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Topical use of monoclonal antibodies as a multipurpose prevention technology offering contraception and decreased transmission of HIV-1 and trichomonas vaginalis

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Dissertation

**TOPICAL USE OF MONOCLONAL ANTIBODIES AS A MULTIPURPOSE
PREVENTION TECHNOLOGY OFFERING CONTRACEPTION AND
DECREASED TRANSMISSION OF HIV-1 AND *TRICHOMONAS
VAGINALIS***

By

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B.S., Davidson College, 2013

Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

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DEDICATION

This work is dedicated to the large group of people in my life that lived this “grad school chapter” alongside me and have helped me grow and thrive during this time. In particular, I would like to dedicate this work to the amazing Manuel E. Baldeón and Mónica Vaca, my dad and mom, whose unwavering support, love, and belief in me could be felt all the way from Ecuador. I would also like to include Alexis Baldeón and Raquel Baldeón, the siblings who both tested and maintained my sanity, but who, overall, made Boston feel like home. These pages would not have been possible without you.

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I've always heard the saying, "it takes a village". In my case, it took a dedicated army of incredible people to get me where I am today.

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through my highest highs and lowest lows. And naturally, to my family and family-like friends, Rosie Kosinski and Tina Lisk, thank you for listening patiently, cheering loudly, and loving unconditionally.

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ABSTRACT

New transgenic antibody production platforms enable cost-effective, rapid manufacturing of antibodies for clinical applications. Our lab is investigating a human monoclonal antibody, the Human Contraceptive Antibody (HCA), produced in *Nicotiana benthamiana*, as a candidate for a topical Multipurpose Prevention Technology offering both contraception and protection against sexually transmitted infections. HCA was developed from a sperm-agglutinating antibody isolated from plasma cells of an infertile woman. The antibody targets a GPI-anchored glycoprotein, CD52g, produced specifically by epithelial cells in the male reproductive tract (MRT). Due to its GPI anchor, the hypermobile

protein coats sperm as they migrate through the MRT. In this study, we tested HCA's specificity and contraceptive properties under physiologically relevant conditions *in vitro*. We demonstrated that HCA quickly and potently agglutinates sperm in physiological conditions at concentrations $>6.25 \mu\text{g/mL}$. Sperm concentration, soluble CD52g found in seminal plasma/whole semen, or prolonged exposure to the low pH found in the vaginal tract did not affect agglutination time. We also determined that CD52g incorporates into other cells present in the MRT, including human immunodeficiency virus (HIV-1) infected cells (lymphocytes, macrophages) and the parasite, *Trichomonas vaginalis* (TV), and that HIV virions produced from CD52g-coated cells incorporate CD52g in the virus particle membrane. HCA alone did not agglutinate HIV or TV. Both pathogens, however, appeared to co-agglutinate with sperm when co-cultures were treated with HCA. The trapping of pathogens in sperm agglutinates resulted in decreased TV adherence to MatTek tissue and vaginal epithelial cells, and a modest neutralization of HIV-1 in TZM-bl assays. These data indicate that HCA is a promising candidate to achieve contraception and decrease the male-to-female transmission of STI pathogens.

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LIST OF ABBREVIATIONS

ADCC.....	Antibody dependent cell-mediated cytotoxicity
ADCP.....	Antibody dependent cellular phagocytosis
AIDs.....	Acquired immunodeficiency syndrome
AMPs.....	Antimicrobial peptides
APC.....	Antigen presenting cell
ARV.....	Antiretroviral
BCA.....	Bicinchoninic acid assay
BCE.....	Before common era
bnAbs.....	Broadly neutralizing antibodies
C'.....	Complement
CDC.....	Center for disease control
CHO.....	Chinese hamster ovary
CVL.....	Cervicovaginal lavage
DC.....	Dendritic cells
EV.....	Ebola virus
FRT.....	Female reproductive tract
FTC.....	Emtricitabine

GFP.....	Green fluorescent protein
GPI.....	Glycosyl phosphatidyl inositol
HCA.....	Human Contraceptive Antibody
HepB.....	Hepatitis B
Hex.....	Hexamer
HIV-1.....	Human immunodeficiency virus
HPV.....	Human papilloma virus
HSV.....	Herpes simplex virus
INSTI.....	Integrase strand transfer inhibitor
IPCP-HTM.....	Integrated Clinical/Pre-clinical program
IPM.....	International Partnership for Microbicides
IVR.....	Intravaginal ring
IUD.....	Intrauterine device
KBP.....	Kentucky BioProcessing
LA.....	Lactic acid
LARC.....	Long acting reversible contraceptives
LPS.....	Lipopolysaccharide
mAbs.....	Monoclonal antibodies

MAC.....	Membrane attack complex
MIC.....	Minimal inhibitory concentration
MHM.....	Multipurpose handling media
MPT	Multipurpose prevention technology
MRT.....	Male reproductive tract
NK	Natural Killer
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI.....	Nucleoside reverse transcriptase inhibitor
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PI.....	Protease inhibitors
PrEP	Pre-exposure prophylaxis
RIPA.....	Radioimmunoprecipitation assay buffer
RT	Room temperature
SC.....	Sperm
Se.....	Semen
SEM	Scanning electron microscopy
SHIV.....	Simian human immunodeficiency virus

SIV _{mac}	Simian immunodeficiency virus
SP	Seminal plasma
STI.....	Sexually transmitted infections
T-DNA	Transfer DNA
TDF.....	Tenofovir
TEM.....	Transmission electron microscopy
T/F.....	Transmitter- Founder
TLR.....	Toll-like receptor
TV	<i>Trichomonas vaginalis</i>
TZ	Transformation zone
UN	United Nations
US	United States
VEC	Vaginal epithelial cells
WHO	World Health Organization
ZV.....	Zika virus

Chapter 1 - Introduction

Humans have long set themselves apart from the rest of the animal kingdom through a variety of behaviors and unique physiology; one notable example is reproduction. Female humans are regularly fertile throughout the year, and consequently, we have been actively developing ways to prevent conception. Evidence for contraceptive methods dates to the early BCEs ranging from spermicides in Egypt—where honey, sodium carbonate, and crocodile dung were mixed and applied intravaginally—to the regular intake of lead and mercury based drinks in China ^{1,2}. These contraceptives ranged from benign but ineffective, to extremely dangerous; they also did not protect against sexually transmitted infections (STIs). In fact, there is only one contraceptive product on the market to date that protects from STIs: the condom ³.

The male condom is perhaps the most well-known contraceptive method with early forms being documented throughout history. A version of the condom is mentioned in the *Iliad* where King Minos used a bladder of a goat to prevent transmission of disease as his semen contained “serpents and scorpions” that led to his queens’ illnesses and deaths ⁴. This simple story illustrates the condom’s significant public health value: contraception and disease protection.

Unsurprisingly, the condom was embraced early on by both sex workers and clients in brothels. Even the Italian author Giacomo Casanova recognized that, despite the discomfort due to the presence of the condom, it prevented the contraction of dangerous diseases like syphilis, which can lead to dementia, paralysis, and death if left untreated^{4,5}. The condom reached its current form in 1885 after the advent of rubber enabled its mass production⁴. Since then, materials have changed, lubricants have been added, and quality control has improved, but the condom's original form has not undergone significant innovation⁶; it also remains the only reversible contraceptive product on the market available for males. In contrast, female-centered contraceptive products underwent a significant revolution in the 20th century resulting in over 10 female-centered products by 2009^{1,3,7}. There are both on-demand and long acting reversible contraceptives (LARCs), many of which regulate menses hormones, but only the female condom, like the male condom, protects from STIs^{3,8}. Despite the large number of contraceptive products available, over 40% of pregnancies worldwide are unplanned, and recently the global prevalence of STIs has increased⁹⁻¹². Both trends indicate a need for a multipurpose prevention technology (MPT) product that offers both contraception and STI protection.

The Contraceptive Gap

Public Health Context

According to a meta-analysis, approximately 40% of pregnancies worldwide are unintended. The trend is reflected in countries in both the developing (39%) and developed (47%) world hinting at a global phenomenon reaching beyond resource and education disparities¹³. A rapidly growing population, concerns about climate change, and subsequent concerns about available environmental resources have made the need for family planning an even greater priority⁷. According to recent United Nations World Population Prospects, the global population is currently 7.5 billion, and median projections suggest that the world could reach 10 billion by 2050, if fertility and life expectancy rates remain consistent^{14,15}. Should fertility rates vary by even +0.5 children, the population could reach 11 billion^{16,17}. Additionally, data shows that 214 million women of reproductive age in the developing world wishing to avoid pregnancy do not have access to modern forms of contraception^{18,19}. In fact, recent United Nations (UN) estimates indicate that in some countries, only 60% of contraceptive needs are met¹⁹ (Figure 1-1). The lack of contraceptive resources

hampers people's reproductive right to freely decide if and when to have children¹⁹.

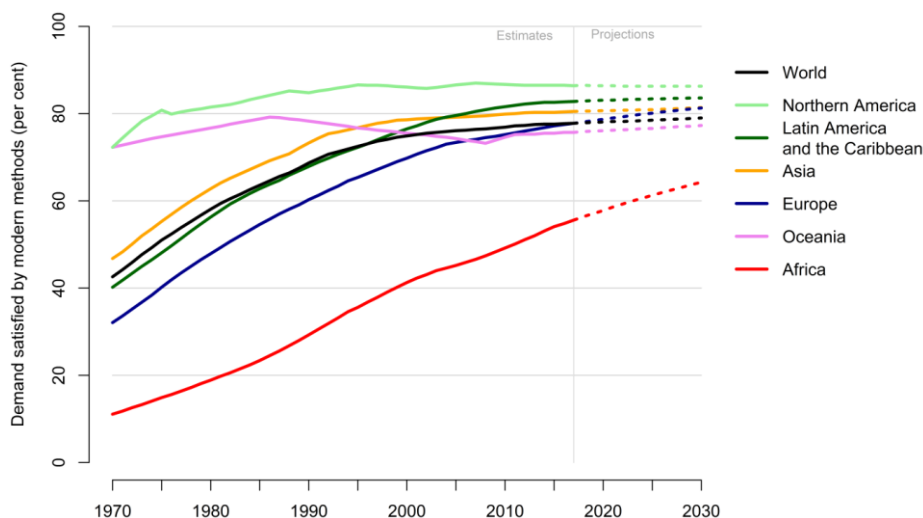


Figure 1-1- Contraceptive demand satisfied by modern methods (percentage), by region. From *World Family Planning 2017 – Highlights*, by United Nations, Department of Economic and Social Affairs, Population Division. ©United Nations 2017. Reprinted with the permission of the United Nations

The Accessibility Gap

Accessibility, both physical and economical, can impact the public health contribution of contraceptives. A lack of accessibility is currently the limitation with most products on the market: over half of the current contraceptives require a medical professional to prescribe or place the prophylactic^{3,10}. In places where medical attention is scarce or where healthcare facilities that dispense contraceptives require extensive travel, acquiring contraception may not be an option^{10,19,20}. A study in Bangladesh found women are significantly less likely to

use contraceptives if they must travel for more than 30 minutes to acquire a product^{10,21}. In contrast, contraceptive accessibility, achieved by decreasing the cost of contraceptive and making LARCs readily available, was found to decrease unintended pregnancies. The CHOICE study, specifically, found a 4-fold decrease in adolescent pregnancy rates in St. Louis, Missouri, United States (US)²²⁻²⁵ when LARCs were made directly available to this young population, providing evidence for the need of a low-cost, over-the-counter, contraceptive.

The Side Effect Gap

In places where contraceptives can be readily acquired, side effects are an additional barrier to contraceptive use. Most products available are hormonal regulators of menses—estrogen and progesterone analogs prevent the cycling of the follicle-stimulating hormone and the luteinizing hormone, thus preventing ovulation and changing the viscosity of cervical mucus preventing sperm passage.²⁶ The systemic administration of hormones, while highly effective at preventing pregnancy (90-99.8% effective)^{3,27}, has the potential to induce mild to severe side effects including headaches^{28,29}, modified libido²⁷, and an increased risk of cardiovascular problems and deep vein thrombosis^{30,30-32}. These side effects are largely eliminated with a Levonorgestrel-intrauterine device (IUD)

that is placed in the uterus and releases hormones locally causing fewer side effects³³. Non-hormonal options also exist but these are often less effective, such as the use of diaphragms and fertility-awareness based methods, or require medical placement, such as copper IUDs or implant placement³⁴. The increased use of Levonorgestrel-IUDs in places where it is available is demonstrative of the need for a non-systemic, reversible, contraceptive.

The Discretion Gap

A final barrier to the use of contraceptives is discretion¹⁰. Some contraceptives are not readily accepted worldwide or their use may have stigma attached. A partner's input or permission may be required to use contraceptives and teenagers may require parental approval limiting access in a sexually active population^{10,19,20,35-37}. The lack of discretion is the greatest limitation of the male and female condom. The condom is part of basic family planning programs due to wide availability, accessibility, and ease-of-use^{9,38,39}. As an on-demand barrier method, it has no side effects. Nonetheless, its use requires both partners' consent, and it is often avoided due to reported decreased pleasure during coitus^{6,35,40,41}. Despite the lack of discretion, it is the only prophylactic that provides protection against both unplanned pregnancy and STIs. Consequently,

there is market demand for an additional easy-to-use, accessible, cost-effective, discreet, non-barrier contraceptive that also protects from STIs.

The Microbicide Gap

The condom is a physical barrier that does not permit passage of sperm or STI pathogens into the male or female reproductive tract. In addition to physical hindrance, STI transmission can also be curbed through the use of microbicides. Microbicides are small molecules or biologics that when applied in the vagina or rectum can prevent the transmission of disease by killing or neutralizing infectious pathogens. Currently, there are no effective microbicides on the market, but their absence on shelves is not due to a lack of research.

Lessons from Previous Microbicide Development

The year 1992 signaled the start of determined research for safe and effective microbicides targeting the human immunodeficiency virus (HIV), gonorrhea, and chlamydia. Several microbicide research groups such as the International Working Group on Microbicides and the International Partnership for Microbicides were formed, and various new compounds were tested (Table 1-1).

Table 1-1-Microbicide compounds tested in clinical trials⁴²⁻⁴⁹

Microbicide Compound	Agent Classification	Pathogen Target	Phase III clinical trials	Result
Nonoxynol-9	Surfactant	HIV-1 Chlamydia	Yes	Failed
Pro2000	Polyanion	Broad	Yes	Failed
Ushercell	Polyanion	HIV-1	Yes	Failed
BufferGel	Acidifier	Broad	Yes	Failed
Carraguard	Polyanion	HIV-1 HPV	Yes Yes	Failed (62% protection)
Lactobacillus suppositories	Acidifier/biologic	HIV-1	No	63% protection in macaques
Amphora/Acidform	Acidifier	<i>Neisseria gonorrhoeae</i> HSV	Yes but for contraception	Protection in animal models
SAVVY (C31G)	Surfactant	HIV-1	Yes	Failed
CAPRISA/VOICE	Antiretroviral (Tenofovir and Truvada)	HIV-1	Yes	Failed due to poor adherence
RING/ASPIRE	Antiretroviral (Dapivirine)	HIV-1	Yes	Protected up to 61% in women

Microbicide development has focused on either mimicking/strengthening the FRT's natural immune defense, inhibiting pathogen interaction with its target cell, or directly affecting pathogen viability and reproduction^{43,45,49,50}. BufferGel, Acidform (amphora), and *Lactobacillus* suppositories, were intended to maintain

the vaginal tract's protective low pH^{43,49,51-53}. Moench, *et al* (2005) demonstrated that BufferGel at a pH 4.0-4.5 decreased HIV-1 infected PBMCs viability to 4% in less than 10 minutes *in vitro*, even in pH-neutralizing seminal plasma⁵².

Unfortunately, while both BufferGel and Acidform passed Phase I and II safety trials⁵⁴, they were not found to significantly prevent STI transmission in Phase III trials. Similarly, polyanion microbicides, such as Pro2000, were found to interact with positively charged surface proteins on pathogens and cells disrupting electrostatic interactions. HIV-1's envelope protein, gp120 has a positively charged variable loop (V3) responsible for binding to the CD4 receptor on its target cell, the first step in the virus' replication cycle^{55,56}. *In vitro*, Pro2000 successfully disrupted V3-CD4 interaction, and subsequently protected macaques infected intravaginally with simian-HIV (SHIV; SIV expressing HIV-1 envelope)^{57,58}. But, Phase III trials were unsuccessful; SHIV relies on CXCR4 as co-receptor to establish infection, and most *in vitro* and pre-clinical trials with Pro2000 used R4-tropic virus. Sexually transmitted HIV-1 isolates which have a negatively charged or neutral V3 loop⁵⁹, are predominately R5-tropic viruses requiring CCR5 as the co-receptor,. While Phase III clinical trials were

unsuccessful, Pro2000 highlighted the importance of mimicking physiologically relevant conditions in microbicide development⁵⁸.

Nonoxynol-9 (N-9), a well-known surfactant and spermicide, was one of the first broad-spectrum microbicide tested for targeting HIV-1, chlamydia, *Trichomonas vaginalis*, and gonorrhea^{49,60}. N-9 disrupts the plasma membrane of cells. In the case of sperm cells, it leads to their immobilization and death. Similarly, N-9 disaggregates the envelope of HIV-1 and other pathogens with phospholipid membranes rendering the organisms neutralized^{46,61}. *In vitro*, N-9 achieved HIV-1 neutralization at concentrations of 1-10 µg/mL⁴⁹. Furthermore, when the microbicide was tested on macaques infected intravaginally with Simian Immunodeficiency Virus (SIV_{mac})⁶² or chlamydia⁶², N-9 successfully prevented infection. In stark contrast, N-9's failure in Phase III clinical trials found an increased transmission of STI transmission, including HIV-1 and gonorrhea^{60,63} due to N-9s cytotoxicity^{46,60,63}. The surfactant led to the formation of ulcers and the disruption of epithelial cell integrity, the vagina's primary form of protection, with repeated use^{61,64,65}. Various other broad-spectrum microbicides have followed suit, failing in Phase III clinical trials mostly due to a lack of

efficacy. In recent years, microbicide research has resurfaced, with a particular focus on HIV-1.

Current Microbicide Development

Microbicide research has recently focused on the use of antiretrovirals (ARVs) to prevent sexual transmission of HIV-1, specifically male-to-female sexual transmission. ARVs prevent retroviruses from replicating by targeting key steps in the viral replication cycle⁵⁵, and the term is primarily used to describe anti-HIV-1 drugs. The recent CAPRISA/VOICE and RING/ASPIRE studies described below have used HIV-1-specific ARVs as microbicides, administered with distinct delivery systems, but have had limited success.

The CAPRISA 004 Phase IIb clinical trial tested the efficacy of 1% Tenofovir gel, administered intravaginally, in preventing sexual transmission of HIV-1. Tenofovir (TDF) is a nucleotide reverse transcriptase inhibitor (NRTI). While the study found a decrease to up to 54% in HIV-1 acquisition in trial participants, this level of protection was only found in women with high levels of adherence^{47,66}. Similar results were found in the VOICE trial, which had over 5000 participants in three African countries, and compared the protection offered by daily oral use of antiretrovirals TDF and Truvada (a combination of

Emtricitabine (FTC) and TDF), and 1% TDF gel. No significant difference in protection was found between treatment groups and the placebo group due to the participants' low adherence to the products⁶⁷. In contrast, the Partners Pre-Exposure Prophylaxis (PrEP) study—which enrolled serodiscordant heterosexual couples from Kenya and Uganda and investigated the prophylactic use of once-daily TDF and TDF-FTC in preventing the transmission of HIV-1, found a 67-75% decrease of HIV-1 incidence in participants with high adherence in both TDF and Truvada groups⁶⁸. Additionally, there was little evidence for developing therapeutic resistance with daily use of these ARVs^{47,67-69}. Altogether, these early ARV trials provided valuable evidence for the use of ARVs as microbicides, and also brought to light the importance of product acceptability and adherence in microbicide development.

The RING and ASPIRE trials emphasized the need for product acceptability suggesting that effective microbicides must have similar criteria to contraceptives: they must be accessible, discreet, and convenient for consistent use^{70,71}. The RING and ASPIRE are “sister” trials that tested the microbicide efficacy of Dapivirine, a non-nucleoside reverse transcriptase inhibitor (NNRTI), when administered with a silicone intravaginal ring (IVR)⁷²⁻⁷⁴. While adherence

in the ASPIRE trial was inconsistent, there was a 56% increase in protection to HIV acquisition in groups with high adherence. Altogether, the CAPRISA and RING/ASPIRE trials suggest that intravaginally administered, topically applied Dapivirine offered microbicide protection against HIV-1 with strict adherence. These results are reflected in the subsequent HOPE and DREAM studies in which trial participants were permitted to choose their microbicide product. 95% of women in the DREAM study opted for the IVR, and mathematical modeling (due to a lack of an adequate placebo group in these open extension studies) suggest HIV-1 incidence was reduced by 63% in this group^{75,76}. The IVR is both discreet and convenient to use and, as study participants in the RING and ASPIRE studies inserted their own ring, is accessible without a medical professional.

Overall, these Phase II and III trials provide evidence that topical use of ARV drugs can prevent sexual transmission of HIV-1; nevertheless, the need for more broad spectrum microbicides has become pressing as STIs are globally on the rise with more than 1 million new infections occurring each day worldwide⁷⁷. A renewed interest in microbicidal research is also pressing as global incidence of antibiotic resistance increases, limiting treatment options⁷⁸⁻⁸¹. An ideal

microbicide would therefore be accessible, discreet, designed for easy adherence and effective against multiple pathogens.

Vaginal Histology and Immunity

The female reproductive tract (FRT) is a mucosal tissue with complex histological structures, and a strong reliance on both innate and adaptive immunity. The lower FRT, consisting of the vagina and ectocervix (Figure 1-2), must balance protection against pathogens with the safe passage of sperm, and maintenance of commensal microbiota- which are crucial to FRT immunity and health⁸²⁻⁸⁴. The primary form of protection in the lower FRT is the formidable epithelial barrier consisting of a thick stratified squamous epithelial layer approximately 25 cells thick (150-200 μm)^{83,85}. The epithelium is also known to be immunologically active, expressing varying levels of extracellular toll-like receptor (TLR)s 1-6, and intracellular TLR-9 that recognize pathogen associated molecular patterns (PAMPs) on bacteria, protozoa, fungi, and viruses^{86,87}. In response to pathogen recognition by these TLRs, the cells secrete pro-inflammatory cytokines, such as anti-viral interferons, chemokines, such as MCP-1, and anti-microbial peptides (AMPs)⁸⁸. AMPs are particularly prevalent in the lower FRT and consist of defensins, cathelicidins, protease inhibitors,

lysozymes, and lactoferrin, all of which inhibit or neutralize pathogens. AMPs are stored in lamellar bodies and secreted by metabolically active epithelial cells in response to PAMP recognition⁸⁵. AMPs are particularly concentrated on the apical layer of the vaginal epithelium: the stratum corneum.

While the basal layer of the vaginal epithelium is metabolically active with tight junctions contributing to the integrity of the stratified barrier^{85,88}, as the epithelial cells move towards the vaginal lumen they differentiate into the stratum cornea (Figure 1-3). Epithelial cell differentiation and cornification results in the extrusion of the epithelial cells' nuclei and organelles and increases glycogen stores in the cells^{85,89,90}. The cornification of the epithelial layer also leads to a loss of tight junctions, permitting the passage of small molecules⁸², and protecting the tissue from abrasion during intercourse⁸². The loosely joined cornified cells are a double-edged sword and could enable the penetration of small pathogens. As a result, in addition to the AMP stores in the stratum corneum and the TLRs found in the basal epithelium, mucins (and the resulting mucus) contribute another layer of protection to the epithelial barrier. Mucins are O-linked glycoproteins that are secreted or associated with the plasma membrane of epithelial cells in the vaginal tract and other mucosal tissues^{91,92}.

They interact with antibodies and trap pathogens and immunocomplexes while also contributing to lubrication and pH buffering of tissue⁹¹⁻⁹⁴. Finally, the glycogen stored in the stratum corneum supports a final component in the vaginal tissue's immune protection: the microbiota. The vaginal microbiota, usually dominated by *Lactobacillus sp*⁹⁵, produce lactic acid (LA) lowering the vaginal pH to ~3.5 pH⁹⁶⁻⁹⁸. Both the low pH, as shown with the BufferGel *in vitro* studies⁵², and the lactic acid itself are shown to have virucidal and microbicidal effects⁹⁹. Additionally, the same bacteria produce hydrogen peroxide as a metabolite at concentrations shown to neutralize viral and bacterial pathogens *in vivo*, providing an additional line of defense^{83,100}. Microbiota dysbiosis, similarly, has been correlated with decreased epithelial integrity and increased infection of sexually transmitted pathogens^{95,101}.

The epithelial barrier, mucosal layer, and microbiota efficiently protect the FRT from pathogen penetration, however, cells also play a crucial function in immune protection. In the lower FRT, innate immune cells predominate. Natural Killer (NK) cells, $\gamma\delta$ T cells, neutrophils, dendritic cells (DCs), macrophages, and Langerhans cells line the basal layer of the epithelium and the lamina propria^{82,88,102}. Antigen presenting cells (APCs), such as dendritic cells and

macrophages, actively play a role in pathogen detection since their projections extend into the vaginal lumen and sample the vaginal microenvironment^{103–105}. While adaptive immune cells are scarcer in the FRT, T cells are found in the lamina propria of the vaginal tract with CD4+ T cells, B cells/plasma cells, and secreted antibodies contributing to immune protection closer to the ectocervix^{102,106}.

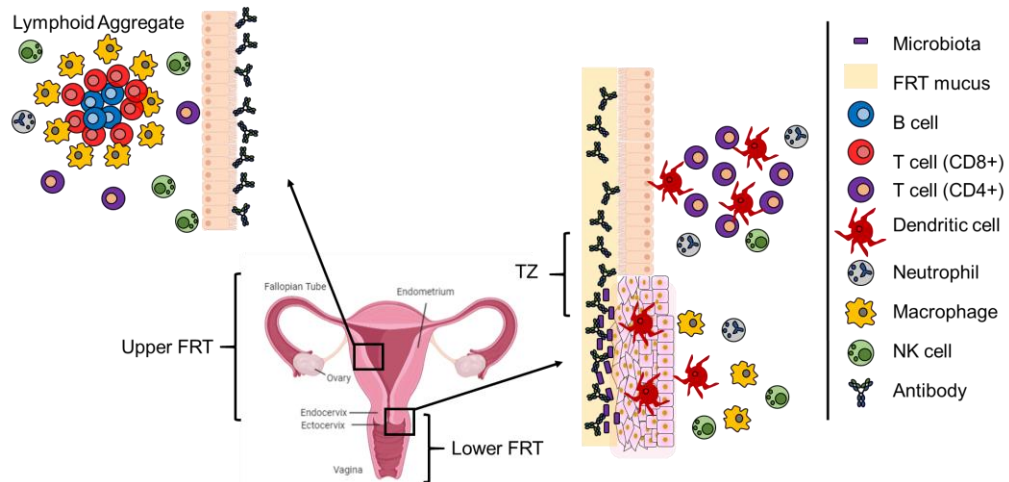


Figure 1-2-Schematic of female reproductive tract histology and immunology. Adapted from Ngyuen, (2014). *Nature* with BioRender

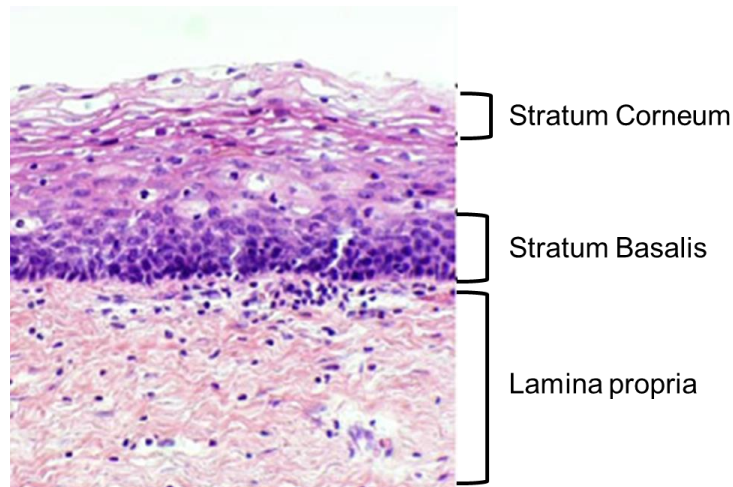


Figure 1-3-Vagina epithelium histology. Hematoxylin and eosin staining of vaginal tissue. Adapted from Anderson, 2014.

The upper reproductive tract consists of the fallopian tubes, uterus, endometrium and endocervix (Figure 1-2), whose primary function is to facilitate the passage of sperm cells and maintain a semiallogenic fetus, while also protecting against infection. The upper FRT consists of a monolayer of columnar epithelial cells relying heavily on intracellular adherence molecules, tight junctions, and desmosomes to maintain tissue integrity and prevent the unregulated passage or penetration of small molecules and pathogens from the lumen¹⁰⁷. The endocervix, additionally, is known to have a ciliated epithelium known to mechanically carry cervical mucus towards the ectocervix and vaginal lumen. The endocervix, like the lower FRT actively secretes mucins, and studies

have found a higher concentration and diversity of mucins in the endocervix compared to those found in the vaginal tract (Mucins 1-6 vs Mucins 1 and 4 in the vagina). Reflecting the dichotomy of the upper FRT function, the mucins and mucus not only prevent the continued advance of pathogens from the lower FRT, but also play a crucial role in facilitating the passage of sperm into the uterus^{91,92,108,109}.

Both innate and adaptive immune cells line the lamina propria of the upper FRT and Table 1-2 summarizes their distribution. However, the extensive nuance in both the protection and immunotolerance mediated by these immune cells is beyond the scope of this dissertation. As a brief overview: IgG and IgA secreting plasma cells in the endocervix are responsible for antibody production in the FRT^{82,88,102,110}. The mechanotransduction of mucus from the cervix also serves to move antibody to the lower FRT¹⁰² contributing to pathogen protection in the vagina, as do the macrophages and neutrophils present throughout the FRT. NK cells, in contrast, found in both the endocervix and endometrium illustrate the diverse functionality of immune cells; while NK cells are responsible for antibody dependent cell cytotoxicity along the FRT, they also mediate implantation and tolerance of the semi-allograft fetus in the

endometrium^{82,111}. Finally, lymphoid aggregates, composed of T cells and B cells surrounded by macrophages, are characteristic of the uterine endometrium, and are not found anywhere else in the FRT. Their size and cell composition, much like the endometrial lining, varies according to the menses cycle, highlighting, the role hormones play in the FRT immune system, thoroughly discussed in Wira *et al*, (2015) and Taneja, (2018)^{102,110,112,113}.

The upper and lower tract are connected by the transformation zone (TZ) where the stratified squamous epithelium of the ectocervix transitions to the columnar epithelial monolayer of the upper FRT. The TZ, consequently, is particularly vulnerable to pathogens, with Draper *et al*. (1980), using scanning electron microscopy to show that *Neisseria gonorrhoeae* preferentially attached to the TZ compared to the stratified squamous epithelium or endocervical epithelium¹¹⁴. While mucins and antibodies continue to offer luminal protection in the TZ, the thick epithelial barrier characteristic of the lower FRT is absent¹¹⁵. This is accompanied by an increased population of adaptive immune cells including CD8+ T cells and CD4+ cells. While these lymphocytes provide an additional immunological barrier, CD4+T cells are target cells for HIV-1. Transcytosis of virus particles has been shown in monolayers of columnar

endometrial epithelial cells. Gupta *et al* (2013) demonstrated that HIV-1 specific IgG antibody increased the transcytosis of infectious virions across the epithelial barrier at neutral or low pH¹¹⁶. Transcytosis at the TZ and the increased prevalence and close proximity of CD4+ cells in the area make this tissue particularly vulnerable to the sexual transmission of HIV-1^{106,117}.

Table 1-2- Immune cells in FRT tissues^{106,118-120}

Upper reproductive tract	
Tissue	Immune cells
Fallopian tube	granulocytes, NK cells, macrophages, B cells, T cells
Endometrium	granulocytes, mast cells, NK cells, macrophages, B cells, T cells
Endocervix	granulocytes, NK cells, macrophages, B cells, T cells
Upper reproductive tract	
Ectocervix	granulocytes, NK cells, macrophages, dendritic cells, B cells, T cells
Vagina	granulocytes, NK cells, macrophages, B cells, T cells

Sexually Transmitted Pathogens

Global Health Statistics

Sexually transmitted diseases have been on the rise in recent years prompting global concern. There are more than 30 different pathogens known to be sexually transmitted worldwide including bacteria, viruses, and parasites/protozoa. The eight most prevalent have been grouped into four

“curable” infectious pathogens: *Treponema pallidum* (Syphilis), *Neisseria gonorrhoeae* (Gonorrhea), *Chlamydia trachomatis* (Chlamydia), and *Trichomonas vaginalis*; and four “incurable” viral pathogens: herpes simplex virus (HSV), human papillomavirus (HPV), hepatitis B virus (HepB), and the human immunodeficiency virus (HIV)^{77,121}. Pathogens considered “curable” can be cleared from the male and female reproductive tract with the help of antibiotics; although gonorrhea has recently made public health headlines due to the increased prevalence of antibiotic resistant strains^{79,122}. Altogether, the WHO estimated in 2016 that over 376 million people were infected with one of these four treatable pathogens (Table 1-3)^{77,121}. In contrast, while antiviral therapies exist for the four most prevalent viruses listed in Table 1-3, they are not curative. Rather than facilitate pathogen clearance, treatment for these viruses controls the progression and manages the morbidity of chronic infection. Approximately 1.2 billion people are estimated to be infected with at least one of these prevalent viral pathogens^{77,121}. The rate of infection and the high global prevalence of these pathogens indicate the need for prophylactics to curb the spread of disease, especially for those without cure. Preventing infection is preferable to treatment:

these STIs can be asymptomatic resulting in chronic infection and morbidity, or access to treatment could be limited for some populations^{121,123}.

Table 1-3-Most prevalent sexually transmitted pathogens^{77,121,124}

Pathogen	Prophylactic/Treatment	Global Burden
Curable/non-viral STIs	Responsible for 376 million infections per year	
* <i>Trichomonas vaginalis</i>	Metronidazole/Tinidazole	156 million cases
<i>Treponema pallidum</i>	Penicillin	6.3 million cases
* <i>Neisseria gonorrhoeae</i>	Ceftriaxone/Azithromycin/ Doxycycline	87 million cases
<i>Chlamydia trachomatis</i>	Azithromycin/Doxycycline	127 million cases
Incurable/non-viral STIs	~1.2 billion people infected	
Hepatitis B	Vaccine available	240 million infected
Herpes Simplex Virus	Acyclovir/Famciclovir/ Valacyclovir	500 million infected
HIV	NRTI/INSTI/NNRTI/PI	36.9 million infected
Human papillomavirus	Vaccine available	290 million infected

*Demonstrated antibiotic resistance

NRTI=nucleoside reverse transcriptase inhibitor/ INSTI=integrase strand transfer inhibitor/NNRTI=non-nucleoside reverse transcriptase inhibitors/PI=protease inhibitors

STI Epidemiology and Impact

STIs disproportionately affect certain populations. Women are generally more susceptible to acquiring STIs than their male counterparts (Figure 1-4A)³⁹.

Despite the robust immune response mounted by the FRT, it is often not enough

to clear STIs and reinfection is not uncommon¹²⁵⁻¹²⁷. Furthermore, STIs can be asymptomatic: approximately 70% of women with chlamydia and trichomoniasis are unaware they are infected. Asymptomatic chronic infections in females can cause pelvic inflammatory disease, pre-term delivery, and infertility. If STIs remain undetected during pregnancy, the infection can be passed on to infants during parturition^{77,121,128}. STIs are also more prevalent among certain minority, racial, and ethnic groups in the US (Figure 1-4B)³⁹. The CDC tracks three STIs at a national level and federally require notification of new cases: chlamydia, syphilis, and gonorrhea. Recent CDC data have indicated that the rate of infection of all three of these STIs is on the rise (Table 1-4) with a record breaking 2.3 million new cases occurring in 2018^{12,129,130}. The rise of STIs is a public health concern because of the increased incidence of antibiotic resistance and the morbidity that accompanies infection.

Table 1-4- Rates of rising cases of CDC reportable STIs

Sexually Transmitted Pathogen	No. of Cases in 2017	Percent increase since 2016
Chlamydia	1,708,569	6.9
Gonorrhea	555,608	18.6
Primary and Secondary Syphilis	60,644	10.5

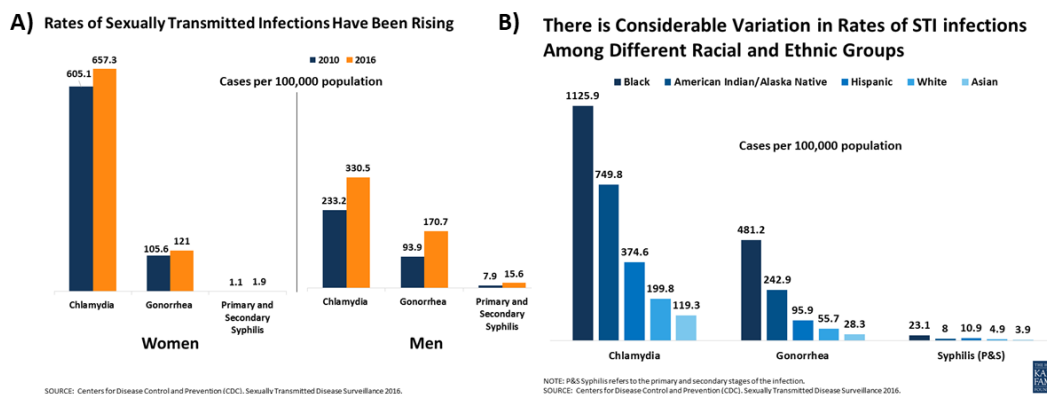


Figure 1-4- USA populations susceptible to STIs. A) Rates of sexually transmitted infections in male and women populations from 2010 and 2016. B) Rates of STI infections amongst different races and ethnicity in the United States. Figures adapted from The Kaiser Family Foundation Report: *Payment and Coverage for the Prevention of Sexually Transmitted Infections (STIs)*. Licensed under the Creative Commons License.

The widespread prevalence of STIs and the consequent morbidity has severe economic impact. Disability-adjusted life-years (DALY) are used to measure the health impact of diseases and chronic infection by quantifying the loss of health due to the disease. DALYs include years of life lost and years of life lived with disease-related disability/morbidity. According to recent data, the eight most prevalent STIs (not including HIV-1) are accountable for 12 million DALYs worldwide, or 12 million healthy years of life lost¹²³. The exact financial burden globally is unknown, and the most recent estimates of direct cost come from the US. The meta-analysis indicated that the 19.7 million cases of STIs reported in 2008 resulted in \$11.0-20.6 billion of total medical costs, adjusted to the 2010 dollar¹³¹. Costs associated with sexually transmitted HIV-1 was

responsible for the highest percentage of economic burden (81%). This, and other public health studies, suggest that the life-time medical costs and DALYs of STIs are a greater financial public health burden than programs and products contributing to STI prevention would cost^{25,121,132,133}.

In addition to the breadth of people affected and the financial public health costs, STIs are problematic due to the difficulty in diagnosing them, treating them, and the continuous discovery of more sexually transmitted pathogens. The CDC and the WHO both report that most STIs cases are undiagnosed or undetected, especially in low and middle income countries (LMICs)^{77,121}. The low-level of reporting can be due to a lack of screening; stigma, shyness, or concerns about sexual health^{24,70,134}; or the asymptomatic nature of some of the most prevalent STIs. Furthermore, the recent epidemics of Zika (ZV) and Ebola virus (EV) revealed that these viruses could be sexually transmitted, and that virions could be found in the MRT for outwards of 2 years post-infection resolution¹³⁵⁻¹³⁷. The emergence or re-emergence of STIs is not limited to viruses. *Mycoplasma genitalium*, like *Trichomonas vaginalis*, was considered a minor pathogen. Now, however, *Mycoplasma genitalium*'s antibiotic resistance is rising and evidence for its role in infertility, urethritis, adverse birth outcomes

and increased STI/HIV-1 susceptibility in women has become apparent^{122,138-140}. As previously mentioned, the emergence of antibiotic resistant strains of gonorrhea has also increased the urgency to develop prophylactic options for STIs^{79,80,122}. In the US, the percentage of gonorrheal isolates with elevated microbial minimal inhibitory concentrations (MIC) has increased from 2.5% to 4.4%, with strains completely unresponsive to antibiotics reported in the UK and eastern Europe^{80,141}. Equally problematic is a lack of treatment altogether for other pathogens. Many STIs are able to be self-resolved in the FRT or treated with antibiotics, but others remain unpreventable or incurable. Additionally, the presence of one STI increases the likelihood of contracting another: Syphilis, HSV, *Trichomonas*, and gonorrhea have all been reported to increase the risk of HIV-1 infection 2-3 fold^{127,139,142,143}. Interestingly, attempts at targeting these curable STIs and decreasing their prevalence in communities at high risk of HIV infection did not decrease the incidence of HIV-1 transmission^{77,144,145}. Between on-going emergence of STI pathogens and limited resources in their screening, diagnosis, and treatment, there is clearly an urgent need for a product to prevent STI transmission.

Factors Complicating the Development of Prophylactics for STI Prevention

As previously mentioned, the FRT is a formidable mucosal tissue. The FRT's epithelium forms a physical barrier preventing the penetration of pathogens; epithelial mucin production encourages pathogen trapping; cytokines, chemokine, and antimicrobial peptide production contributes to pathogen neutralization; and healthy microbiota symbiotically play a role in the FRT's immune defense^{84,102,146,147}. Antibodies—whether by neutralization, trapping, or effector functions such as ADCC—prevent infection in hosts that have been previously exposed to a pathogen's antigens through sexual transmission or vaccination^{148,149}. Finally, immune cells, including DCs, macrophages, PMNs, NK and T cells, can be found interspersed in the epithelium and lamina propria (Figure 1-2) contributing to surveillance, phagocytosis, and elimination of pathogens through adaptive immunity^{82,106}.

The immunology of the FRT also remains under investigation. The ability of the FRT to simultaneously sustain an environment that protects symbiotic microbiota, permit the passage of sperm and implantation of a semi-allogenic fetus, while actively defending the body from pathogens is not thoroughly understood. The changing FRT environment during puberty, menses, coitus,

pregnancy, and menopause increases the complexity of FRT immunology and prophylactic development. For instance, estradiol has been shown to augment the expression of polymeric immunoglobulin receptors (pIgR) responsible for the transport of secretory IgA across the epithelium in the uterus but decreases expression in lower FRT epithelium^{149,150}. Similarly, the secretory stage of menses is considered the most vulnerable period of STI transmission as adaptive and innate immune response are negatively regulated in the FRT to increase the possibility of implantation¹¹³. These hormonally dictated immune response can decrease the success of preventative therapies such as vaccines^{113,148}.

Finally, vaginal microbiota also plays an active role in protecting the FRT against infections and complicates prophylactic development. *Lactobacillus sp.*, a common vaginal microorganism, produces lactic acid which creates an acidic environment hostile to many STI pathogens¹⁰⁰. In contrast, anaerobic vaginal organisms such as *Prevotella sp.* and *Sneathia sp.*, can induce vaginal inflammation directly (through the secretion of lipopolysaccharides (LPS), for example) or by outcompeting commensal microbiota and depleting their protective functions (such as hydrogen peroxide production)¹⁵¹. The secretion of proinflammatory cytokines (such as TNF- α) in response to microbiota dysbiosis, and the resulting

vaginal inflammation, decrease vaginal tissue integrity and increase the risk of STI infection, such as HIV-1¹⁵²⁻¹⁵⁵. Additionally, *Gardnerella vaginalis* another anaerobic species associated with decreased vaginal health, was shown to metabolize and inactivate the antiretroviral agent Tenofovir when it was administered as a vaginal microbicide in the CAPRISA trial¹⁵⁶. All these factors must also be taken into account for both vaccine development and topical microbicides in human FRT.

Current Approaches to STI prevention

There have been intense efforts focused on curbing the spread of STIs. The most widespread method is the modification of behavior. The use of the condom—the only product on the market that offers on-demand protection for STIs—has been prevalently featured in sexual health classes and public health policies, especially in the fight against the sexual transmission of HIV-1^{6,36,157,158}. Additional behavior-based practices include regular STI screening, (allowing for the early detection and treatment of disease) or modification of treatment guidelines; for instance, beginning treatment with stronger antibiotics to prevent antibiotic resistance^{141,159-161}. These practices, while effective if strictly adhered to, are often not followed precisely enough across populations to decrease the

incidence of STIs. As a result, research has been focused on developing products that can be used to prevent infection. Vaccines are the most evident, cost-effective form of preventing the transmission of disease¹⁶²⁻¹⁶⁴.

Vaccines are usually comprised of inactivated or non-infectious antigens that elicit a primary adaptive immune response. Vaccines induce the priming, activation, antigen-tailoring response and memory development of effector cells (T cells or B cells) that enable a faster and stronger immunological response upon infection or re-infection¹⁶⁵⁻¹⁶⁷. A 2015 study modelling the efficacy of different vaccines in China predicted that even a modest vaccine offering 50% protection with only 30% coverage in the Sichuan province alone could prevent 22,000 new infections between 2020 and 2030¹⁶⁸. Vaccines currently exist only for two STI pathogens, HepB and HPV. The efficacy of the HPV vaccine in women in the US decreased the incidence of the most common HPV morbidity, cervical cancer, from 55% to 33%^{133,169}. The data also evidence herd immunity contributing to the decreased rates of cervical cancer even in unvaccinated demographics¹⁶⁹. Similarly, another study found that within four years of introduction into regular vaccine schedules for females between the ages of 11-12, a 56% decline in HPV prevalence was found¹³³.

The success of the HepB and HPV vaccines has encouraged on-going research to develop vaccines against other prevalent STI pathogens. A hurdle in development, nonetheless is that correlates of protection—biomarkers that measure the host's response to vaccination and predict efficacy—are not necessarily defined for all infectious agents^{170,170}. The development of a Chlamydia vaccine has advanced in recent years due to the discovery of novel nanocarrier adjuvants. Early Chlamydia vaccines, with inactivated pathogen, surprisingly, were found to increase infection and exacerbate symptoms. Research done by Stary *et al*, (2015) in a murine model found that exacerbated infection was most likely due to a wave of regulatory T cells instead of the IFN-producing CD4+ cells needed for protection. Stary *et al* (2015) also found that mucosal vaccination (intrauterine) using the nanocarrier adjuvant was necessary to effectively induce the effector and resident memory T cells required to protect mice upon Chlamydia reinfection¹⁶⁶. In contrast, systemic vaccination only provided partial protection or worsened infection^{162,166,171,172}. Vaccines for STI pathogens HIV-1 and *Trichomonas vaginalis* are also under investigation. The APPROACH clinical trial is one of the latest HIV-1 vaccine trials using a mosaic Ad26/Ad26 and gp140 HIV-1 vaccine. Initial results, released in 2018, showed

67% protection in macaques challenged vaginally with SHIV_{mac}, the highest protection level in a vaccine trial to date¹⁶³. For *Trichomonas vaginalis*, vaccines have been developed for *Trichomonas* strains that infect bovine and murine models; however, these vaccines do not translate to the *Trichomonas* species that infect humans¹⁷³. While advances for STI vaccines are evidently being made, progress is comparatively slow and an alternative approach is desperately needed. Topically applied microbicides promise to fill this product void and contribute to curbing STI transmission^{44,45,51,65,174,175}.

Topically applied microbicides neutralize or kill pathogens in the vagina or rectum. As previously discussed, microbicide development has mainly focused on HIV-1 as a single target. HIV-1 microbicides have ranged from Tenofovir gel (clinical trial CAPRISA 004) to preventative oral administration of ARVs including TDF and FTC (VOICE clinical trial)^{47,156,176}. And PrEP, while not a topical microbicide, has become the latest prophylactic available on the market that provides protection against the sexual transmission of HIV-1. Similarly, single-target microbicides for *T. vaginalis* have also been explored. Like PrEP, oral metronidazole has been tested as a microbicide but an insignificant difference in pathogen clearing time was achieved. Similar results were found

with vaginally administered metronidazole, although there was symptomatic relief for women who presented with trichomoniasis symptoms^{177,178}. A promising antimicrobial peptide was also developed in the early 2000s that demonstrated impressive microbial properties against *T. vaginalis* in a murine model, known as D2A21. D2A21 protected 90% of mice from protozoal adherence to vaginal epithelial cells. D2A21's development was discontinued for *Trichomonas vaginalis*; interestingly, it is currently in Phase III clinical trials as an antibiotic in burn patients^{177,179}.

In summary, there are multiple approaches and on-going research studies focused on preventing the sexual transmission of pathogens. Our lab is specifically interested in exploring the use of monoclonal antibodies as topical microbicides and contraceptives. This dissertation focuses on the development of a multi-pathogen microbicide using HIV-1 and *Trichomonas vaginalis* as model pathogens.

HIV-1 Transmission and Infection

HIV-1 is a single-stranded, positive-sense RNA lentivirus responsible for chronic infection, ultimately leading to the acquired immunodeficiency syndrome (AIDS) (Figure 1-5)^{55,180}. The global HIV/AIDS pandemic began in the

early 1980s, and the first form of treatment--an antiretroviral drug known as azidothymidine (AZT) that inhibited HIV-1's reverse transcriptase—was approved in 1987^{55,181}. Since the beginning of the epidemic, the virus has cost approximately 35 million people worldwide their lives, and approximately 37 million people today are living with HIV globally¹⁸². There are two different types of HIV: HIV-1 and HIV-2. HIV-2 is generally characterized by lower transmissibility and a slower, decreased likelihood of progression to AIDS^{183–185}. It is predominantly found in West Africa. HIV-1, in turn, with its high infectivity and generally quick progression to AIDS when left untreated, is a global epidemic¹⁸⁶. The scope of this thesis is focused on HIV-1.

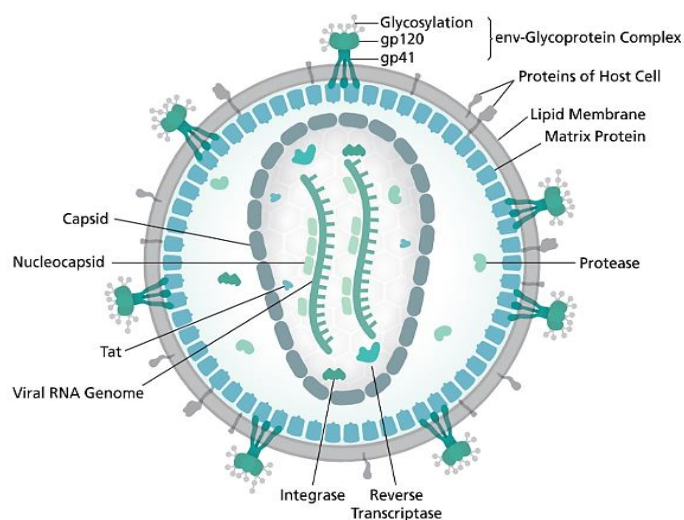


Figure 1-5- Schematic of a mature human immunodeficiency virus. Image by Thomas Spletstoesser (www.scistyle.com)

HIV-1 can be sexually transmitted between individuals through exchange of bodily fluids including blood, semen, and vaginal/anal secretions, and vertically transmitted between mother and child during pregnancy, at the moment of birth, or through breast feeding^{175,187-189}. Young women, girls, and sex workers in particular are disproportionately affected by HIV-1. Young women between the ages of 10 and 24 are twice as likely to be HIV-infected than their male counterparts. Female sex workers are 12 times more likely to acquire HIV-1 than non-sex workers¹⁹⁰. Globally, over half of the people living with HIV-1 are females (~19.1 million), and sexual intercourse with an infected male is the primary reason for infection. The risk of HIV-1 infection in male-to-female transmission of HIV-1 ranged 0.08-0.30% per sex act; in comparison, female-to-male transmission ranges from 0.04-0.38% per sex act¹⁹¹⁻¹⁹³. Risk of transmission is also dependent on viral loads, STIs, and vaginal health or inflammation^{77,194-197}. Baggaley *et al* (2018), in particular, found that gender differences affect the rates of HIV-1 transmission with receptive anal risk of transmission in women (3.38% per sex act) being significantly higher than in men (0.75%)^{191,198}. Additionally, the low rates of sexual transmission per coital act are likely due to the “bottleneck”

phenomena that occurs during sexual transmission¹⁹⁹. Despite the high genetic diversity of HIV-1 found in infected individuals, only a few 'transmitter-founder' (T/F) viruses are responsible for establishing infection in the new host. These viruses have unique genotypic and phenotypic characteristics that enable the pathogen to be successfully transmitted from the donor to the recipient. Selection factors resulting in a T/F can include: 1) blood/genital tract genetic compartmentalization leading to distinct viral diversity in the MRT and (albeit a little more inconsistently) in the FRT; 2) The infected patient's genital fluid which may contain lectins or autologous antibodies that prevent the transmission of specific viruses to the recipient; 3) Glycosylation on Env protein, where less glycosylation is associated with increased likelihood of transmission, particularly evidenced in HIV-1 Clades A, C, and D. 4) Availability of target cells in the recipient's mucosa that encourage replication, such as activated CD4+ T cells; and 5) "genotype fitness" of the virus that determines viral characteristics such as IFN-resistance (secreted by vaginal epithelial cell) and the rapid replication needed to establish infection^{199,200}.

HIV-1 targets CD4+ cells in humans, leading to their depletion and consequently compromising the host's immune system. Figure 1-6 summarizes

the virus' replication cycle within a CD4+ T cell. Briefly, the V3 loop on HIV-1's gp120 binds to CD4 on the target cell. Binding causes a gp120 conformational change which enables the subsequent binding of Env to CXCR4 or CCR5^{56,59}. After co-receptor binding, the virus's gp41 proteins facilitate the fusion of the viral envelope with the cell's plasma membrane. Upon entering the cell, viral RNA is uncoated and reverse transcribed into DNA with a viral reverse transcriptase. The newly synthesized DNA is then integrated into the host cell's DNA where it becomes a provirus. The provirus can remain in the host cell without continuing the replication cycle, resulting in latency and chronic infection^{55,201}. HIV-1 relies heavily on its host cell to aid in its replication: when the provirus undergoes transcription, viral RNA is remade and exported into the cytoplasm. There, viral proteins are translated and all viral components are assembled at the plasma membrane. The virus buds from the cell surface; full viral maturity is only reached upon protease activity, otherwise the virus remains non-infectious^{55,201,202}.

Figure 1-7 depicts male-to-female transmission of HIV-1 through the vaginal tract as an example of mucosal transmission of the virus²⁰³. Semen contains both free HIV-1 virions (cell-free transmission) and HIV-1 infected

CD4+ T cells, and to a lesser extent, macrophages (cell-associated transmission)^{188,204}. Upon entering the vagina, cell-free transmission is established if the virus penetrates the stratified epithelial or simple columnar cell barrier^{102,164}. While formidable, this barrier is not impenetrable¹¹⁵. As previously mentioned, the TZ is susceptible to infection by R5-tropic HIV-1 due to the thinning of the epithelial layer and the high expression of CCR5 on CD4+ T cells prevalent in the lamina propria directly beneath the epithelial barrier^{83,85,117}. Inflammation or lesions in the tissue can also facilitate virus penetration through the epithelial layers into the lamina propria where CD4+ target cells are found^{115,203}. Dendritic cells not directly infected by HIV-1, can capture the virus with surface proteins such CD169 and DC-SIGN^{104,105,205,206}. These cells bind HIV and inadvertently shuttle the virus through the epithelial barrier where it encounters local CD4+ T cells and macrophages, establishing a local infection^{164,180,203,207}. Completely penetrating the epithelial barrier, nonetheless, is not always necessary to establish local infection. Vaginal epithelial dendritic cells, found in the epithelium rather than the lamina propria, have been shown to be R5 HIV-1 cell targets and can potentially play a role in establishing infection¹⁰⁴. The newly infected cells or virus-carrying APCs can subsequently migrate to the lymph

nodes where CD4⁺ cells are readily found and infection will disseminate systemically.

Cell-associated HIV-1 transmission is expected to be 100-1,000-fold more efficient than infection by cell-free virus^{208,209}. In male-to-female HIV-1 transmission, cell-associated infection is attributed to seminal leukocytes, such as T cells and macrophages, ubiquitously found in semen (Se)²¹⁰. HIV DNA PCR was used to quantify HIV infected cells in semen, and the prevalence of infected cells ranged between 21-65%, with up to 80,000 copies/mL found in some samples²¹⁰⁻²¹³. Presence of productive, infectious virus from seminal leukocytes was successfully determined *in vitro*^{214,215}. Cell-associated transmission in the FRT can occur when HIV-infected cells adhered to epithelial tissues via ICAM-1 (or E-Cadherin)- β -integrin interactions^{210,216-218} and release virions towards the epithelium^{210,219}. The subsequent transcytosis of virus across the epithelial barrier facilitates productive infection of underlying CD4⁺ cells. Additionally, cell-to-cell transmission can occur in the FRT tract as 1) infected seminal leukocytes encounter female tract CD4⁺ target cells in the tissue or 2) transmigrate through the epithelium into the lamina propria disseminating infection²²⁰⁻²²². Infected cells can directly transfer infection to bystander target cells via the formation of

virological synapses. Cell-to-cell adhesion molecules stabilize the formation of the synapse initiated by CD4-gp120 interactions between an uninfected and infected cell^{220,223}. The virological synapse then allows for the directed transfer of HIV-1 particles between cells and protects the virus from the host's immune response, such as broadly neutralizing antibodies (bnAbs)²²⁰.

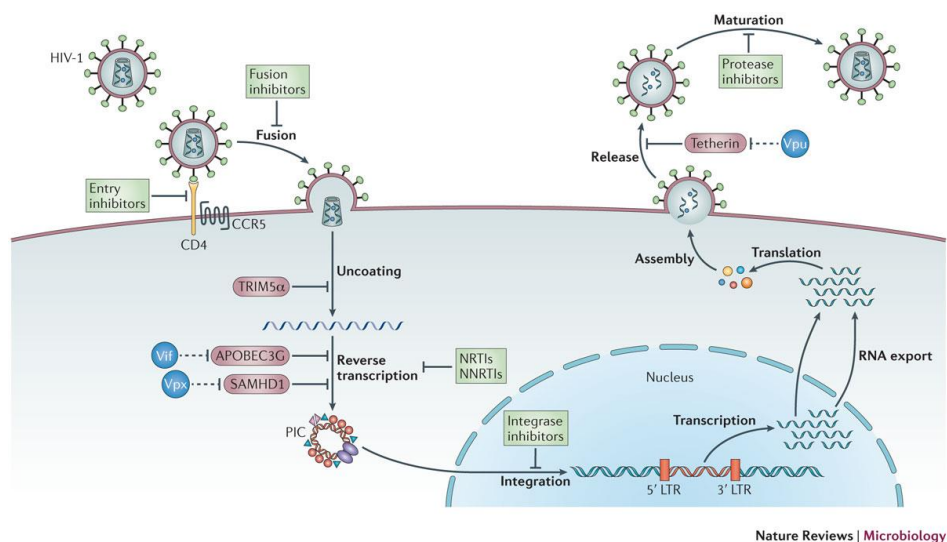


Figure 1-6- HIV-1 replication cycle. The figure depicts the major steps of viral replication including fusion, uncoating, reverse transcription, integration, transcription, translation, assembly, release, and maturation. Green boxes indicate ARV targets. Reprinted with the permission from: Barré-Sinoussi, Ross, & Delfraissy, (2013). *Nature Reviews Microbiology*

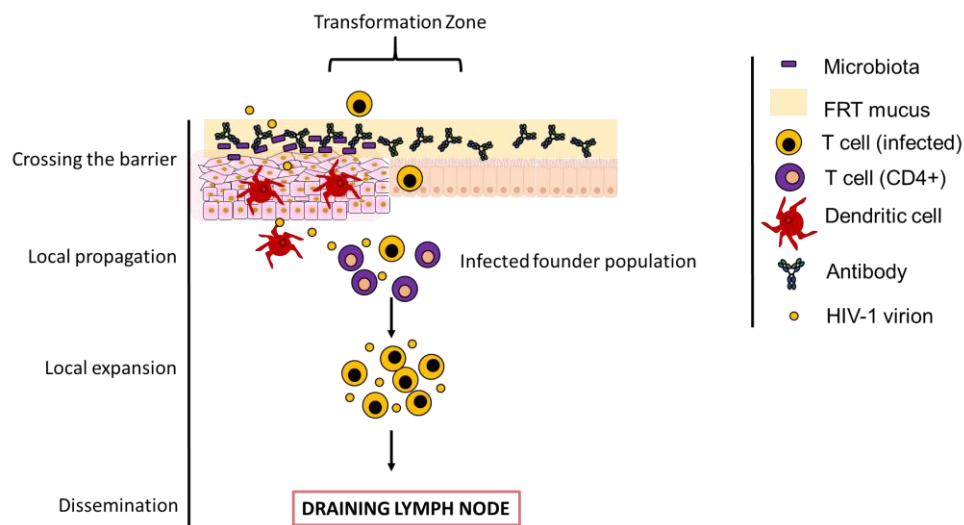


Figure 1-7- Schematic of HIV-1 infection. Infection is established when free virus or infected cells penetrate the FRT epithelial barrier and infect a founder population of cells in the lamina propria or interact with a carrier APC. The infected cells will then expand and disseminate establishing systemic infection. Image adapted from Haase, (2010). *Nature* with help from BioRender

Trichomonas vaginalis Transmission and Infection

Trichomonas vaginalis is a prevalent STI pathogen first discovered in 1836²²⁴. *T. vaginalis* (TV) is a parasitic protozoan that is typically 10-20 μm long and approximately 2-14 μm wide (Figure 1-8, left panel)^{225,226}. The anaerobic parasite is flagellated and usually pear-shaped in its free swimming form, replicating by longitudinal binary fission (Figure 1-8, right panel)²²⁶. Humans are the only known hosts of TV and approximately 70% of cases are asymptomatic^{173,225,227,228}. Until recently, TV was easily cured with metronidazole

but resistance to this only form of treatment has emerged^{81,229}. While men are likely to clear the infection in days, infection in women can persist for years. Chronic infection can result in reproductive pathologies including preterm delivery, pelvic inflammatory disease, and increased susceptibility to HIV-1 infection^{81,230}. Reasons for the disparity of infection between sexes is unclear with increased iron in the FRT due to menses being the primary hypothesis^{231,232}.

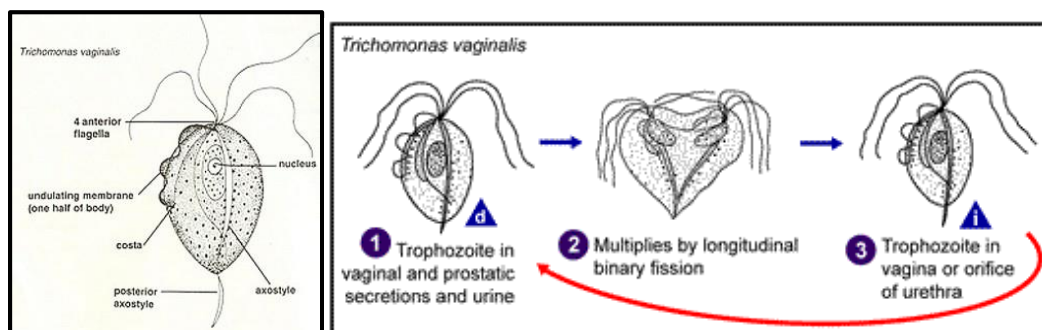


Figure 1-8- *Trichomonas vaginalis* structure and reproductive cycle. Left panel depicts *Trichomonas vaginalis* morphology (Image taken from <http://bioweb.uwlax.edu/>). Right panel depicts *Trichomonas vaginalis* replicate cycle. Longitudinal binary fission happens both in free swimming and adhered protozoa, adapted from the CDC²³³.

TV infection occurs when the free swimming pyriform of *Trichomonas vaginalis* comes into contact with vaginal epithelial cells in the female reproductive tract^{226,234}. In a process not quite fully understood, the protozoa adhere to the vaginal epithelial cells and become amoeboid morphologically. The

only surface molecules on epithelial cells known to mediate attachment, Galactin-1 and Galactin-3, interacts with TV's lipophosphoglycans (LPG)^{228,235,236}. The recent sequencing of TV's genome and subsequent protein studies show BspA-like proteins may mediate TV's adherence to epithelium. BspA proteins are a class of surface adhesion proteins of Bacteroidals with Leucine-rich repeats²³⁷⁻²³⁹. Finally, TV adhesion proteins AP65, AP51, AP33 and AP23, have also been shown to mediate attachment to vaginal epithelial cells (VEC) *in vitro*. Antibodies blocking these proteins decrease adherence to epithelial cells. These adhesion proteins, interestingly, do not play a role in the pathogen's specificity to VEC²⁴⁰

Nevertheless, once adhered, *T. vaginalis* phagocytoses host epithelial cells, surrounding microbiota, and even immune cells. The protozoa are also known to secrete serine and cysteine proteases to break down the epithelial cells' extracellular matrix further, facilitating parasite adherence and mobility. TV's phagocytosis of host cells and vaginal microbiota enable its survival in, what is for them, a nutrient-scarce environment. Phagocytosis of cells and bacteria provides basic macronutrients needed for survival. Due to the pathogen's low production of lipids, it particularly relies on bacteria and eukaryotes as an exogenous source^{228,237}. Finally, phagocytosis of *Lactobacillus sp.* increases the

lumen's pH making it slightly more basic; TV survival and proliferation are optimal at pH 6²⁴¹⁻²⁴³. Despite the presence of phagocytosing parasites and the resulting cellular damage, *T. vaginalis* causes only a mild, barely perceptible inflammatory response^{225,228,229,244}. *T. vaginalis*' consumption of local microbiota and the decreased integrity of the epithelial layer as a result of infection, make the vaginal tract more vulnerable to sexually transmitted pathogens including chlamydia, and gonorrhea. In particular, it is known to increase the rates of HIV-1 infection 2-3 fold, making this pathogen a global concern^{77,228,231}.

Monoclonal Antibodies as MPT Candidates

History of Monoclonals as Contraceptive Candidates

In the mid-1980s, the WHO sponsored two workshops to identify sperm and trophoblast specific antigens with the intention of finding suitable antigens for a contraceptive vaccine. Over 29 laboratories submitted over 100 monoclonal antibodies (mAb), out of which only three antisperm antibodies and two anti-trophoblast antibodies were found to display sufficient tissue specificity²⁴⁵. Public health interest in a contraceptive vaccine eventually declined as this approach is considered irreversible, but monoclonal antibodies (mAbs) as contraceptive and

microbicidal agents still have considerable potential, especially in the case of HIV-1 where no cure or vaccine exists^{65,174}.

Antibodies for Clinical Application

Therapeutic monoclonal antibodies have recently become popular for treating a wide range of diseases. There are currently over 70 FDA-approved mAbs for various clinical applications^{175,246,247}. Due to genetic engineering, antibodies can be completely human or humanized, allowing for repeated dosage and continuous treatment due to low immunogenicity and the avoidance of “serum sickness”²⁴⁸. Antibodies that contain different heavy chains, belong to different isotypes (Figure 1-9). The Fc portion of each isotype provides the antibody with different effector functions subsequently affecting how the rest of the immune system engages with a given pathogen. IgG1, for example, can bind complement through the classical pathway, engage mononuclear cells in Antibody Dependent Cell-Mediated Cytotoxicity (ADCC), and enable efficient phagocytosis^{249,250}. The abundance of IgA in genital secretions indicates that this isotype is well-suited for mucosal protection²⁴⁹. IgA efficiently entraps pathogens in the mucosa and prevents them from reaching their target cells²⁵¹. IgA (specifically secretory IgA) is also highly resistant to proteases abundantly found

in FRT and has documented ADCC engagement with neutrophils. Both IgG and IgA antibodies also encourage phagocytosis through opsonization, facilitating pathogen clearance^{148,252}. Finally, both IgG and IgA antibodies can also directly neutralize pathogen. This mechanism is best exemplified in HIV-1 broadly neutralizing antibody, VRC01, which binds to HIV-1's surface protein gp120 preventing it from binding to its CD4 receptor target cells and establishing infection. While the antibody has a direct neutralizing effect on the pathogen, the antibody's Fc can continue to engage with receptors on effector cells as described above²⁵³. While HIV-1 infected human patients have predominantly IgG broadly neutralizing antibodies, IgA bnAbs have also been documented^{253,254}. These characteristics make both of these antibody isotypes potential candidates for targeting bacteria and viruses in mucosal tissues.

Isotypes can, therefore, be strategically used and antibodies can be engineered to have high potential as a topical microbicide, contraceptive, or multipurpose prevention technology. Furthermore, an antibody based MPT with fully human antibodies promises low immunogenicity, especially when topically applied to the vaginal tract instead of systemically²⁴⁸. Evidence for the effectiveness of antibodies in mucosal tissues is already found *in vivo* where

studies have shown that broadly neutralizing antibodies (bnAbs) can prevent transmission of low-titer SHIV²⁵⁵. Furthermore, antibodies are versatile and can work alongside other antibodies, small molecules, and biologics without losing activity. This collaborative property makes mAbs excellent candidates for an MPT. Mab treatments, however, can be expensive, limiting MPT accessibility; new technologies and antibody producing platforms can help circumvent this limitation.

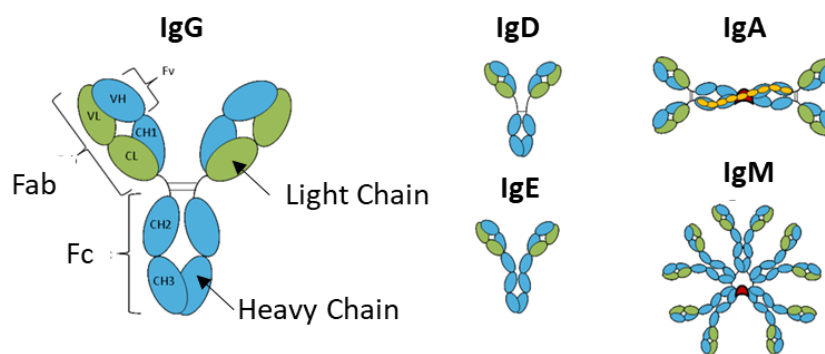


Figure 1-9- Schematic of antibody isotypes. Image adapted from absoluteantibody.com.

Production Platform for Monoclonal Antibodies

Nicotiana sp. was first described as an antibody production platform in 1989^{256–260}.

Interest in this alternative plant-based platform resulted from an interest in decreasing antibody costs and production times, and increasing overall

production^{256,261}. At the time, antibodies produced in mammalian cells (like first generation Chinese hamster ovary [CHO] cells) took approximately 40 days to manufacture and cost between \$50-100/g to produce. Using *Nicotiana*, the whole production process, from transfection to antibody purification, takes 10-12 days and the resulting antibodies cost ~\$20/g (MappBio, personal communication)²⁶¹. In addition to shortened manufacturing timelines and decreased production costs, *Nicotiana* is an attractive production platform due to the ease of genetic manipulation with *Agrobacterium sp*^{256,259,261,262}.

Agrobacterium tumefaciens is transformed with heavy and light chain plasmids of the desired antibody. The transformed bacteria are then sprayed onto the leaves of *Nicotiana* where they transfect the plant. Bacterium attachment to plant cells is still poorly characterized, but the bacterium's flagella and unipolar polysaccharide A (UPP) have been implicated. The wounded plant cells secrete phenolics, hormones, and saccharides that are detected via bacterium membrane sensor protein, VirA, and the sensor protein in the cytoplasm, VirG. Once the VirG is phosphorylated, the process to transfer the transformed DNA from the bacterium to the plant begins. Downstream Vir proteins (such as VirB), activated by interactions with VirA and VirG, result in the formation of the type

IV secretion system (T6SS). T6SS is responsible for shuttling the plasmid DNA from the bacterium into the plant cell. The plasmid is accompanied by bacterial proteins that subsequently process the T-DNA (transfer DNA) so that it is imported and integrated into the plant cell nucleus and subsequently translated (Figure 1-10)²⁶³. The transfected leaves produce antibody over the course of a week. Leaves are then crushed and the antibody purified from the pulp using Protein A sepharose beads^{264,265}. The ease of genetic manipulation of *Nicotiana* also allows for the establishment of glycan homogeneity. Plant-specific glycoforms, such as those containing fucose and xylose, can be easily knocked down in the antibody-producing leaves through RNAi, permitting a homogenous humanized glycosylation of antibodies^{261,262}. Since glycosylation is known to affect antibody Fc function, any changes in glycosylation might result in decreased antibody stability or interfere with ADCC or antibody-complement activity^{266,267}. By ensuring homogenous glycosylation on plant-derived human antibodies, the resulting antibodies will have decreased immunogenicity and predictable, consistent performance, and thus constitute a widely accessible MPT.

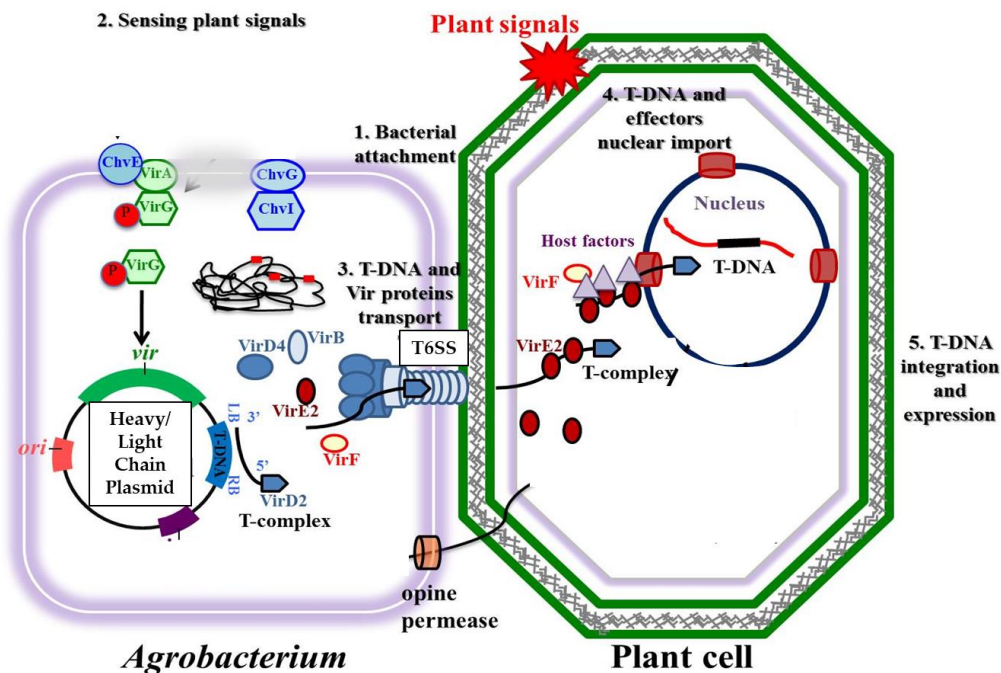


Figure 1-10- *Agrobacterium* transfection of *Nicotiana*. Image depicts all 5 steps of *Agrobacterium* expression of a transformed plasmid. Image adapted from Hwang (2017), *The Arabidopsis Book*.

CD52g as an Antibody Target for MPTs

One of the protein targets submitted to the WHO workshop, CD52g, was targeted by an anti-sperm antibody that demonstrated high specificity and efficiently blocked sperm function^{245,268,269}. CD52g is a GPI-anchored glycoprotein produced in the male reproductive tract (MRT) by epithelial cells lining the caudal epithelium, the vasa deferens, and urethra²⁷⁰. CD52g—also known as CD52w, SAGA-1, seminal CD52 I and II, and CD52mrt—is a 12 amino acid long protein identical to the lymphocyte GPI-anchored protein, CD52^{268,271,272}. The

distinguishing characteristic between these proteins is their *N*-linked glycans (Figure 1-11A)²⁶⁸. Sperm antibodies with high specificity distinguish between the two different glycosylation patterns despite the identical peptide sequence. CAMPATH, commercially known as Alezitumab, is able to detect the amino acid backbone on both CD52g and CD52 (Figure 1-11B)^{273,274}.

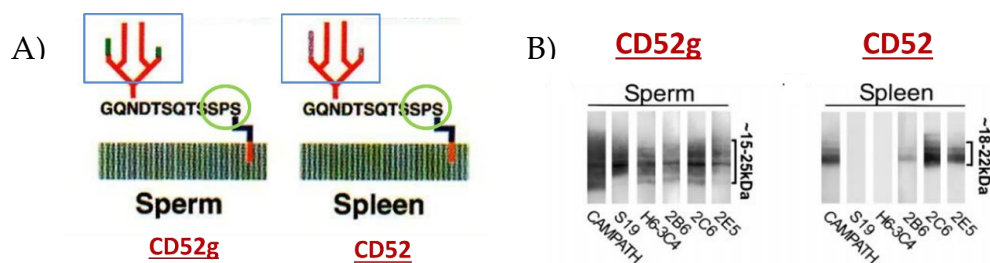


Figure 1-11- Comparing CD52 and CD52g structure and Anti-CD52g specificity. A) A diagram of the CD52g protein. The plasma membrane is depicted in green and the GPI anchor is highlighted in red. The identical 12 amino acid protein core is depicted on both CD52 and CD52g. The green circles depict CAMPATH targets. Blue boxes highlight the *N*-linked glycosylations of both proteins. B) Silver staining of sperm lysates with anti-sperm specific antibodies. S19 and H6-3C4 only detect CD52g not CD52, despite structural similarities. Figures adapted from Herr, 1999.

CD52g is uniquely produced by epithelial cells in the MRT, yet is found coating the heads and tails of mature sperm cells^{274,275}. The GPI anchor of CD52g provides the protein with the hypermobility needed to reach its target cell²⁷⁶⁻²⁷⁸. The exact mechanism of protein transfer is unknown. CD52g is found in soluble form in seminal plasma (SP), and it is likely that soluble CD52g can be found on seminal extracellular vesicles and transferred to sperm and other phospholipid

bilayers, like human lymphocytes²⁷⁹. Additionally, as sperm mature and migrate along the MRT, CD52g can “flip” onto their plasma membranes from direct contact with CD52g+ epithelial cells (Figure 1-12)²⁸⁰. Similarly, the function of CD52g is unclear, but it is thought to be involved in sperm maturation^{275,281}, immune (complement) protection of sperm in the FRT^{271,279}, and the coagulation and liquefaction of semen²⁸², a phenomenon thought to improve sperm mobility in the FRT.

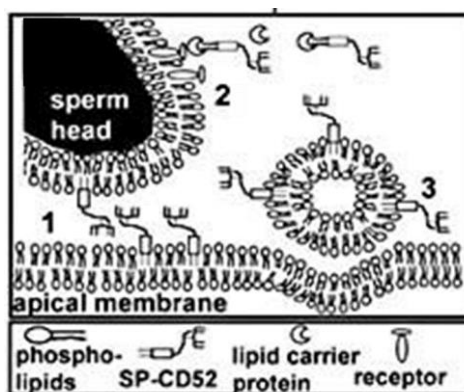


Figure 1-12- CD52g transfer mechanisms. Mechanisms of CD52g membrane transfer. 1) Depicts GPI anchor flipping due to physical contact of plasma membranes. 2) Lipid carrier hypothesis, little evidence has supported this hypothesis since the 1990s. 3) Hypothesis of extracellular vesicular transfer. Image adapted from Diekman, (2001).

Isojima’s laboratory in Japan was the first to characterize an anti-sperm antibody. The IgM antibody, H6-3C4, was isolated from a peripheral blood B cell isolated from a woman with infertility²⁸³. In 1999, John Herr published a mouse IgG hybridoma based on Isojima’s work and patented the antisperm antibody,

S19^{245,268}. It was later determined that H6-3C4 and S19 both target CD52g²⁷². Upon exposure to these antibodies, sperm agglutinate leading to sperm immobility and, in the case of Isojima's patient, infertility. This agglutinating property can be harnessed for contraception. In the early 2000s, the idea of a topical contraceptive and microbicides regained traction, eventually leading to the production of the "Human Contraceptive Antibody" (HCA)^{43,284-286}. HCA is the fully human IgG1 version of Isojima's H6-3C4 produced in *Nicotiana benthamiana*. Our lab collaborates with MappBio, the biopharmaceutical company producing HCA, in order to explore its potential as a female topical contraceptive and broad-spectrum microbicide.

Dissertation Objectives

Two major problems associated with reproductive health in women are 1) high prevalence of unplanned pregnancies and 2) sexually transmitted infections. In recent years, ~40% of pregnancies worldwide were unplanned despite multiple contraceptive products being available on the market demonstrating a gap in contraception use^{9,13}. Additionally, the World Health Organization estimates that 360 million treatable sexually transmitted infections are acquired annually and close to 1.2 billion people are living with a chronic viral infection⁷⁷. This demonstrates an unmet need for improved STI protection. A readily accessible, easy-to-use, discreet multipurpose prevention technology combining contraception and microbicide protection is therefore needed globally.

In recent years, there has been a global effort to develop microbicides (compounds that neutralize or kill sexually transmitted viruses and bacteria in the vagina or rectum) for use as MPTs. Two recent phase III clinical trials, the RING and ASPIRE studies, used vaginal rings that release antiretroviral drugs (ARVs) to prevent male-to-female sexual transmission of HIV-1. The results, published in 2016, demonstrated a 37% general decrease in the risk of HIV-1.

Final interviews from these clinical trials highlighted the participants' interest in adding a contraceptive component to the product^{48,72}. Our laboratory's goal is to develop an MPT that will provide contraception and protection against various STI pathogens through the use of *Nicotiana* plant-derived humanized monoclonal antibodies (mAbs-N)²⁸⁴.

A mAb-N being investigated by our lab for MPT use, the Human Contraception Antibody (HCA) is directed against CD52g, a sperm antigen originally identified in patients with infertility and studied for its contraceptive properties^{269,283}. CD52g is a GPI-anchored, N-linked glycoprotein produced by epithelial cells in the male genital tract²⁷⁰. This ~25 kDa glycoprotein is secreted into seminal plasma and is embedded into the plasma membrane of sperm cells. Anti-CD52g-N rapidly induces sperm agglutination *in vitro* suggesting contraceptive properties. In addition, our preliminary studies also indicate that CD52g inserts into the phospholipid bilayer of somatic (non-sperm) cells in semen, such as HIV-infected leukocytes and STI pathogens (e.g. *Trichomonas vaginalis*) making it a potential target for the prevention of STI pathogens. Based on these findings we hypothesize that **topical use of CD52g-targeting antibodies**

may provide contraception and broad protection against several STI pathogens.

Aim 1- Investigate the contraceptive effects of HCA-N

“Human Contraceptive Antibody” (HCA-N), a human monoclonal antibody specific for CD52g, has previously been shown by our lab to potently agglutinate human sperm. We are interested in developing this antibody as a topical contraceptive and plan to investigate its contraceptive properties in physiologically relevant *in vitro* conditions.

Hypothesis: HCA will effectively agglutinate sperm despite donor-to-donor variability. As a topical contraceptive, HCA will be able to withstand the low pH conditions of the vaginal tract.

Aim 2- Investigate whether CD52g anchors into the sexually transmitted pathogens HIV-1 and *Trichomonas vaginalis* and whether HCA treatment prevents transmission of disease.

Due to the hypermobility of GPI anchored proteins, CD52g can potentially transfer to the phospholipid bilayer of HIV-1 enveloped virions, infected lymphocytes, and *Trichomonas vaginalis*. The agglutinating property of HCA could potentially agglutinate the pathogens preventing transmission of disease.

Hypothesis: CD52g will be found on the surface of HIV-1 and *Trichomonas vaginalis*. HCA treatment will lead to the direct agglutination of pathogens, preventing STI transmission or co-agglutination with sperm in semen.

Chapter 2 - Production and characterization of a human antisperm monoclonal antibody for topical contraception in women

In lieu of this chapter I have submitted the manuscript submitted to Science

Translational, but formatted for this dissertation

Title: Production and characterization of a human antisperm monoclonal antibody for topical contraception in women

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Introduction

At least 40% of all human pregnancies worldwide are unplanned¹³, indicating a need for more family planning education, contraceptive availability, and product diversity. Currently, the most widely used reversible contraceptives are barrier methods, which have a high degree of user failure, and hormonal methods, which are often discontinued due to side effects^{3,28,34,287}. A low-risk, non-hormonal, on-demand, discreet, woman-controlled method could provide an important contribution to the contraception field. Furthermore, this approach could garner even wider acceptance if incorporated into a multipurpose prevention technology (MPT) product that not only provides contraception, but also affords protection against the human immunodeficiency virus (HIV) and other sexually transmitted infections (STIs).

Antisperm antibodies are commonly detected in the sera and genital secretions of infertility patients and are thought to impede conception by agglutinating, trapping in mucus, and/or immobilizing sperm²⁸⁸⁻²⁹¹. One antisperm antibody found in infertility patients is directed against a low molecular weight male reproductive tract (MRT)-specific glycoprotein, CD52g²⁹². The antigen is expressed and secreted by epithelial cells lining the lumen of the

epididymis, and to a lesser extent the vas deferens, and seminal vesicles²⁶⁹. The CD52g protein core is a 12 amino acid peptide sequence, CD52 (CAMPATH-1 antigen), also expressed by lymphocytes²⁹³. The difference between the MRT and lymphocyte forms of CD52 is a unique *N*-linked glycan (g), which is present only on the MRT protein. This unique glycan can be specifically targeted by antisperm antibodies²⁶⁸. CD52g also has a glycosylphosphatidylinositol (GPI) anchor that enables its incorporation into the plasma membrane of sperm as they transit the epididymis²⁶⁸. The glycoprotein is also found in soluble form in seminal plasma (SP) and in semen exosomes^{279,294}. While the exact function of CD52g is unknown, it has been implicated in sperm maturation, protection of sperm from immune responses in the female reproductive tract, and the coagulation and liquefaction of semen^{271,275,281,282,295}.

H6-3C4, the first reported monoclonal antibody directed against the *N*-linked glycan epitope on CD52g, was developed by Isojima *et al.* using immortalized B-cells from a woman with infertility and a high titer of sperm-immobilizing antibodies²⁸³. Mouse monoclonal antibodies that react with the CD52g MRT-epitope have also been developed, including 2C6^{296,297} and S19^{298,299}. These, and other antisperm mAbs, were submitted from various laboratories

worldwide for evaluation by a World Health Organization (WHO)-sponsored antisperm vaccine workshop in 1987. The anti-CD52g antibodies scored very well in functionality and specificity tests, and the CD52g sperm antigen was identified as a potential contraceptive vaccine candidate²⁴⁵. Interest in antisperm mAbs has recently re-emerged due to the introduction of new commercially-viable mAb production platforms, and the possibility that passively administered mAbs, unlike a vaccine, could constitute a reversible contraceptive method. Antisperm mAbs could be used in vaginal products, such as films and rings for contraception, and combined with microbicidal agents to provide multipurpose protection against unplanned pregnancy and sexually transmitted pathogens. In other words, a multipurpose prevention technology (MPT)³⁰⁰.

Our research team used a modification of the H6-3C4 sequence to manufacture a human antisperm mAb in *Nicotiana benthamiana* plants, a platform used for the production of mAbs and other proteins for clinical applications^{264,301}. We have designated this antibody the “Human Contraception Antibody” (HCA). In this report we describe the manufacture of HCA, its specificity, and its performance in sperm functional assays.

Materials and Methods

Study Design

This study was designed to assess the potential of HCA as a topical contraceptive by evaluating its specificity and efficiency in physiologically relevant conditions *in vitro*. HCA is an antisperm mAb, once associated with infertility and adapted as a contraceptive antibody. We determined agglutination efficiency via the time HCA took to agglutinate sperm in various conditions, as well as measuring the progressive sperm that escaped agglutinates after mAb treatment. Experiments were replicated a minimum of three times, with two to three trials conducted for each data point. Donor variability was taken into account with a minimum of three study participants being used for the Agglutination Kinetic Assay and Sperm Immobilization tests, and six study participants used for the Sperm Escape Assay. For all experiments, semen samples met WHO semen parameters³⁰². For the Agglutination Kinetics Assay, the 120 second limit to determine agglutination was selected since literature studies show sperm can reach the endocervix post insemination or ejaculation between 3-5 minutes³⁰³⁻³⁰⁵; we wanted to be under this threshold. Because the assay depends on observation, two semi-blinded, trained technicians observed agglutination to

avoid bias. Investigators were not blinded for the Sperm Escape Assay. For the Luminex panel, if a replicate of a given cytokine was above or below the level of detection (after modifying sample dilutions), it was excluded from analysis. Control tissues were run in duplicate while experimental tissues were run in triplicate. Because S19 is a patented, commercialized agglutinating antibody, it was used as a control until HCA demonstrated faster agglutination, then it was excluded from further experiments due to sample limitations and sperm viability.

Antibodies

Human Contraceptive Antibody (HCA)

The human anti-CD52g mAb was produced by Mapp Biopharmaceutical Inc. (San Diego, CA, USA) from the sequence of H6-3C4, a human antisperm mAb^{292,306}. Genes containing the variable region sequences of H6-3C4 were synthesized (Life Technologies; San Diego, CA, USA) and subsequently cloned into TMV and PVX plant expression vectors³⁰⁷ containing codon-optimized human lambda and human IgG1 constant regions. The vectors were then transformed into *Agrobacterium tumefaciens* strain ICF320 (Icon Genetics; Halle/Saale, Germany). *Nicotiana benthamiana* plants, genetically modified to

produce mammalian *N*-linked glycans³⁰¹, were grown for four weeks in an enclosed growth room (20-23°C) and used for vacuum infiltration as previously described²⁶⁴. Seven days post-infiltration, antibody was extracted from the leaf tissue and purified by Protein A chromatography as previously described^{264,265}. The IgG1 HCA was aliquoted and stored at -80°C.

S19

Mouse anti-CD52g mAbs were obtained from the supernatant of MHS-8 hybridoma cells originally established by John Herr (HB-12144, ATCC)^{269,272,308}. Healthy MHS-8 hybridomas were grown in complete Isocove's Modified Dulbecco's Medium (IMDM) (ATCC Manassas, VA, USA) with 10% fetal bovine serum (FBS), 1% Penicillin Streptomycin, 1% L-Glutamine 200 mM (Gibco, ThermoFisher Scientific, Waltham, MA, USA). For antibody collection, hybridomas were seeded at confluency in IMDM without FBS and cultured until cells died³⁰⁹. The supernatant was collected and concentrated with a 15 mL, 50-kDa centrifuge filter (UFC905096, Millipore Sigma, Burlington, MA, US) at 3,000g for 40 minutes. Concentrated antibody was washed twice with fresh IMDM, spinning the filters each time at 3,000g for 30 minutes. S19 antibody was

resuspended in PBS and quantified by nanodrop. Antibody was stored in Multipurpose Handling Media (MHM; FUJIFILM Irvine Scientific; Santa Ana, CA, USA) at 4°C.

CAMPATH-1

CAMPATH-1 is a commercially available rat mAb, clone YTH34.5, directed against a peptide epitope on CD52 (MA5-16999, ThermoFisher Scientific, Waltham, MA, USA).

Tests of Antibody Specificity

Immunofluorescence Assay

Anti-CD52g mAbs were tested for specificity on washed human spermatozoa and human peripheral blood mononuclear cells (PBMCs, negative control). Cells were air dried on slides, fixed with acetone, and incubated with 25, 50 or 100 µg/mL of HCA, S19 or CAMPATH mAbs. VRC01-N, a human IgG1 mAb produced in *Nicotiana* and directed against the HIV-1 envelope glycoprotein gp120, was used as an isotype-matched negative control antibody for HCA. A mouse mAb directed against CD3, a prominent T lymphocyte surface protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as an isotype-

matched control antibody for S19. Primary antibodies were detected with a 1:200 dilution of fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA). Coverslips were mounted on slides with Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA)

Immunohistology

Antibodies were tested for reactivity on a variety of human reproductive tract tissues (cauda epididymis, vas deferens, vagina) and tonsil. Paraffin-embedded tissues were sectioned at 5 μm , de-waxed, hydrated and subjected to an antigen retrieval step. Antigen retrieval was carried out by immersing the sections in a citrate buffer (pH 6) and then placing the tissues in a pressure cooker that was heated to 125°C for 30 seconds (Biocare Medical, Concord, CA). The sections were incubated with Cy-3 labelled HCA or VRC01 (conjugation kit: Abcam, Cambridge, MA, USA) at a 1:20 dilution overnight at 4°C. Finally, the sections were mounted in an anti-fade medium containing DAPI, as a nuclear counter stain. All sections were examined under an Olympus microscope (Olympus

America Inc. Melville NY) fitted with epi fluorescence imaging and images were captured with a digital camera.

Scanning Electron Microscopy (SEM)

Sperm that had been treated with HCA or PBS (control) were air dried on coverslips and fixed in ½ strength Karnovskys fixative for 48 hours. Coverslips were then rinsed in PBS and dehydrated in a series of ethanol concentrations- 35%, 50%, 70%, 95% (1x for 10 minutes) and 100% (3x for 10 minutes) before critical point drying in a Tousimis semi-automatic Critical Point Dryer.

Coverslips were mounted on aluminum stubs and coated with chromium in a GATAN Ion Beam Coater. Images were taken with a JEOL 7401F Field Emission Scanning Electron Microscope.

Western Blot

Human sperm (SC), SP, and PBMCs were lysed in radioimmunoprecipitation assay (RIPA) buffer and used as antigen targets in the western blot assay. For denatured blots, 20-30 µg of protein were denatured with SDS. Samples were loaded onto a 4-15% gradient polyacrylamide gel (4561084, Bio-Rad, Hercules,

CA, USA), and after electrophoresis, proteins were transferred to PVDF membranes. The blots were blocked with 5% powdered milk and washed with 0.1% TBS-T. All primary antibodies were used at a concentration of 10 µg/mL; anti-mouse and anti-rat secondary antibodies were used at a 1:20,000 dilution, and the anti-human secondary antibody was used at a 1: 5,000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were developed in ECL substrate (ThermoFisher Scientific, Waltham, MA, USA). To cleave the N-linked glycan epitope, sperm lysates were treated with PNGaseF according to the manufacturer's protocol (New England Biolabs, Ipswich, MA, USA). Native blots were run similarly in Towbin Buffer without SDS.

Tests of Antibody Function

Semen Analysis and Sperm Preparation

Healthy men between the ages of 18-45, with sperm samples meeting the 5th edition of the WHO's human semen characteristics for fertility provided the semen samples used in this study. This study was approved by the Boston University Medical Campus Institutional Review Board. Participants provided written informed consent for the study. The samples were processed within one hour of collection, after liquefaction. Live sperm were separated from the rest of

the sample by passage through 90% ISolate density gradient medium (FUJIFILM Irvine Scientific; Santa Ana, CA, USA). Briefly, whole semen was layered over an equivalent volume of Isolate. The sample was centrifuged at 300g for 20 minutes, and the pellet containing live, motile sperm was resuspended in MHM. Sperm count and motility were assessed by Computer Assisted Software Analysis (CASA; Human Motility II software, CEROS II, Hamilton Thorne). Both whole semen and washed sperm were used for the functional assays described below.

Agglutination Kinetics Assay

Antibody was diluted in MHM. Two microliters of sperm suspension were pipetted and evenly spread on a 9mm well microscopy slide (ThermoScientific, Waltham, MA, USA), and an equivalent amount of antibody was added.

Antibody and sperm were mixed by quickly pipetting three times and sperm agglutination was observed in real-time on an Olympus inverted microscope.

“Time to 100% agglutination” is the time elapsed for the sperm cells visible in a single 10x field to become completely agglutinated. Agglutination was timed and recorded by two trained independent readers. A minimum of three replicate

reads was conducted for each data point. The assay was repeated a minimum of three times using different donor samples.

Sperm Escape Assay

A modified sperm swim-up assay was used to assess the number of free-swimming sperm following treatment with HCA. MAbs were diluted in MHM, and 30 μ L were added to upright 0.2mL PCR tubes. 30 μ L of sample were then added to the antibody and mixed three times by pipetting. Tubes were placed at a 45° angle and the mixture was incubated for 5 minutes at room temperature (RT) to allow agglutinated and immotile sperm to settle towards the bottom of the tube. A 2.5 μ L sample was taken from the top millimeter of the mixture and placed in a 4-chambered slide (Microcell 15424, Vitrolife, San Diego, CA, USA). The sample was analyzed by CASA to determine both the total sperm concentration and the number of progressively motile sperm that escaped agglutination.

Complement-Dependent Sperm Immobilization Assay

Washed sperm with an initial motility >90% were adjusted to a concentration of 25-40 x10⁶ cells/mL in MHM. Human serum, separated from fresh whole blood by centrifugation, was used as the complement source. Heat-inactivated (HI) complement (heated to 56°C for 30 minutes) was used as the control. Five µL of complement or HI-complement were incubated with 2.5 µL of sperm suspension for 5 minutes at RT. Next, 25 µL of HCA were added in a series of two-fold dilutions (3.125 - 0.20 µg/mL) and mixed; 3 µL of the suspension were transferred to 4-chambered slides in duplicate and the slides were incubated in a humidified chamber at 32°C for 1 hour. Sperm motility was assessed using CASA. A minimum of three trials were performed using different donors.

Testing HCA Activity under Physiological Conditions

Whole semen vs. washed sperm

To compare the ability of HCA to agglutinate sperm in whole semen and in washed sperm, the semen samples were divided into two fractions: 1) whole unprocessed semen, and 2) washed sperm cells processed as described above, and resuspended in MHM to the same concentration as that in the original semen sample. Semen from two to three donors were pooled for these

experiments. Agglutination Kinetics and Sperm Escape Assays were run as described above.

Effect of sperm concentration

Motile sperm were isolated from semen using ISolate, and resuspended in MHM at concentrations ranging from 2 to 100×10^6 /mL.

Effect of low pH

To determine whether HCA withstands the low pH conditions often present in the human vagina, the pH of MHM was lowered to 3.5 using D/L-lactic acid (Sigma Aldrich, MA, USA). HCA was incubated in pH 3.5 or MHM with neutral pH for 0, 2, 4, and 24 hours at 37°C. Following incubation, the low pH MHM was neutralized by addition of HEPES Buffer (Invitrogen) and neat seminal plasma. The antibody was then used in Agglutination Kinetics Assays.

Preclinical Safety Assessment

MatTek EpiVaginal tissue cytokine response assay

The EpiVaginal Tissue™ model (MatTek Corporation, Ashland, MA, USA) was used to study potential proinflammatory effects of HCA and HCA immune

complexes, as previously described³¹⁰. For this assay, a total of 100 μ L of different combinations of HCA (100 μ g/mL), semen and complement (C') were added to the upper chamber (apical side) of the EpiVaginal Tissue™. Tissues were incubated at 37°C for 6 hours after which the antibody/sperm/complement suspensions were washed off, and 80 μ L of MatTek EpiVaginal Tissue Media was added to the upper chamber. After 24 hours, apical and basal supernatants were collected and cytokines were measured using a custom 6-plex ProcartaPlex Luminex kit (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA).

Statistical Analysis

GraphPad Prism (Version 7.03; GraphPad Software Inc.; San Diego, CA, USA) was used for statistical analysis and graphing of data. Data were subjected to analysis of variance (ANOVA). A significant ANOVA was followed by post hoc multiple comparison tests. For the Sperm Immobilization test, a *t*-test, with a correction for multiple comparisons, was used. Differences were considered to be statistically significant when $p < 0.05$.

RESULTS

HCA Production and Characterization

HCA Yield

The “Human Contraception Antibody” (HCA) was produced via agrobacterium transfection of *Nicotiana benthamiana* with the H6-3C4 variable region sequence and an IgG1 Fc sequence. The monoclonal antibody (mAb) was purified on Protein A columns; concentrations of antibody ranged from 0.4 to 4 mg/mL.

Immunohistology and Western Blot Assays

HCA antigen specificity was ascertained by immunohistology and western blot. Both live and acetone-fixed washed human sperm were processed with HCA, S19, CAMPATH or VRC01 for indirect immunofluorescence microscopy. S19, a once commercialized anti-CD52g, and CAMPATH, a mAb specific to the amino acid core of CD52g and lymphocyte CD52, were positive antibody controls. VRC01, a *Nicotiana* produced HIV-1 specific IgG1 mAb, was the isotype control for HCA. HCA, S19, and CAMPATH reacted with the entire sperm surface-from head to tail. VRC01 showed no reactivity (Figure 2-1A-D,

respectively). HCA, S19 and VRCO1 did not react with PBMCs (Figure 2-1E-H) whereas CAMPATH did, as expected (Figure 2-1C).

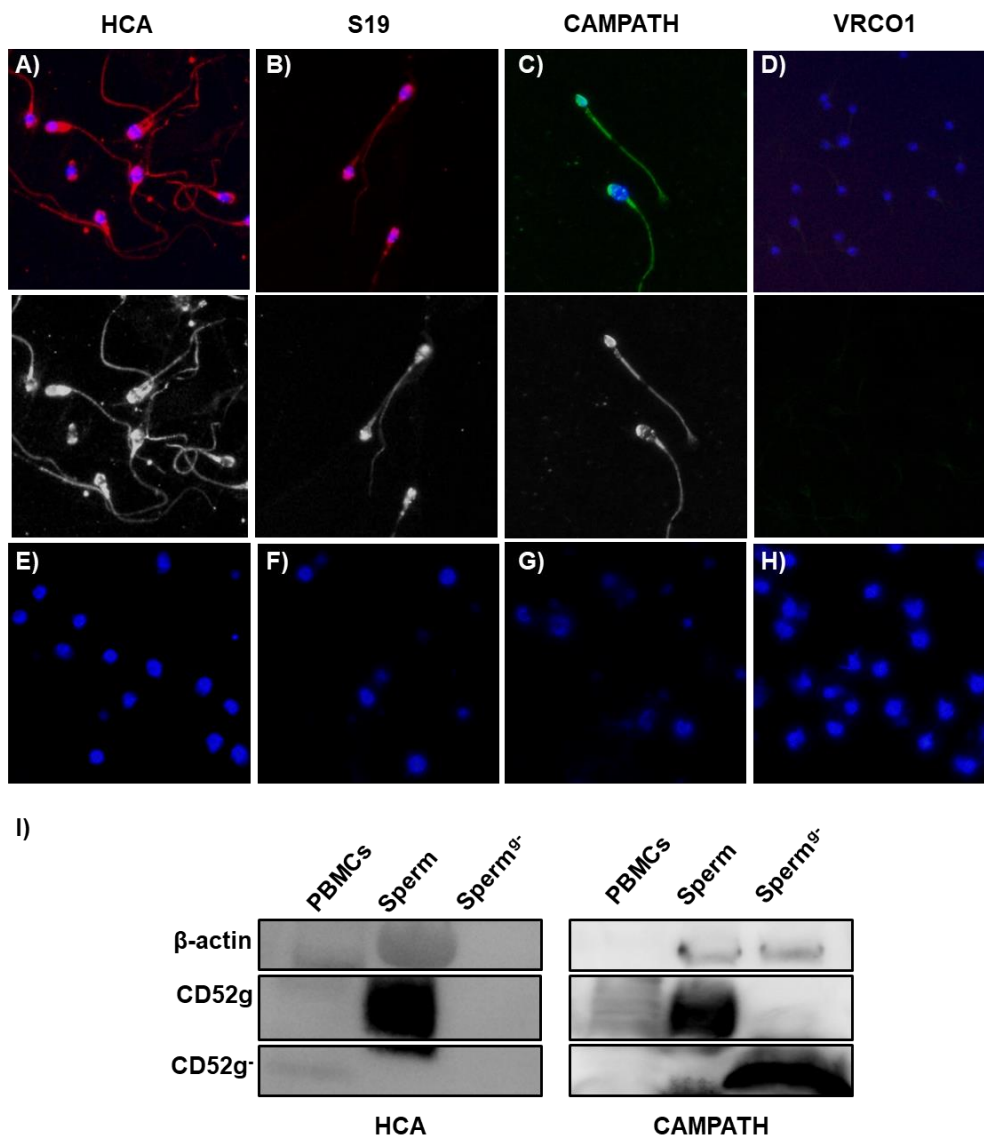


Figure 2-1- *Nicotiana benthamiana* produced HCA is specific to CD52g on sperm. Immunofluorescence of sperm cells with A) HCA antibody, B) S19 antibody, and C) CAMPATH antibody. D) VRCO1 is an isotype control for HCA. Immunofluorescence of PBMCs with E) HCA antibody, F) S19 antibody, G) CAMPATH antibody, and H) VRCO1. I) Denatured western blot of HCA (left panel) and CAMPATH (right panel). PBMCs are a negative control. HCA reactivity

disappeared following treatment with PNGaseF, an enzyme that removes *N*-linked glycans (Sperm^g).

In the western blot assay, HCA detected a ~15-25 kDa band in sperm lysates—the expected size of CD52g—which disappeared following treatment with PNGaseF, an enzyme that cleaves *N*-linked glycans and removes the carbohydrate epitope on CD52g (Figure 2-1I, left panel). CAMPATH, which targets the amino acid sequence of CD52g, also reacted with the ~15-25 kDa band in sperm lysates. After PNGaseF treatment of sperm, CAMPATH detected a lower MW band representing CD52g without *N*-glycans (Figure 2-1I, right panel). Tissue immunohistology was also used to demonstrate HCA specificity. Cy3-conjugated HCA reacted with epithelial cells in the human cauda epididymis and its luminal contents containing sperm (Figure 2-2A), but did not react with human vaginal or tonsil tissue (Figure 2-2B-C). Cy3-conjugated VRC01 (isotype control) did not react with any of the tissues (Figure 2-2D-F).

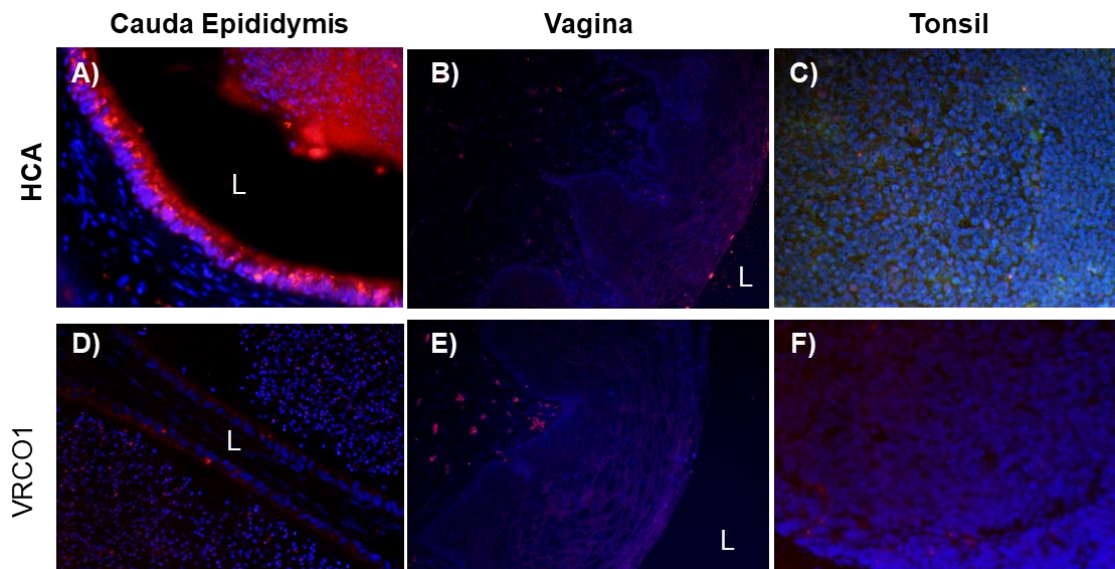


Figure 2-2- *Nicotiana benthamiana* produced HCA is specific to CD52g in male reproductive tract tissue. Immunohistology staining of directly conjugated Cy3-labelled HCA (red) in the A) human cauda epididymis, B) vaginal and C) tonsil tissue. D-F) VRC01 isotype control staining of the same tissues. L= lumen

Inhibition of Sperm Function

Sperm agglutination assay

At a concentration of 100 $\mu\text{g/mL}$, the highest concentration tested, HCA agglutinated 100% of sperm in under 15 seconds (Figure 2-3A and Figure 2-4C). Effective agglutination time varied with HCA concentration; at the lowest concentration tested, 6.25 $\mu\text{g/mL}$, 100% sperm agglutination occurred within 60 seconds (Figure 2-4C). At a concentration of 100 $\mu\text{g/mL}$, S19 agglutinated sperm within 30 seconds, whereas CAMPATH and VRC01 did not agglutinate sperm (Figure 2-3A). Scanning electron microscopy of HCA-treated sperm revealed a mixed agglutination pattern: head-to-head, head-to-tail, tail-to-tail (Figure 2-3B and C).

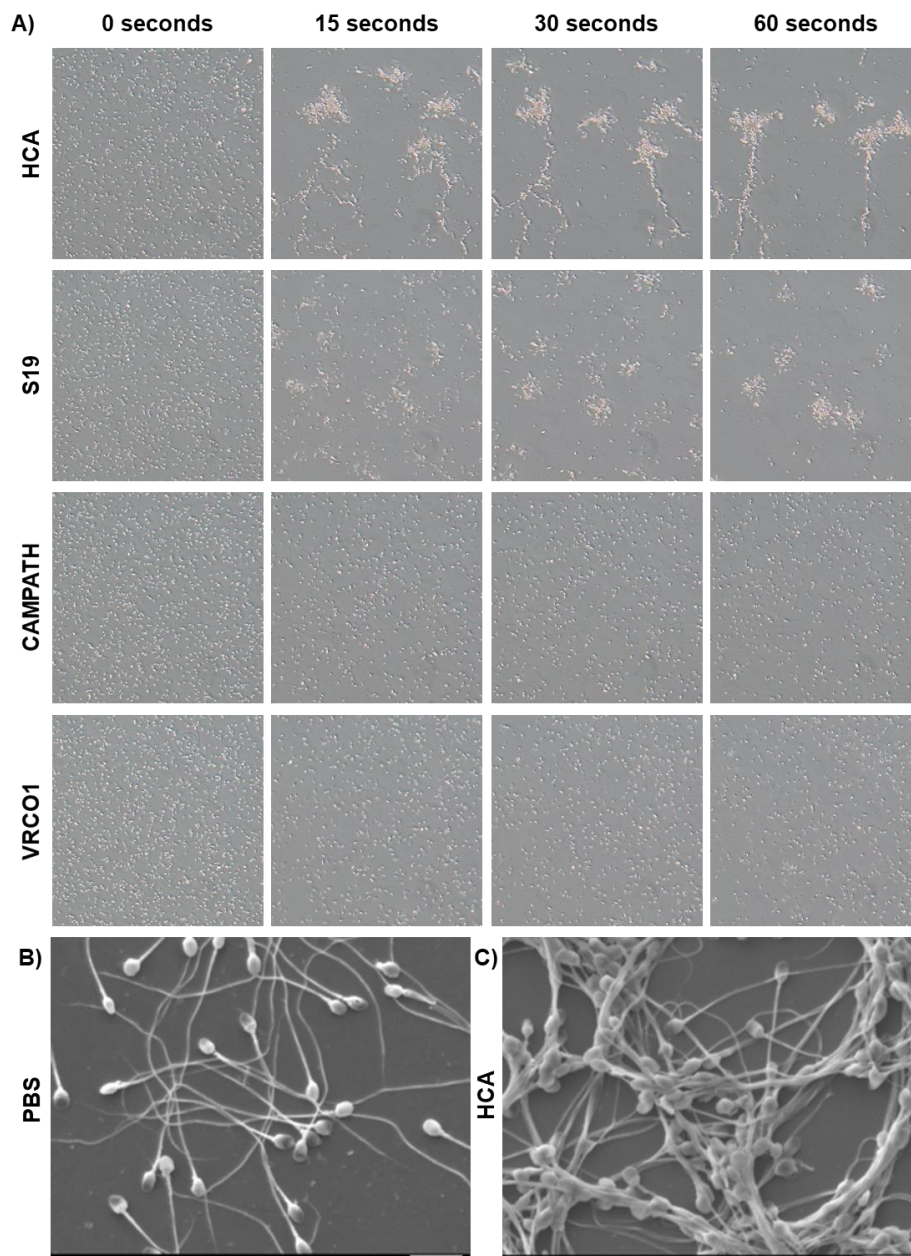


Figure 2-3- HCA rapidly agglutinates washed human sperm A) 100 $\mu\text{g}/\text{mL}$ of HCA was added to washed sperm cells ($100 \times 10^6 / \text{mL}$). Sperm agglutination was monitored using an inverted microscope (10X). B) SEM images of PBS exposed sperm and C) HCA agglutinated sperm

Sperm escape assay

We modified the classical sperm swim-up assay to evaluate the number of progressively motile sperm that escape from the agglutinates formed when sperm is treated with HCA. Following the addition of HCA, agglutinated and immotile sperm sink to the bottom of the tube, whereas sperm with progressive motility can be detected in the upper fraction of the sperm/antibody suspension (Figure 2-4A, left panel). These progressively motile sperm are the antibody “escapees” that could potentially fertilize an ovum. When added to washed sperm suspensions, HCA concentrations of ≥ 6.25 $\mu\text{g}/\text{mL}$ reduced the number of progressively motile sperm in the swim-up fraction to 0-3% of the media control (Figure 2-4A, right panel). There was a slight increase in the number of escaped sperm when HCA was added to whole semen, which contains soluble CD52g. At the lowest mAb concentration tested (6.25 $\mu\text{g}/\text{mL}$), 15% progressively motile sperm escaped from the agglutinates, compared to media samples. At higher mAb concentrations (12.5-100 $\mu\text{g}/\text{mL}$), this number was between 2-5% in whole semen (Figure 2-4A).

Complement-dependent sperm immobilization

A modified form of the Sperm Immobilization Test was also used to determine whether HCA induces complement (C')-dependent sperm immobilization^{311,312}, a phenomenon cited as an effector mechanism reinforcing antibody-mediated infertility. HCA potently agglutinates sperm at concentrations $\geq 6.25 \mu\text{g/mL}$; therefore, the highest HCA concentration tested in this experiment was $3.125 \mu\text{g/mL}$ which enabled visualization and quantification of C'-dependent immobilization of individual sperm using the CASA. With active human complement, there was a significant decrease in motile sperm despite low antibody concentrations ($p < 0.0001$); significant sperm immobilization was observed at HCA concentrations as low as $0.78 \mu\text{g/mL}$ (Figure 2-4B). In the presence of heat-inactivated complement, HCA did not immobilize sperm; the percent of motile sperm was similar to that of media controls across all HCA concentrations.

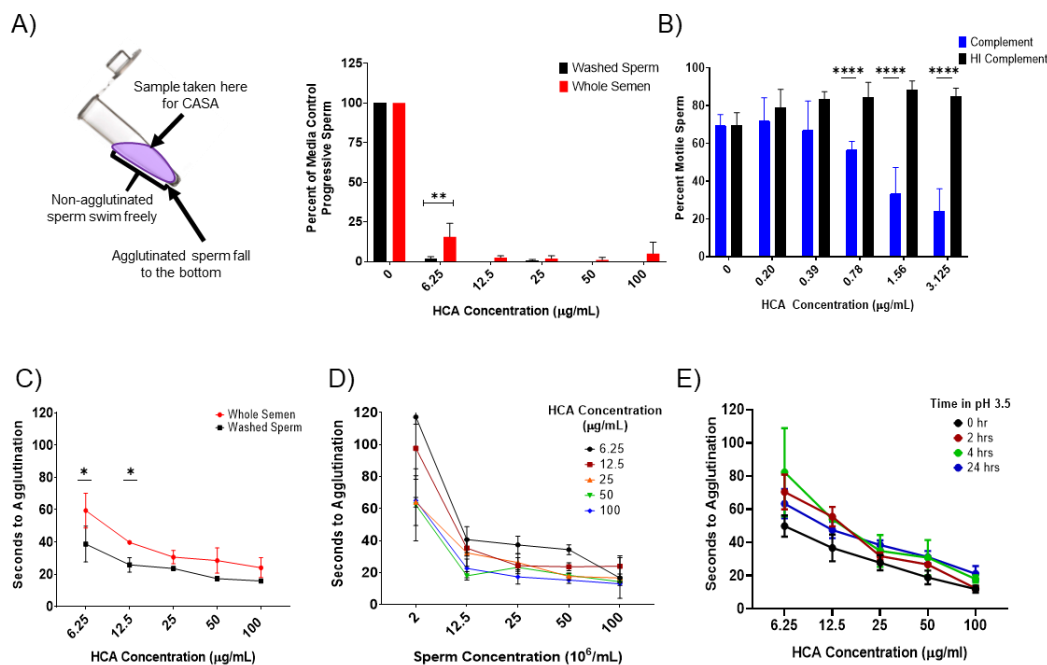


Figure 2-4 HCA agglutinates sperm in physiologically relevant conditions *in vitro*. A) Left panel is a schematic of the Sperm Escape Assay, the modified swim-up assay that measures progressive sperm that escape HCA induced agglutination. Right panel is a Sperm Escape Assay comparing whole semen and washed sperm cell agglutination. Y-axis depicts the percentage of progressive sperm present in the HCA-sperm samples compared to the progressive sperm found in the media (no antibody) control. Black bars indicate washed sperm data and red bars indicated whole semen data. Each bar indicates the average of three trials \pm SD. B) Complement-Dependent Sperm Immobilization Assay. Heat-inactivated complement was used as a control. Graph depicts the percentage of motile sperm found in the sample compared to the total number of sperm in the same sample, **** $p < 0.0001$. Black bars indicate immobilization with heat-inactivated complement and blue bars indicate immobilization with active human complement. Each bar indicates the average of three trials \pm SD, in duplicate. C) Agglutination Kinetics Assay comparing agglutination of whole semen and washed sperm cells. "Seconds to Agglutination" is the amount of time taken for all the sperm in a 10x field of view to fully agglutinate. Each data point is the average of three trials \pm SD (* $p < 0.05$). D) Agglutination Kinetics Assay comparing agglutination of different washed sperm concentrations. Each data point is the average of three trials \pm SD. E) Agglutination Kinetics Assay comparing HCA agglutination after prolonged incubation in 0.5% D/L lactic acid (pH 3.5) for 0, 2, 4, 24 hours. Each data point is the average of 3 trials \pm SD. All graphs are representative of 2-3 independent experiments, and 3- 6 participant samples.

HCA Kinetic Activity Under Physiologically Relevant Conditions

Washed sperm vs. whole semen

Since soluble CD52g in seminal plasma could compete with sperm CD52g for antibody binding, we tested the ability of HCA to agglutinate sperm in whole semen versus washed sperm preparations using the Agglutination Kinetics Assay. At HCA concentrations $\geq 25 \mu\text{g/mL}$, there was less than a 10 second difference between sperm agglutination times in whole semen compared to washed sperm. At lower antibody concentrations ($\leq 12.5 \mu\text{g/mL}$) time to agglutination was significantly slower in whole semen. At $6.25 \mu\text{g/mL}$ the time to 100% agglutination in whole semen was 65 seconds versus 40 seconds for washed sperm and the time to agglutination became highly variable (Figure 2-4C). We also compared the ability of HCA to agglutinate sperm in whole semen versus washed sperm preparations using the Sperm Escape Assay (Figure 2-4A). At HCA concentrations $\geq 12.5 \mu\text{g/mL}$, the number of progressively motile sperm in the swim-up fraction was less than 5% that of media-treated sperm, with both whole semen and washed sperm preparations. Both assays demonstrate that HCA agglutinates sperm in whole semen despite the presence

of soluble CD52g in seminal plasma. There was no significant effect on HCA's potent sperm agglutination activity at concentrations $>12.5 \mu\text{g/mL}$.

Effect of sperm concentration

In addition to soluble CD52g found in SP, agglutination time can also be affected by sperm concentration. Antibody-induced agglutination could be less efficient at low sperm concentrations because antibody-antigen interactions occur at a lower frequency; at higher sperm concentrations effective agglutination could require higher antibody concentrations. Sperm concentrations in human ejaculates across the population can vary from 0 sperm/mL (azoospermia) to greater than 200×10^6 /mL. Men with sperm concentrations $\leq 15 \times 10^6$ /mL are oligospermic, a condition associated with reduced fertility^{302,313,314}, whereas men with $\leq 1 \times 10^6$ sperm/mL are generally considered infertile. We therefore tested the ability of HCA to agglutinate sperm over a wide range of physiological sperm concentrations. As shown in Figure 2-4D, HCA concentrations $\geq 6.25 \mu\text{g/mL}$ effectively agglutinated sperm within one minute at sperm concentrations between 12.5×10^6 to 100×10^6 /mL, indicating that sperm concentrations within the fertile range agglutinate effectively in the

presence of HCA. At the lowest sperm concentration tested, 2×10^6 /mL, effective sperm agglutination occurred within 70 seconds at HCA concentrations ranging from 25-100 $\mu\text{g}/\text{mL}$. but took 120 seconds at the lowest HCA concentration (6.25 $\mu\text{g}/\text{mL}$).

Effect of low pH

HCA can potentially be used as a topical product in the vaginal tract, and must therefore withstand low pH conditions. Vaginal secretions of reproductive-aged women have an average pH of ~ 3.5 due to the production of lactic acid (LA) by *Lactobacillus* species that colonize the vagina^{96,97}. To determine whether HCA can withstand low pH, we incubated HCA for 0, 2, 4, and 24 hours at 37°C in 0.5% LA diluted in MHM. Since low pH affects sperm viability, the pH of LA-treated HCA was neutralized to pH 7.5 by addition of seminal plasma before use in the kinetic agglutination assay. While untreated HCA agglutinated sperm the fastest, LA-treated HCA at concentrations $\geq 12.5 \mu\text{g}/\text{mL}$ effectively agglutinated sperm in under one minute, and there was no significant difference across the various LA-treatment time points. At the lowest antibody concentration tested (6.25 $\mu\text{g}/\text{mL}$), sperm agglutinated in under 80 seconds and had increased

variability (Figure 2-4E). These data indicate that HCA retains activity at the low pH conditions typically found in the human vagina.

Preclinical Safety Test

We tested the effects of HCA and HCA immune complexes in an *in vitro* model of vaginal integrity and inflammation. Combinations of antibody, semen and human complement were added to the apical side of MatTek™ EpiVaginal tissues³¹⁰ and incubated for 6 hours. After washing the apical side of the tissues and allowing cytokines to be secreted into fresh media for 24 hours, the apical and basal supernatants were collected and cytokine concentrations were measured by Luminex (Figure 2-5A). Our previous studies, and the literature, have shown that semen alone increases levels of IL-1 β and IL-6 in apical and basal supernatants, but not HCA or complement^{315,316} (Figure 2-5B). Notably, HCA alone and HCA in the presence of complement did not induce upregulated cytokine secretion in the epithelial cells when compared to the media control. Histology of the treated tissues showed that all tissues were morphologically intact after treatment with HCA/HCA immune complexes.

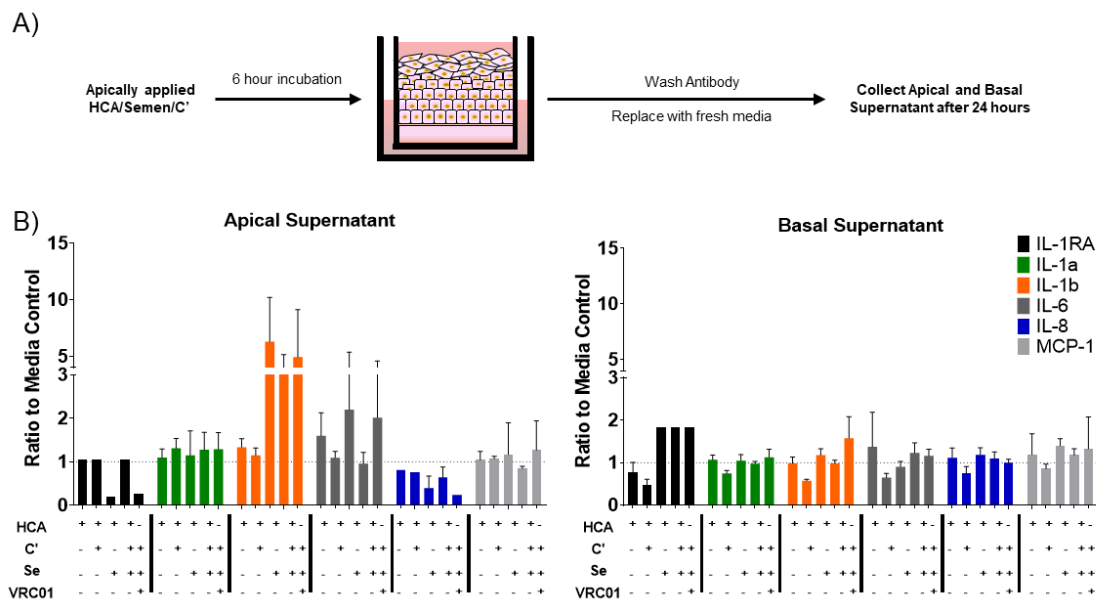


Figure 2-5- HCA does not increase proinflammatory cytokines in EpiVaginal tissue supernatants. A) Schematic of MatTek irritation assay where HCA/Semen/Complement were applied to the apical surface of EpiVaginal tissue. B) Left panel depicts cytokines secreted into the apical supernatant of tissue normalized to the media control. The right panel depicts cytokines secreted basally by the MatTek tissue normalized to the media control. VRC01 is an isotype control. Experiments were performed in duplicate. Graphs are representative of 2 individual experiments.

Discussion

The contraceptive revolution produced a variety of effective contraceptive methods and products, but a large gap remains^{2,7}. Today, approximately 40-50% of pregnancies worldwide are unintended^{3,9,13,17}. In the United States alone, 46% of unintended pregnancies are due to inconsistent or incorrect use of available contraceptives, and 50% are due to the non-use of birth control products⁹. These numbers demonstrate the need for contraceptives that are easier to use, more

widely available, discreet and budget-friendly, with fewer side effects, and suited to diverse patient needs.

Recent advances in the production of human mAbs have led to the introduction of over 70 EMA/FDA-approved mAbs for a number of clinical uses^{246,247}. Our team recently completed a Phase I clinical trial in women that tested the safety, pharmacokinetics and *ex vivo* efficacy of a vaginal film, MB66, that released broadly neutralizing, *Nicotiana*-produced human anti-HIV and HSV-2 IgG1 mAbs for topical STI protection. The MB66 vaginal film was found to be safe, and the concentration of active mAbs present in vaginal secretions after four hours of film insertion were sufficient to prevent HIV and HSV-2 infection *ex vivo* (median >1 mg/mL)³¹⁷. With MB66 as a potential topical microbicide, we wanted to investigate a topical contraceptive for use with the anti-HIV and HSV mAbs for the potential development of an MPT.

In this study, we have adapted an antisperm mAb, once associated with human infertility²⁸³, for potential use as a novel, reversible, topical contraceptive. We have shown that it is possible to produce a high-quality, anti-CD52g human IgG₁, known as the Human Contraceptive Antibody (HCA), using a *Nicotiana* platform. HCA specifically targets an epitope on the N-linked glycan of CD52g, a

male reproductive tract specific GPI-anchored protein produced by epithelial cells and found on the plasma membrane of sperm. Immunofluorescence studies showed that HCA reacted with the head and tail of sperm, as predicted. Further antibody specificity was determined by a lack of reactivity of HCA with lymphocyte CD52 (a protein sharing the peptide sequence of CD52g but exhibiting distinct glycosylation); tonsil tissue, rich in lymphocyte CD52; and vaginal tissue. PNGaseF-treated sperm lysates, also lacked HCA detection since the enzyme cleaves the glycan epitope from the peptide core. The pre-clinical safety of HCA was also demonstrated by the conserved tissue integrity of a human vaginal tissue model and the absence of inflammatory cytokines secreted by the vaginal tissue model after prolonged topical exposure to HCA and HCA immune complexes. With antibodies, including IgG1, found in mucosal secretions as natural mediators of disease protection, we were not expecting to see an immunogenic response^{82,84,106,318,319}. Altogether, these pre-clinical results demonstrate that HCA is specific to its intended target, suggesting safety in vaginal topical application.

The efficacy and potency of HCA under a variety of physiologically relevant conditions were demonstrated by sperm agglutination and swim-up

studies. HCA at the highest concentration tested (100 $\mu\text{g}/\text{mL}$) agglutinated 100% of sperm within 20 seconds. At concentrations $\geq 6.25 \mu\text{g}/\text{mL}$, HCA consistently agglutinated sperm in whole semen and washed sperm preparations in less than 60 seconds. Effective agglutination was additionally observed across a wide range of sperm concentrations (2×10^6 to $200 \times 10^6 /\text{mL}$), and Figure 2-3D reiterated that mAb concentrations, rather than sperm concentration, determines “Time to Agglutination”. HCA also continued to agglutinate effectively after prolonged exposure to LA pH 3.5, a condition generally found in the human vagina. The modified swim-up Sperm Escape Assay reflected similar efficacy. Fewer than 3% of individual progressively motile sperm, when compared to the media control, were detected in sperm preparations after treatment with HCA at concentrations $\geq 6.25 \mu\text{g}/\text{mL}$. Complement-mediated sperm immobilization was also observed at extremely low HCA concentrations ($\geq 0.78 \mu\text{g}/\text{mL}$) suggesting an additional sperm-inhibiting mechanism in mucosal tissues. While not in the scope of this study, HCA has the potential to interact with vaginal mucins and hence mucus-trap sperm that escape agglutination. Altogether, these data on HCA potency are promising and demonstrate the feasibility of topical application of HCA for contraception.

HCA is a highly effective contraceptive antibody that could address some of the current gaps in the contraception field^{6,35,36,40,320}, however costs and delivery methods need to be kept in mind. One of the limitations of antibody therapeutics, is their high cost⁹. Nevertheless, new production platforms such as *Nicotiana*, second generation CHO, and fungus are projected to bring the cost of mAbs down to approximately \$10/gram, thereby making it feasible to use mAbs for contraception and MPT applications. Bispecific and multivalent monoclonal antibodies are also currently being engineered to have even greater potency³²¹, which could bring the cost down further as less product will likely be needed to reach clinical effectivity. Due to the potential low cost of the mAb and its safety profile, HCA as a topical contraceptive could be accessible to patient populations worldwide, overcoming a limitation with current contraceptives³²²⁻³²⁴.

This study does not address delivery methods for a topical contraceptive. HCA administered via film, if pharmacokinetically similar to MB66, can maintain contraceptive concentrations for up to 24 hours (MB66 median= 32.2 µg/mL). Vaginal films are considered an “on-demand” product. For a long acting reversible contraceptive, a common request for contraceptive products, HCA can be administered with an intravaginal ring (IVR). End-product user’s needs must

be kept in mind during product delivery development for HCA to fully address the contraceptive gap.

Finally, one of the most attractive components of HCA is its potential to be combined with other antibodies or small molecules, without altering its efficiency. The condom, currently, is the only product on the market offering both contraception and STI protection. HCA's ability to work in tandem with other molecules, such as MB66 antibodies, opens the door to a topical, non-barrier multipurpose prevention technology, a currently unaddressed global need.²⁶⁴⁻²⁶⁷

Chapter 3 - HCA as a candidate for microbicide protection

Introduction

This chapter investigates HCA as a topical microbicide to prevent male-to-female sexual transmission of CD52g+ *Trichomonas vaginalis* and HIV-1.

Trichomonas vaginalis has a plasma membrane composed of a phospholipid bilayer, akin to sperm and seminal cells that readily take up GPI-anchored CD52g, the target for HCA. The recent sequencing of *Trichomonas vaginalis*' genome demonstrated expression of proteins needed for ceramide phosphoinositol (CPI) anchor biosynthesis, but not for GPI-anchor biosynthesis, explaining the documented absence of GPI-anchored proteins in TV^{237,329-331}. However, the absence of GPI-anchored surface proteins on TV, which are otherwise found in protozoa³²⁹, does not suggest that exogenous GPI-anchored CD52g cannot embed in the TV's plasma membrane. Similarly, HIV-1 is a virus obtaining its envelope from the plasma membrane of its CD4+ host cell. GPI-anchored proteins have been shown to insert into enveloped viruses, either through 1) budding from host cells since GPI-anchored proteins are known to accumulate at sites of viral budding (Figure 3-1) or 2) by direct embedding of soluble GPI-anchored proteins into the viral envelope, a process termed

“painting”^{202,277,332}. We hypothesize that these CD52g+ virions and parasites can be targeted by HCA, leading to agglutination, inactivation, and/or elimination.

To determine if HCA can potentially prevent the sexual transmission of HIV-1 and *Trichomonas vaginalis*, we first determined whether CD52g can coat these pathogens. *Trichomonas vaginalis* was incubated with seminal plasma (containing soluble CD52g), and the resulting CD52g coating of the pathogen was verified with immunofluorescence and western blot. HCA agglutination of CD52g+ TV was investigated using the CASA and a modified version of the swim-up assay described in Chapter 2. TV adhere to vaginal epithelial cells upon infection. HCA’s role in preventing TV’s adherence to its target cells was studied using MatTek™ EpiVaginal tissue and VK2/E6E7 epithelial cell models. HIV-1 virions were coated with CD52g by budding from SP-treated, CD52g+ HEK293T cells and by viral painting with SP. Coating was verified with “antibody capture plates” and colloidal gold staining and transmission electron microscopy. HCA agglutination of CD52g+ virions with sperm was determined by p24 concentrations found in virus-sperm supernatants. HCA neutralization of HIV-1 was evaluated by modifying Montefiori’s TZM-bl neutralization assay³³³.

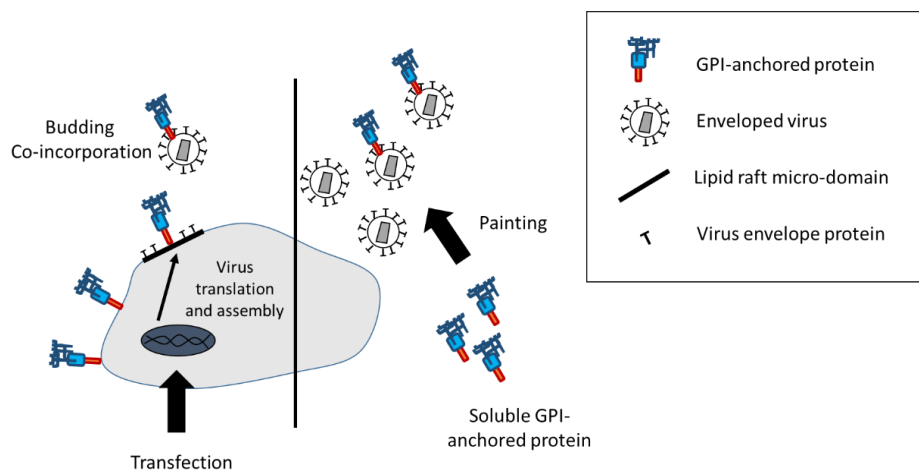


Figure 3-1- Schematic depicting GPI-anchor hypermobility in painting and budding incorporation in enveloped viruses. Painting was used for TV, and budding for HIV. Figure adapted from Dangerfield 2008.

Methods

Antibodies

The human anti-CD52g mAb, HCA, the human broadly neutralizing mAb, VRC01-N, and MB66 film (containing VRC01-N and HSV8) were produced by Mapp Biopharmaceutical Inc. (San Diego, CA, USA) in *Nicotiana benthamiana*, as described in Chapter 2.

Trichomonas vaginalis Experiments

Trichomonas vaginalis culture method

T. vaginalis were cultured in TYM broth supplemented with 10% FBS and 1% Pen/Step, 1x dilution of Diamonds Vitamin Tween 80 solution 40x (58980C, Millipore Sigma, Burlington, MA, USA) and grown in a polystyrene flat bottom 10 mL culture tube with a screw cap (62407-076, VWR, Radnor, PA, USA). *T. vaginalis* were passaged every 48 hours with a 1:1000 dilution; they were incubated at 37 °C under anaerobic conditions.

Trichomonas vaginalis CD52g "painting"

Trichomonas vaginalis were incubated $5-7 \times 10^6$ in 5 mL of pooled seminal plasma or media (negative control) with a 1x dilution of Diamonds Vitamin Tween 80 solution for 2 hours at 37°C. Protozoa were washed twice in PBS. Washed *T. vaginalis* were then resuspended in media.

VK2/E6E7 cell culture

Vaginal epithelial cells, VK2/E6E7 were cultured according to ATCC's instructions in Keratinocyte-Serum Free medium (17005-042, ThermoFisher

Scientific, Waltham, MA, USA). The media includes 0.1 ng/mL human recombinant EGF, 0.05 mg/mL bovine pituitary extract. Additional calcium chloride was added per ATCC instructions (final concentration, 0.4mM). VK2 were trypsinized and neutralized with complete RPMI (10% FBS, 1% Pen/Strep, 1% L-glutamine), spun and plated for passage.

EpiVaginal culture

EpiVaginal™ tissues were acclimated for at least 24 hours post-delivery at 37°C, 5% CO₂, and cultured according to the manufacturer's instructions (VEC-100-FT, MatTek Corporation, Ashland, MA, USA). Transepithelial resistance (TEER) assessments were performed to confirm tissue integrity. Only tissues with TEER values higher than 400 ohms were used. Media was replaced an hour before performing adherence assays.

Immunofluorescence

Trichomonas vaginalis were incubated in CMFDA (1:500 dilution) for 20 minutes at RT before CD52g painting. TV were resuspended in PBS, air dried and subsequently fixed in methanol or acetone. Samples were rehydrated in 1x TBS,

blocked, and stained with 100 µg/mL of primary antibody for 1 hour at RT.

Secondary was diluted 1:200 and incubated with samples at RT for 1 hour.

Coverslips were mounted with DAPI-containing mounting medium. HCA was used to detect CD52g and VRC01-N was used as an isotype control.

SDS-PAGE

Painted TV were washed in PBS and resuspended in RIPA buffer with 0.5%

Sodium Orthovanadate, 0.5% PMSF in DMSO, and 1% protease inhibitor cocktail

(sc-24948, Santa Cruz Biotechnology, Inc. Dallas, TX, USA). Lysed sample was

freeze-thawed before spinning out cell debris at 400g at RT. Protein

concentration of the lysed sample was determined via bicinchoninic acid assay

(BCA) (23227, Thermo Scientific, Waltham, MA). SDS-PAGE was run with 10-20

µg of sample lysis loaded onto a 4-12% gradient gel (4561084, Bio-Rad, Hercules,

CA, USA) in commercial running buffer (1610732, Bio-Rad).

Western blot

The gel was transferred to a PVDF membrane with commercial transfer buffer

(1610734, Bio-Rad). The blot was blocked in 5% dry milk overnight, and

incubated in 10 µg/mL of primary HCA in BSA. HRP-conjugated anti-human secondary was diluted 1:2000. The membrane was stripped and activated with methanol prior to Ponceau staining (59803, Cell Signaling Technology, Danvers, MA, USA).

Agglutination studies

Direct agglutination.

Trichomonas vaginalis were painted with CD52g from SP as described above. Cells were washed twice in *T. vaginalis* media. The CASA was modified to detect *Trichomonas vaginalis* by modifying light and progressive/motile settings. The TV agglutination assay is performed similarly to the Sperm Escape Assay. Briefly, mAbs were diluted in TYM media and 30 µl were added to 0.2 mL PCR tubes. Thirty µL of sample were then added to the mAbs and mixed three times by pipetting; the mixed sample was placed at a 45° angle. A 3 µL sample was taken from the top of the PCR tube and placed in a 4-chambered slide after a 5-minute incubation at RT. The CASA was used to count *T. vaginalis*.

Trichomonas vaginalis co-agglutination

TV were painted with SP as described above. Protozoa were washed twice in MHM. Pelleted *T. vaginalis* were resuspended in either whole semen or washed sperm cells. MAbs were diluted in MHM and 30 μ L were added to 0.2 mL PCR tubes. Thirty μ L of co-cultured sample were added to the mAbs and mixed three times by pipetting; the PCR tube was then placed at a 45° angle and incubated for 5 minutes at RT. A 3 μ L sample was taken from the top of the sample, placed in a 4-chambered slide, and analyzed by CASA.

Adherence studies

Two different tissue culture models were used for adherence models: MatTek EpiVaginal Tissue and VK2/E6E7 cells.

For the EpiVaginal MatTek tissue assay, *Trichomonas vaginalis* were painted with seminal plasma, stained with CMFDA (1:500 dilution), and washed and resuspended in serum-free MatTek tissue media. Washed sperm cells and antibody were also resuspended in serum-free MatTek tissue media. Antibody was placed on the apical side of MatTek tissue and incubated for 1 hour before removal. Sperm and TV were then added and allowed to incubate for 1 hour at

37°C. The apical side of the tissue was washed 3x with warmed PBS and the tissues were fixed with formaldehyde at 4°C, overnight. The tissues were then placed apical side down in DAPI mounting media in glass bottom plates (P35GC-1.5-10-C, MatTek Corporation, Ashland, MA, USA). Tissue was imaged using a Leica SP5 confocal microscope (Training and access courtesy of Cellular Imaging Core, Boston University School of Medicine).

The VK2 epithelial cell model was prepared 24 hours before running the assay. Coverslips were used to grow the VK2 monolayer. A hydrophobic pen (S200230-2, Dako Agilent, Santa Clara, CA, USA) was used to draw four rectangular wells on the cover slips. A piece of tape (for labeling) was affixed to 0.5 cm of the coverslip (Figure 3-2). The coverslips were sterilized with UV light in a cell culture hood for 1 hour. VK2 cells were trypsinized, neutralized with complete RPMI, and resuspended in their media after washing. Cells were seeded at 125,000 cells per well and allowed to adhere overnight; media was replaced the following morning. All cells and antibodies were resuspended or diluted in the Keratinocyte Serum-Free Media. *T. vaginalis* adherence was determined as described above for MatTek tissue with the exception that HCA was not washed

off the monolayers. *T. vaginalis* was allowed to adhere for an hour at 37 °C.

Coverslips were gently washed with PBS and fixed with acetone. Samples were rehydrated in 1x TBS for 5 minutes before mounting in DAPI mounting media onto a glass slide.

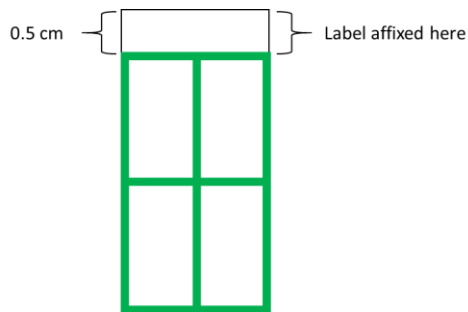


Figure 3-2- Schematic of customized coverslip wells for VK2 monolayer culture. Green represents hydrophobic pen demarcations

HIV-1 Experiments

CEM cell culture

CEMs, a T cell line were cultured in complete RPMI. Passages were maintained at 300,000 cells/mL. Cells were activated at 1×10^6 with 10 $\mu\text{g}/\text{mL}$ of PHA (00-4977-03, ThermoFisher Scientific, Waltham, MA, USA) for 48 hours before HIV-1 infection.

PBMC cell culture

Peripheral Blood Mononuclear Cells (PBMCs) were acquired from blood packs provided by healthy donors (NYBiologics, Southampton, NY, USA) and isolated with Ficoll (17144002, GE Healthcare Life Science, Marlborough, MA, USA). Cells were stored at -80C until use. Frozen stocks were thawed and washed with complete RPMI at 50x the frozen stock volume. Cells were activated upon thawing for 48 hours and allowed to rest for 24-48 hours.

HEK293T cell culture

An aliquot of HEK293T was generously provided by the Henderson lab. Cells were cultured in High Glucose Dulbecco's Modified Eagle's Media supplemented with 10% FBS, 1% Pen/Strep, and 1% L-Glutamate.

HIV-1 strains

The following strains of HIV-1 were used for these studies. Plasmids or viral supernatants were generously provided by Luis Agosto, Ph.D. and the AIDS Reagents Program. This work was facilitated by the Providence/Boston Center for AIDS Research (P30AI042853).

- NL43-IRES-GFP-nef+
- NL43-GAG-eGFP
- Q23-17
- TRJO

HIV-1 CD52g "painting"

Virus was pulsed with pooled healthy SP or media for 3 hours on a shaker at 37°C.

HEK293T transfection

HEK293T cells were passaged once, at a minimum, after thawing before being used for transfection and were not kept for more than 5 weeks. Cells were plated at 0.5×10^6 cells approximately 24 hours before transfection. Media was replaced 8 hours before transfection. HIV-1 DNA plasmid was prepared with PEI in a 3:1 ratio. At 22 hours post transfection, HEK293T cells were pulsed for 2 hours with SP. Adherent cells were washed with warm 1x PBS and non-adherent cells were spun out of SP, washed once, and re-plated. Viral supernatant was collected 40 hours post transfection.

CEM infection with Q23-17 and SP pulsation

In a 96 well plate, 300,000 cells per well were plated and spinoculated with Q23-17 virus for 2 hours at 1200g at RT. Spinoculated cultures were incubated for 30

minutes at 37 °C. Cells were washed in cold 5% FBS in PBS and re-plated at 10,000 cells/well in warm complete RPMI.

PBMC infection with TRJO

PBMCs were activated with 10 µg/mL of PHA and 20 µg/mL of IL-2 for 48 hours. Cells were placed in IL-2 supplemented media for 24-48 hours before infection. Cells were infected at a concentration of 1×10^6 cells/mL in 500 µL in DEAE supplemented media. Cells were washed at RT with 5% FBS in PBS before being placed into culture. Cells were incubated at 37 °C for 4 hours and cells were resuspended every hour.

PBMC infection for immunogold Transmission Electron Microscopy (TEM)

PBMCs were activated with PHA and IL-2 as described above. Cells were pulsed with SP at 36 hours post-infection for 2 hours, washed 2x, and allowed to incubate for 4 hours before staining. Cells were centrifuged at 300g, and, on ice: blocked with FcR block for 30 minutes (NB309, Innovex Biosciences, Richmond, CA, USA), blocked with 5% FBS in PBS for 30 minutes; reacted with HCA for 60 minutes, and washed twice with 10% FBS before adding colloidal gold-

conjugated anti-human IgG secondary (109-205-088 and 109-195-088, Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) at a 1:20 dilution per manufacturer's instructions. Following a 60 minute incubation in secondary antibody, cells were washed in PBS, fixed in 2.5% Paraformaldehyde, 5.0% glutaraldehyde, 0.06% picric acid in 0.2 M cacodylate buffer, and further processed (embedded, sectioned and visualized) by the Harvard Imaging Core.

Immunofluorescence

Cells were transfected or infected with a GFP-labelled HIV-1 and were air-dried on slides and fixed in acetone. Samples were rehydrated in 1x TBS, blocked and stained with 1) Cy3-conjugated HCA and HSV8 for 3 hours at room temperature or 2) unconjugated primary antibody incubated for 1 hour at RT, washed, and stained with Cy-3 conjugated anti-human IgG secondary. Samples were mounted with DAPI-containing mounting medium. HCA was used to detect CD52g, and the HSV8 mAb was used as an isotype control.

Viral plate capture

Non-tissue-treated 96-well plates (351172, Falcon, NYC, NY, USA) were coated with antibody diluted in PBS and serially diluted 2-fold. Plates were incubated at 37 °C overnight. Plates were washed with PBS before blocking with 5% FBS in PBS for 1 hour at 37 °C. Blocked plates were washed with 1x PBS before adding virus sample. Virus supernatant was allowed to incubate with plate-bound antibodies for 3 hours at 37°C before being transferred to p24 ELISA plates to quantify unadhered virus.

HIV-1/Sperm cell co-culture

Virus was added to washed sperm cells and aliquots of the cell suspension were added to serially diluted antibody in MHM. The antibody/virus/sperm mixture was allowed to incubate for 10 minutes at RT before being gently spun down at 300g for 5 minutes at RT to pellet cells. The supernatant was removed and uncaptured virions still in suspension were quantified by p24 ELISA.

p24 ELISAs

Non-tissue culture treated 96-well plates were coated with 20 µg/mL of HIV-1 polyclonal antibody (3957, NIH AIDS Reagent Program) diluted in PBS and incubated at 37°C overnight. Plates were washed with PBS prior to blocking in Blocking Buffer (5% FBS in 1xPBS) for 1 hour at 37 °C. The samples tested were diluted 1:1 with sample diluent (10%FBS, 0.5% Triton-X in PBS) and added to capture antibody for 2 hours at 37 °C. Anti-p24 primary detection antibody (183-H12-5C, NIH AIDS Reagent Program) was diluted in sample diluent and incubated for 1 hour at 37 °C. HRP-conjugated anti-mouse IgG secondary antibody was treated similarly (sc-2005, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Plates were washed 3x with wash buffer (0.2% Tween in PBS) between each of these steps. 1X TMB was added to the plate and incubated at RT until the lowest concentration of the standard turned a pale blue. Reaction was neutralized with 4M Sulfuric Acid (S25898, Fisher Scientific, Hampton, NH, USA).

Neutralization assays with TZM-bl cells

HIV-1 neutralization assays

Antibody and virus were diluted to desired concentrations in complete DMEM media. Antibody and virus were incubated for 30 minutes at RT in a clear bottom, black 96-well plate (3603, Fisher Scientific, Hampton, NH, USA). 10,000 TZM-bl cells, suspended in DMEM with 20 µg/mL of DEAE, were added to each well. TZM-bl cells were infected with virus for 46-48 hours before lysis.

Galactosidase production (a measure of HIV infection) was assessed with a chemiluminescent kit following manufacturer's instructions (T1011, ThermoFisher Scientific, Waltham, MA, USA)³³³.

HIV-1/Sperm neutralization assays

TZM-bl cells were plated at 10,000 cells/well 16 hours before infection. Media was replaced the following morning with 20 µg/mL of DEAE added. Antibody was diluted in tissue culture-treated v-bottom, 96-well plates (3894, Corning, Corning, NY, USA). Previously titrated HIV-1 was added to washed sperm cells or media (negative control); the mixture was then added to the antibody dilutions, and transferred to the plated TZM-bl cells. For HCA-VRC01 antibody

concentration, VRC01 was serially diluted, while HCA was kept at a concentration of 50 µg/mL. Virus and cells were incubated overnight before being replaced with fresh DMEM. TZM-bl cells were lysed 46-48 hours post-infection and read with a chemiluminescent kit

Semen Analysis and Sperm Preparation

Healthy men, between 18-45 years old with sperm samples meeting the WHO's semen characteristics for fertility, provided the samples for this study as approved by the Boston University Medical Campus Institutional Review Board. Participants provided written informed consent for the study.

Samples were processed within one hour of collection. For *Trichomonas vaginalis* studies, live sperm were separated by passage through 90% ISolate density gradient medium (FUJIFILM Irvine Scientific; Santa Ana, CA, USA) as described in Chapter 2. For HIV-1 co-culture studies, sperm were pelleted from seminal plasma by centrifugation at 350g for 20 minutes at room temperature (RT). Cells were resuspended in assay medium, and sperm count and motility were assessed by CASA.

Statistical Analysis

GraphPad Prism (Version 7.03; GraphPad Software Inc.; San Diego, CA, USA) was used for statistical analysis and graphing. Data were subjected to analysis of variance (ANOVA). A significant ANOVA was followed by post hoc multiple comparison tests as recommended by software settings. Differences were considered to be statistically significant when $p\text{-value} < 0.05$.

Results

CD52g Coats the Surface of Trichomonas vaginalis

To determine the effect of HCA on *Trichomonas vaginalis*, we first had to determine whether CD52g inserted onto the plasma membrane of the protozoa. This was determined by immunofluorescence (IF) and western blot assays. While flow cytometry was attempted, TV did not survive the staining, and fixing cells before staining led to conflicting results (data not shown). Immunofluorescence of pulsed TV is shown in Figure 3-3A. DAPI was used to stain protozoal nuclei. CMFDA is a green cytoplasmic dye which requires enzymatic activity in the cytosol, hence staining live cells. CD52g was detected with HCA primary and Cy3-conjugated secondary antibodies. VRC01, an HIV-1 gp120 specific antibody,

was used as an isotype control. As shown in Figure 3-3, following incubation in seminal plasma, TV were painted with CD52g and detected with HCA antibody. *Trichomonas vaginalis* incubated for 2 hours in fresh TYM media and SP-treated TV stained with VRC01 (negative control), were not positive in the immunofluorescence assay.

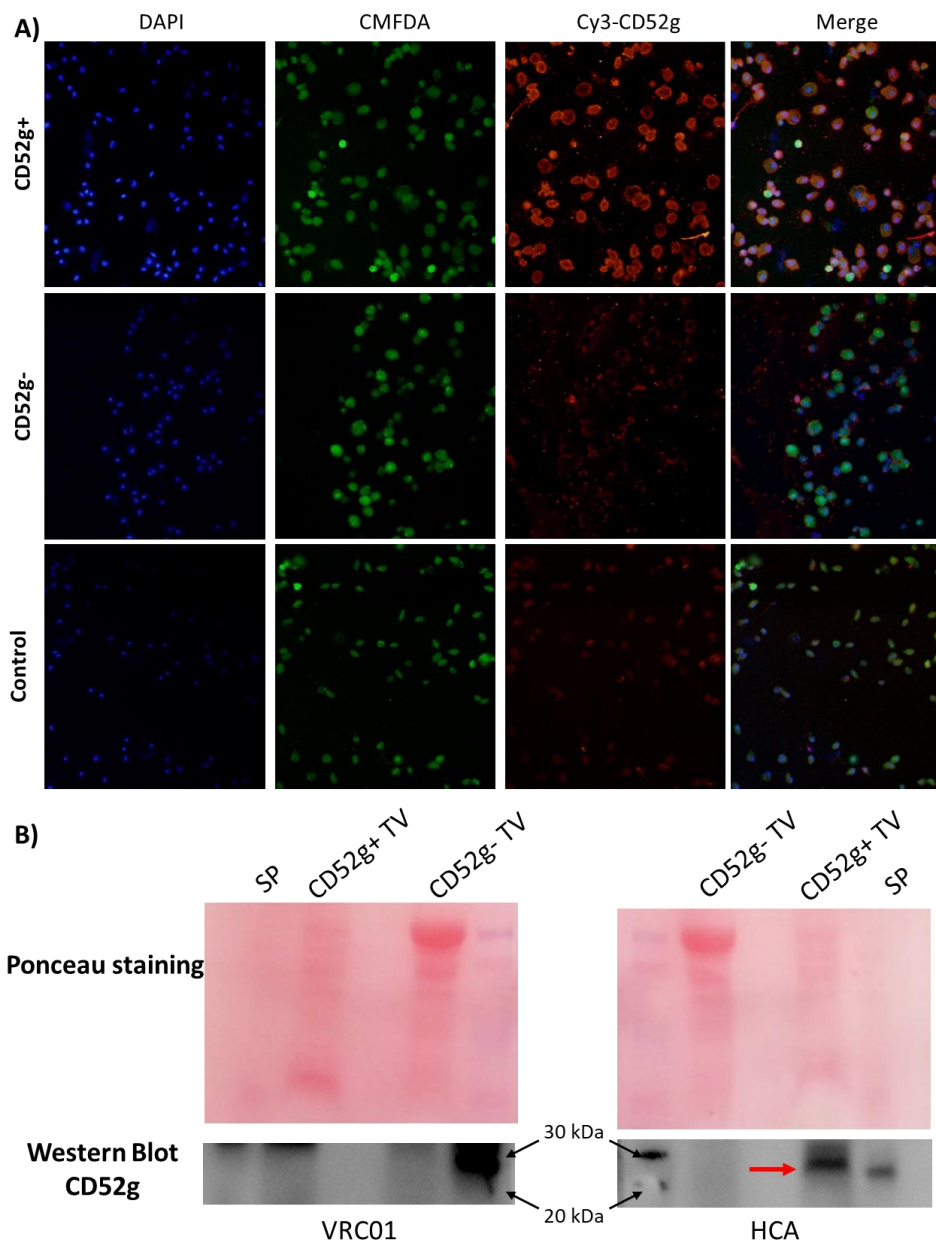


Figure 3-3- HCA coats the plasma membrane of *Trichomonas vaginalis*. A) Immunofluorescence of *Trichomonas vaginalis* pulsed for 2 hours in SP (CD52g+, red) or media (CD52g-). CMFDA (green) is a cytoplasmic stain. DAPI nuclear stain is blue. VRC01 was used as an isotype control. Red arrow indicated CD52g band. All images at 20x magnification. B) Top panel is a Ponceau staining of PVDF membrane of Western blot (bottom panel) of pulsed and lysed *Trichomonas vaginalis*. VRC01 was used as an isotype control.

Western blot was used to confirm the presence of CD52g in SP-treated protozoa. Painted TV were washed in PBS three times before being lysed for western blot, to thoroughly remove SP. Figure 3-3B (bottom panel) depicts a ~25 kDa band, highlighted with a red arrow. The band is the expected size of denatured CD52g. As shown in the western blot panel of Figure 3-3B, only SP (containing soluble CD52g) and SP treated TV had a ~25 kDa band when blotted with HCA primary. Media-treated TV reacted with HCA and VRC01-N blotted membranes (an isotype control) did not show 25 kDa CD52g band. Commercial antibodies for TV were not suitable for western blot and typical loading controls (such as actin) were found to be ineffective for TV western blotting. Therefore, Ponceu stain, a negatively charged red dye that reacts with positively charged amino acids, was used to confirm protein loading on our PVDF membrane prior to blotting (figure B, top panel).

HCA Agglutinates CD52g+ Trichomonas vaginalis in Whole Semen

After CD52g insertion was determined, HCA's ability to agglutinate *Trichomonas vaginalis* was evaluated using a modified version of the swim-up assay. The CASA was modified to detect individual protozoa (Figure 3-4A). TV motility could not be reliably evaluated by the CASA due to software constraints

specific to sperm, such as cross beating frequency, which relies on sperm head and flagella movement. However, altering the parameters on specimen size and light reflection enabled the CASA to detect individual and agglutinated *Trichomonas vaginalis* and correctly distinguish them from sperm cells, as shown in Figure 3-4B.

A)

Set-up	Value
Head Area (μm^2)	Min: 34/Max:245
Elongation (%)	Min: 25/Max: 100
Allow Advanced Tail detection	False
Head brightness min	225
Static tail filter	False
Cell travel max (μm)	10
Enable Mobile Static Collision Avoidance	False
Motile cells require a tail	False

B)

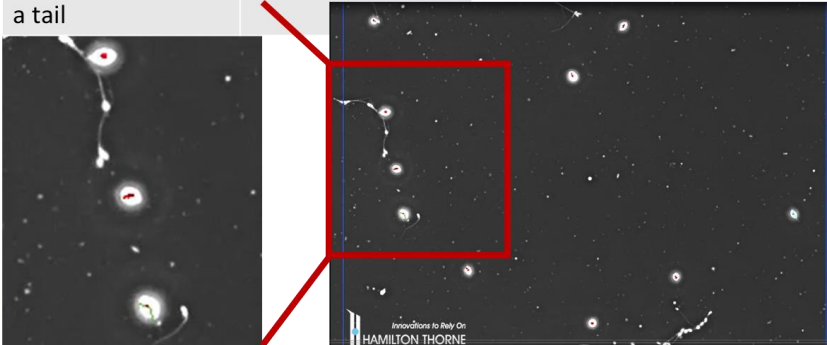


Figure 3-4-CASA settings for *Trichomonas vaginalis* detection. A) Detection setting for TV. B) CASA image showing *Trichomonas vaginalis* detection by CASA (represented by red dots on the protozoa) while excluding sperm at 10x magnification.

The use of the CASA permitted the implementation of a modified “sperm escape assay”, as shown in Figure 2-4. Unlike sperm cells, *Trichomonas vaginalis* did not agglutinate in the presence of HCA even when painted with CD52g (Figure 3-5A). Agglutination was assessed at a variety of TV concentrations, prolonged incubation with antibody and even gentle spinning to encourage protozoal/antibody interactions (Data not shown). We next co-agglutinated *Trichomonas vaginalis* in the presence of washed sperm and whole semen. As shown in Figure 3-5B, there were fewer free-swimming CD52g+ TV in co-cultured samples upon HCA treatment. A dose response was observed with a ~50% reduction of free TV (compared to media control) observed at 100 µg/mL and only a ~30% reduction found at 6.25 µg/mL. To a lesser degree, a modest dose response was also found with free swimming CD52g- *Trichomonas vaginalis*, but was not statistically significant. The decrease of CD52g+ TV in sample taken from HCA treated co-cultures was more pronounced when whole semen samples were used in co-agglutination. Fewer CD52g- TV were also found at higher HCA concentrations in TV-Semen co-culture samples (Figure 3-5C). These data suggest that co-agglutination with sperm could play a role in hindering *Trichomonas* mobility in the presence of HCA.

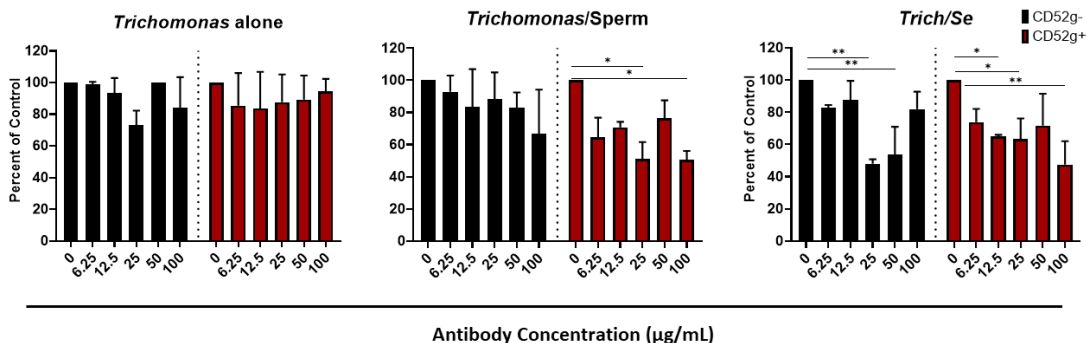


Figure 3-5- *Trichomonas vaginalis* agglutination alone, with sperm cells, and whole semen. A) *T. vaginalis* escape assay with SP pulsed and media pulsed *T. vaginalis*. B) *T. vaginalis* escape assay with both SP pulsed and media pulsed *T. vaginalis* agglutinated with washed sperm. C) SP and media pulsed *T. vaginalis* escape assay with whole semen. Black bars indicate media pulsed (CD52g-) *Trichomonas vaginalis*. *p-value<0.05, **p-value<0.01. Red bars indicate SP pulsed (CD52g+) *Trichomonas vaginalis*. Graphs are each representative of three experiments.

HCA Blocks Adherence of TV to Vaginal Epithelial Cells

Trichomonas vaginalis infection occurs when the protozoa adhere to epithelial cells in the FRT. Adherence studies were done both in MatTek Epivaginal tissue and VK2 cells. Figure 3-6 shows the MatTek tissue results. *Trichomonas vaginalis* were treated with CMFDA and painted with SP as described above. The protozoa were subsequently added to the apical side of the tissue, with or without sperm, and with or without HCA pre-treatment. The cells were allowed to adhere to the tissue for an hour; the tissue was gently washed to remove any unattached TV. The apical side of the tissue was then imaged, and the individual fluorescent TV were manually counted in each field of view.

Manual counting was preferred since the amoeboid form of adhered *Trichomonas* sp. exhibited variable particle size and was not easily quantified by ImageJ. As shown in Figure 3-6A, fewer SP-treated *Trichomonas vaginalis* adhered to the epithelial cells than media-treated TV. TV adherence was unaffected by the presence of HCA or sperm alone in the tissue model. Interestingly, decreased pathogen adherence was found in CD52g- TV in the combined presence of HCA and sperm. Washed sperm, not whole semen, was used since the presence of semen was found to prevent *Trichomonas* adherence (data not shown) *in vitro*. Due to the lower adherence found in SP-treated TV, the assay was insufficiently powered to detect significance; nevertheless, like CD52g- TV, CD52g+ TV adherence was reduced in sperm co-culture in HCA-treated tissue. Adherence was also studied with a confluent VK2 monolayer grown on a coverslip. Quantification with VK2s was facilitated with ImageJ, since VK2 and TV nuclei were visibly different sizes allowing TV nuclei to be consistently counted. HCA, unlike in the MatTek tissue model, was not washed off after an hour incubation since there was no evidence for antibody uptake in the cell monolayer (data not shown). In VK2 monolayers, unlike MatTek tissues, there was a significant decrease in adherence for (Figure 3-6B) both CD52g+ and CD52g- TV in the

presence of the antibody alone. Like in the MatTek adherence assay, CD52g+ TV adherence was reduced. Similarly, the decreased TV adherence in the combined presence of HCA and SC was reflected in both CD52g+ and CD52g- TV.

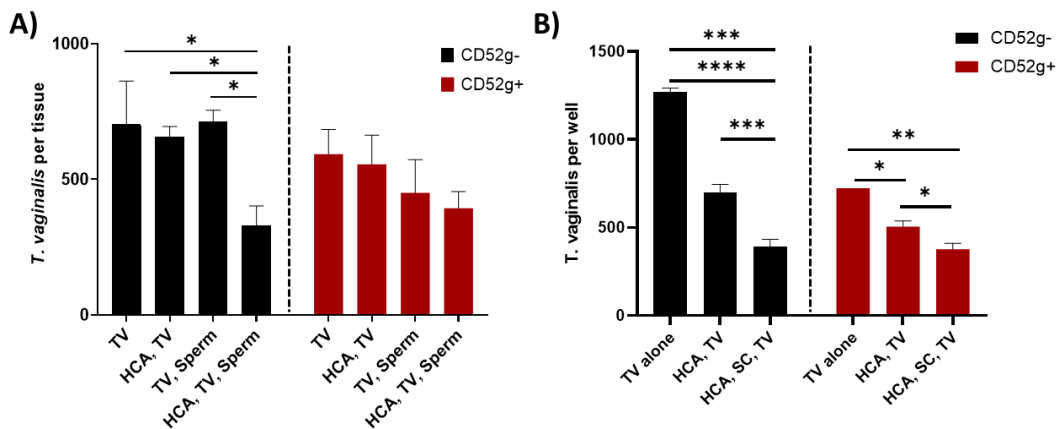


Figure 3-6-*Trichomonas vaginalis* adherence assays. A) *T. vaginalis* adherence assays with MatTek tissue. TV=*T. vaginalis* pulsed in media; TV+=*T. vaginalis* pulsed in SP. Graph is representative of n=2 experiments. B) *T. vaginalis* adherence assay on VK2 monolayer. Black bars indicate media pulsed (CD52g-) *Trichomonas vaginalis*. Red bars indicate SP pulsed (CD52g+) *Trichomonas vaginalis*. ****p-value<0.0001; ***p-value<0.0005; **p-value<0.005, *p-value<0.05. Graph is representative of n=2 experiments.

Producing CD52g+ HIV-1 Virions

HIV has a phospholipid envelope thereby enabling the incorporation of GPI-anchored CD52g possible. To determine whether HIV-1 picks up CD52g during budding from CD52g-coated host cells (e.g. HIV-infected cells in the male genital tract and semen), we transfected NL43-IRES-GFP-nef+ and NL43-GAG-eGFP virus plasmid into SP-treated HEK293T cells. Twenty-four hours post-

transfection, HEK293Ts were pulsed with seminal plasma. Cells reacted poorly to SP treatment, lifting off within an hour pulsation (data not shown). Unadhered cells were spun out of the SP and washed before being reintroduced into culture; HEK293T cells survived seminal plasma treatment and re-adhered; however, SP pulsation did decrease HIV-1 production, and higher volumes of virus were needed to obtain levels of infection similar to those of media controls. Flow cytometry was then used to determine transfection efficiency. As shown in Figure 3-8, SP-treatment caused background in flow cytometry. If the 12.9% background is disregarded, the transfection efficiency is ~50% in SP-treated NL43-GAG-eGFP transfected cells versus a rate of 60% transfection found in media-treated cells. The decreased transfection efficiency is also readily apparent by immunofluorescence in the decreased number of HEK293Ts with GFP-tagged HIV-1 (Figure 3-7). HEK293T cells were still coated in CD52g (detected with HCA primary and Cy3 secondary) at the time of virus harvest as shown in Figure 3-7; CD52g was only detected on SP-pulsed HEK293T cells.

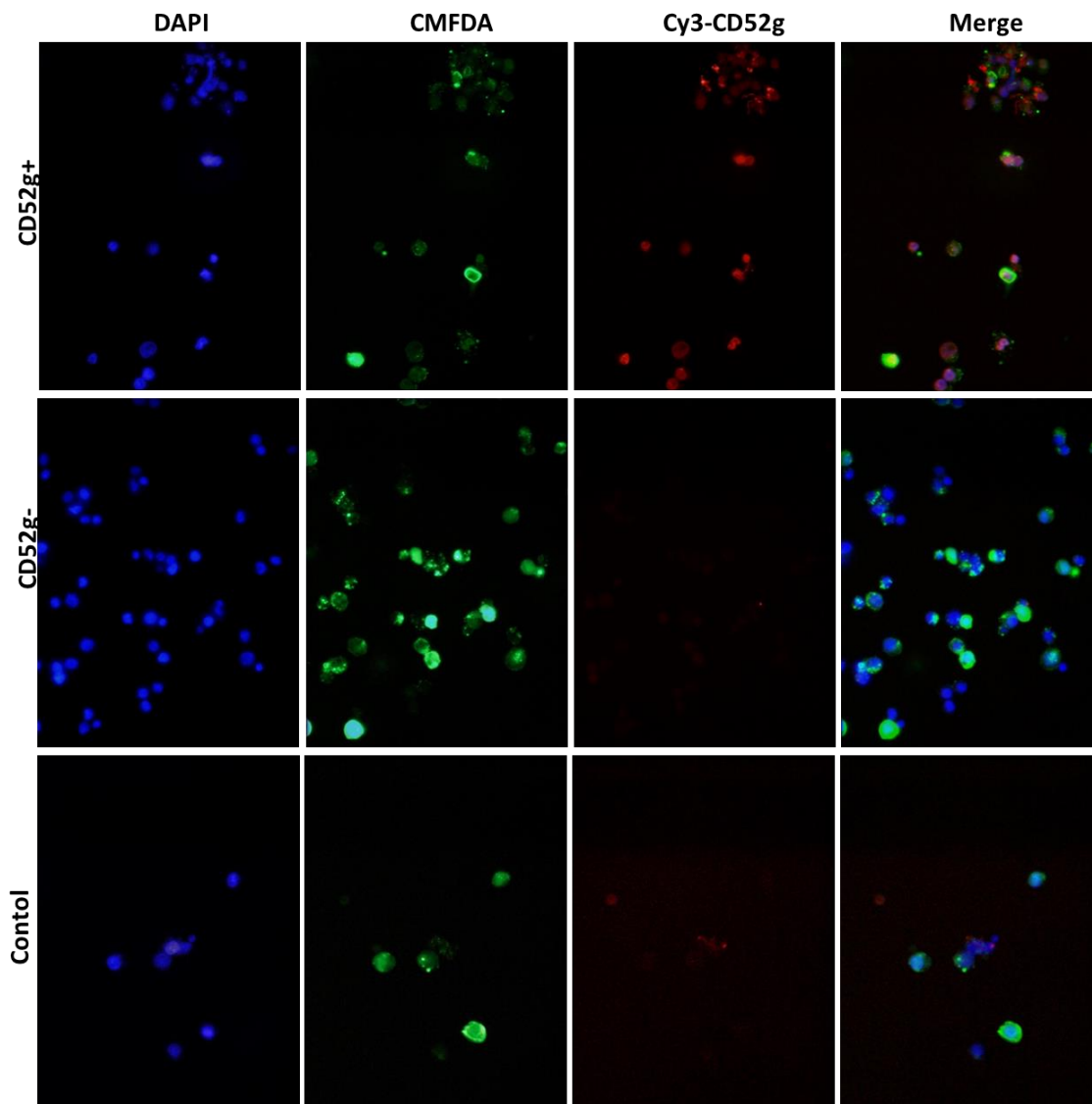


Figure 3-7-HCA coats the plasma membrane of transfected HEK293T cells. A) Immunofluorescence of HEK293T cells pulsed for 2 hours in SP (CD52g+, red) or Media (CD52g-). Green Fluorescence indicates GFP-tagged GAG protein of HIV-1. DAPI nuclear stain is in blue. VRC01 was used as an isotype control. All images were at 20x magnification.

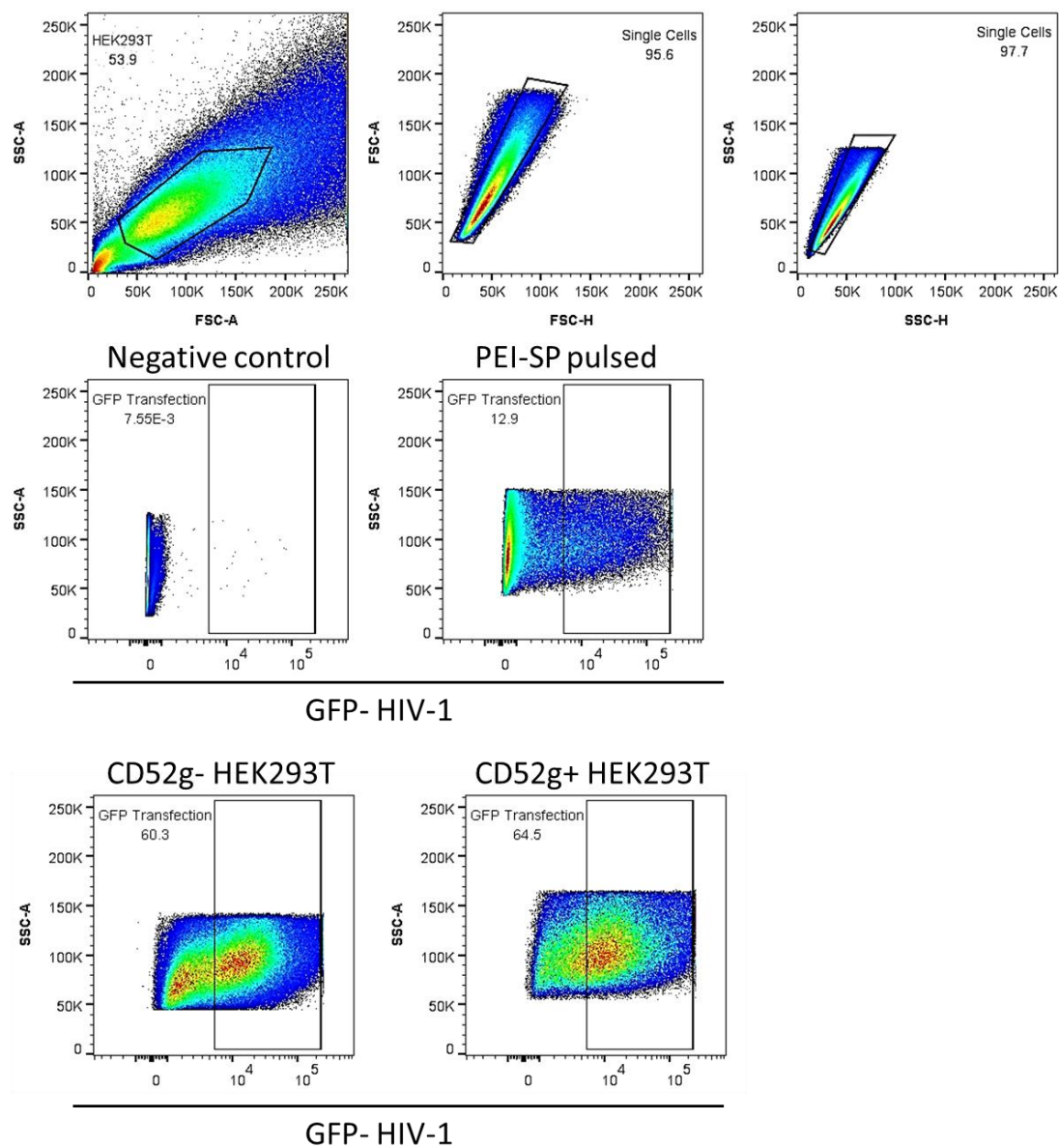


Figure 3-8-Transfection efficiency of SP pulsed HEK293T cells compared to media controls. GFP tagged virus (NL43-GAG-eGFP) allowed easy detection of transfection of HEK293T cells. PEI-SP pulsed mock transfected cells were included to show background induced by SP.

Confirming CD52g on HIV-1 Envelope

While troubleshooting virus production, it was necessary to come up with a proxy measure for testing the presence of CD52g on the HIV-1 envelope. We therefore coated plates, referred to as “capture plates” with either HCA, HSV8 (an isotype-matched negative control antibody), or VRC01 (an isotype-matched positive control antibody specific for gp120 on HIV-1). The supernatant from the antibody capture plates was subsequently quantified with a p24 ELISA. If the virus was captured by the immobilized antibody on the plate, less virus would be found in the supernatant. As shown in Figure 3-9A and B, p24 found in the viral supernatant was significantly decreased with CD52g+ virus captured by VRC01 and HCA (p-value < 0.00005), but not with HSV8. For CD52g- virus, a p24 decrease was only found in the supernatant incubated with immobilized VRC01 (p-value < 0.05), suggesting that CD52g does coat the surface of HIV-1 virions produced from SP-treated HEK293T cells. There was no significant dose response found for each antibody so only the 25 µg/mL data is shown. A western blot was also run on concentrated virus, and CD52g was readily detected in lysates from the SP-pulsed virus (Figure 3-9C).

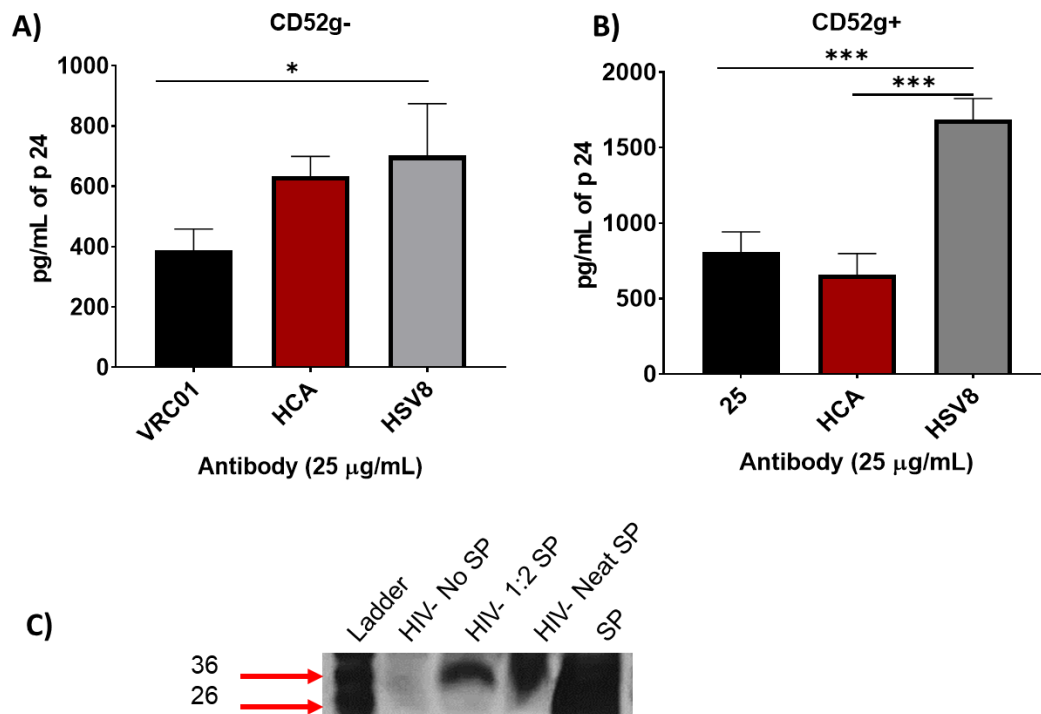


Figure 3-9-Proxy measures for CD52g detection. Capture plate supernatant quantified with p24 ELISA assay from A) virus made with SP-treated HEK293T and B) media-treated HEK2913T. Graphs are representative of three experiments. C) Western blot of concentrated SP-pulsed HIV-1. CD52g was detected with HCA. *p-value<0.05, ***p-value<0.00005

CD52g+ HIV-1 Production with T cells

HEK293T cell transfection is the preferred method for virus production in the lab, but we also wanted to confirm that PBMCs could be coated with CD52g and produce virus, as this would likely be a mechanism of CD52g virion coating in the MRT. We ran a time course to determine how long CD52g protein

remained on the plasma membrane of SP-pulsed T cells (CEM data shown). SP-pulsed cells were washed and cultured in complete RPMI. At specific time points, cells were fixed and either plated for IF or saved for flow cytometry. As shown in Figure 3-10, cells retained CD52g, detected with directly conjugated Cy3 HCA, for up to 8 hours post-SP treatment (Panel A). Flow cytometry showed similar results, although positive CD52g staining was found at 24 hours (Figure 3-10C).

Infected CD52g+ PBMCs were prepared for TEM in order to determine if CD52g could be found on HIV-1 transmitter-founder virions. Cells were infected before incubation in SP, since data showed that SP pulsation during infection delayed virus production for 24 hours (data not shown). Infected cells were used for TEM instead of free virus to facilitate staining and imaging. TEM images of CD52g+ infected PBMCs are shown in Figure 3-10. Panel D shows positive staining of CD52g+ sperm with arrows demonstrating 12 nm colloidal gold staining, a positive control. Panel B and D show colloidal-gold staining of virions and empty vesicles, respectively. However, the plasma membrane of the PBMCs had little labelling and only a few HIV-1 virions were labelled. While preliminary TEM images suggest that CD52g is found on the surface of the HIV-

1, colloidal gold labeling needs to be improved to detect CD52g on the plasma membrane of PBMCs in order to confirm the result.

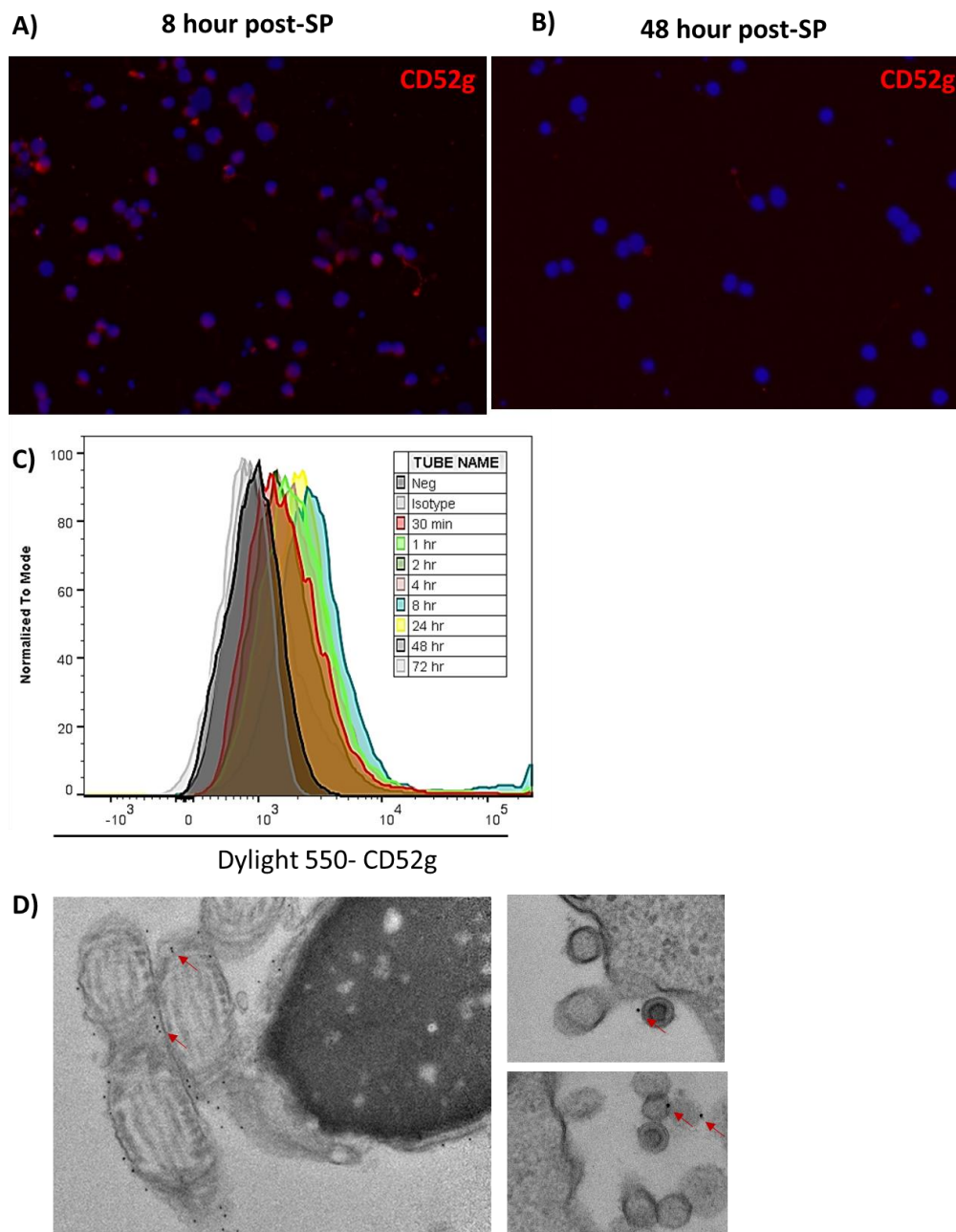


Figure 3-10- CD52g coats T cells and is detectable on the envelope of virions and vesicles. A) Immunofluorescence of CEMs 8 hours post-SP pulse and **B)** 48 hours post-SP pulse. 20x

magnification. Cy3 staining indicates presence of CD52g. C) Flow cytometry of CD52g on CEMs fixed at different time points Post-SP pulse. D) TEM of CD52g+ sperm, T cells, and TRJO virus at 25,000x magnification. Virus particles zoomed in for visualization. Red arrows point to colloidal gold 12nm.

HIV-1 Co-agglutinates with Sperm When Treated with HCA

Once CD52g coating had been ascertained, we determined whether HCA could neutralize CD52g-coated virus. As shown in Figure 3-11A, both CD52g+ virus and CD52g- virus were neutralized by VRC01 in the TZM-bl assay, but not by HCA or HSV8 (isotype control). To determine whether HIV-1 co-agglutinates with sperm in the presence of HCA (similar to TV), CD52g+ and CD52g- HIV was mixed with sperm and treated with VRC01, HCA, and HSV8 in an experimental set-up similar to the “sperm escape assay” and the TV co-agglutination assay. The sperm were gently spun down and the free HIV-1 in supernatant was quantified with a p24 ELISA. If the virus was captured by the antibody or co-agglutinated with sperm cells, then less p24 should be found in the supernatant. As shown in Figure 3-11B and C, both VRC01 and HCA treated co-cultures have decreased p24 in the co-culture supernatant when compared to HSV8. This trend was found whether or not the virus was coated with CD52g. The decreased p24 values in VRC01 treated cultures, while unexpected, are likely

due to the fact that p24 ELISA plates are coated with an HIV-1 polyclonal antibodies and VRC01 antibody decreased HIV-1 capture hence decreasing p24 values. Again, no significant dose response was observed so only the data from 25 $\mu\text{g}/\text{mL}$ are shown.

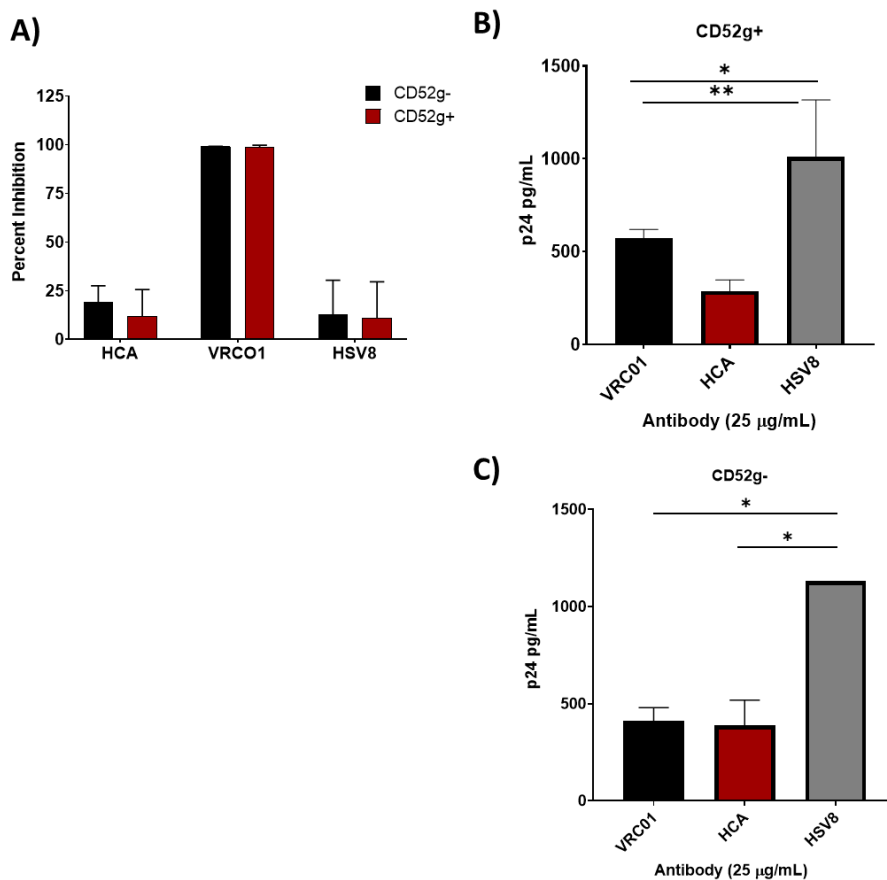


Figure 3-11-HCA does not neutralize virus but co-agglutinates HIV-1 in the presence of sperm.

A) CD52g+ and CD52g- NL43-IRES-GFP virus was neutralized at 100 $\mu\text{g}/\text{mL}$ for VRC01, HCA, and HSV8. Black bars indicate media pulsed virus, red bars indicate SP pulsed virus. Graph representative of n=2. B) Quantification of sperm and virus co-agglutination. Supernatant from virus/sperm co-culture treated with different antibodies were quantified with a p24 ELISA.

CD52g+ virus or C) CD52g- virus was used. HSV8 is an isotype control. *p-value < 0.05; **p-value < 0.0005. Graphs representative of n=3 experiments

HCA offers Modest Protection from HIV-1 in the Presence of Sperm

HCA trapped both CD52g+ and CD52g- HIV-1 virions in sperm agglutinates, decreasing p24 found in sample supernatant. Additionally, HCA on its own offered no protection in the TZM-bl neutralization assay. We next tested the effect of HCA in HIV-1/sperm co-cultures with an uncoated transmitter/founder virus, TRJO (CD52g-) placed in a seminal cell culture emulating infected semen. The TZM-bl neutralization assay with the HIV-1/sperm co-culture reflected the HIV-1/sperm co-agglutination data from Figure 3-11. Modest protection (measured by percent neutralization) was shown by HCA; the highest percentage of neutralization was 23% (Figure 3-12A) in HCA. In contrast, HSV8 antibody treatment resulted in a maximum neutralization of 8% (p-value < 0.05). The AUC was calculated as described as in Ghulam-Smith *et al*, (2017)¹⁸⁷. VRC01 with high neutralization had an AUC close to 1.0, indicating complete protection while HSV8's AUC was closer to 0.02 showing poor protection. HCA had an intermediate AUC of 0.2 (p-value < 0.05; Figure 3-12B).

Therefore, HIV-1 co-agglutination with sperm when treated with HCA decreases HIV-1 infection of target TZM-bl cells.

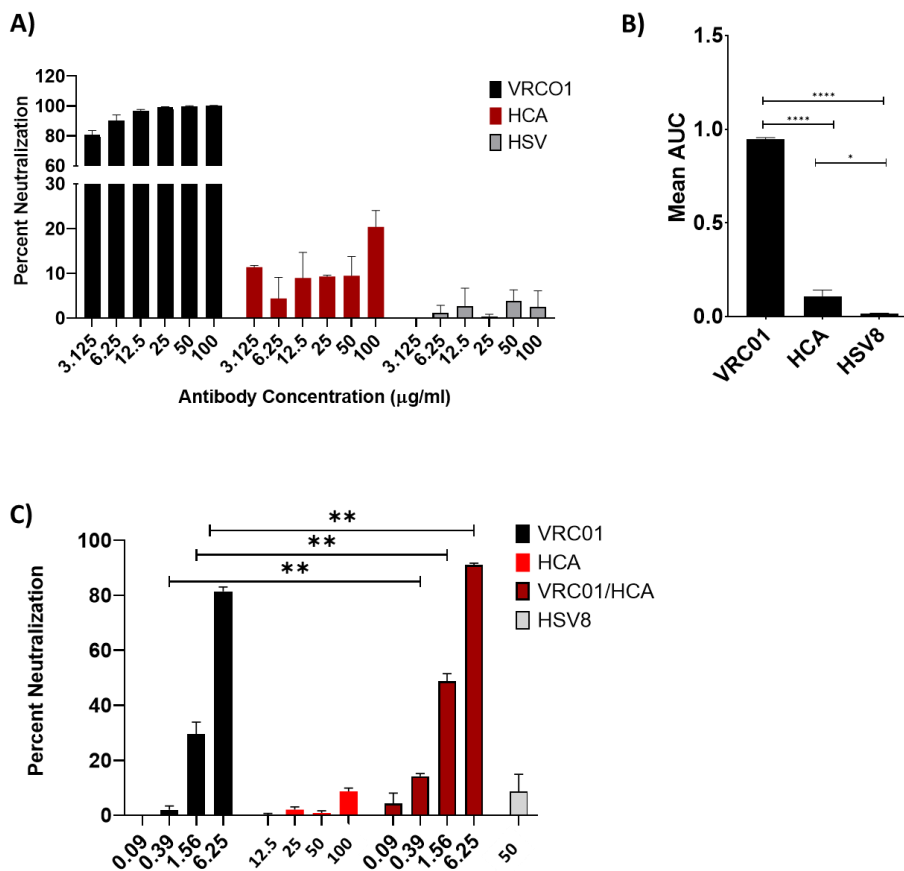


Figure 3-12-Neutralization of TRJO virus in sperm co-culture infection. A) Neutralization curve with VRC01 antibody (black), HCA antibody (red), and HSV8 antibody, isotype control (grey). B) AUC of each antibody. Graph is representative of n=2 experiments. C) Neutralization of TRJO virus in sperm co-culture infection. In VRC01/HCA antibody concentration, VRC01 is titrated, HCA concentration is 50 $\mu\text{g/mL}$. Graph is representative of n=3 experiments ****p-value <0.0001; *p-value <0.05

While HCA does not directly neutralize HIV-1, it does offer modest protection in HIV-1/sperm co-agglutinates. We therefore hypothesized that HCA could enhance the neutralization of VRC01 at low antibody concentrations. As

depicted in Figure 3-12, HCA (at a concentration of 50 $\mu\text{g}/\text{mL}$) rescues some of the protection lost at low levels of VRC01. Approximately 50% neutralization was found at 1.56 $\mu\text{g}/\text{mL}$ of VRC01 when HCA was added, compared to the 29% neutralization found with VRC01 alone. The increased percent neutralization could be due to HIV-1 trapping in sperm agglutinates.

Conclusions

The steady rise of sexually transmitted infections in recent years demonstrates the need for an effective microbicide. The problem is exacerbated as increased incidence is accompanied by rising rates of antibiotic resistance, the discovery of newly identified sexually transmitted pathogens, and unenforceable behavioral practices, such as regular screening and condom use. Currently, only the condom protects from the sexual transmission of most viruses, bacteria, and protozoa; however, its use is often inconsistent^{6,35,36}. While vaccines offer long-term protection against disease without the use of a physical barrier, the complexity of the FRT mucosal immune system has limited product advancement: only HepB and HPV vaccine are available to date. Furthermore, vaccines can be expensive and need to be administered by medical professionals,

limiting access for some populations. Keeping these challenges in mind, an ideal microbicide would be easy to use, encourage adherence, and target a wide breadth of pathogens. While a long-acting product would be preferable, the urgent global need requires that on-demand products be available as well.

In this study, we investigated HCA's ability to protect from sexually transmitted pathogens, specifically HIV-1 and *Trichomonas vaginalis*. The GPI anchor of CD52g provides the protein with hypermobility. This trait is what enables the protein to coat sperm cells after being produced by epithelial cells in the MRT. We found that the hypermobility of CD52g also applied to *Trichomonas vaginalis* and HIV-1; both pathogens were coated with CD52g following exposure to SP. The subsequent lack of agglutination of CD52g+ TV post-HCA exposure is most likely due to the large size of the pathogen: TV is approximately 10 μm long and 7 μm in diameter, compared to the 5 μm length and 3 μm diameter of the sperm head³³⁴⁻³³⁶. Furthermore, the flagella of TV are smaller; and sperm tail-tail and head-tail agglutination has been shown with HCA(Figure 2-3). The size of TV most likely prevents antibody cross-linking, which is needed for agglutination, due to steric hindrance. Additionally, TV are not as motile as sperm, leading to fewer collisions which are necessary for agglutination. HIV-1,

on the other hand, at approximately 120 nm in size, might be too small to be agglutinated, and its movement is limited to Brownian motion. Additionally, CD52g does not play a role in the adherence of HIV-1 or its fusion to CD4+ target cells, and, unlike VRC01, HCA does not interact with CD4 or CCR5/CXCR4 co-receptors. As a result, it has no direct role in viral neutralization.

Our data showed that HCA alone did not have the ability to prevent transmission of CD52g-coated pathogens; nevertheless, some protection was evidenced when the pathogens were co-cultured with sperm or whole semen and treated with HCA. This is likely due to TV and HIV-1 co-agglutinating with the sperm. Sperm, therefore, essentially serve as a “net” trapping the pathogens in the agglutinates and preventing them from reaching their target cells (Figure 3-13). This was observed in both the TV/sperm adherence studies, HIV-1/sperm co-agglutination studies, and the TZM-bl neutralization assays with HIV-1/sperm co-cultures. In both cases, there was a decrease in the pathogen’s ability to infect (or adhere) to their target cells in the presence of HCA and sperm. Hypothetically, the protection offered by the sperm agglutinates trapping pathogens or infected cells could be extended to any STI pathogen making HCA a modest, but broad-spectrum microbicide.

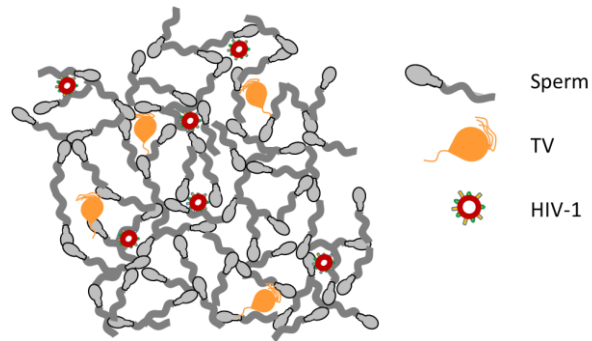


Figure 3-13-Schematic of pathogen-sperm cell co-agglutination in the presence of HCA.

There are limitations in the interpretation of these data and their applications *in vivo*. First, *Trichomonas vaginalis* studies were performed at supraphysiological conditions; the concentration of *T. vaginalis* in semen likely approximates 2500 cells/mL²⁴³. Our experimental concentrations were closer to a 1×10^6 /mL. The increased concentration was used to ensure that a quantifiable number of protozoa adhered to tissues and VK2/E6E7 monolayers or were able to be visualized by the CASA. As previously mentioned, whole semen, which has soluble CD52g, was not used because it completely prevented TV adherence to tissue and monolayers. Additionally, some of our methods had limitations. In the case of the plate capture method, no dose response was found. My committee suggested using beads to remove HIV-1 from the supernatant. Nonetheless, no reduction in p24 concentrations were found. Different buffers, virus

concentrations, beads, and antibody concentrations were attempted, and troubleshooting is still on-going (data not shown). As previously mentioned, TEM data analysis also has to be repeated. While some staining did occur, the protocol needs to be optimized. Finally, antibody agglutination and neutralization were the sole focus of these studies. The Fc region on antibodies enable multiple functions, including opsonization and cellular cytotoxicity which are important mechanisms of pathogen protection. Data from recent vaccine trials suggest that these non-neutralizing effector functions are responsible for eliciting effective immune responses, especially against HIV-1^{163,164,337}. As an IgG antibody, it is possible that HCA could elicit these types of responses in the FRT that have not been studied *in vitro*.

Overall, HCA was not shown to protect target cells and tissues from HIV-1 or *Trichomonas vaginalis* infection but, in the presence of sperm cells, it offered moderate protection most likely due to co-agglutination in sperm “nets”. As a result, HCA could reinforce protection in an antibody-based topical microbicide containing pathogen-specific neutralizing antibodies, in addition to offering contraception.

Chapter 4 - Discussion

Summary

This study explores the use of monoclonal antibodies as candidates for multipurpose prevention technology, combining both contraceptive and microbicidal properties. I started my project by characterizing the activity of the anti-HIV antibody VRC01 in genital secretions (Appendix I), but the main focus of my dissertation research has been the characterization of another MPT candidate antibody, the Human Contraceptive Antibody (HCA). HCA targets CD52g, a glycoprotein synthesized exclusively in the male reproductive tract. CD52g is GPI anchored, leading to protein hypermobility and enabling CD52g to embed in various phospholipid membranes. CD52g embeds in the plasma membrane of sperm and seminal leukocytes, and, as demonstrated by this study, those of some pathogens such as TV and enveloped viruses like HIV-1 (Figure 3-3, Figure 3-10).

HCA is an IgG1 antibody derived from H6-3C4, an agglutinating, anti-sperm, IgM antibody originally isolated from B cells from an infertile woman by Shinzo Isojima²⁸³. His IgM antibody, like our HCA, was known to immobilize sperm in the presence of complement. While the specificity and agglutination

efficacy of H6-3C4 had been extensively investigated, its production and use for the purpose of human contraception had not been previously explored. The human IgG1 HCA antibody was produced by transfection of the H6-3C4 DNA sequence, with an IgG1 Fc, into modified *Nicotiana benthamiana* (tobacco) plants (Δ fucose/ Δ xylose), a cost-effective antibody production platform. In this study, we demonstrated that the IgG1 isotype of Isojima's antibody agglutinates sperm effectively, despite the decreased valency. We also demonstrated that antibody activity and efficacy was not adversely affected by prolonged exposure to low pH, such as that found in the human vaginal environment. Additionally, we demonstrated that HCA could agglutinate sperm effectively in whole semen—which contains soluble CD52g—and in the presence of very low or high sperm concentrations that represent the physiological range of sperm density found in human males. It was necessary to develop new techniques to assess sperm agglutination in the presence of HCA for this study. We developed a kinetic sperm agglutination assay that quantified the time elapsed to 100% sperm agglutination following treatment with different concentrations of HCA, and the "sperm escape assay" which quantified the number of progressive motile sperm remaining in a sample five minute after addition of HCA. Our study, overall, is

the first to investigate *Nicotiana* produced antibodies in the vaginal environment, and to characterize the specificity and sperm agglutination activity of the HCA mAb (Figure 2-1, Figure 2-2, Figure 2-4).

We also investigated the ability of HCA to target STI pathogens, a function which has not previously been investigated. We determined that CD52g, the antigen recognized by HCA, embeds on the phospholipid layers of two model STI pathogens, TV and HIV-1 (Figure 3-3, Figure 3-9, Figure 3-10), evidencing that CD52g non-specifically associates with pathogens in the male reproductive tract, and suggesting that HCA may be active against multiple STI pathogens. The benefit of a ubiquitous target is that a single therapeutic mAb could target a variety of bacteria, viruses, and parasites found in semen. Because the non-pathogen specific target, CD52g, is passively acquired, it is unlikely that pathogens would develop resistance to HCA. While the CD52g glycoprotein was found to embed on the surface of both HIV-1 and TV, HCA treatment alone did not lead to decreased pathogen interaction with target cells or result in inter-pathogen agglutination (Figure 3-6, Figure 3-11, Figure 3-12). However, in the presence of sperm cells, HCA moderately neutralized HIV-1 in a TZM-bl assay. HCA was also found to improve VRC01 neutralization at low concentrations in

HIV-1/sperm co-cultures (Figure 3-12). Our data also showed that both seminal plasma-exposed (i.e. CD52g-coated) and media-treated TV co-agglutinate with sperm after HCA treatment, thus decreasing pathogen adherence to target epithelial cells. This HCA co-agglutination “lattice-trapping” effect could reduce the infectivity of both CD52g+ and CD52g- pathogens found in semen.

Implications

HCA was shown to be an effective contraceptive *in vitro* whose primary mechanism of action is sperm agglutination. HCA could function as a short-acting reversible contraceptive if delivered via intravaginal film, a product produced by our industry partner Kentucky BioProcessing (KBP) and currently undergoing further testing. However, different delivery methods could be used to produce a LARC, such as slow-release intravaginal ring, which the RING and ASPIRE studies have shown to be acceptable by patient populations. HCA, therefore, is a potential candidate for a novel, non-hormonal, non-barrier contraceptive, which would fill an important gap in the contraception field.

Seminal plasma-exposed pathogens appeared to be trapped in sperm lattices upon HCA treatment, *in vitro*. The trapping mechanism suggests that this

potential protection method could be extended to other sexually transmitted pathogens not examined in this study, such as *Chlamydia trachomatis* and *Treponema pallidum*. Additionally, HCA, a primarily contraceptive antibody, could be combined with other microbicides, whether they be small molecules or biologics, to produce a novel MPT product. As mentioned in Chapter 2, we have performed pre-clinical and clinical studies with Nicotiana-produced bnAb VRC01-N and HSV8-N. The film containing both of these antibodies is MB66. The first segment of the Phase I clinical trial of MB66 tested the safety of the intravaginally applied mAb film and determined *ex vivo* function of the antibody after 24 hours in the vaginal tract. The clinical trial data reflected the results of our pre-clinical *in vitro* studies where VRC01-N was incubated with cervicovaginal lavage (CVLs) or seminal plasma (SP) samples and demonstrated no loss of neutralization efficacy against both Q23-17 and BAL (R5 tropic) viral strains (Appendix 1A-B). VRC01-N/MB66 also demonstrated no significant loss of efficacy when exposed to lactic acid, pH 3.9, for up to 8 hours, and showed only a mild decrease in activity after 24 hours (Appendix 1C-E). The first segment of this clinical study included a PK study in which VRCO1 concentrations were assessed in TearFlow and CVL samples at time points up to

24 hours post-film insertion. Antibodies were readily detected in TearFlo and CVL samples for up to 24 hours, but levels returned to baseline after 6-7 days. Segment B recently concluded. The second segment tested film safety with more trial participants and repeat film applications. Participants were requested to administer a film every day for 7 days. Elevated levels of VRC01-N and HSV8-N were found in TearFlo and CVL samples for 24 hours after the last film was applied. Additional studies with macaques have provided promising data for antibody administration using an intravaginal ring. Macaques with slow-releasing pods containing VRC01-N antibody had rings inserted in the posterior vagina, and VRC01-N was detected with TearFlow strips for up to 21 days³³⁸. Overall, the data presented here provides evidence for an antibody-based MPT product containing HCA and VRC01-N antibodies produced in *Nicotiana* plants.

The use of *Nicotiana* as an antibody producing platform is important for MPT development because it enables this product to be affordable and accessible. As mentioned previously, antibodies in *Nicotiana* can be quickly produced (40 days) in large quantities resulting in decreased manufacturing costs. This means that mAb MPTs could be available globally even for populations with limited resources. Storage of the antibody in film form has been

shown in our lab to preserve HCA stability and function at 4 °C which suggests flexibility in storage conditions. Antibody stability in film at RT remains to be tested. If room temperature stability can be demonstrated and low-cost production can be achieved, this film could become a much needed, antibody-based MPT—a valuable product that could curb the rise of STIs while providing the option of contraception, with HCA.

Study Evaluation: Strengths and Limitations

We are grateful to the semen donors who participated in this study. Working with a steady reserve of human samples has simultaneously been one of the greatest strengths and challenges of this study. During the course of the study, we used approximately 20 donors. The variability of our samples was evident in both sperm count (~13 M/mL-180 M/mL) and mobility (<20%-80%), presence or absence of round cells, and sample volume (1 mL-7 mL). Donor-to-donor variability in seminal fluid, such as in the case of cytokine concentrations, has been well documented. In order to minimize any effect a single donor may have on the outcome of an experiment, 2-3 donors were pooled if whole semen was used. Our large pool of donors allowed for different combinations of

samples, generally, to be pooled for each experiment. While pooling controlled for variability in general, there were unique challenges of working with whole semen. Semen and seminal plasma can be cytotoxic to cells *in vitro*. *Trichomonas vaginalis*, PBMCs, T cell lines, and epithelial cells were able to withstand brief exposures to SP. HEK293T cells survived the treatment but became non-adherent and had to be re-plated after the SP was removed. The effect of SP on TZM-bl cells was inconsistent; the cells sometimes survived SP exposure, but other times, lifted off the plate and apoptosed in under two hours. Interestingly, sperm had variable viability in whole semen as well, with motility being conserved best if sperm were isolated and resuspended in MHM. Our lab has demonstrated that a 1/32 dilution of SP allowed for overnight incubation of TZM-bl cells without decreasing viability; however, this diluted the sperm to sub-physiological concentrations. Difficulty preserving viability clearly limited our HIV-1/sperm TZM-bl neutralization studies since whole semen, which would be more physiologically relevant, could not be used. In the case of TV adherence studies, semen/seminal plasma prevented the adherence of the protozoa to epithelial cells, a situation circumvented by the pathogens *in vivo*. Removing SP from sperm cells was necessary to obtain results. It is important to note that semen,

FRT secretions, and the interaction between the two, may have additional protective and/or permissive functions *in vivo* which could not be observed in our *in vitro* experiments. Animal models could theoretically shed light on these interactions, but there are limitations for all available models. In our case, the presence of CD52g in the MRT is only observed in higher primates (great apes, humans). Furthermore, the human vagina has a unique structure and low pH that is not found in any other animal, limiting the conclusions that can be derived from these studies, and favoring *in vitro* work.

Despite the limitations of working with whole semen, we attempted to maintain physiologically relevant conditions to the best of our ability, such as in the case of our pathogens. NL43 virus (R4 tropic) was used initially as a learning tool due to its GFP tag that allowed for infection monitoring during protocol troubleshooting. However, HIV-1 is sexually transmitted by R5 tropic virus produced by infected CD4+ cells. For later studies, TRJO virus was used. TRJO is a Clade B, R5 tropic, transmitter-founder virus. It was selected because of the early Fiebig stage (II) of the male patient from which the virus was isolated³³⁹. It is also known to infect macrophages, which are potential disseminators of infection in the FRT. There are also limitations with this virus selection. The virus

is Clade B which is predominant in the USA and Western Europe but represents only 12% of HIV-1 infections worldwide. Clade C is the most prevalent clade worldwide, and is found predominantly in southern Africa and India¹⁸².

Limitations also existed when working with *Trichomonas vaginalis*. The B7RC2 strain is well-documented WT TV strain and known to adhere to epithelial cells *in vitro* within an hour^{340,341}. *Trichomonas vaginalis*, however, is an obligate anaerobe; VK2/E6E7 cells and MatTek tissues are obligate aerobes. As is the case with most FRT research, the combination of anaerobic and aerobic organism is a challenge for long-term assays. *In vivo*, the lumen of the vaginal tract is known to be a microaerophilic environment permitting the growth of both aerobic and anaerobic organisms. The aerobic vaginal tissue is maintained by the circulatory system. In an *in vitro* setting, it is difficult to combine the two conditions. We circumvented this difficulty by keeping TV adherence to an hour, a time constraint that preserved TV viability. Adherence was also encouraged by sealing MatTek and VK2 cultured plates with parafilm to avoid oxygen exchange in the absence of an anaerobic chamber. These efforts were intended to allow experimental conditions to mimic physiologically relevant conditions as closely as possible in a laboratory setting. Nevertheless, as evidenced by Figure 3-6, this

set-up was not sufficient to improve SP-pulsed *Trichomonas vaginalis* adherence which is decreased compared to media controls. SP also interfered with protozoal adherence, and it is possible that the decreased adherence was not due to the condition of TV post-exposure to SP, but to components of SP itself that remained in the sample despite washing.

One of the greatest strengths of using *Nicotiana*-based antibodies is reflected by the Luminex panel and the MB66 clinical trial: the safety profile of the product is promising. There was no significant increase of pro-inflammatory cytokines by vaginal epithelial cells *in vitro* and only one mild adverse event related to the product documented in the Phase I, segment A clinical trial of MB66³¹⁷. The balance between safety and efficacy has historically been delicate, as evident in the case of nonoxynol-9 (N-9), a commercially available spermicide. N-9 is a surfactant which kills sperm effectively, preventing unplanned conception when used correctly. However, it was also shown to negatively affect vaginal epithelial cell integrity and compromise the barrier's protection against STIs with no microbicide effect⁴⁹. Overall, our *in vitro* and pre-clinical studies indicate that an antibody-based MPT could work in tandem with the FRT

immune response, and could potentially be used in conjunction with other contraceptive or microbicidal compounds for enhanced efficacy.

Future directions

A large portion of this work was dedicated to developing *in vitro* assays that assessed mAb contraceptive and microbicidal efficacy in physiologically relevant conditions. The scope of this study was therefore limited to the agglutination and pathogen-neutralization functions of HCA. Antibodies can have other effector functions which could also play a role in the contraceptive/microbicidal effects of HCA:

1. **Opsonization and phagocytosis (ADCP)**- where antibody coats the pathogen, enabling macrophages and neutrophils to efficiently phagocytose the antibody target;
2. **Complement fixation**- where complement interacts with antibodies bound to their epitope and can result in the formation of the membrane attack complex (MAC) which leads to cell death. Complement in the human FRT has been poorly studied and the magnitude of the protection it offers remains to be understood.

3. **Cell mediated cytotoxicity (ADCC)**- where antibody bound to an infected cell leads to NK cell- induced apoptosis of the target.

HCA, as an IgG1 antibody, has the potential to induce any of these functions in the FRT. Their relevance and potential to enhance the contraceptive and microbicidal properties of HCA remains to be explored. Similarly, and of great concern since CD52g coats the envelope of HIV-1 without neutralizing it, is the possibility that antibody dependent enhancement of infection. This potential adverse effect of HCA remains to be determined.

The function and efficacy of HCA *in vitro* has been determined in this study, but improvements can continue to be made via an improved delivery system or continued antibody engineering. Our current *Nicotiana*-produced antibodies, VRC01-N and HSV8-N, have been incorporated into a film that is manually inserted into the vagina. The film dissolves quickly in the vagina and the released antibody is found in high concentrations for up to 24 hours after a single application without losing neutralization potency. The current form of the potential MPT makes it a short acting, on-demand product. However, incorporation of mAbs into an intravaginal ring with controlled antibody release could convert the MPT into a long-acting product, if adequate antibody levels

can be maintained in the tissue. Promising IVR mAb pharmacokinetics research is already underway in the laboratory of Francois Villinger in the Rhesus macaque model. Furthermore, the possibility of a long acting (3+ month) IVR MPT is being explored by the International Partnership for Microbicides (IPM) where levonorgestrel was added to the Dapivirine ring explored in The Ring/ASPIRE studies. Phase I clinical trials are currently underway⁷³.

Finally, current technology enables antibodies to be engineered in novel, ingenious ways that improve antibody function. Our collaborator, MappBio, recently made an HCA-hexamer (Hex) mAb which contains 5 F(ab')₂ components (Appendix 2, Panel A). Preliminary data indicate that the Hex antibody is a more potent agglutinator of sperm (and possibly pathogens) through increased valency. The engineered Hex antibody significantly outperformed HCA IgG1 at lower concentrations in the agglutination kinetics (Appendix 2, Panel B), and sperm escape assays (Appendix 2, Panel C). The data suggest that the novel Hex antibody could decrease the amount of antibody needed to achieve levels of protection, perhaps decreasing product costs. Furthermore, the data provide evidence that increased valency leads to faster, more robust agglutination, which can also be explored with an IgM isotype of HCA. The safety profile of Hex-like

antibodies remains to be determined; IL-6 was noticeably increased in MatTek tissue after Hex antibody treatment, although no other inflammatory cytokines were increased.

Conclusion

HCA is a powerful sperm agglutinator that performs consistently across donors, and is not significantly impacted by prolonged exposure to the low pH of the vagina. Its microbicidal properties, nonetheless, are mostly limited to pathogen trapping in sperm agglutinates. HCA, however, can potentially work in combination with other *Nicotiana*-produced antibodies, such as VRC01-N, to constitute a topical antibody-based MPT. The resulting MPT could have the potential to fulfill a much needed gap in women's health: a readily accessible, low-cost, non-hormonal, non-barrier contraceptive and microbicide MPT that is reversible and discreet.

APPENDIX 1: VRC01 and MB66 neutralization in the presence of genital secretions and lactic acid

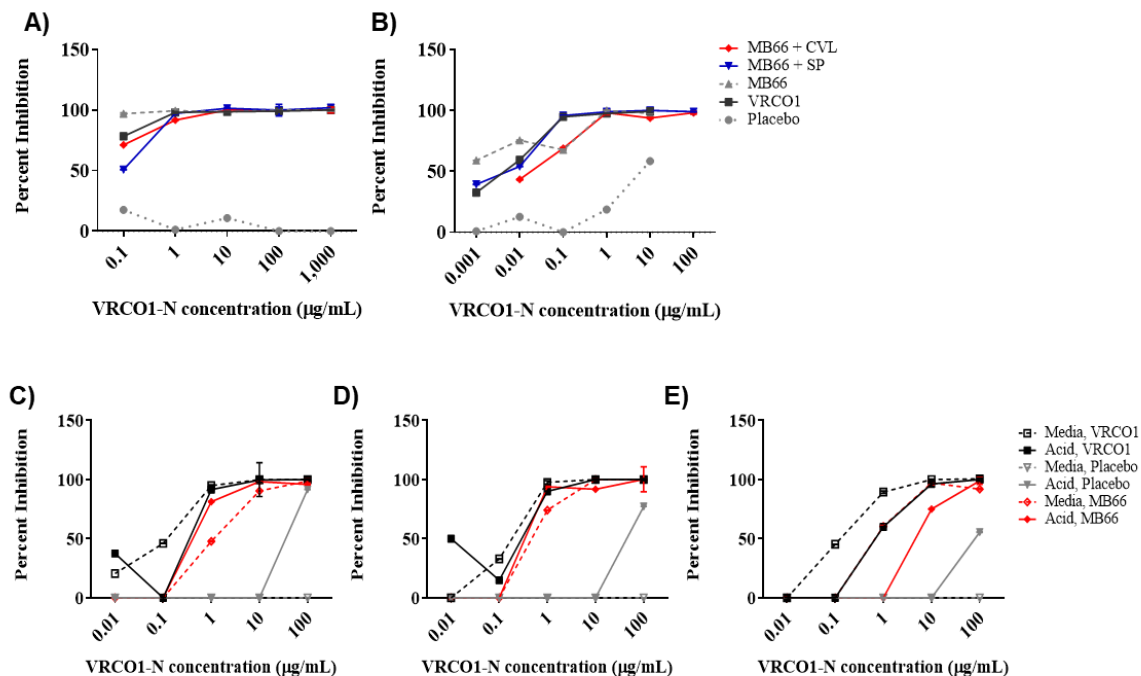


Figure A1.1- Effect of genital secretions on neutralization of HIV-1 by MB66. A modified TZM-bl neutralizing antibody assay was used to evaluate VRC01-N and MB66 neutralization after incubation with CVLs or SP with A) Q23-17 HIV-1 virus and B) BAL HIV-1 virus strain. Graphs are representative of three experiments. C) A modified TZM-bl neutralization assay was used to evaluate VRC01-N and MB66 efficacy after 1% LA exposure for 2 hours, D) 4 hours, and E) 24 hours. Graph is representative of two experiments.

For panels A and B, VRC01 and MB66 were diluted in either TZM-bl media, CVL samples, or SP samples generously provided by the Integrated Clinical/Pre-clinical program for HIV topical microbicides (IPCP-HTM) and approved by Boston University's IRB. Antibody and film were diluted in genital

secretions before being added to virus for 30 minutes and following the TZM-bl neutralization protocol as described in Chapter 3. Antibodies were tested using both Q23-17 (panel A), considered a transmitter-founder virus, and BAL virus, a lab adapted R5 virus (Panel B). As shown by the graphs, 100% neutralization was found for 1 $\mu\text{g}/\text{mL}$ of VRC01 with both BAL and Q23-17 virus. CVL-treated antibody at low concentrations had the lowest percent inhibition at 0.1 $\mu\text{g}/\text{mL}$ when using BAL virus, and SP-treated antibodies had the lowest percent inhibition at 0.1 $\mu\text{g}/\text{mL}$ when using Q23-17. To examine the effect of LA on VRC01 neutralization ability, the antibody was previously incubated with 1% LA for 2, 4, and 24 hours. The antibody solutions were neutralized (pH 7) with 1N NaOH before carrying out the TZM-bl neutralization assay as described in Chapter 3. As shown in panels C-E, neutralization efficacy is mildly decreased after 24 hours at low pH. The LA treated placebo film also shows neutralization at the highest concentration. This was expected as LA has been shown to protect from HIV-1 infection²⁴¹.

APPENDIX 2: Preliminary studies with Hex antibody

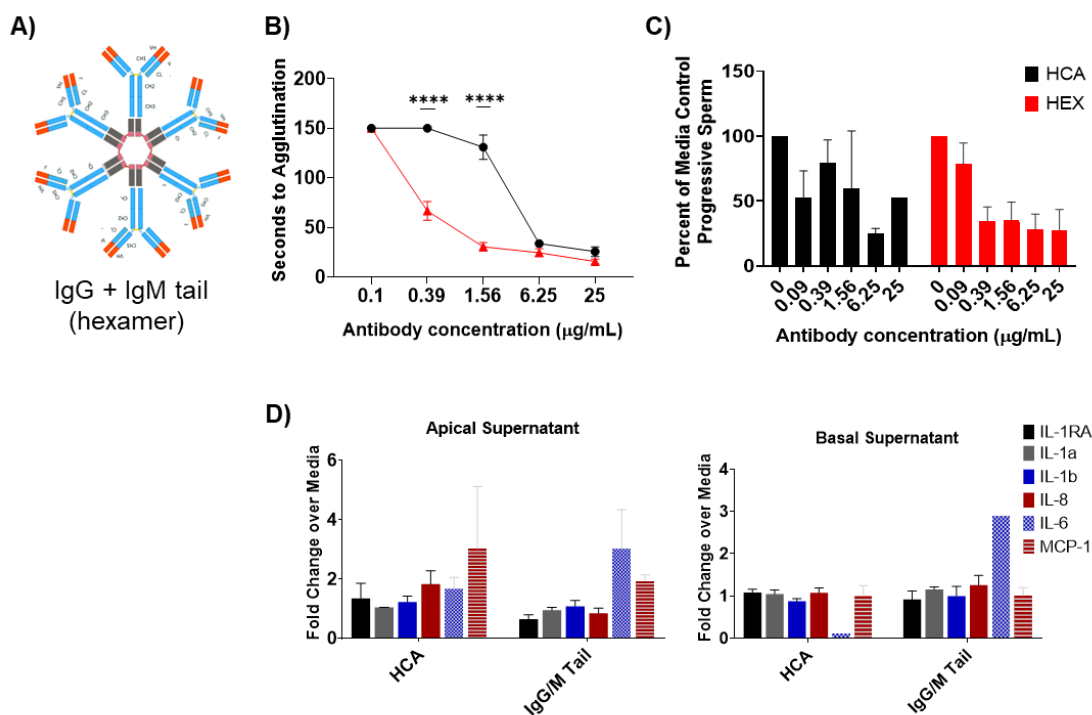


Figure A2.1- Agglutination assays with engineered, multivalent CD52g specific antibody. MappBio produced a multivalent antibody with a predominantly IgG1 Fc joined by an IgM tail. Diagram courtesy of Ellena Nador. B) Kinetic agglutination assay comparing HCA (black) with the hexamer (red). C) Sperm escape assay with Hex and HCA. Graphs representative of two experiments. D) Luminex panel of MatTek tissue apical (left panel) and basal (right panel) supernatants. IgG/M tail= Hex antibody. Graph representative of one experiment.

Mappbio Pharmaceuticals, our collaborators, manufactured a multivalent CD52g specific antibody. The antibody joined six IgG antibodies with an IgM tail to form a hexamer (Hex). We compared our monomer HCA to the multivalent antibody at low concentrations using the kinetic assay (panel B) and the sperm

escape assay (panel C) as described in Chapter 2. The low antibody concentrations were used to better detect the differences between antibody efficacy. HCA performed comparably to the multimer at concentrations ≥ 6.25 $\mu\text{g/mL}$. At lower concentrations, Hex agglutinated up to three-fold faster than HCA. The sperm escape assay also provided evidence that the Hex antibody agglutinated progressive sperm more consistently and efficiently than HCA at lower concentrations. MatTek tissue was used for preliminary assessment of safety by quantifying cytokine production upon epithelial exposure to both soluble HCA and Hex (here denoted IgG/M). As shown in panel D. There is a 2-3-fold increase in IL-6 production detected in both the apical and basal supernatants which was not found in HCA. Further studies must be done to ensure pre-clinical safety with this novel antibody.

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