of Fc mediated functions like ADCC and ADCP are not as dependent on functional and well-formed trimers, elicitation of these types of mAbs could be an important component in a successful HIV vaccine.
Chapter IV

Effector functions elicited by an HIV vaccine in humans (DP6-001).

Introduction

One important finding from the RV144 ALVAC-HIV (vCP1521) prime-AIDSVAX B/E boost vaccine efficacy trial is the identification of immune correlates of vaccine-induced protection from acquisition of human immunodeficiency virus type 1 (HIV-1). Antibodies targeting the Env gp120 V1/V2 region inversely correlated with infection risk, while IgA Env-binding antibodies to Env directly correlated with infection risk (76). Further studies indicated that low plasma IgA Env antibody levels in association with high levels antibody-dependent cellular cytotoxicity (ADCC) were inversely correlated with infection (76).

While the bNabs have been the main focus for HIV vaccine development efforts for several decades, ADCC activities were also suggested in the literature to play important roles in control of established HIV-1 infection as well as protection from initial infection. The majority of such studies have shown an inverse correlation between ADCC and progression of disease or viral load (103). There was an inverse correlation between ADCC titers in milk and transmission to newborns which was not correlated with neutralization (104).
Along with ADCC, an inverse correlation exists between ADCVI and progression to simian AIDS (145). Furthermore, phagocytosis is increased in acute infection, impaired in chronic infection for monocytes and dendritic cells. Blockage of FcγRII results in loss of antibody dependent cellular phagocytosis (ADCP) activity (146). It was shown that non-neutralizing antibodies can reduce plasma viral load in mucosal protection study in a non-human primate (NHP) model and phagocytosis was implicated in passive protection (37).

When investigating how well an adenovirus prime/gp140 boost vaccination regimen compared to un-boosted vaccination in macaques, it was discovered that improved ADCC and ADCVI levels correlated with reduced acute viremia (61). Animals that had greater pre-challenge ADCC, ADCVI and transcytosis inhibitory activity had greater antibody avidity and reduced acute viremia (61). In humans, differences in vaccination efficacy may be due to the Fc mediated functions elicited by each vaccine. The RV144 trial had a similar immunogen to the VAX003 trial, yet the outcomes were different. RV144 was able to elicit highly functional IgG3 antibodies, while the repeated immunization of a subunit vaccine only pushed the VAX003 repertoire into the less functional IgG4 subclass (81). Mounting evidence indicates that more complex interrogations of serology are needed to understand vaccine trial outcomes, and that these interrogations include Fc mediated activities (147).

In recent years, molecularly cloned mAbs from HIV-1 infected patients have been used to provide a more in-depth understanding of the mAb ADCC activity. The mAb A32 is a non-neutralizing antibody isolated from a chronically infected individual that
has been shown to bind to Env on the surface of CD4 cells and mediate potent ADCC activity. Most importantly, A32 can block most of the ADCC activity in a broad range of HIV-1 infected plasma samples (31). Two A32 like mAbs with distinct ADCC potencies were shown to have overlapping epitopes, but differed in their binding angle and ability to mediate ADCC (148). Non-neutralizing epitopes are usually buried on the trimer surface and only accessible after engagement by CD4, which is the case with Fc mediated function by mAb A32 (31).

There is only limited study with ADCC-mediating mAbs produced from human volunteers who received experimental HIV vaccines, almost exclusively from the RV144 trial. These Env-specific mAbs from RV144 preferentially use VH1 gene family and recognize multiple Env epitopes (32). These RV144 non-neutralizing antibodies contrasted with other results that indicate Env binding antibodies with neutralization activity have a lambda chain bias (149). The region implicated with the reduced risk of infection in this trial was the region overlapping with the A32 like epitopes in cluster A, which has been shown previously to be a target of ADCC-mediated viral escape (33, 79, 80). The V2 region in Env was another region implicated in the RV144 trial (76). Four antibodies (CH58, CH59, HG107, HG120) isolated from vaccine recipients have specific binding to this region and mediate ADCC activity (34).

In the current chapter, we assessed Fc-mediated effector functions of a panel of HIV-1 Env specific mAbs isolated from four volunteers who received a DNA prime-protein boost human vaccine in a previously reported phase I clinical trial DP6-001 (85). These mAbs could recognize a wide range of primary HIV-1 Env glycoproteins from
multiple major subtypes. While these mAbs did not exhibit significant neutralizing activity, they demonstrated overall potent Fc-mediated effector functions including ADCC and ADCP. Recent reports start to appreciate the role of Fc mediated Ab function and its ability to distinguish protective vaccine trials from less efficacious vaccine designs (147). Our results from the current study provides valuable information on the specificity and quality of mAbs produced from the second human HIV-1 vaccine trial in addition to RV144. Information learned here can be used as the baseline results to guide the designs of future HIV-1 vaccines to elicit high level, broadly reactive Fc-mediated effector functions.
Results

Absence of neutralizing activities from gp120-specific HmAbs.

Previous reports have shown serum neutralization from the volunteers of DP6-001 vaccine trial (85), so an in house neutralization panel was performed for the cloned hmAbs, along with Dr. M Seaman’s laboratory. The majority of cloned DP6-001 hmAbs did not exhibit a neutralization titer, with the exception of the V3 hmAb TA6 which could weakly neutralize tier 1 subtype B pseudotyped virus based on a neutralization assay conducted in house at UMMS or at Dr. M Seaman’s laboratory (Table 4.1).
Table 4.1: Neutralization titers of human mAb TA6 by TZM-bl assay

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*Data represent IC50 values in µg/mL.*
ADCC activities with DP6-001 sera and hmAbs.

We next studied the presence and levels of Fe-mediated antibody activities in either immune sera or hmAbs from DP6-001 volunteers (Figure 4.1, 4.2). After multiple immunizations including protein boost, gp120-specific ADCC activities can be seen in all volunteers, and is sustained out to 36 weeks which was the last time point we included in the ADCC study. ADCC activity is seen against both subtypes B and AE gp120 antigens (Figure 4.1A). Most significantly, ADCC activities can be detected by using either the assay format with target cells coated with recombinant gp120 antigens, or the infection format with viruses expressing HIV-1 Env antigens (Figure 4.1A and 4.1B). ADCC levels in cells with viruses expressing HIV-1 Bal Env showed lower ADCC activities than the viruses expressing HIV-1 CM235 (Figure 4.1B). The levels of ADCC activities from volunteer ABL040 sera (Group C of DP6-001 trial) were lower than other three volunteers, most likely due to the fact that Group C volunteers did not receive the second protein boost (Figure 4.1B).

Most of the DP6-001 hmAbs generated from these volunteers also showed high levels ADCC activity (Figure 4.2, 4.3). The hmAbs that have shown broad gp120 binding also showed broad ADCC activities. Those with narrow binding specificity (mainly B subtype) (EA1, CC3C2, TA6, TA7) also had poor ADCC activity. TB7 is the one hmAb that had broad gp120 binding profile but lacks ADCC activity, making all hmAbs produced from this Group C volunteer with poor ADCC activity (Figures 4.2D, 4.3D). In the RFADCC assay, which was the chosen assay for use in house because of its ease of use and robustness, the mAbs were confirmed to be able to bind to antigen on the surface
of the target cells (Figure 4.4). The Duke ADCC assay measures granzyme B activity, a product of the effector cells, while the RFADCC assay measures GFP loss caused by membrane permeability. These are separate but connected actions by the effector cells, which secrete perforin and granzyme B to mediate cell killing. When the relative ADCC activities were compared against the positive control hmAb A32, some of the DP6-001 hmAbs (GB1, EA7 and CC685) had ADCC activities against selected subtypes of gp120 antigens close to that achieved by A32 (Figure 4.2E, 4.3E, 4.5A). The DP6-001 hmAbs were also tested against cells infected with two infectious molecular clone (IMC) viruses, Bal and CM235. Levels of specific killing were lower than seen with gp120 coated cells, though GB1 was still able to mediate decent ADCC against both IMC viruses (Figure 4.5B).

Figure 4.1: Serum ADCC titers for select volunteers against gp120 coated cells from a Gran-Toxi-Lux ADCC assay (panel A) and infected cells from a Luc based ADCC assay (panel B). ADCC with sera from select DP6-001 volunteers against autologous Bal and AE in (A), and both autologous Bal and heterologous CM235 in (B).
Figure 4.2: The ADCC reactivity of 13 HmAbs with a panel of autologous DP6-001 gp120 proteins in the Duke ADCC assay. HIV-1 ENV gp120 proteins were derived from the following isolates: 92UG037.8 (A2), 92US715.6 (B), Ba-L (Bal), 96ZM651 (CZM), 93TH976.17 (AE). (A) hmAbs from ABL034 (Group A). (B) hmAbs from ABL001 (Group B). (C) hmAbs from ABL003 (Group B) (D) hmAbs from ABL040 (Group C). (E) hmAb Controls.
Figure 4.3: The ADCC reactivity of 13 HmAbs with a panel of autologous DP6-001 gp120 proteins in the RFADCC assay. HIV-1 ENV gp120 proteins were derived from the following isolates: 92UG037.8 (A2), 92US715.6 (B), Ba-L (Bal), 96ZM651 (CZM), 93TH976.17 (E). (A) hmAbs from ABL034 (Group A). (B) hmAbs from ABL001 (Group B). (C) hmAbs from ABL003 (Group B) (D) hmAbs from ABL040 (Group C). (E) hmAb Controls.
Figure 4.4: The binding of mAb to autologous gp120 on the surface of target cells in RFADCC assay. HIV-1 ENV gp120 proteins were derived from the following isolates: 92UG037.8 (A2), 92US715.6 (B), Ba-L (Bal), 96ZM651 (CZM), 93TH976.17 (E). (A) hmAbs from ABL034 (Group A). (B) hmAbs from ABL001 (Group B). (C) hmAbs from ABL003 (Group B) (D) hmAbs from ABL040 (Group C). (E) hmAb Controls.
Figure 4.5: ADCC activity of human mAbs in the Duke ADCC assay. (A) The max % GzB activity of 13 HmAbs with a panel of autologous DP6-001 gp120 proteins. HIV-1 ENV gp120 proteins were derived from the following isolates: 92UG037.8 (A2), 92US715.6 (B), Ba-L (Bal), 96ZM651 (CZM), 93TH976.17 (AE). (B) The max % specific killing of 13 HmAbs with a cells infected with IMC viruses Bal and CM235.
**ADCP activities with DP6-001 sera and hmAbs.**

Another important Fc mediated function that has been explored in the current study is antibody-dependent cellular phagocytosis (ADCP). As seen with binding antibody and ADCC analyses, sera from DP6-001 volunteers were able to mediate ADCP against all 5 autologous gp120 proteins (Figure 4.6). Also consistent with ADCC results is the observation that the group C volunteer ABL040 has reduced activity, most likely due to the missing second protein boost in the original human vaccine study (85).

The majority of DP6-001 mAbs are capable of mediating ADCP against the entire range of vaccine autologous gp120 antigens. Like the results of the ADCC study, two hmAbs with narrow gp120 binding (EA1 and CC3C2) also had lower levels of ADCP activity. Also similar to ADCC results, while TB7 had relative broad binding antibody profile, its ADCP activity was also low. Interestingly, TA6 and TA7, while having narrow binding breadth and low ADCC activity, still showed strong ADCP activity against multiple subtypes of gp120 antigens, which could be explained by the high amount of gp120 coated on the beads, which may overcome the low affinity binding of TA6 and TA7 for non-B clade antigens (Figures 4.7, 4.8).

When the endpoint titer for ADCP or Max ADCP Score is used, DP6-001 hmAbs showed quite impressive ADCP activities (Figure 4.8). They are even higher than hmAb A32 which is known for having extremely high ADCC activity against many subtypes of gp120 antigens. In contrast, hmAbs VRC01 and PGT128 showed higher ADCP activities than A32.
Figure 4.6: Plasma ADCP response for four DP6-001 volunteers against gp120 coated fluorescent beads. ADCP with sera from 4 volunteers, day 0 and day 210 bleeds, against beads coated with the following autologous gp120 isolates: 92UG037.8 (A2), 92US715.6 (B), Ba-L (Bal), 96ZM651 (CZM), 93TH976.17 (AE).
Figure 4.7: The ADCP reactivity of 13 HmAbs with a panel of autologous DP6-001 gp120 proteins. HIV-1 ENV gp120 proteins were derived from the following isolates: 92UG037.8 (A2), 92US715.6 (B), Ba-L (Bal), 96ZM651 (CZM), 93TH976.17 (AE). (A) hmAbs from ABL034 (Group A). (B) hmAbs from ABL001 (Group B). (C) hmAbs from ABL003 (Group B) (D) hmAbs from ABL040 (Group C). (E) Normal human Ig (NHIgG) was used as negative control.
Figure 4.8: The Max ADCP reactivity of 13 HmAbs with a panel of autologous DP6-001 gp120 proteins. HIV-1 ENV gp120 proteins were derived from the following isolates: 92UG037.8 (A2), 92US715.6 (B), Ba-L (Bal), 96ZM651 (CZM), 93TH976.17 (AE). Normal human Ig (NHIgG) was used as negative control.

In summary, it can be seen that binding antibody profile and ADCP activity of DP6-001 hmAbs most closely correlate (Table 4). The broad gp120 binding reactivity is mirrored in the ADCP activity. The position where we see ADCC activity drop off is with the mAbs that have B clade specific gp120 binding (EA1, CC3C2, TA6 and TA7). Two B clade specific mAbs EA1 and CC3C2 have low to no ADCC activity against any clade of gp120, including the subtype B ADCC targets that they bind to in ELISA. The one exception to this is TB7, which has moderate but broad gp120 binding, but no ADCC activity (Table 4.2).
Table 4.2: Comparison of relative activity for gp120 ELISA binding, ADCC and ADCP among DP6-001 hmAbs.

<table>
<thead>
<tr>
<th>Subject</th>
<th>mAbs</th>
<th>Function</th>
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<td></td>
<td></td>
<td></td>
<td>A2</td>
</tr>
<tr>
<td>ABL034</td>
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<td>Binding</td>
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<tr>
<td>(Group A)</td>
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<td>ADCC</td>
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<td></td>
<td></td>
<td>ADCP</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>HH2D11</td>
<td>Binding</td>
<td>+++</td>
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<td></td>
<td></td>
<td>ADCC</td>
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<td></td>
<td>ADCP</td>
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</tr>
<tr>
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<td>HH4E4</td>
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<td></td>
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<td>TB7</td>
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<tr>
<td></td>
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<td>ADCP</td>
<td>+</td>
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- >5 DC50 (Binding), <5 Max GZB% (ADCC), <5 Max (ADCP)
+ 0.5-5 DC50 (Binding), 5-10 Max GZB% (ADCC), 5-10 Max (ADCP)
++ 0.05-0.5 DC50 (Binding), 10-15 Max GZB% (ADCC), 10-15 Max (ADCP)
+++ 0.005-0.05 DC50 (Binding), 15-20 Max GZB% (ADCC), 15-20 Max (ADCP)
++++ <0.005 DC50 (Binding), >20 Max GZB% (ADCC), >20 Max (ADCP)
Discussion

Analysis of non-neutralizing antibody responses in RV144 volunteer immune sera showed that ADCC was a correlate of reduced infection risk in the group with low IgA C1 binding titers (76). At the same time, the knowledge in this area is limited due to the limited availability of human mAbs that are generated from non-HIV infected individuals. More studies are needed to understand Fc effector functions in antibody responses from volunteers who received different experimental HIV vaccines.

One of the central activities that have been associated with protection in the RV144 trial is ADCC. The RV144 panel of mAbs isolated from vaccine volunteers lacked neutralization activity, but had a significant level of ADCC activity against autologous antigen (32). This pattern is also observed among mAbs characterized in this current study. Another similarity between RV144 and DP6-001 mAbs is that both panels preferentially used the VH1 germline locus and recognize multiple clades, while both sets have a low level of SHM (32). Some key differences between the two panels of mAbs are that the RV144 mAbs lacked linear epitopes and had inconsistent binding to autologous antigen, but consistent ADCC activity. For the subset of mAbs in the current study that had the most consistent ADCC activity, gp120 binding was universally broad and potent, with potent ADCC present in the absence of the enhancing modifications A32 has (150, 151). These mAbs recognize a mixture of linear and possible conformational epitopes. These characteristics are possibly due to the vaccine formulation utilized in DP6-001, which included a 5-valent gp120 prime and boost, while RV144 only had two envelope
proteins in the vaccine. Although there were other differences between the vaccine formulations such as a viral vector prime compared to a DNA prime, the most likely factor in epitope differences would be the antigens. The one linear epitope seen in RV144 ADCC mAbs was V3 (CH23), which was also present in our panel (TA6 and TA7). Both sets of V3 mAbs were clade specific in their ADCC activities. The one shortcoming in exploring these Fc mediated responses was the failure to extend the breadth of binding seen in ELISA to the ADCC and ADCP assays by using heterologous antigen in these assays. Testing more antigens would bolster the argument that this polyvalent vaccine was able to elicit poly-functional antibodies with activity not just for autologous antigen.

A smaller panel of RV144 mAbs (CH58, CH59, HG107, HG120) were specifically isolated because of their V2 epitope that was correlated with protection in vaccine recipients and their ability to mediate ADCC against RV144 vaccine strains (34, 76). Epitopes that can mediate ADCC are diverse, and as seen in RV144 and DP6-001, vaccines are able to elicit ADCC to multiple epitopes (30, 32, 34, 76). When antibodies against the two regions implicated in protection in RV144 (C1 and V2) were tested together, synergy was observed that increased the potency of CH58 to clinically relevant concentrations (36). Because the epitope and activity of Fc mediated functions like ADCC and ADCP are not as dependent on functional and well-formed trimers, elicitation of these types of mAbs could be an important component in a successful HIV vaccine.

Along with ADCC, another Fc mediated function that was explored was ADCP activity. Recent studies have started to look into comparisons between vaccine trials and the differences in serology that may help with distinguishing which protocol will be best
With the group of mAbs that displayed the highest breadth of gp120 binding and ADCC activity, ADCP activity was also broad and potent in the current report. Our study is one of the few to start looking into a more complete description of the Fc mediated functions of mAbs elicited by vaccines in humans. Although the value of non-neutralizing activity is still being explored, some evidence indicates that there is immune pressure that HIV tries to circumvent during chronic infection. It has been shown that during chronic infection, FcγRII is downregulated on immune cells, which is specifically associated with a loss in ADCP activity (146). While groups have looked at comparisons of ADCP activity in sera, our data will among the first to start to look more specifically at mAbs (152). Even without neutralization, the ability of mAbs to broadly bind gp120 is associated with breadth in ADCP activity in the current study. This multi-clade mAb elicitation may be an important factor to consider in future vaccine design. Recent studies in macaques have given evidence for this stance. Utilizing both SIV and HIV immunogens, poly-functional antibody responses and antibody titer were correlated with increased sterilizing immunity (82). Both the macaque protocol and DP6-001 vaccine regimen utilized a protein boost. In the Macaque study, the protective efficacy was linked to the protein boost, which conferred both poly-functional antibodies and protective efficacy. The groups that lacked a protein boost had reduced levels of both of these parameters (82).

Of interest in the field of Fc mediated function is the positive control that has been consistently used across most studies. Although rarely noted, most utilize a modified version of the A32 monoclonal antibody. This modified version is officially
referred to as A32-AAA, which indicates that it has 3 amino acid modifications in the Fc domain that increases its binding affinity for Fc receptors, and consequently has greater Fc mediated activity (150, 151). Our mAbs, along with most antibodies produced in vivo and by vaccination, do not have this advantage. In the RFADCC assay and ADCP assay, usage of the native A32 antibody gives an indication that the DP6-001 antibodies are equal or greater in effectiveness and breadth to A32 when no modifications have been introduced to the Fc portion of the mAb, as would be the situation in a natural environment like a human vaccine trial. Modification of DP6-001 mAbs Fc to contain this amino acid motif would most likely increase the measured ADCC activity, though the relevance when looking at vaccine induced responses is questionable.

The group C mAbs have a unique circumstance that may be affecting how the mAbs developed with this immunization strategy display effector functions. This regimen was a high concentration DNA boost compared to the other two arms, but it did not receive a second protein boost like the others did. With the limited number of mAbs produced, no definitive answers can be made, but some interesting inferences are possible. The ADCC and ADCP activities with the sera from the ABL040 volunteer are all reduced when compared to the other two groups. This pattern is also partially found in the mAbs, with them being either clade specific, or in the case of TB7, a broad ELISA binder, but reduced levels of ADCC and ADCP. This type of data could point to the importance of the second protein boost in a prime/boost regimen. Therefore, it is exciting to demonstrate in the current chapter that broadly cross-reactive and poly-functional
mAbs indeed can be elicited in healthy human volunteers with the polyvalent DNA prime/protein boost HIV vaccine formulation DP6-001.
Chapter V

Materials and Methods

Plasma and cellular samples from DP6-001 vaccine recipients.

All trial participants in the completed multi-gene polyvalent HIV vaccine DP6-001 phase I clinical trial gave written informed consent as previously described (85). Samples were collected and tested according to protocols approved by the institutional review board (IRB) at the clinical trial site of this study. The vaccinees received polyvalent DNA vaccine (5-valent gp120s and one Gag) prime at weeks 0, 4, and 12, and 5-valent gp120 proteins protein boost formulated with QS-21 as adjuvant at weeks 20 and 28 (Groups A and B) or Week 20 (Group C) (85). The DNA vaccine was given by intradermal injection (ID) in Group A (1.2 mg) and by intramuscular injection (IM) in Groups B (1.2 mg) and C (7.2 mg). The protein boost was given by IM (0.375 mg) in all three groups. HIV-1 ENV gp120 were derived from the following isolates: 92UG037.8 (A2), 92US715.6 (B), Ba-L (Bal), 96ZM651 (CZM), 93TH976.17 (AE)

Plasma and peripheral blood mononuclear cell (PBMC) samples involved in the current study were from Subjects 034 (Group A), 001 and 003 (Group B), and 040 (Group C). The plasma samples used for ADCC assays were collected at Week 0 (pre-bleed) and Week 30 (post protein boosts). The PBMCs used for Env-specific mAb isolation were collected after two protein boosts from subject 001 (Week 30-32), 003 (Week 36) and 034 (Week 36), and after one protein boost from Subject 040 (Week 30) due to the early termination of Group C after one protein boost (84).
**Isolation of Env-specific single B cells.**

The cryopreserved PBMCs from the above vaccinees were thawed and processed in two ways for gp120-specific single B cell sorting. The first method had the freshly thawed PMBCs cultured in media (RPMI 1640 with 15% FBS) overnight in a 37°C CO2 incubator. Then, B cells were isolated by magnetic bead separation (MACS Human B Cell Isolation Kit II, Milteny Biotec, San Diego, CA). The gp120 protein probe for B cell staining was mixture of 5 gp120 proteins: A2 (clade A 92UG037.8), Bal and B (clade B Bal and 92US715.6, respectively), Czm (clade C 96ZM651) and E (clade E 93TH976.17), which were autologous to the gp120 DNA and proteins in DP6-001 vaccine formulation. The gp120 proteins were first biotinylated and then conjugated to Streptavidin-APC (Invitrogen) or Extravidin-PE (Sigma) as previously reported (22). The B cells were stained with PE-conjugated gp120 protein (5 µg/mL), APC-conjugated gp120 protein (5ug/mL), anti-human-CD19 APC-Cy7 (1:200) and Via-Probe (a dead cell marker, BD Biosciences, San Jose, CA). Gp120+/CD19+/7AAD- cells were sorted with a FACSArray (BD) into 96 well PCR plates containing a lysis buffer (RNaseOut, Invitrogen 5x First Stand Buffer, DTT, Igepal). Plates were immediately spun down and frozen on dry ice.

The second method involved bulk sorted B cells isolated from freshly thawed volunteer PBMC cells. After B cell isolation, cells were incubated with gp120 and cell markers as described above. Cells were then sorted into a single tube by FACSArray with
the same conditions as above. For the EBV conditions, in addition to EBV supernatant (previously titrated), 2.5 µg/mL CpG and 5µM CHK2 inhibitor was added to the stimulation media. The R848 conditions mixed in 1 µg/mL R848 and 10ng/mL recombinant human interleukin 2 to the stimulation media. The rabbit protocol used an activated rabbit splenocyte supernatant as the stimulation factor. And finally the human cytokine protocol used a combination of cytokines and CpG in the stimulation media (100ng/mL Blys, 10 units/mL IL-2, 1 unit/mL IL-4, 10ng/mL IL-6, 25 units/mL IL-10 and 1 µg/mL CpG). The B cells were incubated with two separate feeder cell lines, either EL4B5 cells or CD40L 293T cells. When EL4C5 cells were used, B cells were seeded at clonal dilutions in 96 well round bottom culture plates with 5x10⁴ irradiated (7,500 rads) EL4C5 feeder cells per well in medium B (RMPI 1640, 15% FBS, 2% HEPES, 0.1% 2-mercaptoethanol, 1% Penicillin/streptomycin). When CD40L 283T cells were used, B cells were seeded at clonal dilutions in 96 well round bottom culture plates with 5000 irradiated (7,500 rads) CD40L 293T feeder cells per well in complete medium (RMPI 1640, 15% FBS, 1% HEPES, 1% sodium pyruvate, 1% NEAA, 1% Penicillin/streptomycin). After a week of culture in a 37ºC 5% CO₂ incubator, supernatants were tested for gp120 binding, and positive wells were used for cloning of Ig variable regions.

**Isolation of V(D)J immunoglobulin regions.**

The single B cells sorted in the PCR plates were directly amplified by reverse transcription (RT) and nested PCR of the variable Ig region. In contrast, for the gp120-binding positive wells from the bulk sorting and clonal culture, RNA was extracted and
taken for RT-PCR. The Ig heavy chain variable (VH) and light chain (lambda or kappa chain) variable (VL or VK) regions were isolated by RT-PCR and using the RT and nested PCR primers according to a previous report, and shown in Table 2.2 (105).

RT was performed using SuperScript III (Invitrogen) according to manufacturer’s instructions, with primer concentrations of 10uM for each heavy chain primer and 25 µM for each light chain primer. The nested primers were situated on the edge of the VH, VL or VK of immunoglobulin genes, with primers that contain overlap sequence with an Ig overlap cassette system(105). Outside (Ext) and the internal (Int) primers for VH nested PCR were forward primers covering VH1-VH6 and reverse primers covering IgA, IgD, IgG and IgM. Outside and internal primers for VK nested PCR were forward and reverse primers covering VK1-VK7. Outside and internal primers for VL nested PCR were forward and reverse primers covering VL1-VL8. The internal primers for VH, VK or VL had the 5’ tags overlapping with the linear expressing cassette leader sequence (C fragment), CH (H fragment), CK (K fragment) or CL (L fragment), respectively, for overlapping PCR to construct the linear expression cassettes (105).

The nested PCR utilized QIAGEN’s HotStarTaq plus kit. The first PCR used 5ul of RT product and followed manufacturer’s instructions with the addition of MgCL2. The second PCR used 2.5ul of PCR 1 product and replaced MgCL2 with QIAGEN Q buffer in the reaction. PCR products were then cleaned up with a QIAGEN PCR cleanup kit for sequencing analysis.

Variable region sequence from heavy and light chain cloning was analyzed by IMGT/V-QUEST (153). Parameters that were collected included functionality of
sequence, H, K or L designation, allele designation for V, D and J, CDR and FR lengths, % identity to germline, and junction amino acid sequence.

**Construction of linear Ig expression cassettes.**

After identification of 400-500 bp bands from RT-PCR, sequencing was done with primer CL-F681 TCTGGGTCCAGGTTCCACTGGTGAC to confirm functional Ig sequence. After sequence verification, variable region fragments were cloned into an immunoglobulin expressing linear cassette by overlapping PCR (105). The 5’ fragment of the cassette contains a CMV promoter and Ig leader sequence, while the IgH, IgK or IgL 3’ fragment contains the constant region of Ig heavy chain (CH), light chain (CK) or lambda chain (CL), respectively, and a polyadenylation signal. The overlap PCR was done with iProof DNA Polymerase (Bio-Rad, CA) according to manufacturers’ instructions. Equal amounts of VH, VK or VL PCR product, 5’ promoter region fragment, and the 3’ constant region CH, CK or CL fragment were mixed with dNTPs, HF buffer, forward and reverse primers, and polymerase. After overlap PCR product of heavy, kappa or lambda chain was verified by agarose gel electrophoresis, the paired IgH/IgK or IgH/IgL fragments were used for transient transfection of 293T cells to produce antibody in transfection supernatant. The supernatant was used for screening for gp120 binding, as described in ELISA. Clones of interest were cloned into a mammalian expression vector, pJW4303, for large scale production in 293F cells.

**Expression and production of recombinant mAbs.**

For large scale production, equal copy number amounts of heavy and light chain plasmids were transfected with PEI into Freestyle 293F cells (Invitrogen). After two days
of expression, cell culture supernatant was harvested and human IgG purified with an
AKTA FPLC using Protein A HP columns (GE Healthcare).

**HIV-1 gp120 protein production.**

Recombinant HIV-1 gp120 was produced from transient transfection of Freestyle
293F cells. HIV-1 gp120 plasmids (92UG037.8 (A2), 92US715.6 (B), Ba-L (Bal),
96ZM651 (CZM), 93TH976.17 (E), JR-FL, 93MW965 (C2), 92UG021 (D), consensus
AE (AE)) were used to transfec 293F cells with 293Fectin (Invitrogen), and after two
days of expression, cell culture supernatant was harvested and gp120 protein was purified
over a lectin column.

Biotinylation was performed by transferring biotin to the lysine residue in the
Avitag sequence (LNDIFEAQKIEWHE) at the C-terminus of gp120. A commercially
available biotin ligase system was used to biotinylate the Avitag sequence of the DP6-
001 autologous gp120s (A2, B, Bal, CZM, E) (Avidity, CO). After biotinylation, the
protein was conjugated to either Extravidin-PE (Sigma) or SA-APC (Invitrogen) (105).

**ELISA.**

Supernatant from B cell clonal culture, transient transfection of 293T cells with
paired IgH/IgK or IgH/IgL linear cassettes or purified mAb was screened for binding in
sandwich enzyme-linked immune-absorbent assay (ELISA) format. In 384 well
microtiter plates (Corning, NY), 20ul of goat anti-human IgG-Fc (2 µg/mL) (Southern
Biotech, catalog no. 2048-01) diluted in PBS (Gibco) was incubated overnight at 4°C.
Plates were washed five times with wash buffer (PBS containing 0.1% Triton-X) and
blocked for 1 hour at 37°C in block buffer (PBS containing 4% whey and 5% powderd
milk). Plates were washed again, and serial dilutions of mAbs were added to wells, diluted in dilution buffer (PBS containing 4% whey). After incubation of mAbs for 1 hour at room temperature, plates were washed. Biotinylated gp120 or peptide were diluted in dilution buffer at 1µg/mL and added to the wells. After incubation of gp120 or peptide for 1 hour at room temperature, plates were washed. Streptavidin-conjugated horseradish peroxidase (Vector Laboratories, CA) was diluted in dilution buffer at 500 ng/mL and added to the plate for 1 hour at room temperature. Plates were washed and developed for 5 minutes at 37°C in 20µl of a 3,3′5,5′-tetramethylbenzidine substrate solution (Sigma). The reaction was stopped with 20µl of 2N H2SO4. Plates were read at 450 nm.

For testing the breadth of binding in ELISA, a ConA ELISA format was utilized. In 384 well microtiter plates (Corning, NY), 20µl of ConA (1:100 dilution) diluted in PBS (Gibco) was incubated at room temperature for 1 hour. Plates were washed five times with wash buffer and 20 µl gp120 (1µg/mL) was incubated overnight at 4°C. Plates were washed and blocked for 1 hour at 37°C in block buffer. Plates were washed again, and serial dilutions of mAbs were added to wells, diluted in dilution buffer. After incubation of mAbs for 1 hour at room temperature, plates were washed. Biotinylated goat anti-human IgG (H/L) (Vector Laboratories) were diluted in dilution buffer at 1µg/mL and added to the wells. After incubation for 1 hour at room temperature, plates were washed. Streptavidin-conjugated horseradish peroxidase (Vector Laboratories) was diluted in dilution buffer at 500 ng/mL and added to the plate for 1 hour at room temperature. Plates were washed and developed for 3 minutes at room temperature in
20µl of a 3,3’5,5’-tetramethylbenzidine substrate solution (Sigma). The reaction was stopped with 20µl of 2N H2SO4. Plates were read at 450 nm.

For the ELISA using glycan modified forms of gp120, PNGase F (NEB) was used to remove glycans. The protocol from NEB was followed to remove glycans, either with or without the heat and denaturing solution. Without denaturisation, partial de-glycosylation is done and conformation intact. With denaturisation, the gp120 is fully de-glycosylated and in a denatured form.

Both human and rabbit mAbs were used in ELISA. Previously cloned human mAbs were obtained through the NIH AIDS reagent program. Rabbit mAbs were created in lab by Michael Vaine, and epitopes determined by Yuxin Chen.

**Peptide Microarray.**

Peptide libraries (designed by Dr. Shixia Wang) consist of 15-mers overlapped by 12 amino acids were printed on glass slides by JPT (Berlin, Germany), covering the full length of consensus gp160 Env from clade A, B, C, D, CRF01_AE, M; and gp160 Env sequence from NL40 and B33. Briefly, the peptide microarray was performed using a Tecan HS400 Pro Hybridization WorkStation. Arrays were incubated with hmAbs diluted in Superblock with Tween 20 blocking buffer for 2 hours at 30°C with agitation. After wash, the arrays were incubated for 45 minutes at 30°C with Goat anti-Human DyLight650 (Thermo Scientific) secondary antibody (1µg/ml final concentration) diluted in Superblock with Tween 20 blocking buffer. PBS containing 0.1% Tween was used for each washes between all steps. Arrays were scanned using an Agilent DNA Microarray Scanner. Images were analyzed using Genepix Pro 7 software (Molecular Device).
Virion Binding.

Virus Production: The day before production, $2 \times 10^6$ 293T cells were plated into a T75 flask. The day of production, culture media was replaced with 7 mL DMEM with 10% FBS. pSG3ΔEnv plasmid (10µg) was co-transfected with 2.5 ug VSV-G plasmid and 2.5 ug HIV Env of choice using the PEI transfection reagent. The next day, media was replaced with 10 mL DMEM with 10% FBS. After two days of virus production, supernatants were harvested by spinning at 1000 rpm for 5 minutes to clear cellular debris and stored at -80°C.

Virus Titration: ELISA plate coated with 50 µl anti-p24 mAb, AG3, at 1ug/mL for 1 hour and a gag standard in duplicate. Plates were washed 5 times with PBS-T and blocked overnight in whey dilution buffer with 4% powdered milk. Virus preps were lysed in .5% Triton-X in PBS for 1 hour at 37°C. Serial dilutions in PBS were plated, beginning at 1:100 dilution. Plates were washed 5 times in PBS-T and then 50uL of serial diluted viral lysate was added to wells. Plates were incubated for 1 hour at RT and washed 5 times in PBS-T. Bound p24 was detected with a polyclonal rabbit sera by ELISA.

Confirmation of Env/VSV expression on HIV virions: Virus preps were lysed in .5% Triton-X in PBS for 1 hour at 37°C and serially diluted, beginning at 1:10 in PBS. Lysate was coated directly onto ELISA plates and incubated for 1 hour at 37°C. Plates were washed 5 times in PBS-T and blocked overnight in whey dilution buffer with 4% powdered milk. Plates were washed 5 times in PBS-T. Bound HIV Env was detected with
polyclonal rabbit sera by ELISA. VSV-G was detected with mouse anti-VSV-G mAb P5D4 (Sigma #V5507) at 1:1000 dilution by ELISA.

Virus Capture/Competition: ELISA plates were coated with 50 µl of anti-human Fc (Southern Biotech) at 5 ug/mL and incubated for 1 hour RT. Plates were washed 5 times with PBS-T and blocked overnight in PBS with 1% BSA. Test mAbs were diluted in DMEM 5% FBS culture media, starting at 10ug/mL and serially diluted 1:5. Virus was diluted to 40ng/mL p24 and combined with mAbs. Virus/mAb mixture was incubated for 1 hour at room temperature. ELISA plates were washed twice in PBS-T and 3 times in sterile PBS. 50 µl virus/sera mixture was added to the ELISA plate and incubated for 3 hour at RT. ELISA plate was washed 5 times with sterile PBS. 10,000 TZM-bl cells per well in 200 µl DMEM 5% FBS media were added to the ELISA plate, and incubated for 48 hours at 37C°. After the two-day incubation, plate was washed once in PBS and 40 µl luciferase lysis buffer was added. The plates were incubated for 5-10 minutes until cells are lysed and then 30uL lysates were transferred to white luciferase plates. Developed luciferase assay as described in neutralization.

Octet Qke analysis.

Human mAb binding kinetics to gp120 proteins were measured by ForteBio Octet Qke based on biolayer interferometry. The antibody being tested was loaded on to either a Protein A or Protein G coated sensor tips at 10µg/mL diluted in ForteBio kinetics buffer. After capture, tips were washed in kinetics buffer and a baseline measurement recorded. The tips were then incubated in wells containing serial dilutions of gp120
(300nM-0.4nM) to record the on rate. After this, the off rate was measured by moving the tips into wells with kinetics buffer. The antibody binding kinetics and KD values were analyzed by the ForteBio Data Analysis software package v7.1 using a 1:1 binding model.

**Neutralization Assays.**

Pseudotyped virus neutralization assays were performed using previously optimized system in the TZM-bl cells (154). Pseudotyped virus was produced by co-transfection in HEK 293T of pSG3dEnv HIV-1 backbone plasmid and a plasmid expressing the gp160 gene of the HIV-1 being tested. After 2 days of virus production, cell supernatants were clarified and titrated on TZM-bl cells using a 10-fold increase in luciferase levels above background levels as the dose. Titrated virus was incubated with serially diluted mAbs for an hour at 37°C in a 96 well culture plate. After incubation, 10^5 TZM-bl cells with 20μg/mL DEAE dextran was incubated with the virus/mAb mixture at 37°C for 2 days. Cells were then lysed and luciferase added according to the manufacturer’s instructions (Promega). Relative Light Units (RLUs) were collected by running plates on a Wallac luminometer, and neutralization was calculated as follows:

\[
\left\{ \frac{1}{(1- (\text{Average sample RLUs}/\text{Cell Control RLUs}))} \right\} \times \frac{1}{(\text{Virus Control RLUs}/\text{Cell Control RLUs})} \times 100 \quad (\text{where RLU=relative light units}).
\]

**ADCC assays.**

(i) ADCC against gp120-coated target cells. The GranToxiLux (GTL) assay was used to detect the ADCC activities of human MAbs and plasma directed against
CEM.NKRCCR5 cells (NIH AIDS Reagent Program, Division of AIDS) coated with recombinant gp120 proteins (from strain A2, B, Bal, Czm, or AE) which were included as part of the vaccine immunogens as described previously (155). Cryopreserved human PBMCs from an HIV-seronegative donor with the homozygous 158V/V genotype for Fc gamma receptor IIIa were used as the source of effector cells (156) at an effector-to-target cell ratio of 30:1. Data are reported as the proportion of cells positive for proteolytically active granzyme B (GzB) out of the total viable target cell population (percent GzB activity) after subtraction of the background activity observed in wells containing effector and target cells in the absence of antibodies or plasma. ADCC endpoint titers were determined by interpolation of the dilutions of sera that intercepted the positive cutoff (8% GzB activity) using Graph-Pad Prism (version 6.0) software (GraphPad Software, Inc.).

(ii) ADCC against HIV-1-infected target cells. The ability of DP6-001 hMAbs and plasma to direct the killing of CEM.NKRCCR5 cells infected with subtype AE HIV-1 isolate CM235 (GenBank accession no. AF259954.1; Agnès Chenine, U.S. Military HIV Research Program) and subtype B isolate Bal (GenBank accession no. AY426110) infectious molecular clone (IMC) viruses containing a Renilla luciferase (Luc) reporter gene (157) was measured using a Luc-based ADCC assay according to a previously described procedure (34, 158). Cryopreserved PBMCs were used as a source of effector cells (effector-to-target cell ratio, 30:1). Killing was measured as a reduction in luminescence (ViviRen assay; Promega) compared to that of control wells containing target and effector cells in the absence of antibodies. ADCC endpoint titers were
determined by interpolating the concentrations of serum that intercepted the positive
cutoff (15% specific killing).

(iii) The RFADCC assay was performed according to a modified form of the
protocol George Lewis (159). Target cells were a modified CEM-NKR cell line that
expressed cytosolic GFP and a fusion protein of CCR5 with a snaptag signal. One million
target cells in 60ul complete media are incubated with 10ug gp120 and Alexa647 snaptag
substrate (NEB 1:200) for 25 minutes at 37C. After target cells and incubated and washed,
5000 target cells are added to each well of a 96 well V bottom plate with serial dilutions of
antibodies being tested. After a 15 minute incubation, PBMC effector cells are added at a
50:1 ratio of effector:target cells. Plates are incubated at 37C at 5% CO2 for 2 hours. Plates
are then washed and fixed, and wells read on a FACS machine. When binding of antibody
to gp120 on the surface of target cells is being measured, Brilliant Violet 421-Goat anti-
human (Biolegend, 1:200) is incubated with cells after the ADCC incubation period.
ADCC is measured by the loss of GFP in Alexa647 positive target cells.

ADCP assays.

Antibody dependent cell phagocytosis (ADCP) was measured by a modified form
of a previously described method (160). Biotinylated gp120 (5 µg) was incubated with 1
µl streptavidin-fluorescent beads (FluoSpheres® NeutrAvidin® Labeled Microspheres,
1.0 µm, Yellow-Green Fluorescent (505/515), 1% solids, Invitrogen) that were diluted
100-fold for 25 minutes at room temperature in the dark. THP-1 cells were plated in a 96-
well U-bottom plate (250,000 cells per well), and serial dilutions of mAbs were added.
The bead-gp120 mix was then further diluted in PBS/BSA 5-fold, and 50µL was added to cells, incubated for 3 hours at 37°C, and washed at low speed. Cells were then assayed for fluorescent bead uptake by flow cytometry using a BD Biosciences LSR II.

Phagocytic score was obtained by the formula (% phagocytosis/MFI), and normalized by division by $10^6$. 
The study of HIV vaccines has in the past couple of years shifted back to exploring the role of antibodies in vaccine elicited protection after the failure of T cell based immunization strategies. Multiple bnAbs have been isolated for HIV infected patients. This allows for the elucidation of epitopes that are vulnerable to antibodies and conserved enough to allow cross clade neutralization. Along with the isolation of bnAbs, the RV144 trial has shown that vaccination is able to afford limited protection, a milestone in the field. The project that I have undertaken is to develop a system to isolate mAbs from vaccine volunteer samples and study the multiple effector functions mAbs have developed as a result of vaccination.

One of the first reasons for the project was to confirm previous studies that looked into the neutralizing capacity of sera from the DP6-001 DNA prime/protein boost regimen conducted in humans. The study used sera from the vaccine volunteers to show that a broad range of viruses could be neutralized. With the isolation of mAbs from these same volunteers, the neutralizing capacity of the sera can be isolated and studied. With the isolation of the mAbs, further characterization of the neutralization profile from these volunteers can be performed. The lack of enough sera could have a limiting factor on the amount of epitope studies performed, but with a mAb that recapitulates the neutralization
of the sera, further epitope characterization studies can be completed, with very detailed and specific epitopes identified that correspond with neutralization.

Before the project was undertaken, one question that needed to be answered was the number of antibodies that would be needed to recapitulate the breadth of neutralization present in the sera. In infected individuals, there has been shown to be both single mAbs that confer the majority of neutralization breadth, and multiple antibodies that confer this breadth (27, 28, 52, 161). When multiple mAbs are isolated from the vaccine samples, assays can be performed to determine if one or multiple mAbs are needed for breadth of neutralization. It may be that the vaccine elicits multiple antibodies with a specificity for each immunogen present in the polyvalent vaccine, in which case each antibody could have a narrow range of neutralization restricted to the subtype that the antibody was selected on. The other possibility is that one or a few antibodies were selected that could bind multiple parts of the vaccine and each mAb itself has a breadth of neutralization.

To this end, my project was to develop enough neutralizing antibodies to identify the major neutralization component from the sera. Unfortunately, I was not able to identify any neutralizing antibodies besides a weakly neutralizing V3 specific mAb, TA6. This antibody was clade specific, in contrast with the majority of other antibodies that had a broad antigen binding in ELISA, along with a breadth of ADCC and ADCP activity to multiple antigens. Because the sera neutralization and antigen binding in the DP6-001 trial was broad, TA6 is unlikely to be a major component in the activities seen in sera. TA6 does give insight into a different aspect of humoral responses to HIV vaccination.
Along with another newly isolated hmAb from DP6-001 (TA7), there are other V3 specific mAbs that derived from the same pairing of heavy and light chain germline genes. MAbs 311-11D and 1334 were both isolated from HIV infected patients and derived from the same germlines as TA6 and TA7 (126, 162). Of most interest is that even with the same epitope, the CDR3 is highly divergent between the 4 mAbs. This may indicate that there is an intrinsic binding profile in this heavy and light chain paring that confers binding to the V3 epitope of HIV-1. This type of intrinsic binding is also seen for CD4bs mAbs which use the VH1-2 and VH1-46 germlines, which gives the possibility that stimulating the correct germline can be a beneficial vaccine strategy (26, 163, 164).

A possible reason as to why TA6 was the only neutralizing mAb isolated in this panel is that the number of mAbs isolated was low. Expanding the panel to greater numbers will most likely yield a larger number of neutralizing antibodies with greater breadth. A hint that this would be the case is in a secondary panel of DP6-001 mAbs developed by another group in lab that contained a CD4bs mAb and a glycan specific mAb, both of which neutralized pseudotyped virus from multiple clades when tested by the Harvard group. This panel was even smaller than the one presented, indicating that neutralizing antibodies can be isolated from these volunteer PBMC samples.
Figure 6.1: Crystal structure of BG505 SOSIP.664 HIV-1. Crystal structure based on a stabilized trimer (PDB 4ZMJ), and a single gp120 monomer is shown. Epitopes mapped onto the structure include the CD4bs (cyan), the V1V2 loop (blue), the V3 loop (red), and the amino terminal end of C5 (orange). DP6-001 epitopes include the V3 loop (TA6, TA7) and the amino terminal C5 (EA1).
A secondary project to the search for the broadly neutralizing component of the vaccine sera is a search for and comparison of non-neutralizing antibodies. This would include ADCC and ADCP activities. One of the interesting findings in this study was that despite the lack of neutralizing antibodies isolated, most antibodies were able to mediate both ADCC and ADCP. With the renewed interest in Fc mediated functions because of the RV144 trial, this was an encouraging finding. The majority of mAbs had broad ELISA binding profile and they maintained this breadth into the Fc mediated functions such as ADCC and ADCP. Consistent results were found when using gp120 as an immunogen and test material. When the more stringent ADCC assay using HIV-1 infected target cells was tested, ADCC activity drops and can only be seen for a few DP6-001 mAbs. Even with this activity drop, which could be due to the reduced amount of antigen on the surface of an infected target cell, the majority of the mAbs exhibited great breadth on binding, ADCC and ADCP.

Building upon this work can give further insights into developing an effective vaccine for HIV-1. The main goal would be to isolate more monoclonal antibodies, both from the current DP6-001 samples, and any further studies done in humans. For DP6-001, there is still the unanswered question of what type of neutralizing antibodies were elicited from this study. One of the ways that could be utilized to find these types of neutralizing antibodies is to modify the way the mAbs are isolated. The current set of mAbs used gp120 as a bait to isolate gp120 mAbs. This type of bait may miss mAbs that bind to trimer and its associated forms on the surface of the virion. These types of mAbs may have a better chance of being neutralizing because of their ability to bind virions.
Instead of using gp120 as a bait, a micro neutralization assay could be used to test for potential B cells that secrete neutralizing antibodies. Even with the old method of mAb isolation, increasing the numbers of mAbs isolated could help with finding a neutralizing antibody. For future studies, the techniques developed can be used to quickly develop mAbs and test their binding and effector functions for specific activities of interest.

One of the last goals of the project was to develop a system of isolating antibodies for later studies. With the system in place, future vaccine studies in humans can be assessed at the antibody level quickly. This will allow changes in immunogen usage to be compared to the types of antibodies created. All the ideas presented above, (antibody component of neutralization, one vs multiple antibody repertoire in neutralization, epitope usage, non-neutralizing antibodies) can be assessed and compared with the different immunogens or vaccine strategies used. With the development of the isolation of B cells and culture system, the next step is to modify this system to work into a robotics system so that large scale screening can be done. This type of automation would allow for a greater number of samples to be tested, and increase the chance of developing a panel of higher representative mAbs that recapitulates the function seen ins era developed by a specific vaccine regimen.

Along with isolating mAbs from these volunteers, another aspect that could be explored in the mining of the immunoglobulin sequence repertoire in the PBMC samples. Current research in the field has used deep sequencing with samples from HIV-1 infected individuals to expand the sequence information from patients from which known broadly neutralizing antibodies have been isolated (50–52, 124, 127). This information has been
used to infer the un-mutated common ancestors of bnAbs, along with intermediate antibodies that would eventually develop into these bnAbs. This information, along with virus populations in the host, together gives a picture for how bnAbs are developed in humans, and how these antibodies interact with virus. This system can be utilized to study antibody maturation in humans during vaccine studies. Being able to determine how antibodies develop at different stages of immunization will help with understanding the effectiveness of different immunization protocols and how these may be optimized in the future. This information could help determine whether vaccination is targeting naïve B cells or stimulating memory B cells, and whether an immunogen should be optimized for either scenario. Ideally, a combination of mAbs, deep sequencing data, and immunogen information would be integrated to provide a comprehensive view of the effect vaccination has on the humoral system.

From the current set of mAbs, more studies can be performed. For one, the epitopes of most of the mAbs are unknown. One of the main ways to address this would be to develop a competition assay and utilize antibodies with known epitopes. Another way of testing for competition would be in the functional assays. Since the ADCP and ADCC assays need a functional Fc for activity, making Fab fragments would allow for competition of well characterized mAbs. If the Fab fragments bind to the same epitope as a known mAb, then a drop in Fc mediated effector activity should be noticeable. Being able to identify the epitopes developed by DP6-001 would allow for a better understanding of how the DNA prime/protein boost vaccine regimen was interacting in a human host.
An additional factor that was not studied with the current round of monoclonal antibodies was the isotype of each antibody. The current protocol isolated variable regions only and cloned them on to an IgG1 constant region. Knowing the isotype of each mAbs would give a better understanding of the effects the antibodies would have in vivo, and these effects could be tested in the current assays. An initial test would be to use the sera to determine the predominate isotype in the DP6-001 volunteers. This would also be useful in future vaccine testing to test what a protocols effect is on isotype and how they may change. Also, as noted previously, the current set of mAbs utilized an IgG1 Fc region that was not optimized for binding to the Fc receptor, whereas the prototypical ADCC mAb A32 has been. Modifying this region in the DP6-001 mAbs for improved Fc binding would allow for a better comparison between those and A32.

Another region of interest that could be explored is the effect of the autologous effector cell function. While we have developed mAbs and have sera from vaccine volunteers, the functional assays use effector cells from donors not in the vaccine study. This could have the effect of missing key information that the vaccine regimen has elicited in the test volunteers. Combining the full length cloning of mAbs with the natural Fc region and the autologous effector cells could give a more precise view of the response vaccination has had on the immune system.
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