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HIV NEUTRALIZATION THROUGH USE OF ANTIBODIES AND PHARMACOKINETICS OF TOPICAL APPLICATIONS

by

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ABSTRACT

The Human Immunodeficiency Virus Type 1 (HIV-1), a sexually transmitted retrovirus that causes the Acquired Immunodeficiency Syndrome (AIDS), infects over two million people a year. Several methods introduced to prevent HIV-1 transmission, such as condoms, circumcision and antiretroviral drugs, have proven to be partially effective, but more effective approaches are being sought. Topical microbicides are being developed to provide a women-controlled method to prevent the transmission of HIV-1. Unfortunately, most of the candidate microbicide compounds tested to date have either elicited undesirable mucosal inflammation and epithelial lesions leading to increased seroconversions, or have been ineffective. One novel approach currently being explored is the use of monoclonal antibodies as components of topical microbicides. Monoclonal antibodies can be produced inexpensively by transfection into *Nicotiana* plants. We hypothesize that anti-HIV monoclonal antibodies produced in *Nicotiana* (MAb-N) will be effective in neutralizing HIV- 1 when used as topical microbicides at mucosal sites, and set out to test whether they retain their efficacy under physiological conditions. We tested the pharmacodynamics of anti-HIV MAb-N efficacy in Cynomolgus macaques following application of the antibodies in the vaginal compartment. We further studied the ability of

MAb-N to cross through the vaginal epithelium using an EpiVaginal tissue model.

To determine the pharmacodynamics of HIV neutralizing activity after the application of anti-HIV MAb-Ns to the vaginal mucosa, we used a neutralization assay based on HIV-expression in the TZM-bl cell line to test the efficacy of various doses of MAbs in a time course after they had been administered intravaginally in gel form to Cynomolgus macaques. To determine the pharmacokinetics of Mab-N transport across the vaginal epithelium, monoclonal antibodies were added to the apical surface, and a human-IgG ELISA was used to detect Mab-N that had crossed the epithelium into the basal supernatant. Immunohistology was used to confirm and validate ELISA data for evidence of transfer of Mabs across the epithelial layer.

Our results show that anti-HIV MAb-Ns were effective in neutralizing cell-free HIV in TZM-bl neutralization assays. We found that MAb-Ns retained their anti-viral efficacy in monkeys after 4-hours. However, neutralizing activity was decreased after 24-hours and 72-hours, with wide variability in effectiveness between individual macaques. Mab-ns tested in the EpiVaginal tissue model showed minimal transfer of antibodies across the epithelium, ranging from 0.005% to 0.09%. Immunohistological data showed that antibodies applied apically to tissue models concentrated only in the superficial layers of the stratum corneum and did not penetrate the epithelium. Our data indicate that anti-HIV MAb-Ns are effective in neutralizing HIV-1 following vaginal application for at least 4 hours, and that they do not pass through the vaginal epithelium in significant amounts. Our data support their further development as vaginal microbicides.

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ABBREVIATIONS

4E10-N	4E10 monoclonal antibody derived from *nicotiana* plant provided by Mapp Biopharmaceuticals
AIDS	Acquired Immune Deficiency Syndrome
ADCC	Antibody dependent cellular cytotoxicity
ADCP	Antibody dependent cellular phagocytosis
ARV	Antiretroviral
bnAb	broadly neutralizing antibody
bnmAbs	broadly neutralizing monoclonal antibodies
CCR5	Coreceptor for HIV infection
CDR	complementarity-determining regions
CXCR4	Coreceptor for HIV Infection
FcγR	Fc immunoglobulin receptors
FcR	Fc domain receptors
FcRn	Neonatal Fc receptor
HEC	Hydroxyethyl Cellulose
HIV	Human immunodeficiency virus
HIV-1	sub-type 1 strain of human immunodeficiency virus
HSV-2	herpes simplex virus type 2
IACUC	Institutional Animal Care and Use Committee
IVR	In-vitro release
MAb	monoclonal antibody
MAb-N	monoclonal antibody derived from *nicotiana* plant species

PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
pIgA	polymeric immunoglobulin A
pIgR	polymeric immunoglobulin receptor
R5	Denotes virus that uses CCR5 coreceptor explicitly for entry
RANTES	regulated and normal T cell expressed and secreted
RC-101	retrocyclin 1 peptide analogue
SDF	Stromal Derived Factor
SHIV-Ba-L	Simian HIV Ba-L strain
TCLA	T-cell line- adapted
TZM-bl	epithelial HeLa cell line used for HIV neutralization assays
VRC01-N	VRC01 monoclonal antibody derived from *nicotiana* plant species provided by Mapp Biopharmaceuticals
WHO	World Health Organization
UNAIDS	United Nations joint program on HIV/AIDS
X4	Denotes virus that uses CXCR4 coreceptor explicitly for entry

Introduction

According to UNAIDS, the joint United Nations program on HIV/AIDS, the Human Immunodeficiency Virus-type 1 (HIV-1) afflicts approximately 34 million people in the world as of 2011, with 2.5 million new cases of newly infected adults and children each year (UNAIDS, 2012). Based on the Global Report from UNAIDS, sub-Saharan Africa represents an endemic region consisting of 1.8 million of newly infected cases each year, followed by south and southeast Asia with approximately 200,000-300,000 cases each year (UNAIDS, 2012). According to the World Health Organization (WHO), women account for half of the 7,000 new HIV cases that occur each day (Grammen, Augustijns, & Brouwers, 2012). It is necessary to continue to search for therapeutic solutions. However, in order to solve the issues that affect high-risk populations and endemic regions of HIV, improved prophylactic approaches must also be sought. This research explored the possibility of a prophylactic solution through the use of cost-effective monoclonal antibodies produced in plants that can be used topically at mucosal sites to prevent transmission of HIV.

The HIV virus and infection mechanism

The virus is thought to have originated from monkeys in West Africa. HIV-1 is separated into three major groups that are designated M (main), O (outlier), and N (non-M, non-O). Group M is subdivided into subtypes A to D, F1, F2, G to J, and K. Subtypes that originate from a common ancestor are then referred to as *clades* (Pier, Lyczak, & Wetzler, 2004). After initial infection with HIV-1, flu-like symptoms develop transiently;

following the acute infection period, individuals can remain asymptomatic for periods of 2-20 years before they develop symptoms of Acquired Immunodeficiency Syndrome (AIDS).

HIV-1 is an enveloped retrovirus of the lentivirus group that contains two copies of RNA genome. As a retrovirus with an envelope acquired from host cell membranes, HIV is highly susceptible to environmental conditions that affect surface tension and as a result do not transfer through air, dust, or fomites (Ray, CG & Ryan, KG, 2010). Within the outer envelope, the virus contains a bullet-shaped capsid composed of the p24 protein. Because the p24 protein is present in abundance in the virus, it is often used to test for positive infection of HIV through enzyme-linked immunosorbent assays or PCR (Pier et al., 2004). Lentiviruses were given their name due to their ability to persist in latent form in the host over lengthy periods of time. In the case of HIV, the cells affected tend to be those of the immune system. While affecting immune cells such as monocytes, macrophages, dendritic cells, Langerhans cells, and other immune cells, the most devastating infection occurs in the CD4 T cell which plays a major role in immune activation and immune response to bodily infections (Ray, CG & Ryan, KG, 2010).

Entry of HIV into host cells occurs through direct fusion to the host cell membrane governed by the envelope protein complex of the virus. The presence of a precursor protein gp160 is cleaved by host cell enzyme furin leading to surface subunits of gp120 and a transmembrane subunit of gp41 (Chan & Kim, 1998). The gp41 and gp120 proteins exist on the virus surface as a trimer and are responsible for viral entry into target cells. They coexist with other complex-carbohydrate side chains that may also

serve a purpose in allowing the virus to evade immune system antibodies (Pier et al., 2004). Binding of gp120 to target receptors leads to a conformational change of gp41 to a fusion-ready state for viral entry. The viral life cycle is like any other retrovirus in that virus envelope binds to receptors on target cells resulting in membrane fusion and entry of viral capsid. Viral RNA is then reverse transcribed to DNA and integrated into host-cell genome ultimately using cellular machinery to produce viral RNAs and proteins. The virus is then packaged and assembled with host cell membrane and buds off (Pier et al., 2004).

HIV-1 infection requires CD4 receptors on target cell surfaces, but co-receptors such as CXCR4 and CCR5 must also be present in order to for fusion to be successful (Pier et al., 2004). The CCR5 coreceptor has been found to be important in early infection periods while CXCR4 corresponds to advancement of infection to AIDS (Ray, CG & Ryan, KG, 2010). Late-infection virions, can also use another coreceptor, CCR3. Interestingly, CCR5-deficient individuals have been observed to be highly resistant to infection even from repeated exposures to the virus suggesting it can be utilized as a possible therapeutic or prophylactic target (Pier et al., 2004). It has been found that HIV strains that utilize the CCR5 coreceptor can be blocked by a CCR5 ligand such as RANTES, which is released by activated CD8 T cells, while CXCR4 strains of HIV can be blocked with the corresponding SDF (stromal derived factor) ligand, an endogenous cytokine (Chan & Kim, 1998).

HIV affects the human immune system by attacking the CD4 T cells. The destruction of CD4 T cells prevents the immune system from mounting adequate

defenses against infections thus leading to Acquired Immune Deficiency Syndrome (AIDS). Acute infection begins by transport of virus to lymph nodes by dendritic cells and Langerhans cells. Lymphocytes that cluster with infected dendritic cells become highly activated and undergo intense replication of HIV-1. There is a spike in viral replication that occurs 3-6 weeks after initial infection and virion count in the blood can reach millions per milliliter of blood. CD4 lymphocytes also rise in numbers to counter the infection. It is at this stage that 60% of people develop a non-specific response such as fever, pharyngitis, lymphadenopathy, headache, rash, nausea, or diarrhea. Antibody testing for HIV-1 during the earliest stage of infection is negative due to the delay in humoral response, but virus detection by p24 antigen ELISA or polymerase chain reaction (PCR) assay is possible. During the chronic asymptomatic infection stage, a steady-state equilibrium is reached between viral load and CD4 T-cell numbers in the blood. Over time the CD4 T cells decrease leading to late stage of infection, however, this chronic infection period can last many years (Pier et al., 2004). Specific events of the immune system following HIV infection include polyclonal B-cell activation, increased T cell turnover due to T cell activation, and increased levels of inflammatory and cytokines and chemokines in the blood serum (Douek, Roederer, & Koup, 2009). Although the immune system reacts as expected, immune activation of T cells promotes HIV replication in these cells, which leads to the production of more virus and T cell death. The high turnover rate of T cells leads to diminished pools of memory T cells and inflammatory damage of lymphoid tissues (Douek et al., 2009). The activation, infection, and loss of the central pool of memory CD4 T cells eventually progresses the disease

state to AIDS. At this late stage of infection, CD4 T cells rapidly decrease and viral loads increase. Once T cell counts drop below 200 cells per ul, opportunistic infections usually result in mortality (Pier et al., 2004).

Transmission

Transmission of HIV generally occurs through sexual intercourse and maternal to fetal transmission. Other methods of HIV transmission include the sharing of needles by drug users, and infection of healthcare workers by needle sticks or exposure of mucosal surfaces to infected blood. Different routes of transmission have different infection rates. Vertical transmission from mother to the fetus or newborn carries a risk of 1 in 3, insertive rectal intercourse with an infected partner carries a risk of 1 per 100 exposures, while female-to-male heterosexual activity is lower at 1 per 1,500 (Pier et al., 2004). It has been found in non-human primate models (NHP) that HIV-1 can cross the mucosal barrier and establish populations of infected cells within hours (Haase, 2010). Virus then propagates from these founder populations continuing onto the secondary lymphoid tissue. Normally, vaginal secretions are acidic and can inactivate HIV; however, semen neutralizes the acidic pH environment of the vagina leading to potential infection at ectocervical, endocervical, and endometrial mucosal sites (Hladik & Doncel, 2010). HIV prevention strategies should focus on the initial phases of infection at the mucosal level (Haase, 2010). Exposure to virus causes recruitment of CCR6+ plasmcytoid dendritic cells, which then recruit macrophages and T cells, which in turn leads to growth of the infected founder cell population (Haase, 2011). Vaginal Langerhans cells and dendritic

cells are thought to play a role in ferrying infectious virions from the mucosa to lymphatic tissues (Hladik & Doncel, 2010). Trapping the virus in the mucus and physical mucosal barriers would therefore be a useful strategy. It is these recent findings that have suggested to researchers to begin looking at preventing transmission at the mucosal level at what is considered the window of opportunity to (neutralizing virus at time and location of exposure prior to infection). However, multiple difficulties must be overcome for strategies targeting the mucosa to work. These include the possibility of HIV disseminating too rapidly, or of HIV being carried within infected cells and therefore protected from the mucosal environment.

To date, circumcision has proven to be effective in decreasing female to male transmission by 50% to 60%, but vaccine and microbicide approaches targeting mucosal HIV transmission have largely been ineffective. The mucosal solutions must be able to penetrate through mucus and mucosal epithelium and retain activity in the mucosal environment. Microbicide concentration, molecular weight, hydrophobicity, release rate, surface area coverage, safety, and efficacy in presence of semen must all be taken into account for such an approach (Hladik & Doncel, 2010). Tenofovir, an antiretroviral drug that blocks reverse transcriptase, was the first microbicide compound to show significant efficacy in a clinical trial; it reduced HIV acquisition by 40% in a Phase IIb trial, with its efficacy related to drug compliance (Hladik & Doncel, 2010).

Antibodies

Antibodies play a major role in immune defense, especially the recognition and effector phases of humoral immunity against foreign antigens. Antibodies can activate the complement cascade, resulting in the lysis of target microbes, and can also block (neutralize) microbe function by binding to structural components such as receptors. Antibodies also play a role in a number of innate immune functions for elimination of pathogens including opsonization of antigens for phagocytic processing, and antibody-dependent cell-mediated cytotoxicity in which antibodies target microbes for lysis by other immune cells.

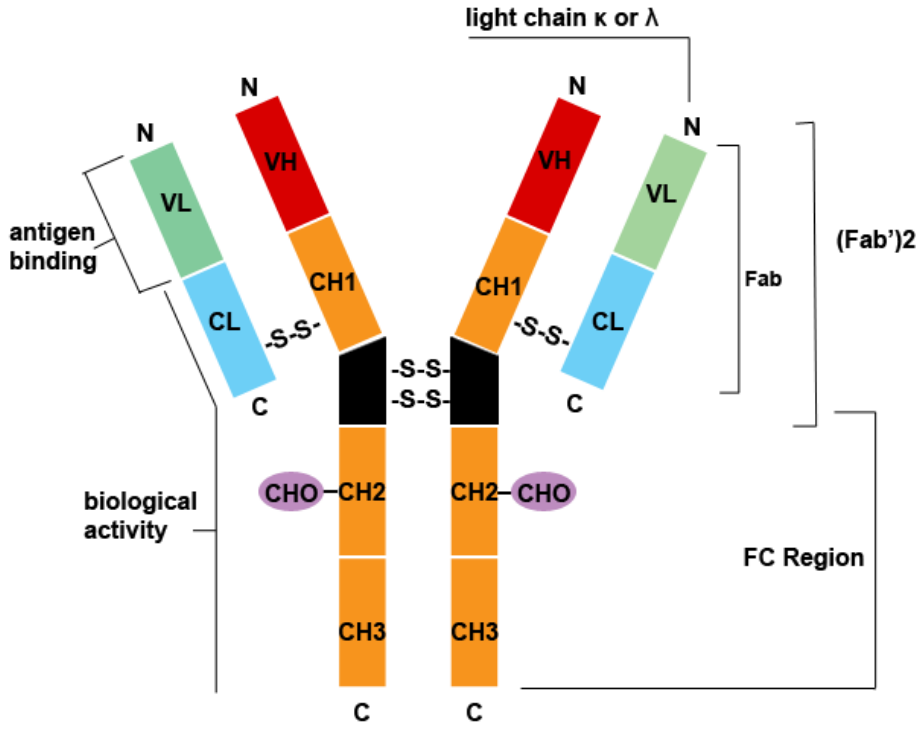


Figure. 1. General Antibody Structure. H denotes heavy chain and L denotes light chain. S-S denotes disulfide bonds. FC region denotes the crystallizable portion of an antibody, while Fab region denotes antigen-binding region. C denotes constant region. V denotes variable region (Abcam, n.d.).

As shown in Figure 1, the antibody has a symmetric structure with two light chains and two heavy chains. The light chain is attached covalently to a heavy chain with a disulfide bond while the two heavy chains are attached to each other with yet another covalent disulfide bond. Within the light and heavy chains are amino terminal variable regions that play significant roles in antigen recognition and carboxyl terminal constant regions that facilitate interaction of effector functions. Only the variable region's constant regions are made up of three or four Ig domains, which comprise of 110 amino acid residues that fold globularly. Variable regions further consist of a heavy chain and a light chain that make up the antigen-binding site. Since there are two sets of variable heavy and light chains, each antibody contains two binding sites. Within the variable heavy and light chains are three hypervariable regions that are held in place by more conserved framework sequences. These three hypervariable regions are known as complementarity-determining regions (CDRs). CDR3 is the most variable of the three hypervariable regions. These CDR regions have been shown through crystallographic analysis to extend from the surface of antibodies as loops to be able to interact with antigens. Thus sequence differences in the CDRs are responsible for the binding variability of antibodies. Another important structural distinction is the Fab (fragment, antigen binding) and Fc (fragment crystallizable). The Fab consists of just the variable light and heavy chain pair while the Fc fragment consists of the heavy chain regions together that are not attached to a light chain. The distinction is important as it leads to innovative approaches to neutralizing antibody research in which it is possible to use just the Fab without the Fc.

Antibody molecules are classified into isotypes IgA, IgD, IgE, IgG, and IgM.

Isotypes IgA and IgG in humans are further subdivided into subtypes IgA1, IgA2, and IgG1, IgG2, IgG3, and IgG4. IgA can be a monomer, dimer, or trimer, and IgM can be a pentamer, while IgE and IgG are monomers and IgD is found on the B-cell membrane. IgG has the longest half-life of all isotypes. IgG and IgA play an important role in mucosal immunity and are being considered as effector molecules in anti-HIV mucosal immune defense in the genital tract.

Two characteristics are critical to antibody neutralization of antigens, affinity and avidity. Affinity is the strength of binding of one antibody site to an antigen epitope. Avidity is the overall strength of attachment to the epitopes by the antibody. This is important in IgA and IgM isotypes as IgA can be a dimer or trimer while IgM can be a pentamer. Since avidity increases geometrically, IgM can have low affinity on its individual monomeric arms, but the overall avidity of the pentameric antibody can be far greater than monomeric binding (Abul K. Abbas & Andrew H. Lichtman, 2003). Developing antibodies against HIV-1 has been met with three major roadblocks. Important neutralizing sites are conformationally protected and thus unable to be targeted. For example, gp120, which is the viral protein responsible for binding to CD4 on target cells, requires a CD4-induced conformational change in order for the binding site to be exposed. A second roadblock is the heavy glycosylation of gp120; surface sugar structures hide viral epitopes from the immune system. The third roadblock is the ability of HIV to rapidly mutate and escape the antibody response. In these ways, free virus can escape antibody defenses and sustain a latent infection for long periods of time (Pier et al., 2004).

Immune Response

Antibodies take advantage of various mechanisms to neutralize foreign bodies often utilizing the Fc region of the antibody. These mechanisms include effector functions such as recruiting B cells, dendritic cell, natural killer cells, macrophages, mast cells, and monocytes. Recruitment of these cells further activates or inhibits functions that can release cytokines; induce granulation, phagocytosis, or oxidative effects. In turn these effector functions can activate the complement system of proteins as well as mannose binding lectin proteins, which result in initiation of the antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis mechanisms. Antibody-dependent cell-mediated viral inhibition has also been used as a term to describe situations in which antibodies result in decreased viral loads. Again they take into account the previously mentioned mechanisms of immune response. Fc domains itself also interact with FcR's (Fc Receptors) which dictate immune response. Besides the typical variables such as titer, affinity, avidity, specificity and polyclonality of antibodies present, glycosylation of Fc domains play a major role in interactions with FcRs. There is also variability in effector function dictated by which immunoglobulin's bind. For example, IgG1 and IgG3 are able to activate complement and bind all FcR's, but IgG2 activates complementation and binds only some FcγR2 genotypes. IgG4 interacts with only FcγR and but does not activate complementation (Ackerman, Dugast, & Alter, 2012). These distinctions could play a significant role in harnessing antibodies to prevent the transmission of HIV-1.

In HIV-specific immune responses, anti-p24 IgG1 is the first significant antibody

to develop. Anti-gp120 IgG3 is the other significant antibody to develop during early infection. Approximately 2 weeks after infection, antibodies to gp41 are also observed. These neutralizing antibodies (nAbs) must constantly adapt to changes in the viral envelope. True broadly neutralizing antibodies (bnAbs) that are able to neutralize a wider range of virus do not form until after years of seroconversion (Ackerman et al., 2012). Recruitment of natural killer (NK) cells that can recognize virus-infected cells leads to elimination of HIV-infected targets by ADCC. Unfortunately, NK cell activity becomes weaker with progressing viral infection. ADCC has been studied as a mechanism that decreases viral load in the acute phase of infection. Other studies have considered the role of ADCC in the course of HIV infection. A study by Baum, et al. at the Multicenter AIDS Cohort Study showed rapid disease progression resulted in lower ADCC against cells coated with gp120 antibodies (Forthal & Moog, 2009). These results, however, were not consistent with other studies, and in the case of the VAX003 HIV vaccine trial lowered ADCC activity was not observed. Macrophages can phagocytose and eliminate opsonized (antibody-coated) HIV without antigenic recognition; however, macrophages of the CD14+, FcγR3+ subset are themselves vulnerable to HIV infection leading to long-lasting viral reservoirs. HIV is also known to change phagocyte structure and function due to loss of FcγR3 and this is speculated to be the cause of decreased antibody-dependent cellular phagocytosis (ADCP) function. It is the ADCP mechanism that allows antibody-opsonized material to be cleared regardless of direct cytotoxicity. Fc receptors themselves play a role in the HIV immune response. Polymorphisms of Fc receptors such as FcγR2a have been shown to correlate with HIV disease progression.

Another type of antibody-dependent immune response to HIV infection is antibody dependent cell-mediated virus inhibition (ADCVI). This mechanism is similar to ADCC and ADCP in that it relies on an antibody and effector cell relationship, but ADCVI activity is measured by impact on viral production by target cells. Cells engaged in ADCVI can directly lyse HIV-infected target cells, or release antiviral cytokines such as Beta-chemokines. Studies have found ADCVI antibodies to have an inverse relationship with plasma viremia, indicating that they may play a role in viral control in vivo (Forthal & Moog, 2009).

Mucosal surfaces provide a physical barrier to viral infection, and can also mount specialized immune defense mechanisms. Since genital and rectal mucosal surfaces are the most common sites of HIV transmission, much thought has been dedicated to generating protective mucosal immune responses. Studies by Hur et al, have shown that a polymeric IgA form of anti-HIV antibody (the type of IgA that is found in mucosal secretions) can inhibit HIV mucosal transmission with greater effectiveness than monomeric IgA or IgG. It has been suggested by recent data that HIV polyclonal antibodies in conjunction with mucus trapping and immobilizing offer better results than mAbs. It is logical that polyclonal antibody would be better suited for this purpose by forming multivalent aggregates more easily (Ackerman et al., 2012). Hur et al transplanted human hematopoietic stem/progenitor cells with an anti-HIV b12-IgA bnmAb gene into a humanized bone marrow-liver-thymus (BLT) mouse model. These mice were protected from virus-mediated depletion of CD4 T cells after HIV-1 challenge. One reason why IgA performs favorably at mucosal sites is that polymeric IgA (pIgA) is

transcytosed from the lamina propria to the mucosal lumen by a polymeric immunoglobulin receptor (PIGR) found on mucosal epithelial cells (Hur et al., 2012). Studies have also been carried out to study the neonatal Fc receptor FcRn in its role in mucosal immune response. FcRn has been found in the epithelial cells of the human intestine, lung, kidney, nasal, vaginal and rectal tissue and is capable of transporting Ig-G associated antigens across polarized epithelial barriers. It has also been observed to transport IgG across epithelial barriers in both human placenta and neonatal intestines in rodents to transfer passive immunity from mother to fetus (Yoshida, 2006). It has been speculated that FcRn could bidirectionally transport IgG between apical and basal environments, and tests have shown FcRn-deficient mice become susceptible to infections by *C. rodentium*, a bacterial pathogen similar to *E. coli* in humans (Yoshida, 2006). FcRn may also affect HIV infection at mucosal sites by transporting IgG to the mucosal surface, and transporting HIV-IgG immune complexes across the mucosa into the lamina propria they could infect or sensitize immune cells. Ye et al, were able to protect mice that were challenged with herpes simplex virus type-2 (HSV-2) by mimicking IgG transfer at mucosal surfaces through fusing of an IgG Fc fragment with an adjuvant CpG. The Fc fragment was attached to an HSV-2 major envelop glycoprotein and delivered by FcRn to instill both humoral and cellular immunity (Ye, Zeng, Bai, Roopenian, & Zhu, 2011). The research additionally showed that FcRn knockout mice that were treated with the glycoprotein-fused IgG were unable to fend off lethal doses of HSV-2 suggesting FcRn as crucial to this novel vaccine delivering mechanism (Ye et al., 2011). Therefore, the approach to antibody design against the HIV-1 virus must take into

account the multitude of variables arising from the antibodies themselves and their interactions with immune response effector functions, particularly those relating to Fc-FcγR interactions.

Current Research Related to this Project

There is constant work towards a vaccine for HIV that acts by inducing antibodies in a way similar to vaccines for other viral diseases such as influenza, measles, or polio. However, methods of testing antibody responses capable of neutralizing HIV continue to be difficult. HIV neutralization assays have numerous variables that can affect the outcome of the test, such as target cell line, density of plated cells, viral dose, antibody dose, duration of infection, cell free virus incubation time, quantitative endpoints and others (Polonis et al., 2008). Early neutralization assays used T-cell line-adapted (TCLA) viruses; virus and antibody were incubated together and then added to CD4+ target cells. Multinucleated cells and cell survival was then quantified to determine whether the virus was neutralized, however, results from such assays proved to be unreliable (Polonis et al., 2008). Assay design turned towards use of peripheral blood mononuclear cells (PBMC) from human donors which hopefully would give results in a more physiological context, however, human donors have different susceptibility to HIV-1 infection. Variability in PBMC infectability resulting from donor genetics could be attributable to CD8+ and CD4+ variability along with genetic polymorphism differences in CCR5 and other chemokine receptors. Added to this was inter-experiment and inter-lab variability in PBMC-based neutralization assays (Polonis et al., 2008). More recent approaches to

testing virus neutralization with antibodies have used “pseudoviruses” that have a cloned HIV-1 envelope gene into defective virus particles resulting in virus that is capable of only one round of infection. These viruses are then used to test antibody neutralization using the epithelial HeLa cell line (TZM-bl) that is genetically modified to express the CD4, CCR5, and CXCR4 receptors as well as a β-galactosidase reporter gene allowing quantification of infection through spectrometry (Polonis et al., 2008) Table 1 shows the comparison of current approaches in HIV neutralization assays and their differences. It has been noted that TZM-bl cells have a higher CD4:CCR5 ratio (1:2) compared to PBMCs (1:20), which can also be a factor in the neutralization assay. Comparisons between neutralization assays using TZM-bl vs. PBMC target cells often show divergent results. For example, the 4E10 mAb has much broader neutralizing activity in the TZM-bl assay than in the PBMC assay. The TZM-bl assay is thought to offer a more uniform method to study inhibition of binding and entry of HIV. However, some studies have shown that a high proportion (up to 85%) of HIV entry into TZM-bl cells occurs as endocytosis which may not be physiological (Polonis et al., 2008). Most TZM-bl assays also include the use of DEAE-dextran which increases HIV entry into endosomal vesicles (Polonis et al., 2008). The endosomal entry issue is not relevant however in the study of bnmAbs, as HIV is inactivated in the endosomes. It is the virus that is not neutralized and infects the cells via surface receptors that is measured by the quantitative assay endpoint. The TZM-bl HeLa cell model thus has evolved as the gold standard neutralization assay to determine effectiveness of bnmAbs (Gilbert et al., 2010).

Table 1. Comparison of HIV neutralization assay using PBMC and TZM-bl

Assay	**PBMC**	**TZM-bl (JC53-bl)**
Cells	PBMC	Epithelial HeLa
Virus	Uncloned primary	Pseudovirus
Assay length	4-6 days	2-3 days
Common endpoint	Extra- or intra-cellular p24	Luciferase activity
Rounds of infection	Multiple or Single	Single
DEAE-dextran used	No	Yes
Measure inhibition of attachment/entry	Yes	Yes
Cell-cell transmission	Potentially	No
Coreceptors used	CCR5, CXCR4, others (CCR5 physiologic)	CCR5, CXCR4 (>2 logs more CCR5 than PBMC)

(Polonis et al., 2008)

Current Pharmacologic Therapies

Early HIV-1 therapeutic agents such as zidovudine acted as nucleoside analog inhibitors of reverse transcriptase. These were unsuccessful in *in vivo* experiments, and increases in CD4 cell counts in patients were short lived. Combination therapy was introduced in the mid-1990's, which proved to be highly successful. Nucleoside analogue inhibitors terminated chain formation during reverse transcriptase, which would lead to depletion of intracellular nucleotides. While nucleoside reverse transcriptase inhibitors used individually resulted in high mutation to resistance, when used in combination with purine or pyrimidine analogues, protease inhibitors, and non-nucleoside reverse transcriptase inhibitors, resistance was avoided and effectiveness was increased. Non-nucleoside inhibitors targeted the early viral infection phase by preventing pro-viral DNA production while protease inhibitors targeted the viral protease necessary for processing

viral precursor proteins. In such a way, they interfered with viral replication in late stages of its life cycle (Pier et al., 2004).

Vaccine development efforts and broadly neutralizing monoclonal antibodies

Past efforts to develop an HIV vaccine have for the most part failed. An early strategy that focused on eliciting an antibody response using monomeric gp120 particles as immunogens produced antibodies that neutralized laboratory strains of HIV that had those specific gp120 monomers, but not primary isolates of virus (Chan & Kim, 1998). Furthermore, antibody responses to gp120 were generated by linear epitopes which are not as effective and ideal as physiologically significant tertiary structure epitopes of gp120 (Chan & Kim, 1998). Other methods of vaccine generation included reverse vaccinology, using crystallographic structure of broadly neutralizing monoclonal antibodies (bnmAbs) that could bind to HIV-1 epitopes, however epitopes used to stimulate bnmAbs were found to be ineffective (Regenmortel, 2012). Specific epitopes that have had some promise and continue to be explored include the membrane proximal external region (MPER) of gp41, which bnmAbs 2F5, 4E10, and Z13e1 have shown to bind to (Hessell et al., 2009). Thus MPER has been pursued as a likely candidate for design as an immunogen.

Other Prophylactic Approaches

Another biopharmaceutical approach for HIV prevention has been the use of topical microbicides. The earliest research in this area explored the idea of non-specific

anti-HIV microbicides. Attempts at topical microbicides included Nonoxynol-9, Savy®, Carraguard®, cellulose sulfate, PRO-2000, and Buffergel®, all of which reached phase 3 clinical trials but failed (Grammen et al., 2012). Nonoxynol-9 caused cytokine release, immune cell recruitment, and changed vaginal flora while also increasing epithelial lesions (Adams & Kashuba, 2012). Savvy® disrupted the envelope membrane of HIV, and was thought to be less toxic than Nonoxynol-9 but both drugs showed more seroconversions in treatment than placebo arms. Likewise cellulose sulfate also showed more seroconversion with use than placebo arms (Adams & Kashuba, 2012). The other nonspecific compounds tested, Buffergel®, PRO2000®, and Carraguard®, were unable to show significant efficacy in trial studies (Adams & Kashuba, 2012). These nonspecific microbicides have thus proven to be ineffective and even dangerous as methods of HIV prevention (Whaley et al., 2010). Other approaches to topical HIV prevention have included the study of RC-101, retrocyclin peptide 1, a peptide analogue from the defensin group of proteins that is able to prevent HIV binding to CXCR4 and CCR5 receptors. The RC-101 peptide has been shown to be promising in macaque challenges and studies of it continue (Adams & Kashuba, 2012). Another promising microbicide candidate has been recombinant RANTES, which has successfully worked in macaques to protect against vaginal SHIV challenges; however it has been difficult to produce in sufficient quantities (Whaley, Hanes, Shattock, Cone, & Friend, 2010). Recombinant RANTES maintains stability at 55-degree Celsius for > 1 month in HEC gel, and is also stable under conditions found in the vaginal environment such as low pH. It is suggested that recombinant RANTES could be incorporated into IVR devices (Whaley et al., 2010).

Recombinant antibodies have also been explored due to their specificity, potency, and safety profile. Nanoparticles have been explored as well in hopes of achieving sustained drug release for periods longer than a day, however, with the presence and turnover of mucus in vaginal environments, one would not expect nanoparticles to penetrate the mucus let alone reach the epithelium (Whaley et al., 2010). It was discovered that the average pore size of cervical vaginal mucus is 340 ± 70nm, which is far greater than the size of HIV particles (Lai, Wang, Hida, Cone, & Hanes, 2009). It has also been observed that HIV-1 adheres to mucus at pH 4; however, when the vaginal environment is neutralized by semen, HIV-1 can readily diffuse through the mucus (Lai et al., 2009). This presents the case for development of mucus-penetrating compounds that can provide protection against HIV within and under the mucus coat.

Research has shifted from non-specific topical microbicides to smaller molecular antiretroviral (ARV) drugs that can be applied at tissue sites of possible transmission such as the vaginal mucosa (Adams & Kashuba, 2012). No matter if the approach is ARV or non-specific microbicides, the pharmacokinetics of drug application to mucosal tissues have been difficult to study. The ARV tenofovir has been tested as a gel and from measurements of interleukin-8 (an indicator of inflammation), data suggests it is well tolerated by the tissue (Kiser et al., 2012). Data from the first large clinical trials conducted [the Chemoprophylaxis for HIV Prevention in Men study (iPrEx) and Partners PrEP studies] using tenofovir to prevent transmission showed modest effects. Tenofovir was able to reduce HIV risk compared to a placebo by 67 percent with daily use (Marrazzo et al., 2013). However, subsequent studies have been less successful primarily

due to poor compliance in the study population (Marrazzo et al., 2013). This suggests that approaches such as vaginal rings that deliver microbicides for longer periods of time may provide a better solution to prevent HIV transmission in the populations of the endemic regions.

Monoclonal Antibodies

Initially, select bnMAbs were isolated from sera of HIV-1 infected individuals and screened against HIV in hopes of designing an effective antibody based vaccine with broadly neutralizing capabilities at low concentrations (Laura M. Walker et al., 2011). The use of high throughput neutralization screening resulted in bnMAbs targeting an epitope on the envelope protein that spans conserved regions of variable loops on the gp120 protein (L. M. Walker et al., 2009). Early bnMAbs such as b12 isolated in 1992 from a phage display library database was able to neutralize 40% of known HIV-1 isolates (Wu et al., 2010a). Data from Walker et al., identified two bnMAbs, PG9 and PG16, which specifically bound to trimeric Env targets suggesting another target epitope for immunogen design, or at least an additional bnMAb to consider in alternative therapeutic approaches. Both PG9 and PG16 offer more insight into vaccine immunogen design due to their possible potency against non-clade B viruses. Non-clade B viruses make up a large percentage of infections outside North American and Europe where treatments are lacking. Two other well-known bnMAbs are 2F5 and 4E10, which are known to act on the MPER region of the gp41 coreceptor (Stamatatos, Morris, Burton, & Mascola, 2009). The antibodies 2F5 and 4E10 were originally IgG3 antibodies collected

from a HIV-1 seropositive asymptomatic patient. They were then modified in hybridomas to IgG1 for longer shelf life and future scalability reasons (Hessell et al., 2009). In the studies of Hessell et al., mabs 2F5 and 4E10 provided complete protection against SHIV infection challenges in rhesus macaques. The 4E10 bnMAb has been shown to be neutralizing across clades at a moderate potency thus suggesting limitations to its effectiveness as a prophylaxis or target for immunogen design (Binley et al., 2004). However, according to more recent data, the 4E10 antibody is highly efficient at inhibiting cell-to-cell viral spreading via mature dendritic cells (Sagar et al., 2012). Finally, another promising bnMAb is VRC01, which targets CD4 binding sites of HIV thus allowing it to be effective in neutralization (Zhou et al., 2010). VRC01 has been shown to neutralize approximately 90% of viral particles suggesting it can be used as a template to further develop more effective versions of bnMAbs (Zhou et al., 2010). In Zhou et al, out of 190 circulating HIV-1 isolates tested for neutralization by VRC01, 173 were neutralized and 17 were found to be resistant. The potency of VRC01 is attributed to its interaction with gp120 in a way that mimics CD4, the cell receptor crucial to HIV-1 cell entry (Wu et al., 2010b). VRC01 has served as a template for rational design of improvements. This has resulted in the development of NIH45-46, a VRC01 variant of increased potency (Diskin et al., 2011). Some of the differences that have made NIH45-46 more potent include a tyrosine at position 74, a tyrosine to serine substation in constant domain region L1, and a four-residue insertion in constant domain region H3 (Diskin et al., 2011). It remains to be seen whether NIH45-46 can be used to further develop more potent bnMAbs for use as prophylaxis or to generate possible targets for

immunogen design. Like combination anti-retroviral therapy, various different bnMAbs are likely to be used in combination with each other for maximum effectiveness.

Taking into consideration HIV-1's ability to evolve rapidly against full-sized antibodies, it has also been hypothesized that mAb approaches may be more effective using smaller antibody fragments. This approach would use fragments 11-15kDa in size which would enable binding to hidden epitopes while maintaining good penetrative properties (Chen & Dimitrov, 2011). Naturally the next step in bnmAb design is the combination of multiple bnMabs to increase the potency and longevity of such a treatment. Already studied *in vivo*, a 10mg/kg dose of a bi-functional fusion inhibitor dose of anti-CD4 mAb's has been shown to maintain a 2000-fold serum concentration over IC90 for a week. This suggests that when combined, a cocktail of such mAbs would be effective and have favorable pharmacokinetic characteristics (Chen & Dimitrov, 2011). Of course the main barriers to HIV-1 therapy has always been the virus' evolutionary capabilities. Oral pharmaceutical approaches have always been favored because of the relatively low costs of production as well as ease of drug administration, despite the toxicities of long-term use of pharmaceuticals. The cost of mAb production has always been a significant barrier as well.

Monoclonal Antibody Production

Current methods of producing monoclonal antibodies begin with the immunization of a mouse model, retrieving immune cells from the spleen, and creating hybridomas by combining the immune cell and cancer cell to create an immortal antibody

producing cell line (Marrazzo et al., 2013). Once the cell line is obtained, two methods are used to produce mAbs. One way is to clone the cell in-vitro, in culture. The second method is to inject the hybridoma cells into the peritoneal cavity of a mouse where the hybridoma multiplies and produces ascites (fluids) containing large amounts of the antibody (National Academy Press, 1999). Harvesting mAbs from ascites comes with its own difficulties. Ascites mAb production takes a long time and there is considerable variability of titers based on production abilities of individual mice. Ascites-produced mAbs may contain mouse proteins and other contaminants requiring costly purification steps. The physical costs of a mouse facility are also often overlooked as an important expense. Ascite production requires daily monitoring of mice to determine when a mouse should be euthanized to limit stress. The Institutional Animal Care and Use Committee (IACUC) have many recommendations in proper procedures for ascite harvesting. Overall animal welfare is of high priority as mice must be monitored for changes in behavior, appearance, body weight, temperature, clinical signs and so on (National Academy Press, 1999). Such procedures have increased costs of mAb production. In-vitro methods have their own issues as well. Hybridoma cells vary in their ability to survive in-vitro environments, and the equipment needed for commercialization is costly. Fetal bovine serum is used in most culture media but can contain bovine immunoglobins, which can contaminate mAb preparations. This has led to the development of serum-free culture media but not without its own issues. Serum-free media increases hybridoma in-vitro adaptation time by 3%-5% because of the increase in cell passages and sometimes hybridomas never adapt to in-vitro environments (National Academy Press, 1999). In-

vitro culture methods can also sometimes produce mAbs that gain or lose sites of glycosylation (National Academy Press, 1999).

Commercial sources currently produce mAbs for diagnostics and therapeutics. The main method of mAb generation by the industry is through in-vitro technology with serum-free media due to therapeutic requirements of very pure and uncontaminated mAbs. They are usually produced in small batches, which is quicker than large-scale production, but costlier. Large-scale production is less expensive but requires more time investment (National Academy Press, 1999).

Initial industrialization of MAb production in 1986 resulted at first in low yields of 1g/L but has since achieved yields of up to 5g/L, which in terms of a 10kL – 25kL bioreactor would equate to 15-100kg of product (Kelley, 2009). According to industry averages, however, even with increased yields MAbs remain an expensive product as cost per gram begins at a minimum of $2,000 per gram with a median of $8,000 and up to $20,000 per gram (Kelley, 2009). MAbs are clinically used at concentrations of 10-15 mg per kg thus leading to high production costs in the clinical realm (Ma et al., 2005).

Complications and inefficiencies of current methods of mAb production have led research to explore the possibilities of plant-based models of monoclonal antibody generation. Manufacture of such mAbs has made use of Agrobacterium as a vector for primary infection of plant based models. The tobacco mosaic virus has also been used in Nicotiana models for mAb production (Hiatt & Pauly, 2006).

One advantage of plant-based MAb production is the range of products producible in plants. Plants as eukaryotes, are able synthesize small peptides,

polypeptides, and complex multimeric proteins that are not possible in microbial solutions. Furthermore, plant chaperones are analogues to mammalian versions and targeting of similar excretory pathways can result in control of glycosylation as well (Ma et al., 2005).

Of course with any transgenic-based model, there is risk of inadvertent introduction to the wild. The plant-based model's routine use of green houses can physically minimize such incidents, as well as the use of specific male-sterile plant lines. Seed dispersal is also controlled by making seed viability dependent on exogenous stimulation to seed germination (Ma et al., 2005). Recent research has looked into the development of MAbs using plant-based models with the pursuit of increased yield being an important driving factor (Ma et al., 2005). Previous plant models using common species such as corn faced issues of long life cycles with low yields and also requiring acres of environmental containment to prevent genetic contamination (Hiatt & Pauly, 2006). More recently, research has taken advantage of the *Agrobacterium*-mediated viral infection model of tobacco plants to produce full-sized IgG antibodies in less than two weeks with yields of 0.5 g of mAb per kg of plant biomass. Initial plant production of heterooligomeric antibodies met difficulties due to the natural ability of plants to defend against viral infection through exclusion of viral genome (Hiatt & Pauly, 2006). The techniques used to produce the antibodies used in experiments carried out in this paper take advantage of *Nicotinia*-based manufacturing pioneered by Mapp Biopharmaceuticals Inc. This model benefits from high yields using transient viral expression to output high amounts of mAbs as well as using a plant model with genetically modified glycosylation

pathways to produce antibodies with mammalian glycoforms (Whaley, Hiatt, & Zeitlin, 2011). The Nicotiana model is scalable without requiring the need to change environmental conditions and because the viral genome is not encoded into the plant pollen, there is no risk of spread of recombinant genes (Whaley et al., 2011). This technique has led to the production of mAbs such as 2G12 that are indistinguishable from mammalian cell-derived derived 2G12. It is estimated that plantibodies can be produced for use in microbicides at a cost of less that one US dollar for a dose of 10mg of purified protein (Whaley et al., 2011). Thus this approach could provide an inexpensive and effective method to prevent HIV transmission in developing countries with a high prevalence of HIV.

Specific Aims.

(1) Assess the effectiveness of broadly neutralizing antibodies against HIV produced by the *Nicotiana* plant species on HIV infection in the TZM-bl HeLa cell model

(2) Determine the time course of mAb neutralizing activity in the vaginal environment

(3) Study transport of nMabs across the vaginal epithelium

The study is expected to:

(1) Provide further insight on the effectiveness of broadly neutralizing antibodies produced by plants against HIV.

(2) Show time course and duration of efficacy of plantibodies in vaginal secretions of Cynomolgus macaques

(3) Determine whether plantibodies are transported across the vaginal epithelium; this mechanism would be a safety concern because, if transported, the mabs could potentially carry HIV into the tissues or enter the systemic circulation.

MATERIALS & METHODS

Neutralization assays with plant-derived mAbs

The TZM-bl HeLa cell line, engineered to express CD4, CXCR4, and CCR5 with luciferase and beta-galactosidase reporter genes, was used for HIV neutralization assays (sourced from the NIH AIDS Research and Reference Reagent Program). Cell propagation media was DMEM 1X high glucose, with 10% heat-inactivated FBS 2% Penicillin/Streptomycin solution, 1% 2 mM L-Glutamine, 1% HEPES (all from Life Technologies). Neutralization assays were carried out by incubation of virus (500IU/well Q23-T/F strain from Dr. Manish Sager) with antibody at decreasing concentrations for 1 hour and then adding TZM-bl cells (10,000 cells / well). Antibody was pipetted into wells starting at 1µg/ml with serial 2-fold dilutions resulting in antibody titer concentrations of 0.5µg/ml, 0.25µg/ml, 0.125µg/ml, 0.0625µg/ml, and 0.03125µg/ml. Black-walled, clear bottom, tissue culture treated 96-well plates (Fisher) were used to incubate the assay for 48 hours. The assay was developed using the Galacto-light plus system (Applied Biosystems) according to kit protocols (20µl lysis buffer, 70µl substrate, 100µl accelerator solution). A Synergy HT (Biorad) plate reader was used to read the developed 96-well plate assay for luminescence levels. Experiments were run in triplicate.

Cynomolgus macaque vaginal lavage antibody test

Cynomolgus macaque cervicovaginal lavages (CVLs) were obtained from Dr. Francois Villanger (Emory University). CVLs were collected prior to antibody treatment from 6 macaques to serve as baseline controls. Macaques were then treated with 2ml of HEC gel containing 1.25mg of VRC01-N, and then CVL was collected 4-hours after treatment. The macaques were allowed to rest and then treated with 1.25mg of VRC01-N with CVLs collected after 24-hours. The macaques were then rested again and treated with 1.25 mg of VRC01-N with CVLs collected after 72-hours. The entire process was further repeated with 5mg and 20mg concentrations of VRC01-N also in 2ml of HEC gel. CVL samples were tested in a neutralization assay, using virus (500IU/well of HIV-1 Q23-T/F strain from Dr. Manish Sager), TZM-bl cells (10,000 cells/well), and 25µl or 50µl of CVL sample diluted to 1:4 or 1:8. Virus and CVL sample were incubated for 1 hour before cells were added. Black-walled, clear bottom, tissue culture treated 96-well plates (Fisher) were used to incubate the assay for 48 hours. The assay was developed using the Galacto-light plus system (Applied Biosystems) according to kit protocols (20µl lysis buffer, 70µl substrate, 100µl accelerator). A Synergy HT (Biorad) plate reader was used to read the developed 96-well plate assay for luminescence levels. Each experiment was run in at least triplicate.

Pharmacokinetic of monoclonal antibody transport across MatTek Epivaginal Tissue

To study the pharmacokinetics of monoclonal antibody transport across the

vaginal epithelium, the MatTek Epivaginal tissue model (VEC-100, Ashland, MA) was used. The tissue is a fully stratified vaginal epithelial layer, grown on tissue culture inserts in 24-well tissue culture plates (Ayehunie et al. 2006). The tissue was incubated in proprietary MatTek media (MatTek Corporation) at 37˚C. Monoclonal antibody was added to the apical side, and basal supernatant was collected after 3-12 hours and tested for antibodies using the Abcam IgG Human ELISA Kit (Abcam). Some experiments were conducted under low pH conditions by adjusting MatTek media to pH 6.0 using HCl. In other experiments, inflammatory conditions were simulated by treating, tissues with TNF-alpha (50µl of a 10µg/ml concentration) for 24-hours prior to loading of Mabs. To measure the inherent permeability of the tissues, 10µl of 0.005g/ml sodium fluorescein was concurrently added to the tissues, and the amount that crossed into the basolateral supernatant was measured on a fluorometer. After incubation with the mabs, tissue was fixed in formaldehyde embedded in paraffin wax, and sectioned for staining. Four steps of Pro-Par submersion, three steps of 100% ethanol submersion, one step of 95% ethanol submersion, and one step of 70% ethanol submersion was used to de-wax the tissue prior to staining, each step carried out for approximately 5 mins. A 1:10 dilution of primary mouse anti-human IgG was applied to the tissues for 1 hour of incubation. Tissue was washed with 10% Tris buffer, and then incubated in the dark with secondary donkey anti-mouse Cy3 labeled antibody for 30 mins at antibody concentration of 1:1,000. Tissue was then counter-stained with anti-fade DAPI solution (Vector Labs). Follow-up staining was done on sections using an enzymatic stain, treating first with primary anti-IgG antibody (DAKO; diluted 1:50, 1 hour incubation),

followed by streptavidin-alkaline phosphatase (DAKO) for 20 mins, and developed with a DAKO proprietary substrate kit. Substrate development was halted with distilled water and sections were counterstained with hematoxylin.

RESULTS

Specific Aim 1: Mab-N Neutralization Efficacy

The plantibody VRC01-N effectively neutralized HIV Q23 virus at a concentrations ≥0.5 µg/ml (Figure 2).

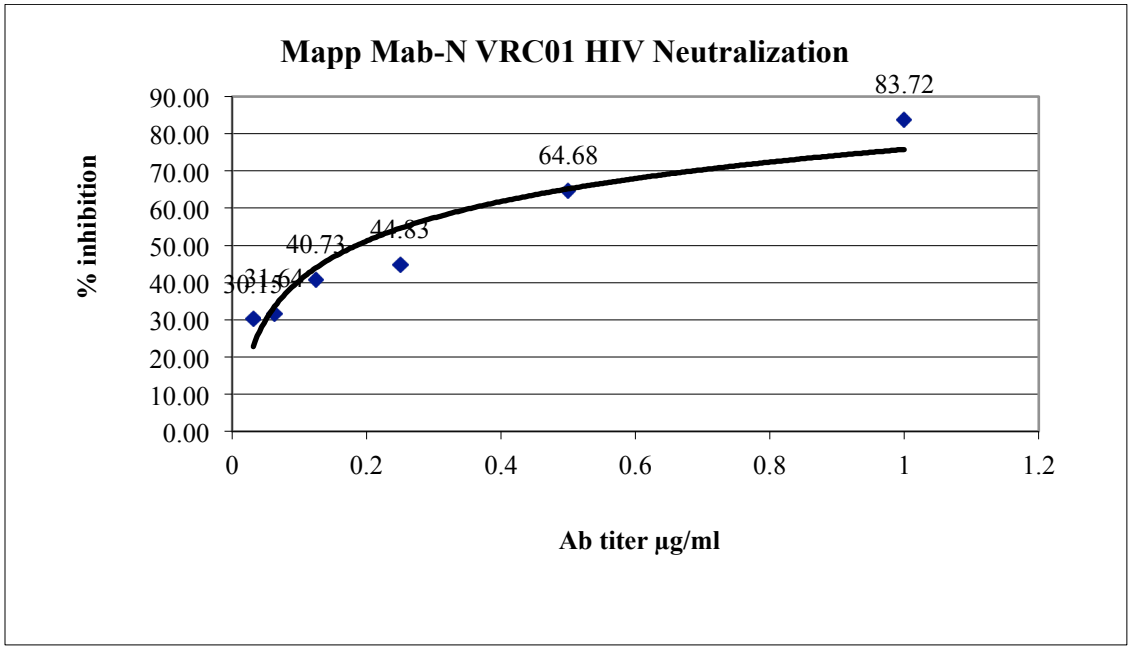


Figure 2. Mapp VRC01-N mAb neutralization of Q23 transmitter/founder HIV strain. Varying concentrations of Mapp VRC01-N mAb were incubated with Q23 virus for one hour, then the mixture was added to TZM-bl cells and cultured for 48 hours. An antibody concentration of 1µg/ml of mAb inhibited HIV infection by 83%. The neutralization assay was carried out in triplicate. The data point labels represent the mean value of the triplicates.

The NIH45-46 mAb was more effective at inhibiting HIV infection in the TZM-bl assay than VRC01 (Figure 3). This was expected as NIH45-46 is a newer construct of a mAb that used VRC01 as a basis for improvement. This suggests that if Mapp were able to produce NIH45-46 in *nicotiana* and use it in place of VRC01, it could increase the anti-HIV efficacy of the microbicide product.

We were able to use the VRCO1 antibody dose response as a standard curve for the macaque cervicovaginal lavage assays (described below).

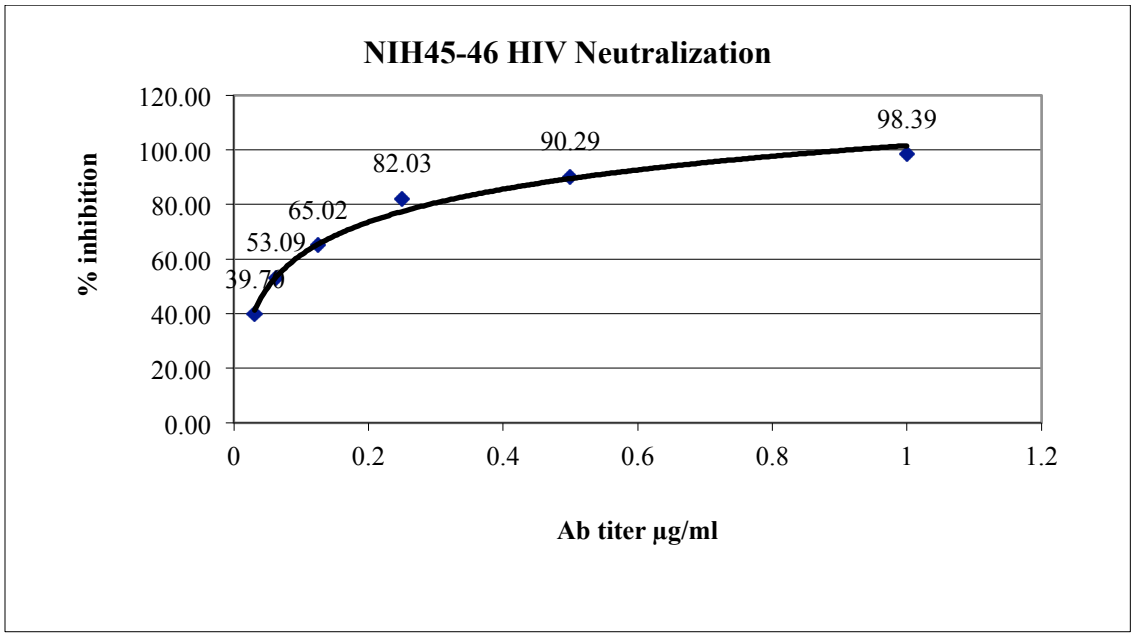


Figure 3. NIH45-46 mAb neutralization of Q23 transmitter/founder HIV strain As a comparison and positive control for Mapp VRC01-N, NIH45-46 was used in the assay. As a modified, enhanced version of VRC01, NIH45-46 shows improved neutralization capabilities against the Q23 transmitter/founder HIV strain. At a concentration of 1µg/ml of mAb, 98% of the virus was inhibited. NIH45-46 showed improved neutralization capabilities at similar doses with Mapp VRC01-N. Neutralization assay was carried out in triplicate. The data point labels represent the mean value of the triplicates.

PGT136 and PG135 were used as negative controls for both VRC01-N and NIH45-46. Results of neutralization assays of PGT136 and PGT135 (Figure 4 and Figure 5) showed that these antibodies were not inhibiting, which was expected, as they are known not be effective in neutralizing HIV.

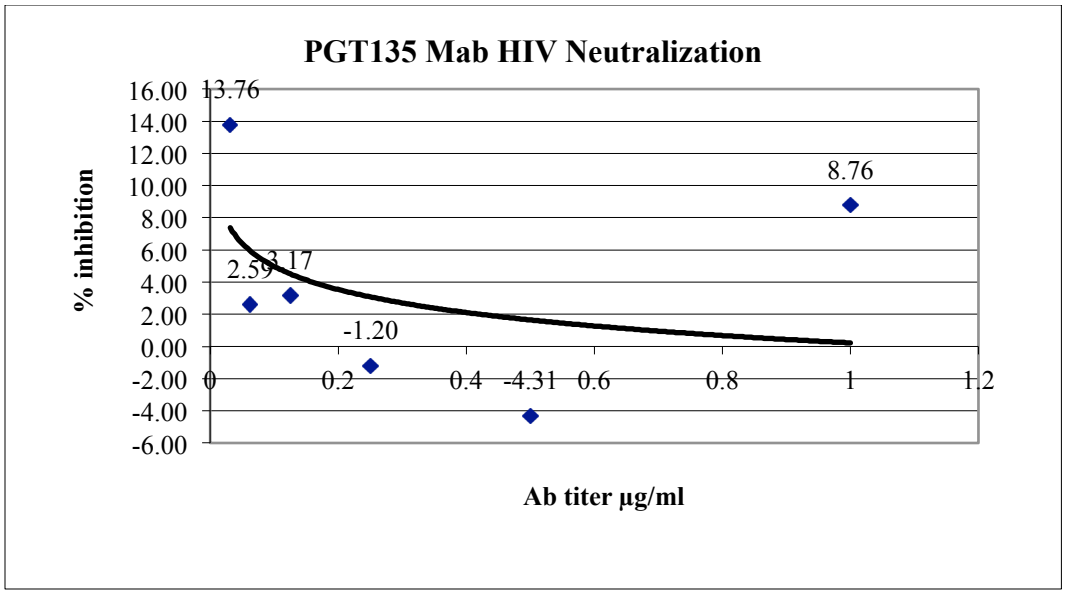


Figure 4. PGT135 mAb neutralization of Q23 transmitter/founder HIV strain. PG135 mAb used as a negative control in the assay. PG135 is known not to neutralize this specific HIV strain. Results show that PG135 is not neutralizing at any concentration of mAb titer. The neutralization assay was carried out in triplicate. The data point labels represent the mean value of the triplicates.

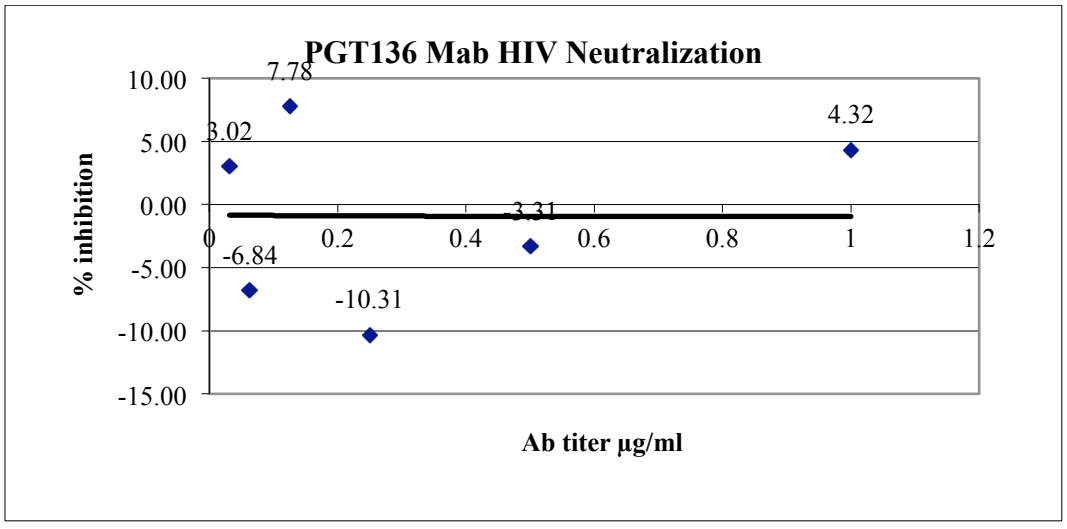


Figure 5. PGT136 mAb neutralization of Q23 transmitter/founder HIV strain. PG136 was used as a second negative control in the assay. PG136 is known not to neutralize this specific HIV strain. Results show that PG136 is not neutralizing at any concentration of mAb titer. The neutralization assay was carried out in triplicate. The data point labels represent the mean value of the triplicates.

Specific Aim 2: Cynomolgus macaque cervicovaginal lavage antibody test

Some of the CVLs obtained for baseline reference from macaques had innate inhibiting ability against HIV virus. The CVLs collected 4 hours following 1.25mg mAb dosing neutralized virus by near 100% (Figure 6), with just one macaque neutralizing at approximately 75%. However with increased doses at 5mg and 20mg, all the CVLs were able to neutralize cell-free virus at nearly 100% efficacy.

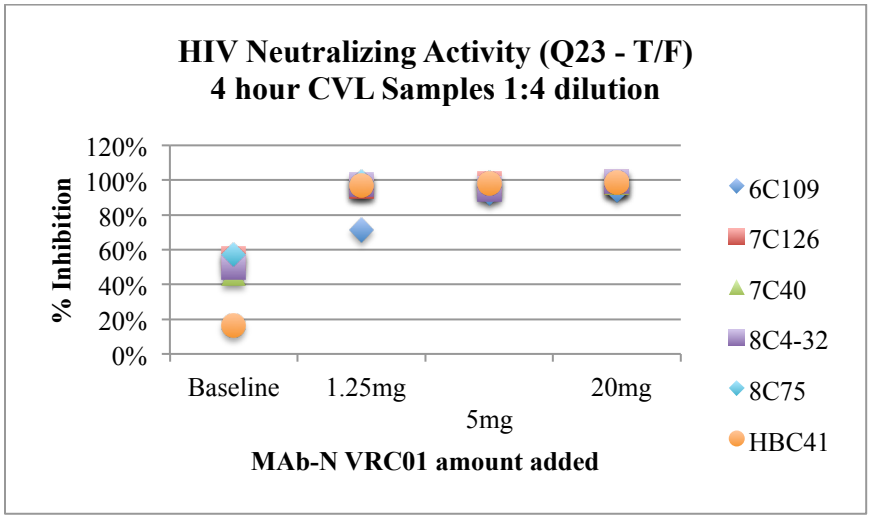


Figure 6. MAb VRC01-N neutralization of Q23 transmitter/founder HIV from 6 Cynomolgus macaque cervicovaginal lavage samples collected after 4-hours of antibody application at varying concentrations. Results show baseline untreated CVLs having moderate innate inhibition activity. CVLs from macaques treated with 1.25mg, 5mg and 20mg of VRC01 mab-N very effectively neutralized HIV.

These results were expected, as these doses were much higher than the 1µg/ml of Mab-N shown to be effective in the first experiment. Examination of the results from CVLs that were collected at the 24-hour time point from all concentrations of administered Mab-Ns however, showed unexpected data (Figure 7).

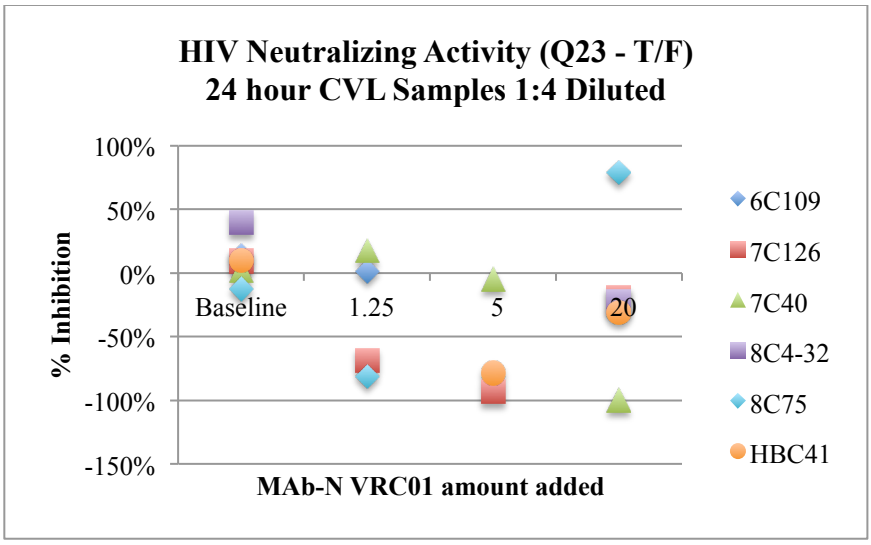


Figure 7. MAb VRC01-N neutralization of Q23 transmitter/founder HIV from 6 Cynomolgus macaque cervicovaginal lavage samples collected after 24-hours of antibody application at varying concentrations. The Baseline CVLs again showed innate inhibition abilities however assay results showed very little inhibition for CVLs collected 24-hours later for doses of 1.25mg, 5mg, and 20mg. Results suggest possible increase in infection of TZM-bl cells.

What we expected was decreased neutralization capabilities given the longer time point. However, from assay results, what can be seen are not only decreased neutralization capabilities but in some macaques the CVLs increased viral infectivity. Increased viral infection was seen as negative inhibition (luminescence was higher than the positive control wells in the assay). Figure 7 has some data omitted due to evidence of cell toxicity during assay incubation. The TZM-bl cell line contains the beta-galactosidase reporter gene and thus when used in conjunction with the Tropix beta-galactosidase system, would result in luminescence upon successful viral infection. Cell toxicity or cell death would result in artificially low luminescence or no response to the luminescence developing substrate at all and thus the reason for removal of a few data points in Figure

7. Only one macaque (8C75) showed HIV neutralization at the 24 hour time point, and only at the highest dose of Mab tested.

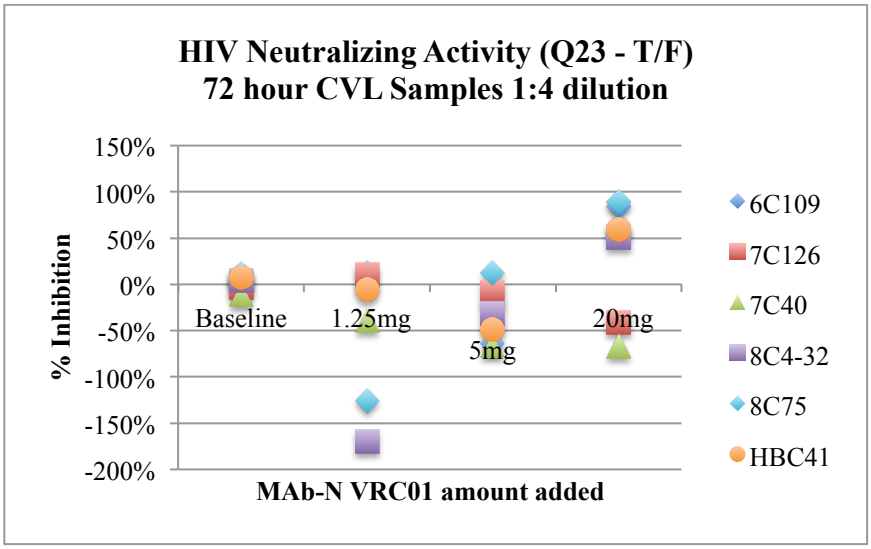


Figure 8. MAb VRC01-N neutralization of Q23 transmitter/founder HIV from 6 Cynomolgus macaque cervicovaginal lavage samples collected after 72-hours of antibody application at varying concentrations; assay carried out twice. Baselines in this assay run did not show any innate inhibition affects. In the 1.25mg and 5mg dose samples, increased infection of TZM-bl cells was observed. In the samples of 20mg doses, results indicate some inhibition is occurring but observations of assay showed cell toxicity, which would indirectly and incorrectly suggest inhibition.

Figure 8 show HIV neutralization of CVLs collected 72 hours after Mab treatment. Macaque 8C75, and two others showed HIV neutralization at the highest mAb dose, others showed a tendency for increased viral infection. It is unclear as to the reasons of why this may be occurring. Figures 9 through 11 show CVL neutralization data when tested at a dilution of 1:8.

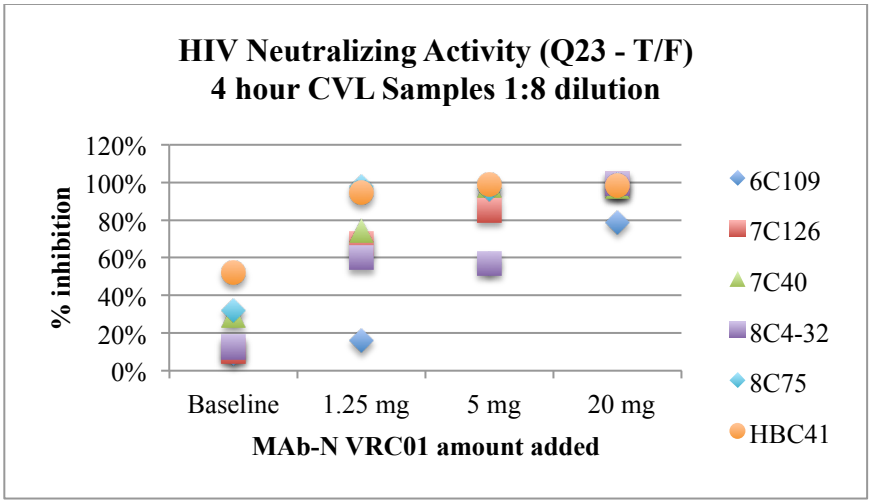


Figure 9. MAb VRC01-N neutralization of Q23 transmitter/founder HIV from 6 Cynomolgus macaque cervical vaginal lavage samples collected after 4-hours of antibody application, diluted 1:8. Results show baseline samples again having innate inhibition of virus. Most CVLs from dosing of 1.25mg, 5mg, and 20mg of antibodies maintain inhibiting activity against virus. Some data were excluded due to TZM-bl cell toxicity.

Figure 9 shows that the mabs in the CVLs retained neutralization activity at the 4 hour time point. Figure 10 shows neutralization data for macaque CVLs collected at the 24-hour time point and diluted 1:8. The CVLs showed less neutralizing ability at the higher dilution, and again showed evidence of increased viral infection or negative inhibition, where detected luminescence was greater the positive control wells.

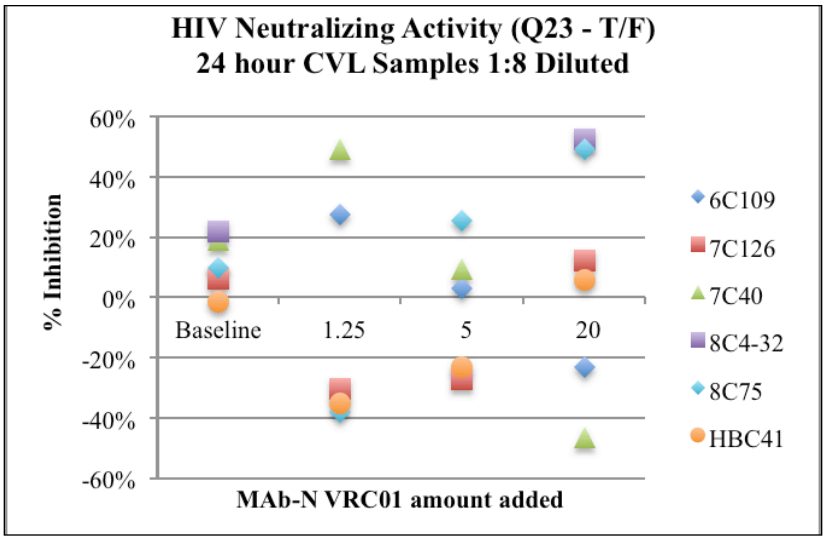


Figure 10. MAb VRC01-N neutralization of Q23 transmitter/founder HIV from 6 Cynomolgus macaque cervical vaginal lavage samples collected after 24-hours of antibody application at varying concentrations, diluted 1:8. Results show baseline CVLs having minor inhibitory effects. CVLs from macaques treated with 1.25mg of mAb showed both successful inhibition of virus and increased viral infection. CVLs from macaques treated with 5mg and 20 mg of mAb show similar results. Some data were excluded due to TZM-bl cell toxicity.

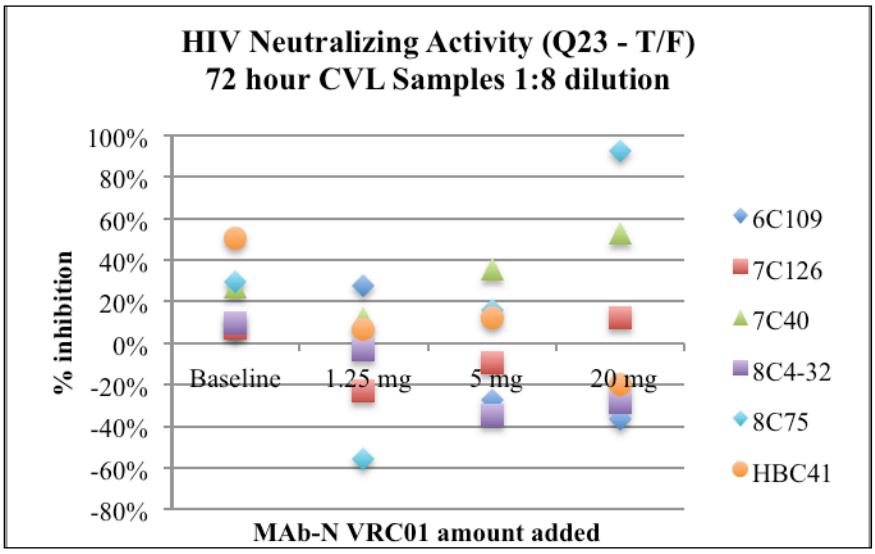


Figure 11. MAb VRC01-N neutralization of Q23 transmitter/founder HIV from 6 Cynomolgus macaque cervical vaginal lavage samples collected after 72-hours of antibody application at varying concentrations, diluted 1:8. Results show baseline CVLs having some innate inhibition. CVLs from macaques treated with 1.25mg of mAb showed both inhibition and increased viral infection. CVLs treated with 5mg and 20mg

showed similar results of exhibiting both inhibition and increased viral infection. Macaque 8C75 shows high viral inhibition in this assay.

Figure 12 shows a comparison of assay results for CVLs from individual macaques collected at the 4-hour time point and tested at 1:4 and 1:8 dilutions. The data for each macaque show neutralization occurring consistently in an upward trend coinciding with increased dose of Mab-N, with the 1:8 dilution data showing less inhibition for certain macaques such as 6C109, 7C126, and 8C4-32. For some macaques such as 7C40, 8C75, HBC41, the higher dilution factor had less effect on neutralization capabilities and at higher doses such as 5mg and 20mg, the dilution had no effect on inhibition. This was an expected result as given the short time point at which CVLs were collected, the Mab-Ns were expected to retain their efficacy at all doses.

Individual Macaque Comparision of CVLs collected at the 4-hour time point, 1:4 and 1:8 dilutions.

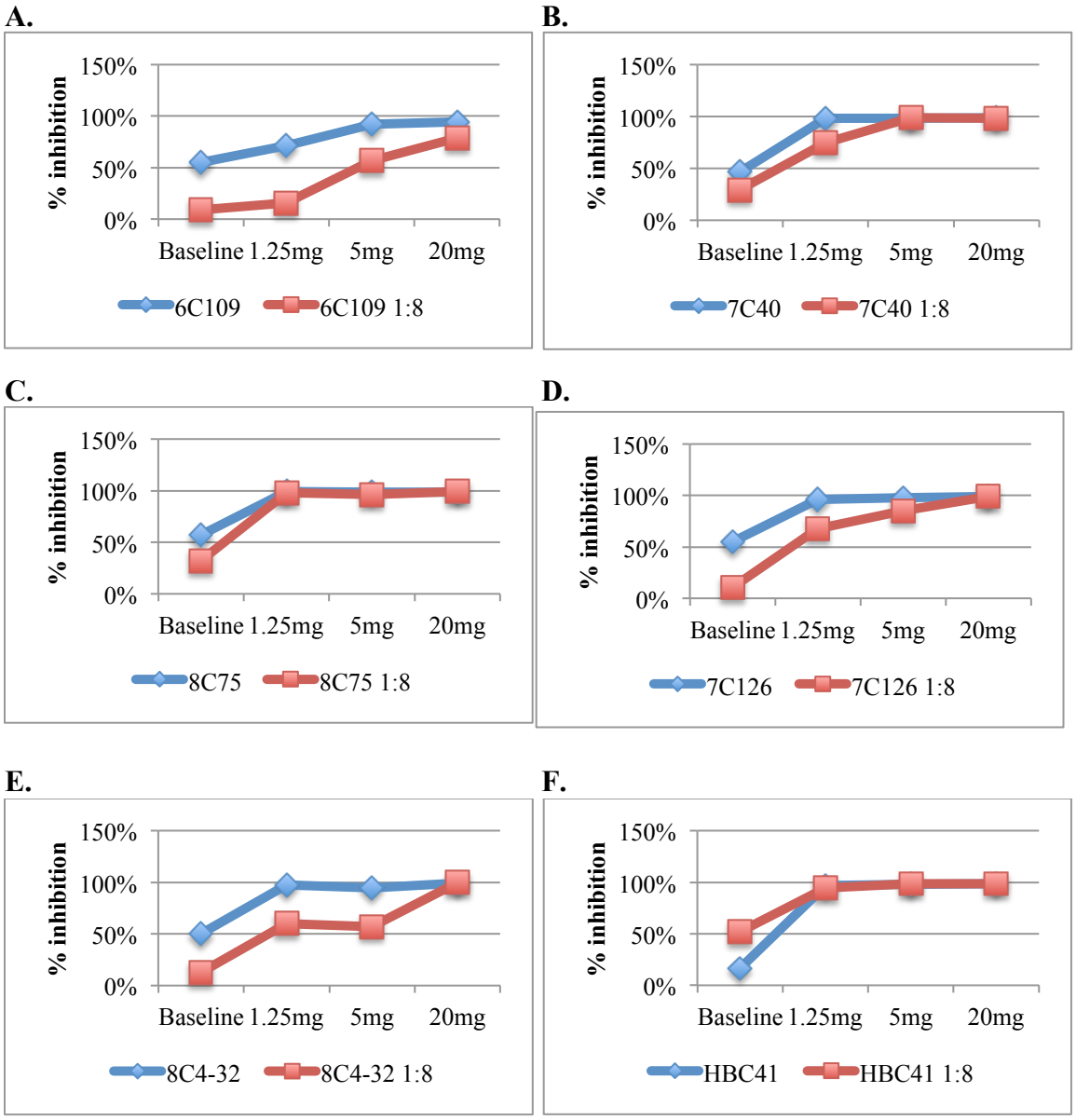


Figure 12. Comparison of individual macaque neutralization assay results, 1:4 versus 1:8 dilutions. CVLs were harvested 4-hours post treatment. Data on individual macaque samples are compared against each other with samples diluted 1:4 and 1:8. The same macaques showed similar neutralizing trends and capabilities even at diluted amounts.

Figure 13 shows a comparison of data from CVLs at the 24-hour time point: two 1:4 dilution assays and one 1:8 dilution assay. It is here at the 24-hour time point that the increased viral infection is seen. Comparison of individual monkeys, specifically Figure 13A, B, C, D, shows similar trends between the three assays suggesting that what is causing the increased infection is sample specific. Furthermore, where increased viral infection is observed in Figure 13A, B, C, D, the effect is decreased at the 1:8 dilution. The repeated pattern strongly supports the accuracy of the data. Figure 13E, and F, show data that is less uniform.

Individual Macaque Comparision of CVLs collected at 24-hour time points, 1:4 dilution to 1:8 dilution..

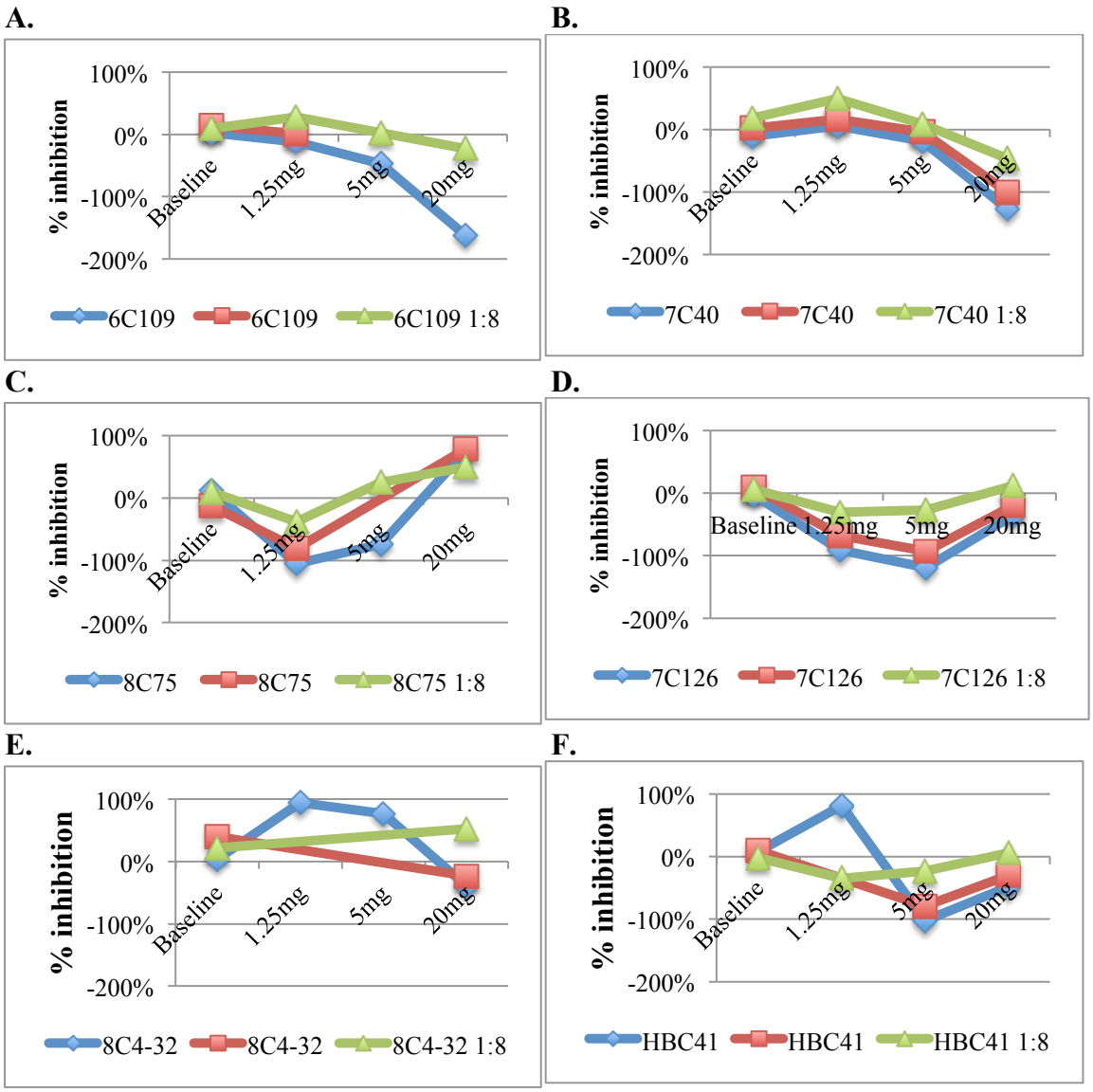


Figure 13. Comparison of individual macaque neutralization assay results. Two runs of 1:4 dilutions and 1 run of 1:8 dilutions. CVLs were harvested 24-hours post treatment. Three macaques showed data points that were similar in neutralization capabilities. The other three macaques showed data points that were not similar in neutralization capabilities. Some data from the post 24-hour treated samples showed increased viral infection. One data point from macaque HBC41 specifically showed 100% neutralization without observed cell toxicity. Other data points were excluded due to observed cell toxicity signifying unreliable data.

Figure 14 shows a comparison of data from CVLs collected at the 72-hour time point: two 1:4 dilution assays and one 1:8 dilution assay. Individual macaques show similar trends of neutralization capability and similar trends of increased viral infection, specifically in Figures 14A, C, E, F.

Individual Macaque Comparision of CVLS collected at the 72-hour time point, 1:4 and 1:8 dilutions.

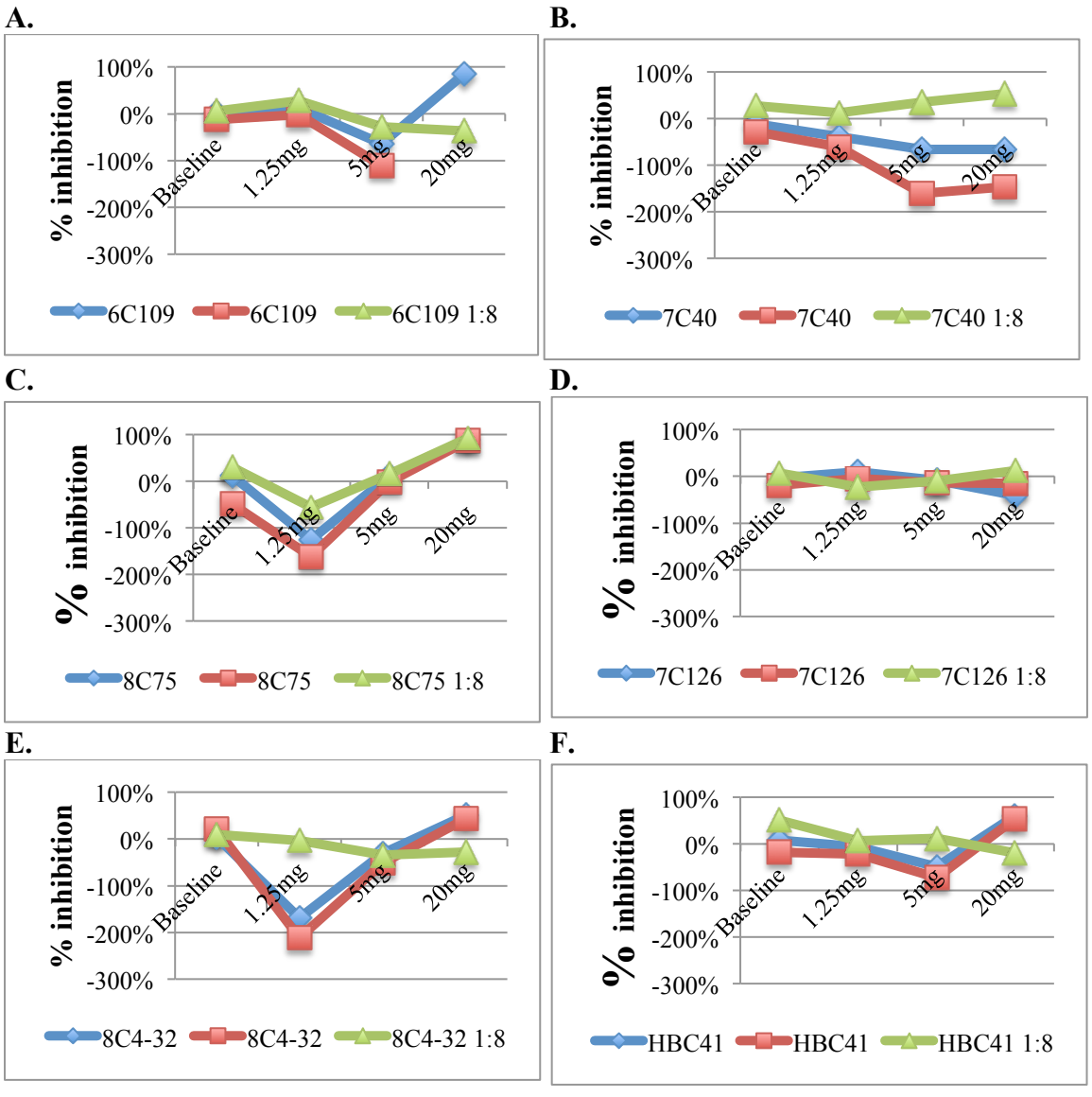


Figure 14. Comparison of individual macaque neutralization assay results. Two runs of 1:4 dilutions and 1 run of 1:8 dilutions. CVLs were harvested 72-hours post

treatment. Macaques 6C109, 7C40, and 8C75 showed data that followed a neutralization trend given the two runs of 1:4 dolutions and 1 run of 1:8 dilutions. Macaques 7C126, 8C4-32, and HBC41, showed data that did not show neutralization trends between the tested 1:4 and 1:8 dilutions. Again all samples showed data points in which increase viral infection occurred.

Specific Aim 3: Mab-N Pharmacokinetics in MatTek Epivaginal Tissue Model

Preliminary tests of Mab-N ability to traverse the vaginal epithelium at both neutral and pH 6 suggested very little transfer, 0.03% of loaded amount (Figure 15). At pH 6, because the absorbance reading of both VRC01-N samples and one 4E10-N sample was greater than the upper limit of the standard, the data suggests that at least 0.03% was transferred. A larger experiment was conducted repeating these preliminary studies and also including TNF-alpha treated tissue, while incubating the tissues with antibodies for 24-hours. Figure 16A and 16B show again that at neutral pH and both pH 6, both Mab-N treated tissue samples showed consistent transfer of approximately 0.03%.

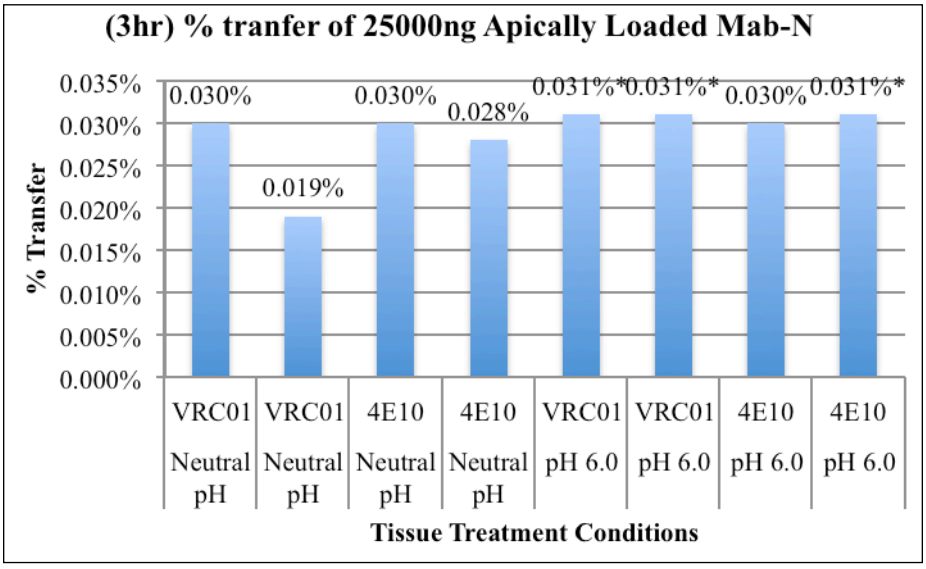
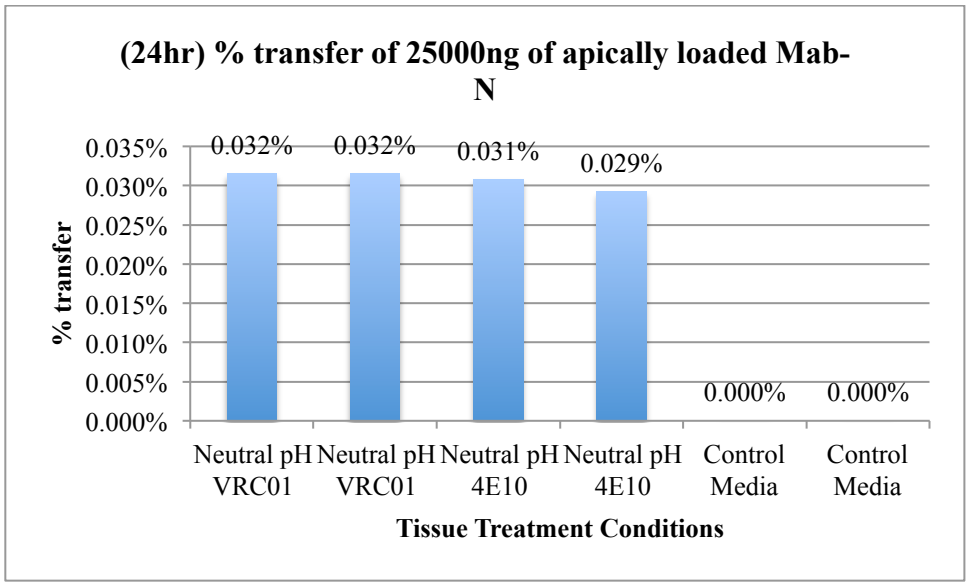


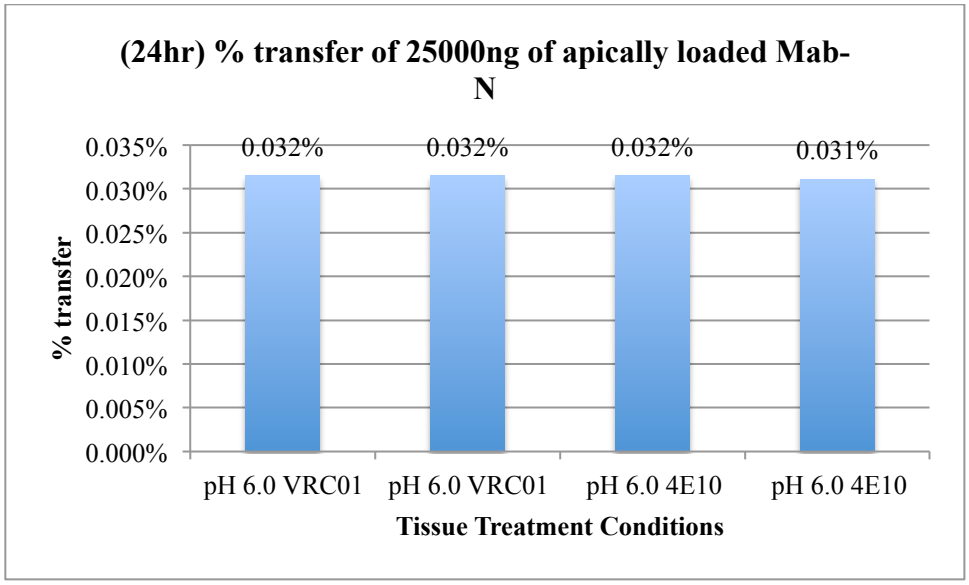
Figure 15. Percent transfer of 25ug of IgG added to apical reservoir detected in basal supernatant after 3-hours. ELISA results from recovered basal supernatant after 3-hours suggests a transfer of 0.03% of loaded IgG. At neutral pH, both Mab-Ns did not

exceed 0.03% transfer of IgG loaded. At pH 6.0, both VRC01-N treated tissue exceeded the highest known standard readings denoted by the asterisk. At pH 6.0, only one VRC01-N treated tissue suggested IgG transfer exceeding 0.031%. Thus the asterisk noted data suggests that at least 0.031% transfer occurred.

A



B



C

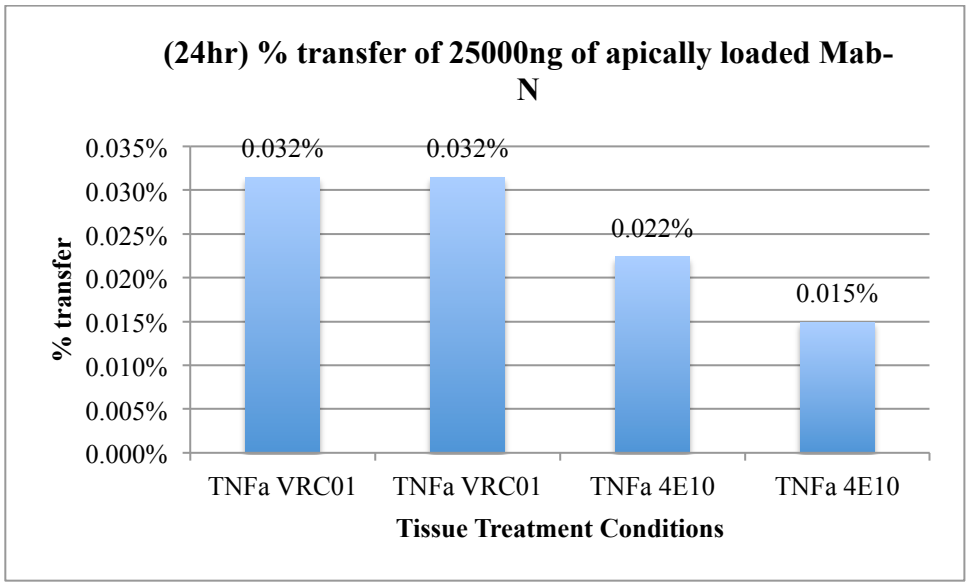


Figure 16. Percent transfer of 25µg of IgG added to apical reservoir detected in Basal supernatant after 24-hours. Panel A shows that at 24-hours, levels of both VRC01-N and 4E10-N in the basal medium do not exceed 0.032% of originally loaded amounts. When the experiment was conducted at pH of 6.0 (Panel B), both Mab-N's again did not exceed 0.032% transfer of apically loaded IgG. When tissue treated with TNF-alpha was used (Panel C), VRC01-N transfer was consistent at 0.032% while 4E10-N showed a lower percentage of IgG transfer at 0.022% and 0.015%.

When TNF-alpha treated tissue was used (Figure 16C), 0.3% of VRC01-N was detected in the basolateral supernatant, while levels of 4E10-N were lower. These data suggest that minimal amounts of Mab-Ns cross the vaginal epithelium. As a comparison, tissue was treated with 5mg/ml and 1mg/ml of IgG purified from human serum for 24 hours (Figure 17). Higher amounts of IgG were observed in basal supernatants, possibly due to the longer incubation time.

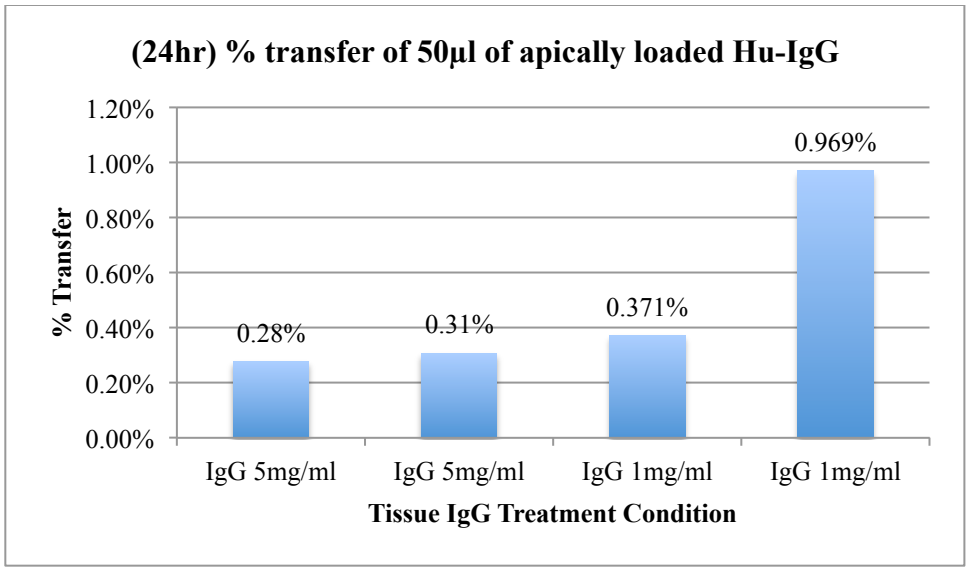
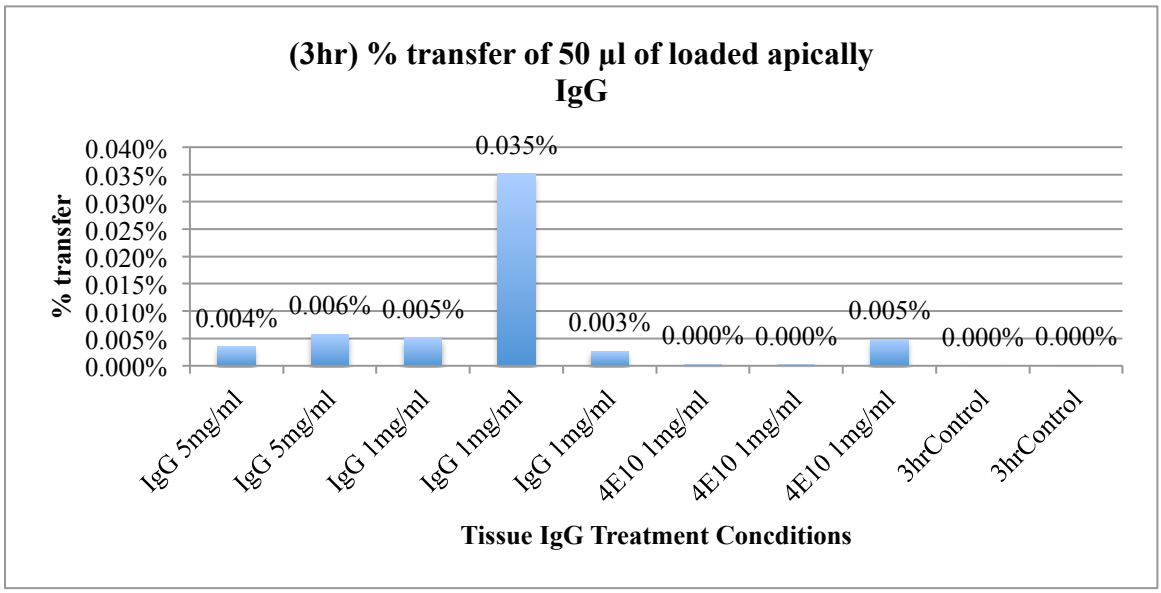


Figure 17. Percent transfer of IgG added to apical reservoir detected in basal supernatant after 24-hours. The experiment was carried out using purified IgG using super-concentrated amounts of purified human-IgG. The data shows 5mg/ml transferring at approximately 0.3% across while 1mg/ml unexpectedly transferring higher at 0.371% and 0.969%.

A larger experiment was conducted this time with a 3-hour time point and 24-hour time point Figure 18). At 3-hours, with high concentrations of purified human IgG and 4E10-N, far lower amounts of IgG transferred across the tissue (approximately 0.005%). One tissue sample of human-IgG loaded with a 1mg/ml concentration managed to show percent transfer across tissue at 0.035%, closer to previous results. At 24-hours, the transfer of IgGs across tissue was inconsistent. At the highest concentration of 5mg/ml the percent transfer of IgG was in 0.05% and 0.095%. Unexpectedly the 1mg/ml showed greater transfer at 0.114%, 0.468%, and 0.460%. Again it was unexpected to see the lower concentrated condition show greater transfer. These results, however, show some correlation with preliminary results from Figure 17. At 24-hours, the 4E10-N, showed consistent of transfer of IgGs around ~0.03% with one sample being 0.045%.

This was inline with previous results from Figure 15,16.

A.



B.

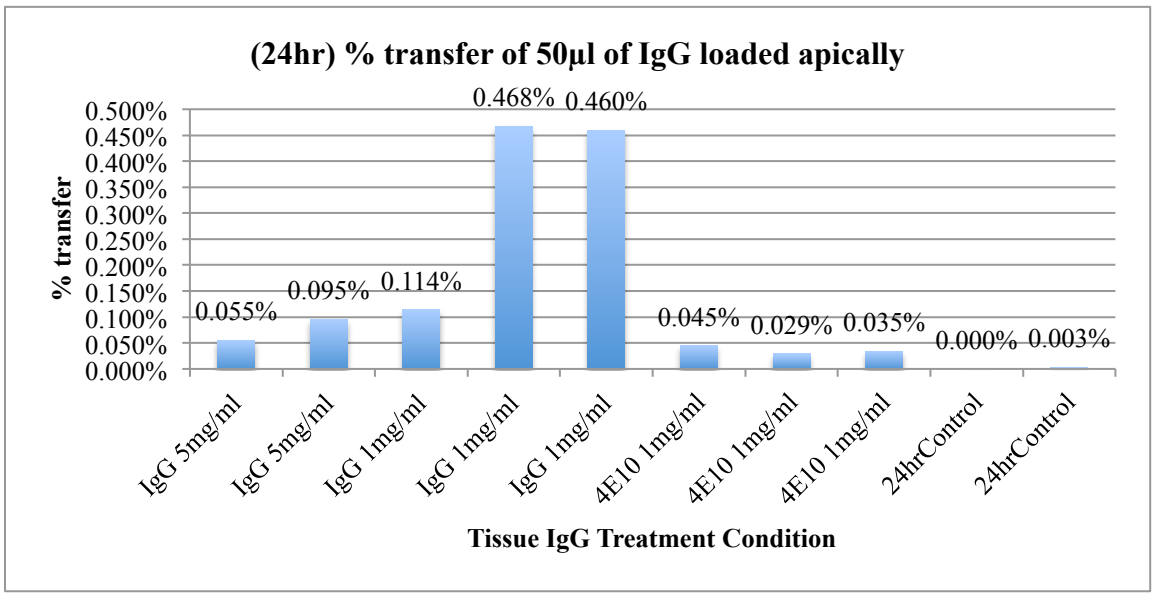
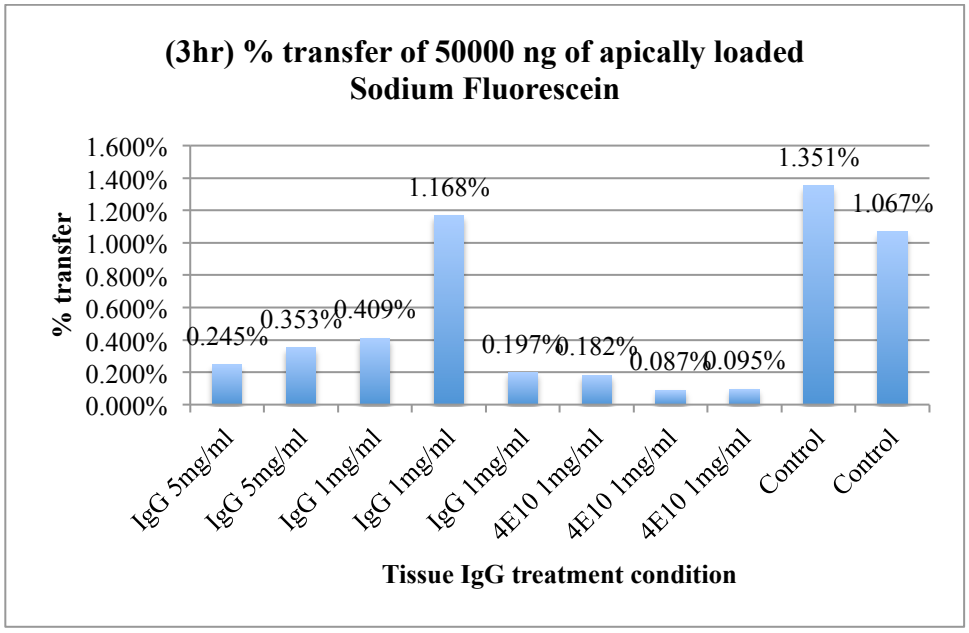


Figure 18. Percent transfer of IgG added to apical reservoir detected in basal supernatant after 3 hours and 24-hours using purified human-IgG at 5mg/ml and 1mg/ml concentrations. The data here for both 3-hour and 24-hour incubation times (Panel A and Panel B) show the 5mg/ml conditions transferring at far lower amounts than Figure 17. Figure 18A shows 3-hour incubation time with maximum transfer of IgG for one tissue using 1mg/ml concentration IgG not exceeding 0.03%. At 3-hours, the data indicate that very little transfer of IgG occurs. At 24-hours the 5mg/ml loaded

concentration showed transfer of 0.05% and 0.09%, while the 1mg/ml loaded concentration unexpectedly showed greater IgG transfer. IgG transfer was greater at the 24-hour time point than at 3-hours.

In order to rule out any inherent leakage of the tissue itself the experiments conducted in Figure 18 were carried out concurrently with addition of 10µg of 0.005mg/ml sodium-fluorescein, a dye that detects tissue leakage. Figure 19 show results from fluorescence readings of collected basal supernatants. At the three-hour time point, the well that showed the most IgG transfer also had the most sodium fluorescein transfer, indicating that this tissue was leaky. At the 24-hour time point, much more sodium fluorescein crossed over indicating that the tissues were much leakier at that time point, and that Ig crossover at the 24-hour time point may be nonspecific.

A.



B.

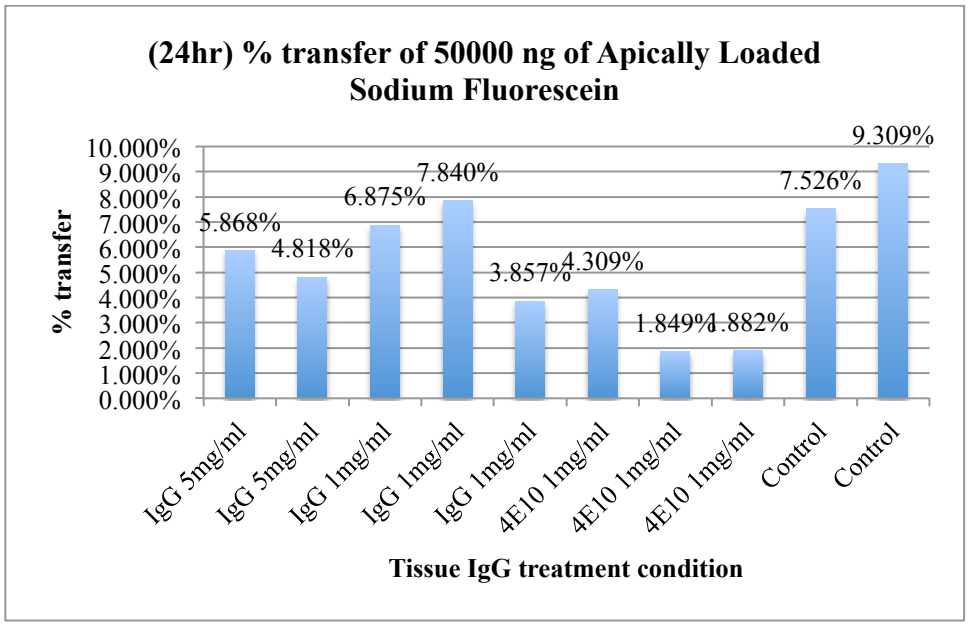


Figure 19. Percent transfer of 50ug of Sodium-Fluorescein loaded apically, 3-hour and 24-hour time points. The data show that at 3-hours the minimum transfer was 0.087% while the maximum was 1.31%. At 24-hours the minimum was 1.85% while the maximum was 9.30%.

The tissue that had been treated with Mabs was processed for immunohistologic detection of IgG in the tissues. Figure 20 shows data for Mab-N 4E10.

A. B.

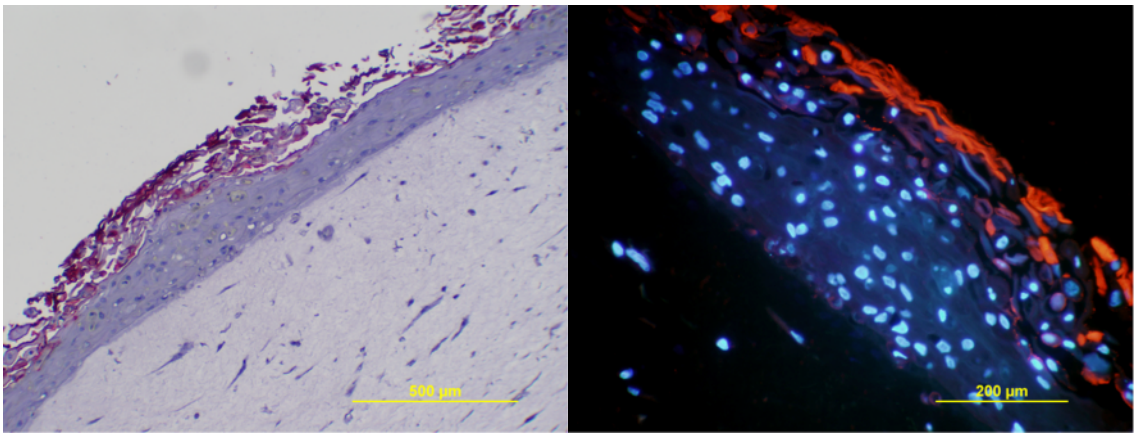


Figure 20. Anti-Human-IgG stain for Mab-N 4E10 in VEC tissue. Immunohistochemistry showed Mab-N concentrated on the apical surface, but no penetration beyond the stratum corneum layer. Panel A is immunohistochemistry using alkaline phosphatase enzyme stain (positive Mabs stain purple), while Panel B is immunohistochemistry utilizing a Cy3 fluorescence stain (positive Mabs stain red) with nuclei counterstained with DAPI.

Figure 20A and B show 4E10-N concentrated along the apical surface of the vaginal epithelial model, but none was detected deeper in the tissue. Figure 21 shows similar data with VRC01-N. Both Figure 20 and Figure 21 confirm the ELISA data showing very little transport of IgG across the vaginal epithelium.

A. **B.**

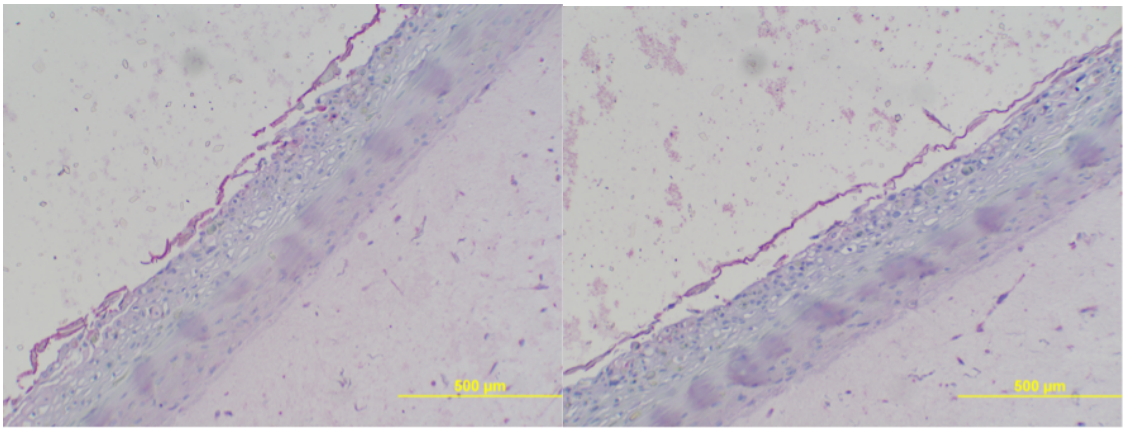
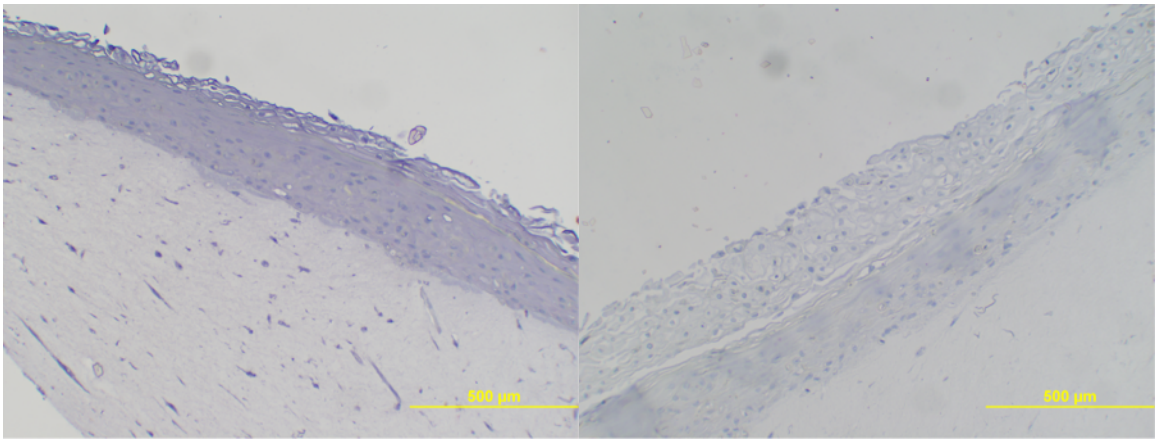


Figure 21. Anti-human IgG stain for VRC01-N. Panels A and B show two stains of the bright purplish-red stained VRC01-N concentrated in the superficial layers of EpiVaginal tissue model. VRC01-N does not penetrate below the stratum corneum of tissue.

Figure 22 presents immunohistology for tissue that was not exposed to IgG (negative control) showing that media does not contain IgG and also that the primary antibody used to detect the presence of IgG does not bind nonspecifically to the tissue. Figure 22C shows immunohistology of experiment loaded with Human-IgG as a positive control showing specific binding of primary antibody to IgG in the stratum corneum.

A. **B.**



C.

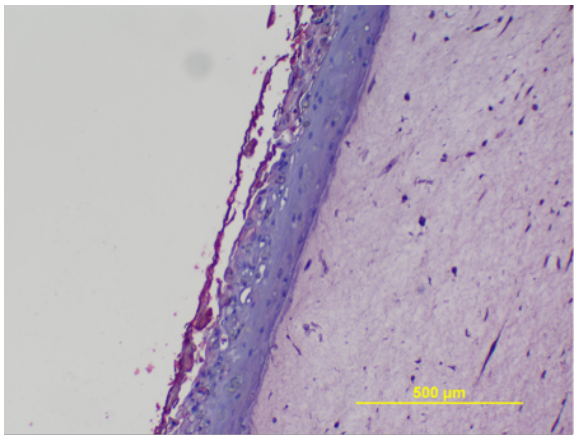


Figure 22. Immunohistology for experiment control and staining control. A. Immunohistology for experiment control and B. staining negative control do not show any non-specific staining of tissue. C. Positive control using human serum showing no penetration of human-IgG below stratum corneum in enzyme staining. (Human IgG shown by dark purple stain).

We also performed immunohistology to detect the IgG transport molecule, FcRn, in

EpiVaginal tissue, as was described in the report by Liu wet al (2011). We detected

FcRN in the basal epithelial cells, but not in cells higher in the epithelium.

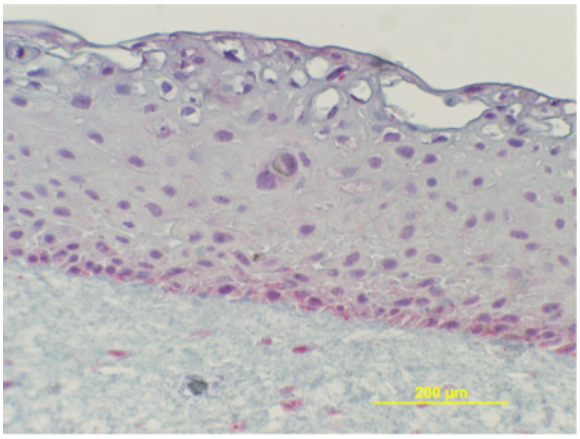


Figure 23. FcRn localization in MatTek EpiVaginal Tissue Model. Immunohistology shows FcRn staining (red) localized in the basal layer of epithelium above the lamina propria. The tissue is counterstained with hematoxylin. Photo courtesy of Dr. Jeffrey Pudney.

DISCUSSION

Recent strategies to block HIV transmission have been focusing on topical microbicides. As a part of a larger project, the specific aims of this thesis focused on investigating the efficacy of Mab-Ns VRC01 and 4E10-N in *in-vitro* and *ex-vivo* HIV infection assays. These studies aimed to characterize the efficacy of Mab-Ns in three separate experiments. In the first experiment, Mab-N neutralizing efficacy was assayed against cell-free virus. In the second experiment, Mab-Ns in CVLs were tested against cell-free virus. CVLs were collected after administration of Mab-Ns in varying concentrations to the vaginal lumens of Cynomolgus macaques for a specific duration of time. Lastly, the pharmacokinetics of Mab-Ns was studied on an EpiVaginal tissue model to insure its inability to penetrate the epithelial layer and enter into system circulation.

Our data show that the neutralizing MAb-Ns performed similarly to Mabs produced by traditional methods and support the continued development of *Nicotiana* derived MAbs in order to seek a more cost effective methods of production. In terms of retention of activity in the vaginal environment, after four hours the mAbs were able to neutralize free virus. However, beyond the four-hour time point antibody activity was reduced and in some cases the assay showed increased viral infection. The increased viral infection was not a result that was expected. This could possibly be attributed to TZM-bl's containing a higher ratio of CD4:CCR5 receptors rather than natural PBMC's which would be an inherent issue with the assay model. Unfortunately an ideal assay model that would better represent PBMCs without the variability of multiple donors does not exist.

The TZM-bl assay at this point in time offers the most consistency for carrying out neutralization assays.

There are some thoughts as to why increased viral infection is being observed in CVLs collected at the 24-hour and 72-hour time points, but not at the 4 hour time point. It is possible that the Mab-Ns are eliciting the release of immune response molecules such as proinflammatory cytokines from macaques that may be increasing viral infection. It is also possible that what we are interpreting as increased viral infection is actually and artifact. One possibility is that the CVLs collected at the 24- and 72-hour time points contain microflora that produce enhanced luminescence readings. These microflora could be missing from the 4-hour samples, because collection of baseline samples entails washing the vaginal cavity with saline. It is also possible that the data are evidence of a mechanisms known as antibody dependent enhancement of HIV-1 virus (Willey et al., 2011). Further research is needed to determine the reason for the observed viral enhancement at the 24- and 72-hour time points.

Our data show that 4E10-N, VRC01-N, and human-IgG will cross the MatTek vaginal epithelial model, but it occurs to a very small degree within a 3-hour time frame, and observed transfer at 24 hours could be due to nonspecific leakiness in the model. The ELISA data is confirmed by immunohistochemistry showing that Mab-Ns are not transferred across the tissue. The Mab-Ns did concentrate in the stratum corneum (top most layer) of the vaginal epithelium, but no evidence of internalization was observed. Based on vaginal epithelial histology, the stratum corneum is comprised of cells that have terminally differentiated to lack nuclei and organelles. Cells at this level are not expected

to contain cell functions able to transport antibody molecules. Furthermore, FcRn (the IgG transport molecule) was located only in the basal layers of the epithelium. Since it is not possible for Mab-Ns to penetrate to the depth of tissue where FcRn is expressed due to tight junctions between the cells, FcRn could not play a role in any active transport of IgG from the apical to basal direction. Our data provide evidence that Mab-Ns do not cross the vaginal epithelium and are safe to use as topical microbicides.

Future directions that stem from this project would be to test the ability of *Nicotiana* derived monoclonal antibodies to block SIV transmission in simian models that would best replicate human physiology. Upon success of those experiments, Phase 1 clinical trials to prove safety of the antibodies in women could be conducted, followed by Phase 2 and Phase 3 (clinical efficacy) trials. Potential applications include films, rings or other types of drug reservoirs that could slowly release the Mab-N cocktails without the need for frequently repeated application. This would negate the non-compliance issues of previous microbicide trials such as those that plagued the VOICE study, and offer easy use for consumers in an over the counter setting.

Conclusion

In summary, we have provided data supporting the efficacy of *nicotiana*-derived monoclonal antibodies in comparison to those produced by conventional methods. Data from cervicovaginal lavages obtained from macaques that had been treated with *nicotiana*-derived monoclonal antibodies showed efficacy in physiological conditions at the 4-hour time point. Our data strongly confirm that *nicotiana*-derived monoclonal

antibodies are not transferred across the vaginal epithelium in an apical to basal direction to potentially enter the systemic circulation. Our work supports the continued study and pursuit of *nicotiana*-derived monoclonal antibodies as a topical microbicide to prevent the transmission of HIV-1.

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VITA

CHIA-WEI THOMAS CHOU

20511 Pacific Dr., Walnut, CA 91789 **Year of Birth**: 1987
Email: thomasch@bu.edu

Education

Boston University
2011-2013: Masters of Arts in Medical Sciences

University of California San Diego
2005-2009: Bachelor's of Science in Biochemistry/Cell Biology

Certifications/Licenses

- Previously held California Pharmacy Technician License #: 101197

Work Experience

Research Experience

Scripps Institute Internship under Dr. Jegla March '08 – Sept '08

- Duties included Transforming and growing colonies to extract DNA. Extracting DNA and digesting and ligating nucleic acids samples for use in ion channel studies. Basic lab duties included bench maintenance and supply, solution supply maintenance.

Employment

Contractor Administrative Assistant March '10 – Oct '10

- Duties include translating between Mandarin and English for Client and Contractor. In order to ensure smooth project transition a schedule had to be made and adhered to prevent delays on site. Client had to be educated on project progress and choice of materials to prevent unexpected delays.

Human Resources Assistant, Panda Restaurant Group, Rosemead, CA Summer, 2005/06

- Duties involved processing Employee Applications using AS400 System and organizing human resources documents for mailing and receiving. Documents processed included confidential employee benefit forms as well as facilitating bank sensitive direct deposit bank requests between employees and accounting department.

Administrative Assistant, Panda Restaurant Group, Rosemead, CA Summer, 2005/06

- Duties involved included revamping Postal communications between the support center and stores through FedEx. An entirely new set of accounts and mailing supplies was established in order to be able to update postal supplies for all stores.

Language Skills

- **English** - Fluent in Reading, Writing, and understanding in English – **Mandarin**, Advanced capability in speaking, Moderate Reading and Writing – **Taiwanese (Hokkien)** – Fluent in speaking and understanding – **German** – Basic level of reading, writing and understanding

Volunteering Work

Clinical Shadowing

- UCSD Health Professional Mentor Program January ’09 - June ‘09
- Scripps Clinic Carmel Valley

AID Summer Program Summer ‘07-‘08

- My job was to analyze student capabilities and devise a teaching plan that would help expose rural students to English. Original methods and applications of introducing English had to be devised that deviated from traditional methods used in local settings.

Kaiser Permanente Volunteer, Baldwin Park, CA

- Organize Paperwork in Various Departments Spring 2004 – Fall 2005