Defining the HIV neutralizing activity of antiproteases within the female genital tract and evaluating the HIV inhibitory mechanism of Serpin B1

by

Lindsay Grace Aboud

A thesis submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements for the degree of:

Doctor of Philosophy

Department of Medical Microbiology and Infectious Diseases

University of Manitoba

Winnipeg, Manitoba, Canada

“Research is the process of going up alleys to see if they are blind.”
~Martson Bates

Abstract

The HIV (Human Immunodeficiency Virus)/AIDS (Acquired Immune Deficiency Syndrome) pandemic continues to be one of the most devastating global health pandemics in history. Without an effective preventative vaccine, research on alternative preventative measures is necessary. Furthermore, with women accounting for approximately 60% of all new annual HIV infections, preventative strategies that provide women with the ability to protect themselves is growing ever more critical. To this end, identifying the role of innate factors within the female genital tract in controlling HIV infection is essential to understand an individual's susceptibility to infection. Furthermore, natural factors expressed in the female genital tract that are capable of inhibiting HIV may prove to be novel candidates for female-controlled microbicide preventative strategies.

The work in this thesis examined the differences in CVL (cervicovaginal lavage) composition between HESN (HIV-exposed seronegative) women and HIV-susceptible women. Distinct differences in the female genital tract (FGT) proteomes, as well as HIV inhibitory activity exhibited by CVL, were observed among women that were highly exposed to HIV compared to women at lower exposure. Furthermore, while HESN women as a group did not show stronger inhibitory effects compared to susceptible women from the Pumwani cohort, it was apparent that CVL from individual women was capable of inhibiting HIV and did so consistently over longitudinal analysis.

These results provided evidence that women in HIV high-exposure areas appear to contain a higher abundance of antiviral factors within their genital fluids compared to women in lower exposure settings. The identification and characterization of these factors may provide additional candidates for use in microbicides.

From the antiproteases that were previously identified as over-expressed within the CVL of HESN women, Serpin B1 exhibited the strongest and most consistent HIV inhibitory activity.

The mechanism for this activity does not appear to be directly against HIV but rather through effects exerted on HIV target cells. Specifically, Serpin B1 alters the proliferative capacity and induces early apoptotic markers on these cells. Proteomic pathway analysis of the proteins over-expressed following Serpin B1 treatment suggests that Serpin B1 may affect these cellular processes through up-regulating the expression of proteins associated with inhibition of the mTOR pathway. This inhibition may be caused by induction of increased production of ROS (reactive oxygen species) by macrophages or through Granzyme A activity, and subsequent dysfunction of the mitochondria, potentially inducing an autophagic state, which may explain the observed increase in early apoptotic markers, yet minimal increase in active apoptosis within Serpin B1 treated cells. However, this would need to be confirmed with further molecular studies.

These results defined a potential mechanism of HIV inhibition for Serpin B1. Specifically, Serpin B1 appears to be altering cell cycle regulation, thereby reducing the efficiency by which HIV is produced from infected cells. Hence, the overabundance of Serpin B1 in the CVL of HESN women may, in fact, be contributing to their protective phenotype against HIV infection. These findings suggest that Serpin B1 could be considered as a candidate in future microbicides. However these findings must be validated in *in vivo* models, and the interactions of Serpin B1 with other antiviral factors, such as other antiproteases, needs to be defined to determine if synergistic activity occurs against HIV, thereby improving any microbicide that Serpin B1 is incorporated into.

Dedications

I would like to dedicate this thesis, and the past six years of work, to my incredible husband, Zaid, without whom I would never have had the courage to undertake graduate school. He had faith in my abilities long before I ever did and because of that, I have achieved more than I could have ever imagined. We have a future set before us that far surpasses anything I had previously deemed possible.

I would also like to dedicate this thesis to my brother and sister. Matt and Tracy, you have been my protectors, cheerleaders, best friends and confidants since day one, literally. You have given me the best gifts in the world (Bella, Ethan, Rylee, Brodie and Hannah) who inspire me on a daily basis to be the best human being possible. It is because of my sincere desire to make them, and you, proud that I have pushed through the tears and frustrations to complete this degree and to continue striving to leave a mark in this world. Thank you for loving me through every obstacle, argument, sickness, adventure, and blessing. You have both played incredibly vital roles in making me the woman that I am (yes that's a good thing) and for that I will always be grateful. I love you and your families more than you could ever possibly know.

Finally, I would like to dedicate this work, as well as all future research in the field, to every single person that has been affected by HIV/AIDS. I pray that in some small way my current and future research will contribute positively to your lives and that you can take solace in knowing that countless people are working tirelessly to ensure that one day, in the not so distant future, we will have a world free from HIV/AIDS.

Acknowledgements

First and foremost, I would like to thank someone who doesn't often get a lot of credit in the science world, God. For those that know my story, you are aware that there is absolutely no way that I would have completed this degree if someone wasn't watching out for me and giving me strength when I had nothing left to give. So thank you, Jesus, for walking with me through the past six years.

Secondly, I would like to thank my supervisors, Dr. Blake Ball and Dr. Frank Plummer whose support throughout this degree has been invaluable. Blake, you have molded me into a scientist that I can be proud of, and given me the confidence to step outside of where I am comfortable into new and exciting adventures. Thank you for trusting my judgment (even when you shouldn't have) and giving me the freedom to make the mistakes that have ultimately made me the scientist that I am today. I look forward to seeing what future collaborations are in store for us.

I would also like to thank my graduate studies committee. Dr. Julia Rempel who is no longer with the University of Manitoba but who contributed immensely to the development of this project. To Dr. Yao who was always the beacon of light in committee meetings and who challenged me to think beyond my comfort zone into the terrifying world of molecular virology. Dr. Burgener, who has helped mold this project from the very beginning, Dr. Douville who is the newest member of the committee but who has put a lot of time and energy into reading and editing this thesis, and lastly, Dr. Rugeles who traveled all the way from Colombia to attend my defence and who took the time to provide feedback on this very lengthy thesis.

Thank you to the members of the Ball/Plummer lab who have been my second family for the past six years. To Bernie and Christine for the immense amount of patience that you have shown me, and the incredible amount of work you do to keep our lab running smoothly. You don't receive nearly the amount of credit you deserve. Aida, thank you for patiently showing me the ropes when it came to PCR. I would still like to meet the person for whom those impossibly small PCR tubes were made. Derek, thank you for all the time you put into trying to get that cell line working (which I never did get to cooperate). To Were for being the first person in the lab to take me under your wing and show me how the lab worked and for being a friend above all else. To Kenzie, Laura, Michelle, Stuart and Max for helping me navigate the mass spectrometry world and for running my samples. To all the lab members, thank you for being my friends and for putting up with me when I was over-stressed and without sleep when my dark side appeared.

Thank you specifically to Dr. Adrienne Meyers. You are an incredible woman and researcher who I cannot thank enough for all of your mentorship, support, and love. You started out as the lab manager and quickly progressed to a friend. I would not have made it through this degree without you. Thank you for talking me off the ledge more than a hundred times and for lending me your shoulder on far too many occasions. You will always hold a special place in my life.

Thank you to Dr. Keith Fowke's laboratory. To Julie, Genevieve, Colin, Andrew and Courtney for all the times, we were conference buddies, for always making yourselves available to talk

through obscure results or to sit down for a break from the craziness of the lab. I know you will all be wildly successful in your future careers and can't wait for our paths to cross again.

To the members of Dr. Yao's lab, in particular, Kallesh and Zhujun, who always made themselves available to explain complex molecular experiments. Kallesh you have become far more than a lab mate to me. It has been an absolute pleasure to watch you grow into the amazing scientist that you are and to now see you so successful and happy with Sowmya in Virginia.

Thank you to the Department of Medical Microbiology and its support staff. A big thank you goes out to Jude, Angela, Sue, Eva, Natasha, Cheryl and Sharon, who make themselves available to the constant questions and needs of the many students that traverse the halls of the department and without whom we would never be able to navigate the mazes of the university administration. Thank you also to the support staff and maintenance at the JC Wilt Infectious Disease Research Centre. Many security and maintenance engineers work tirelessly to ensure we can complete our work in a safe and efficient laboratory.

Thank you to all of the laboratory and clinical staff at the University of Nairobi and the Majengo clinics. Your hard work is what makes this research possible.

Thank you to the participants of the Pumwani and Winnipeg cohorts without whom this work would not have been possible. Thank you for your commitment to progressing the field of HIV research. Thank you to Dr. Jennifer Chan, Dr. Helen Pymar, and Mary Driedger, who so willingly came on board as the medical practitioners in the Winnipeg cohort. Thank you for answering my endless questions and for investing your time and energy into this work.

Thank you to the Allan Ronald Studentship Fund, CAHR, CIHR and the Sir Gordon Wu scholarship for providing funding for me at various points in my graduate program.

Thank you to the pioneers of our research collaboration between the University of Manitoba and the University of Nairobi: Dr. Allan Ronald, Dr. King Holmes, Dr. Herbert Nsanze, Dr. Frank Plummer, Elizabeth Ngugi, Dr. David Haase, Dr. Margaret Fast, Dr. J. O. Ndinya-Achola, Dr. Ian Maclean, Leslie Stanley and Dr. Joanne Embree, amoung many others, for never giving up on a project that would one day change the face of HIV in Kenya and Canada, all the while providing invaluable opportunities to countless students.

Lastly, thank you to every member of my family. Mom, Dad, Zenda and Mom Abdul-Hameed, you are a constant source of guidance and support in my life. Thank you to my entire family for all encouraging me at different times to pursue my dreams and for having faith in my abilities.

TABLE OF CONTENTS

Page

List of Figures

List of Tables

List of copyrighted material / permissions obtained

PUBLICATIONS

Publications resulting from this thesis work:

Lindsay G. Aboud, Kallesh D. Jayappa, Caroline Herrera, Bernard Abrenica, Xiaojian Yao, Francis A. Plummer, Adam Burgener and T. Blake Ball. Serpin B1 inhibits HIV replication through reduced cellular proliferation and enhanced expression of Annexin V. In final editing stage for submission to Journal of Virology.
Includes data from Chapters 6 and 7 of this thesis.

Lindsay G. Aboud, Max Abou, Stuart McCorrister, Garrett R. Westmacott, Francis A. Plummer, Adam Burgener and T. Blake Ball. Molecular pathways associated with apoptosis and reduced protein translation are up-regulated following treatment of PBMCs with Serpin B1.
In preparation for submission to Journal of Virology.
Includes data from Chapter 8 of this thesis.

Lindsay G. Aboud, Francis A. Plummer, Joshua Kimani, T. Blake Ball. Cervicovaginal fluids from women in high-exposure settings inhibit HIV more strongly than women in low-exposure settings and exhibit unique CVL proteomic profiles. In preparation for submission to AIDS Research and Human Retroviruses.
Includes data from Chapter 3 and 4 of this thesis.

Lindsay Aboud, Terry Blake Ball, Annelie Tjernlund, Adam Burgener. The role of Serpin and Cystatin antiproteases in mucosal innate immunity and their defense against HIV. AJRI, 2014; 71(1):12-23.
This is a review article that contains original data.

Other Publications:

Laura M. Romas, Klara Hasselrot, **Lindsay G. Aboud,** Kenzie D. Birse, T. Blake Ball, Kristina Broliden, Adam D. Burgener. A comparative proteomic analysis of the soluble immune factor environment of rectal and oral mucosa. PLoS One, 2014; 9(6): e100820.

Q. Guan, Y. Ma, **L. Aboud**, C.R. Weiss, G. Qing, R.J. Warrington and Z. Peng. Targeting IL-23 by employing a p40 peptide-based vaccine ameliorates murine allergic skin and airway inflammation. Clinical Experimental Allergy, 2012; 42(9): 1397-1405.

"There are two mistakes one can make along the road to truth…
not going all the way, and not starting." ~Buddha

CHAPTER 1: INTRODUCTION

1.1 HIV Pandemic

1.1.1 A Brief History of HIV

On June 5th, 1981, the United States Center for Disease Control and Prevention (CDC) reported a rare lung infection, *Pneumocystosis carinii pneumonia,* in five homosexual men in Los Angeles, California[1]. Three of these men also exhibited low $CD4^+$ T lymphocyte counts[1]. It wasn't until September of 1982 that the term Acquired Immune Deficiency Syndrome was used for the first time, and in June 1984 an HLTV-like virus [later termed human immunodeficiency virus (HIV)] was determined to be the cause of AIDS[2]. Following the development of an efficient enzyme-linked immunosorbent assay (ELISA) blood test in early 1985, at least one case was reported from every region of the world by late that same year[2].

While there are numerous theories of how HIV came to exist, the most widely accepted involves the cross-species transmission of simian immunodeficiency virus (SIV) from its natural host, primates, to humans[3]. HIV-1 group M is the primary cause of the HIV epidemic and is believed to have been transmitted to humans as SIVcpz from chimpanzees in southeastern Cameroon[3]. HIV-2, first discovered in 1986 within patients of Western Africa, is morphologically related to HIV-1 yet genotypically unique and is presumed to have originated in Sooty Mangabeys[4]. Subsequent transmission between humans through sexual, percutaneous and perinatal routes led to rapid dissemination of the virus[3]. With the era of global access the spread of this infectious disease, similar to so many others, has been expansive and rapid.

1.1.2 Global HIV prevalence

HIV continues to be one of the most devastating infectious diseases with an estimated 36.9 million (34.3 million – 41.4 million) people affected at the end of 2014 and 2.0 million (1.9 – 2.2 million) new infections each year[5]. While progress has been made in treatment and preventative options, there remains approximately 1.2 (0.98 - 1.6 million) million people losing their lives to

this disease annually, an improvement from the previously reported 2.1 million deaths in 2005[5,6]. Thus, at least a portion of the World Health Organization's 6th Millennium Development Goal (MDG) has been met, including halting and beginning to reverse, the spread of HIV/AIDS by 2015.

One of the most challenging, yet potentially useful ways of eliminating new infections is by ensuring prophylactic treatment is available to all individuals at risk of acquiring HIV and ensuring all HIV-infected persons are treated. However, identifying individuals at risk and enrolling them into treatment programs has proven to be a significant challenge. Hence, achieving universal access to treatment is still a work in progress, with only 38% of all adults currently living with HIV receiving the necessary treatment and only 24% of children[5]. We continue to be a far way off from the ultimate UNAIDS 90/90/90 goal of ending the AIDS epidemic by the year 2020. The 90/90/90 goal also strives to ensure 90% of all people living with HIV will know their HIV status by 2020, 90% of the people diagnosed with HIV will receive treatment, and 90% of people on ARVs will achieve viral suppression[7]. It is thus imperative that novel forms of prevention and treatment be developed. One such prevention method is microbicides, which would provide women, who account for approximately 60% of all new HIV infections[5], with the power to protect themselves. Identifying naturally abundant, mucosally derived anti-HIV factors to be incorporated into novel female microbicides would be an important step towards this goal.

1.1.3 Prevalence of HIV in Kenya

Of the roughly 36.9 million people living with HIV at the end of 2014, approximately 25.8 million were from Sub-Saharan Africa, and 1.6 million resided in Kenya, of which approximately 58,000 succumb to their infection annually[5,6]. Six percent of the adult population in Kenya is infected with HIV and women (aged 15 years or older) account for 58.6% of these infections, resulting in over 1.1 million orphaned children as a result of HIV[6].

Key populations including commercial sex workers bear a disproportionate burden of the HIV epidemic[8]. In Nairobi, Kenya specifically, the HIV prevalence rate in female sex worker (FSW) populations was determined to be 29.5%[9,10]. Studies have indicated that FSWs engage in high risk behavior including dry sex and anal sex practices, which would increase their risk of acquiring HIV[11,12]. Additionally, the increased prevalence of sexually transmitted infections (STIs) within these women increases their risk[13]. It is, therefore, imperative to continue to develop effective community-based and medical interventions that will provide protection against HIV-1 acquisition within this vulnerable population.

1.1.4 Prevalence of HIV in Canada

According to the Public Health Agency of Canada's most recent surveillance report in December 2013 there was a total of 78,511 Canadians living with HIV, which amounts to 0.22% of the total 35.16 million Canadian population[14]. Two thousand and ninety new HIV cases were reported during 2013, representing a 12.8% decrease in comparison to the previous year. The province of Manitoba accounts for 2.5% of the total number of cases[14]. Furthermore, nationally, women who have acquired HIV through heterosexual transmission account for 29.5% of all new HIV+ test results in Canada[14].

1.1.5 Disproportionate number of women infected with HIV

Sexual transmission, via the genital tract and rectal compartment, continues to be the primary mechanism for the global spread of HIV[15]. While transmission via the female genital tract (FGT) through vaginal intercourse is quite inefficient (rates ranging from 1 in 200 to 1 in 2000 sexual acts), it continues to account for the largest proportion of new HIV infections[15]. Factors that contribute most strongly to this disproportionate number of HIV-infected women include gender inequalities and gender-based violence. Women who experience abuse from sexual partners are 50% more likely to contract HIV[6,16]. Often, women from underdeveloped

countries, including those in Sub-Saharan Africa, are not in a position to negotiate the use of condoms or HIV testing with their partners and are left vulnerable to the spread of HIV and other sexually transmitted infections. Therefore, the development of female-controlled prevention methods, such as microbicides, would prove to be a critical component in the prevention of new HIV-1 infections.

1.1.6 HIV clade differentiation and global distribution

The term HIV-1 does not refer to only one virus but rather to four distinct lineages, identified as M, N, O and P. Each of these groups is the result of separate cross-species transmission events. These groups vary in their global distribution and prevalence rates[17]. Group M accounts for millions of cases of HIV-1 across the world and is, thus, considered the pandemic form, while Group O accounts for only 1% of global HIV-1 infections[18], Group N has only been identified in 13 patients[19], and Group P has only been identified in two individuals. All patients infected with Group O, N, and P HIV viruses originate from Cameroon[19].

HIV-1 Group M is further classified into nine clades (A, B, C, D, F, G, H, J, K) with differing rates of evolution, sequence variation and associated rates of disease progression. Clade D has more rapid disease progression compared to subtypes A and C[20], while clade C is transmitted more readily from mother to child[21]. It has also been suggested that clade C is transmitted via sexual intercourse more easily than subtype A or D[22]. This may explain the 48% global prevalence observed for C compared to 12%, 10%, 6% and 3% for A, B, G and D, respectively[23]. Clades F, H, J and K together account for only 0.94% of global infections[24]. Within Sub-Saharan Africa, 56% of all HIV-1 infections are caused by clade C[24]. Within North America, approximately 94% of all HIV-1 infections are the result of clade B viruses with the remaining infections caused primarily by clade C[24].

HIV-2 is primarily restricted to countries in Western Africa; however, prevalence rates are steadily declining with HIV-1 replacing it in many countries[25]. Infection with HIV-2 is clinically milder than HIV-1 causing much lower viral loads and rare progression to AIDS. However, patients that do demonstrate clinical symptoms do so similarly to those individuals infected with HIV-1[26].

1.2 HIV virology and HIV replication cycle

1.2.1 Structure of HIV

HIV is an enveloped virus containing two identical single-stranded, positive sense RNA genomes[27]. It belongs to the *Lentivirus* genus within the *Retroviridae* family[28]. The HIV genome is made up of nine genes (gag, pol, env, vif, vpu, vpr, tat, nef and rev), encoding 15 proteins[28]. The envelope glycoproteins 120 and 41 (gp120 and gp41) are the cleavage product of the polyprotein gp160. The internal structural proteins result from cleavage of the Gag polyprotein into p24 (capsid), p17 (matrix) and p7 (nucleocapsid). Enzymatic polymerase proteins result from the cleavage of the Pol polyprotein into reverse transcriptase (RT, composed of two subunits, p66 and p51), integrase (IN, p32) and protease (PR, p11). Lastly, the HIV accessory proteins include virion infectivity factor (vif), viral protein r (vpr), viral protein u (vpu), and negative factor (nef), and the regulatory proteins include regulator of expression of virion proteins (rev) and trans-activator of transcription (tat)[27,28].

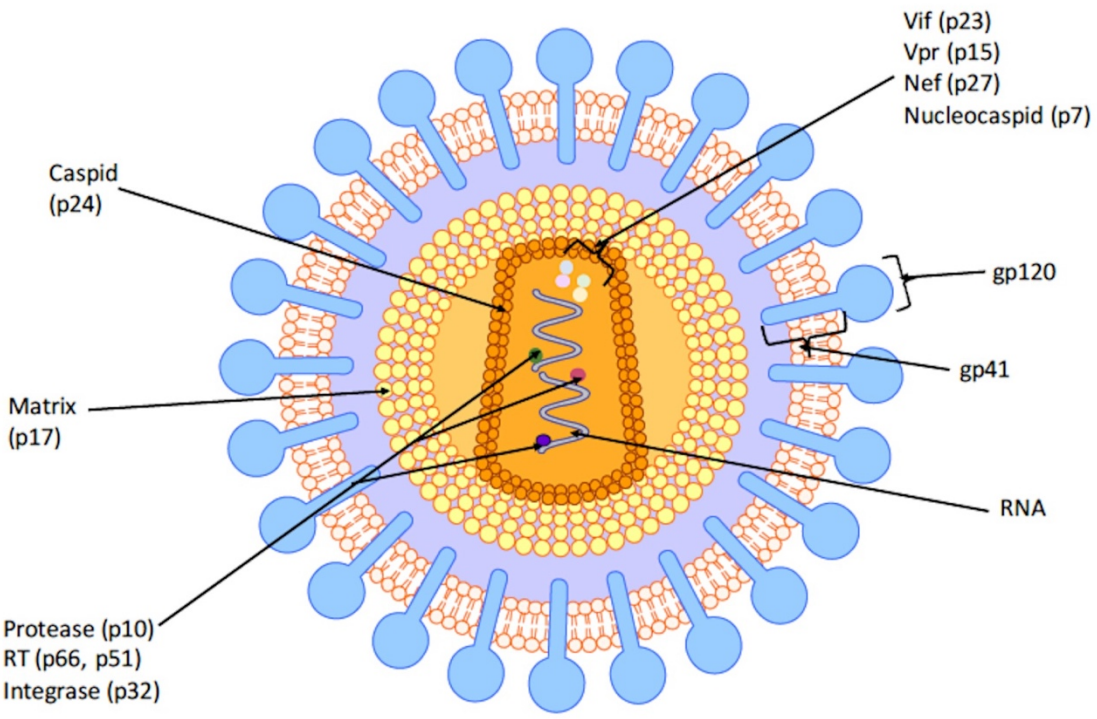


Figure 1: Structure of an HIV virion
HIV virion depicting surface proteins (gp120, gp41), matrix proteins (p17), capsid proteins (p24), two copies of single-stranded viral RNA, enzymes (protease, reverse transcriptase, and integrase) and accessory proteins (vif, vpr, nef and vpu).
Figure was constructed by author using virus structure templates produced by motifolio.com

1.2.2 HIV replication cycle

1.2.2.1 Binding/fusion of HIV to host cells

The envelope of HIV contains only one viral protein, Env, which is generated by host cells as gp160. Following post-translational modification and glycosylation gp160 is cleaved to form gp120 and gp41 trimers[29]. Binding of gp120 to CD4 (located primarily on host T cells, monocytes and macrophages) results in a conformational change in Env allowing gp120 to bind host cell co-receptors, CCR5 or CXCR4. CCR5 is found primarily on monocytes, macrophages and memory $CD4^+$ T cells, while CXCR4 is located on naïve and memory $CD4^+$ T cells[30]. HIV viruses that preferentially use CCR5 (R5-tropic) are considered to be the most common founder viruses, (the virus that establishes infection), compared to X4-tropic HIV strains which preferentially use CXCR4[30,31]. Once gp120 binds to its co-receptor, additional conformational changes occur, allowing gp41 to fuse with the host cell membrane. Fusion of the membranes results in uncoating of the virus envelope and release of the capsid into the host cell cytoplasm[28].

1.2.2.2 Reverse transcription

The HIV core is composed of multiple copies of p24. Within the core are two copies of single-stranded RNA, various accessory proteins, and viral enzymes. Once in the cell cytoplasm, reverse transcriptase converts single-stranded RNA into double-stranded DNA[28]. Both the RNA- and DNA-dependent DNA polymerases are located within the p66 subunit of reverse transcriptase. The RNase H enzyme (also within p66) degrades the viral RNA template upon completion of transcription[28]. Reverse transcriptase lacks a proof-reading mechanism, resulting in a high rate of virus mutation and thus a means for viral adaptation to host evolutionary pressure[28].

1.2.2.3 Nuclear import and integration

Newly formed viral DNA binds to HIV Integrase forming the pre-integration complex (PIC) together with vpr, matrix (p17) , and various host proteins[28]. The PIC enters the nucleus

and binds to the host lens epithelium-derived growth factor (LEDGF)/p75 followed by integration into the host DNA. Host DNA repair enzymes repair the ends of the vDNA forming a fully integrated HIV provirus[28]. Once integrated, the provirus is either transcribed, resulting in infectious viral progeny, or it remains dormant allowing the virus to evade the host immune system for years ("latent infection") as memory T cell reservoirs decay relatively slowly, with a half-life of approximately four years[32].

1.2.2.4 Transcription and translation

The 5′ LTR of integrated proviral DNA contains three binding sites; one for the viral tat protein and two for NF-κB which are recognized by NF-κB and NFAT[33]. Binding of these three factors leads to initiation of provirus transcription[34]. Tat also initiates transcription through binding TAR (transactivation-response region) and pTEFb (positive transcription elongation factor)[35-37]. Due to the overlapping structure of the HIV genome, alternative splicing of the 9 kb pre-mRNA transcript is required, resulting in 40 mRNA splice variants[38,39]. HIV RNA splicing results in the formation of three classes of mRNAs; unspliced mRNAs, which encode Gag or Gagpol proteins; single-spliced mRNAs that encode Env, Tat (p14), Vif, Vpr, Vpu, and multi-spliced mRNAs that encode Rev, Tat(p16) and Nef[40]. Multi-spliced mRNAs can freely move to the cytoplasm, however, binding of Rev to the RRE (Rev-responsive element) is required to transport all unspliced and single-spliced mRNAs to the cytoplasm for translation[39,41].

HIV viral protein synthesis relies exclusively on host cell translation machinery[42]. Viral pre-mRNA is transcribed by RNA polymerase II and is spliced, capped and polyadenylated. Once in the cytoplasm, translation of the splice variants occurs through various mechanisms including scanning, internal ribosome-entry site, frame-shifting, and leaky scanning[42], all of which contribute to the highly variable HIV viral genome.

1.2.2.5 Viral assembly and budding

Following Gag binding to viral genomic RNA (vRNA) via its nucleocaspid and matrix, it is trafficked to the cell membrane thereby initiating viral assembly[28]. The myristoylated end of Gag becomes anchored to the membrane via matrix proteins, initiating recruitment of other HIV proteins to the membrane, where p6 and capsid proteins are responsible for protein-protein interactions that ensure proper virion assembly[28]. Budding of the immature HIV virion occurs through binding of p6 and capsid to factors within the host Endosomal Sorting Complexes Required for Transport (ESCRT) pathway causing release of the virion[28,43,44]. Following release of the immature virion, HIV's aspartic protease forms a dimer and begins cleaving viral polyproteins starting with Gag which results in the condensation and stabilization of the viral RNA[45]. Once all polyproteins are cleaved, the mature virus is formed and is capable of infecting new cells.

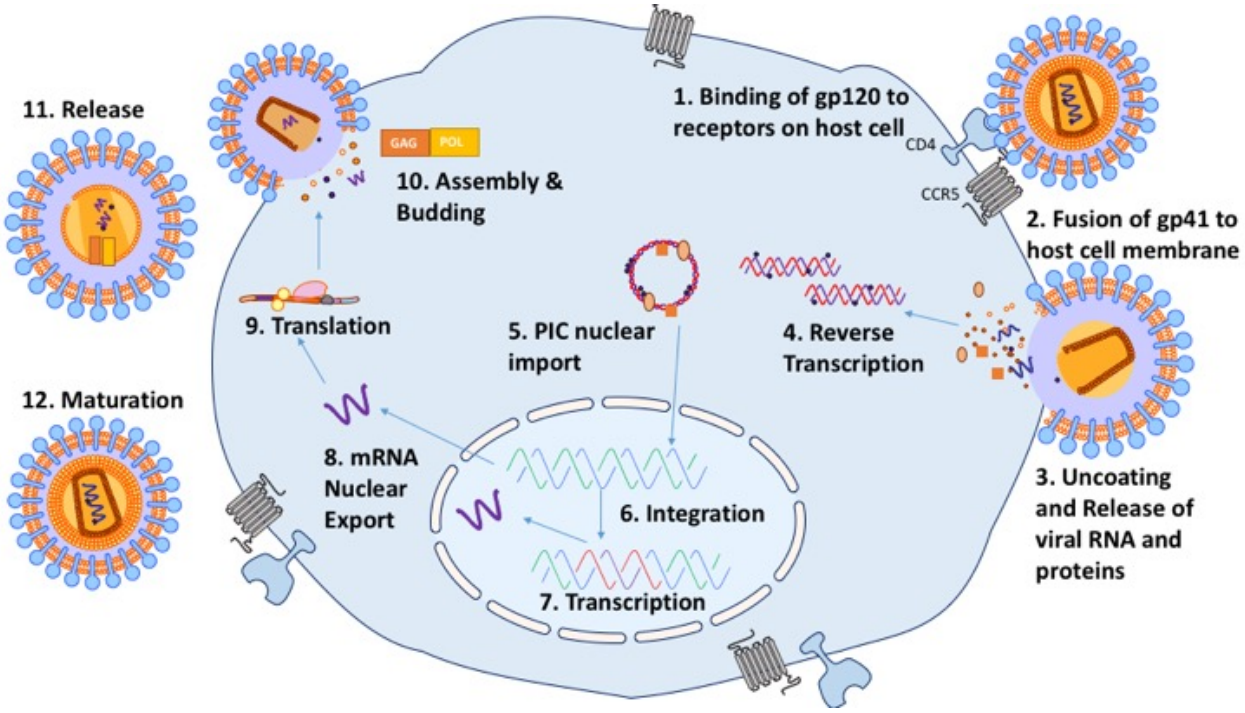


Figure 2: Replication cycle of HIV

1. HIV gp120 binds to CD4 and co-receptors (CCR5 or CXCR4) followed by a conformational change in the structure of the virion which allows for 2. fusion of gp41 with the host cell membrane. 3. The viral capsid becomes uncoated releasing its RNA and proteins into the cytoplasm of the cell. 4. The single-stranded RNA is then reverse transcribed into double-stranded DNA, which 5. binds to integrase, matrix and vpr to form the PIC. 6. The PIC then enters the nucleus where the viral DNA is integrated into the host genome. 7. Viral genes are transcribed into mRNA, which 8. is exported from the nucleus into the cytoplasm for 9. translation of viral proteins. 10. New viral proteins and RNA translocate to the cell membrane where they assemble into immature virions and begin to bud from the cell membrane. 11. New virions are released from the cell membrane. 12. Protease cleaves the polyprotein to form mature viral proteins, resulting in mature virus formation.

This figure was constructed by author using pre-made organelles and virus structures produced by motifolio.com.

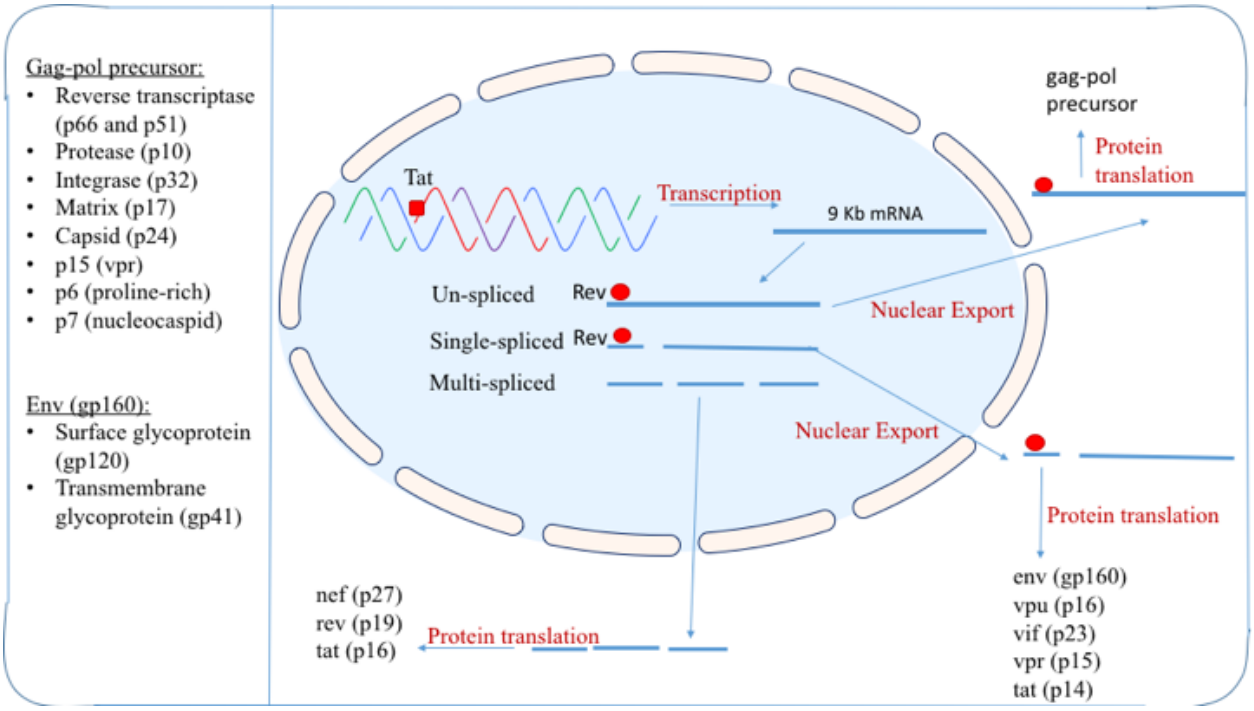


Figure 3: Splicing of HIV mRNA following transcription
HIV's 9kb pre-mRNA transcript becomes processed at multiple splice sites resulting in unspliced, single-spliced, multi-spliced variants. Unspliced mRNA encodes gag and gag-pol proteins which are later cleaved by HIV protease into RT, protease, integrase, matrix, capsid, vpr, p7 and nucleocapsid[39]. Single-spliced mRNA encodes for env which is subsequently cleaved to form gp120 and gp41, tat (p14), vif, vpr and vpu[39]. Multi-spliced mRNA encodes for rev, tat (p16) and nef[39]. Tat regulates the transcription of all viral RNAs and rev transports unspliced and single-spliced RNAs to the cytoplasm where translation occurs[38,39].
This figure was constructed by author using pre-made nucleus and nucleic acid structures produced by motifolio.com. Figure was adapted from Wong et. al., 2013[38].

1.3 HIV pathogenesis

1.3.1 Modes of transmission

HIV transmission occurs through sexual contact, including vaginal, anal, and oral routes and accounts for 75-85% of all known infections[46]. Other routes include vertical transmission from mother-to-child and transmission through needles during injection drug use (IDU) or through contaminated blood products during medical procedures (parenteral transmission)[47]. The risk of transmission is significantly different between these routes. Breastfeeding mothers who are not on antiretroviral medications have a 15-45% chance of passing the virus onto their child during pregnancy, labour or through breastfeeding[53,54], while ARV treatment can reduce the risk of transmission to below 2%[54,55]. Sexual transmission occurs most readily through anal intercourse (0.65%-1.7%)[48,49]. Both oral and heterosexual intercourse have a much lower rate, with efficient transmission occurring in 0.1-0.2% and 0.03-0.5% of sexual acts involving one infected partner, respectively[49,50]. Although infection at all mucosal surfaces has been shown to occur through successful transmission of relatively few founder viruses[51], the variability in viral load within infected bodily fluids as well as the number of available HIV target cells at the site of infection likely account for a large proportion of the variability in viral transmission[46,47].

1.3.2 Disease progression

HIV infection in approximately 70% of infected individuals results in mononucleosis-like symptoms within three to six weeks of initial infection[52]. During this time HIV multiplies and disseminates throughout the body, resulting in high levels of viremia, which induces an immune response within 1-12 weeks[53]. This immune response is insufficient to eradicate viral replication; however, it does significantly reduce the level of viremia[53]. Approximately six months following acute infection, most individuals will enter an asymptomatic period where viral load drops to below 20,000 copies per milliliter (mL), followed by a latent period which, in some cases, results in undetectable levels of virus within the blood[54]. The stable integration and

methylation of viral complementary DNA (cDNA) within host chromosomes allows for persistent infection for the duration of the cell's lifespan[32]. During this latent period, HIV will often continue replicating at low levels within lymph nodes[54]. Within ten years of the onset of latency, if ARV medication has not been administered, the immune system will systematically deteriorate[54], resulting in decreased $CD4^+$ T cell counts and a loss of HIV specific immune responses[54]. The drop in $CD4^+$ T cells may be due to increased permeability of cell membranes caused by HIV; CD4 cell destruction caused by immune activation induced apoptosis in both infected and uninfected bystander T lymphocytes[65,66,67-69]; or can be caused directly by HIV proteins, specifically Env[70], Tat[71], Nef[72] and protease[73,74]; or by perpetual flux of cytokines (IL-6, IL-1α, and TNF-α[67,69]). Also, extensive loss of memory $CD4^+$ T cells within the gastrointestinal tract mucosal-associated lymphoid tissue (MALT), results in a general depletion of HIV-specific immune responses and contributes towards a general dysbiosis of the gut[55]. Lastly, damage of lymphoid tissues, results in reduced T cell restoration processes, reducing T cell replenishment[56].

Acquired immunodeficiency syndrome (AIDS) is diagnosed once an individual's $CD4^+$ T lymphocyte count drops below 200 cells per microliter (μl)[54]. This stage of the infection is characterized by the establishment of opportunistic infections and specific cancers due to loss of $CD4^+$ T helper cell function, which cause a significant proportion of AIDS-related deaths[54].

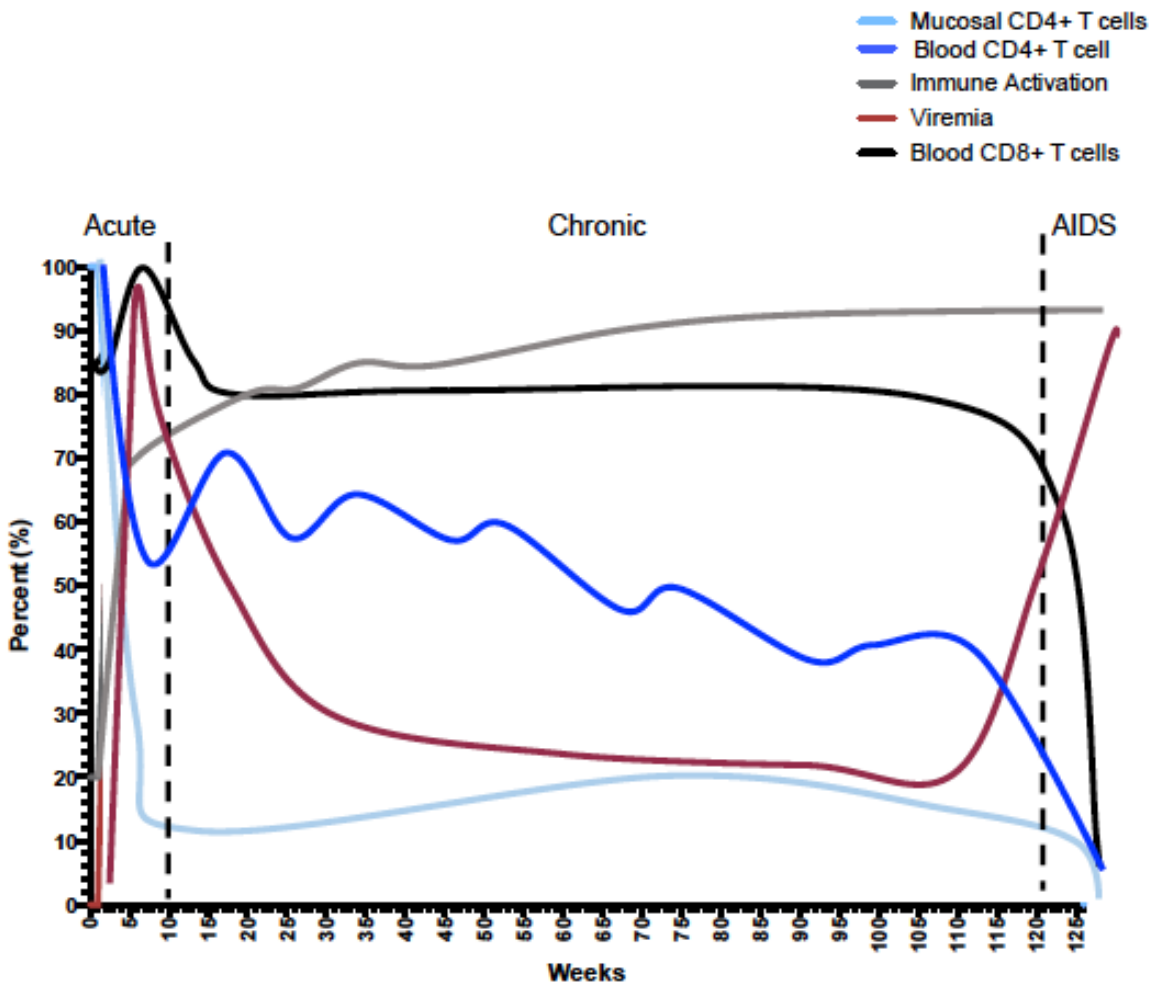


Figure 4: HIV disease progression from acute infection to AIDS
During acute HIV infection mucosal $CD4^+$ T cells begin to decline rapidly, along with blood $CD4^+$ T cells. The latter recover to moderate levels followed by a gradual decline throughout the chronic/latent phase of infection, while those in the mucosal compartments become close to negligible. Also, during the acute phase, viremia rises quickly resulting in an activation of blood $CD8^+$ T cells as well as overall general immune activation which eventually leads to moderate control of the virus and subsequent drop in viremia. During the chronic stage of infection, immune activation remains elevated, which continues to control viremia. However, prolonged infection with HIV-1 and prolonged immune activation results in a gradual decline in $CD4^+$ T cells and eventual dramatic destruction of both number and function of $CD4^+$ and $CD8^+$ T cells in the AIDS stage of infection, allowing the virus to begin replicating rapidly[77].
This Figure was constructed by author and adapted from Grossman et al., 2006[57].

1.4 Female Genital Tract (FGT)

1.4.1 Physiology

1.4.1.1 Structural morphology

Nearly 60% of all new HIV infections occur in women, the majority of which occur via heterosexual transmission in the FGT[5,6,46]. The female genital tract is composed of the upper reproductive tract, which consists of the fallopian tubes, uterus and endocervix; and the lower reproductive tract which consists of the ectocervix and the vaginal vault[58]. The area between the endocervix and ectocervix is referred to as the transformation zone[59]. The vagina is composed of three layers, the deep fibroelastic adventitia, middle muscularis layer and the superficial mucosa layer containing rugae (ridges)[60]. Similarly, the uterine wall is composed of three layers; the perimetrium, the middle myometrium layer composed of smooth muscle, and the superficial endometrium, which is the mucosal lining of the uterine cavity[60]. Additionally, the endometrium is made up of two layers; the stratum functionalis which is the layer that undergoes cyclical changes in response to hormone levels and is shed during menstruation, and the stratum basalis, which is unaffected by hormones and forms a new stratum functionalis following menses[60].

The upper reproductive tract, beginning at the cervical os (opening into the uterus) and extending into the endometrial layer of the uterus, has an epithelial layer consisting of a single layer of columnar cells joined by tight junctions, which forms a mechanical barrier against potential pathogens[61]. Conversely, the lower reproductive tract's mucosa is lined by stratified squamous epithelium, typically 15-30 layers thick, with numerous epithelial layers that function in protection against abrasion and also form a barrier to potential pathogens[61]. Due to the terminally differentiated nature of the superficial layers of the lower reproductive tract and the lack of cellular organelles, these cells lack structural integrity and are regularly sloughed off, and as a result, they are often penetrable by invading microbes. Alternatively, the basal layers of

epithelial cells are metabolically active and undergo active proliferation, and as such, this cellular layer is not as susceptible to invasion by pathogens[62]. In a process known as cervical ectopy, during adolescence and pregnancy, the columnar cells of the endocervix extend over the ectocervix replacing the squamous cells[59]. However, with age or delivery of the baby, these columnar cells are replaced once again by stratified squamous epithelium[63]. The lamina propria beneath the epithelium of both the upper and lower FGT is composed of fibroblasts, blood vessels, and immune cells[58]. The epithelial cells of the FGT produce a glycocalyx, consisting of both glycoproteins and glycolipids, as well as a thick glycoprotein rich mucus, which coats the FGT and acts as a barrier to invading pathogens, including HIV[64].

1.4.1.2 Cervicovaginal fluid composition

The mucosal fluid covering the cervical and vaginal epithelial tissue is made up primarily of water, however, it also contains mucus, secretions from the cervical vestibular glands, endometrial and oviduct fluids, as well as plasma transudate[65]. There is myriad of antimicrobial peptides present, primarily produced by vaginal epithelial cells, cervical glands, and neutrophils[66-68]. Additionally, the high number of commensal microorganisms within the vagina release organic acids and bioactive peptides, which are capable of killing potential pathogens or preventing infection of resident cells[66,69]. Specifically, *Lactobacillus* species are the most common microbial species present in healthy FGTs. These organisms release lactic acid as a metabolic product which creates an acidic environment that is hostile for many invading pathogens[69]. However, this *Lactobacillus*-dominant environment can change rapidly during menstruation, sexual intercourse, pregnancy, menopause and during urinary or reproductive system infections, to one that is colonized by a wide diversity of microorganisms[69]. This colonization by new microorganisms may cause immune activation and, thus, result in CVL that is saturated with inflammatory mediators[70]. Also, the presence of a higher proportion of non-

Lactobaccillus species results in an increased pH and reduced H_2O_2 within the FGT and therefore also within the CVL[69].

1.4.2 Menstrual cycle overview

The average menstrual cycle is 29.5 days and consists of three main phases: the menstrual (menses) phase, follicular (proliferative) phase, and luteal (secretory) phase. Menstruation is generally 4-6 days long. The follicular phase begins at the end of menses and continues until ovulation on day 14-16[71]. During the follicular phase, ovarian follicles continue to grow, a process that commences in the menstrual phase, and the ovaries secrete estradiol. A peak in this hormone is reached on day 12-13, resulting in the thickening of the uterine endometrium[60,71]. Approximately 24-48 hours following the peak in estradiol, a large surge in luteinizing hormone (LH) and a smaller surge in follicle-stimulating hormone (FSH) released from the anterior pituitary gland, together with an increase in progesterone from the ovaries, occurs, resulting in ovulation[60,71]. The luteal phase begins after ovulation and continues until the start of menstruation. During this stage the corpus luteum of the ovary (follicle from which the ovulated egg was released) begins to release estradiol and progesterone, preparing the uterus for implantation. Approximately four days before the start of menstruation the corpus luteum starts to breakdown causing a decrease in the production of estradiol and progesterone[71], resulting in the endometrium beginning to break down, and the onset of menstruation[71].

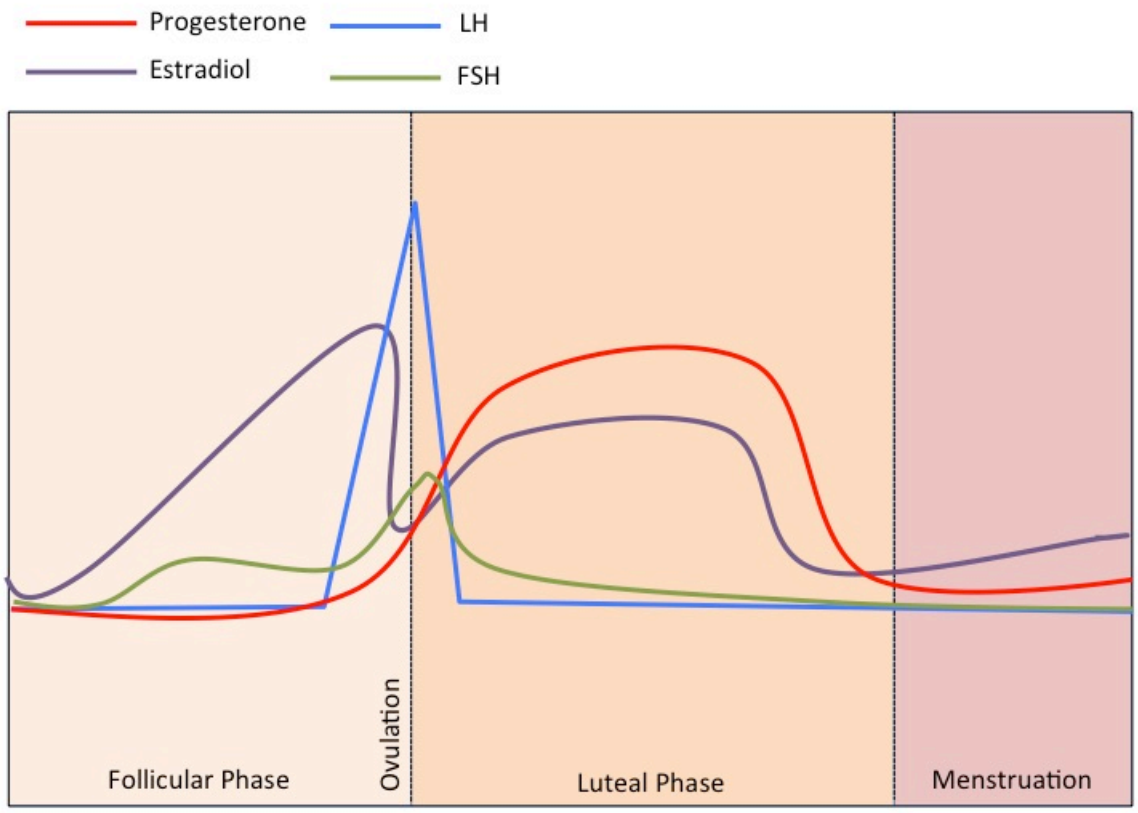


Figure 5: Hormone fluctuations throughout menstrual cycle
Follicular phase begins at the end of menses. During this stage estradiol is continually released by the ovaries eventually resulting in peak levels reached on day 12-13. Approximately one to two days after this peak in estradiol a large surge in LH and a small surge in FSH occurs causing ovulation. Following ovulation, the luteal phase begins which is marked by the steady release of estradiol and progesterone from the corpus luteum. Four days before menstruation the corpus luteum starts to breakdown causing a reduction in the levels of both estradiol and progesterone. This drop in hormone levels causes the breakdown of the endometrium, triggering menstruation.
This figure was constructed by author and adapted from Jones, 1997. [71]

1.4.3 Routes of mucosal HIV acquisition

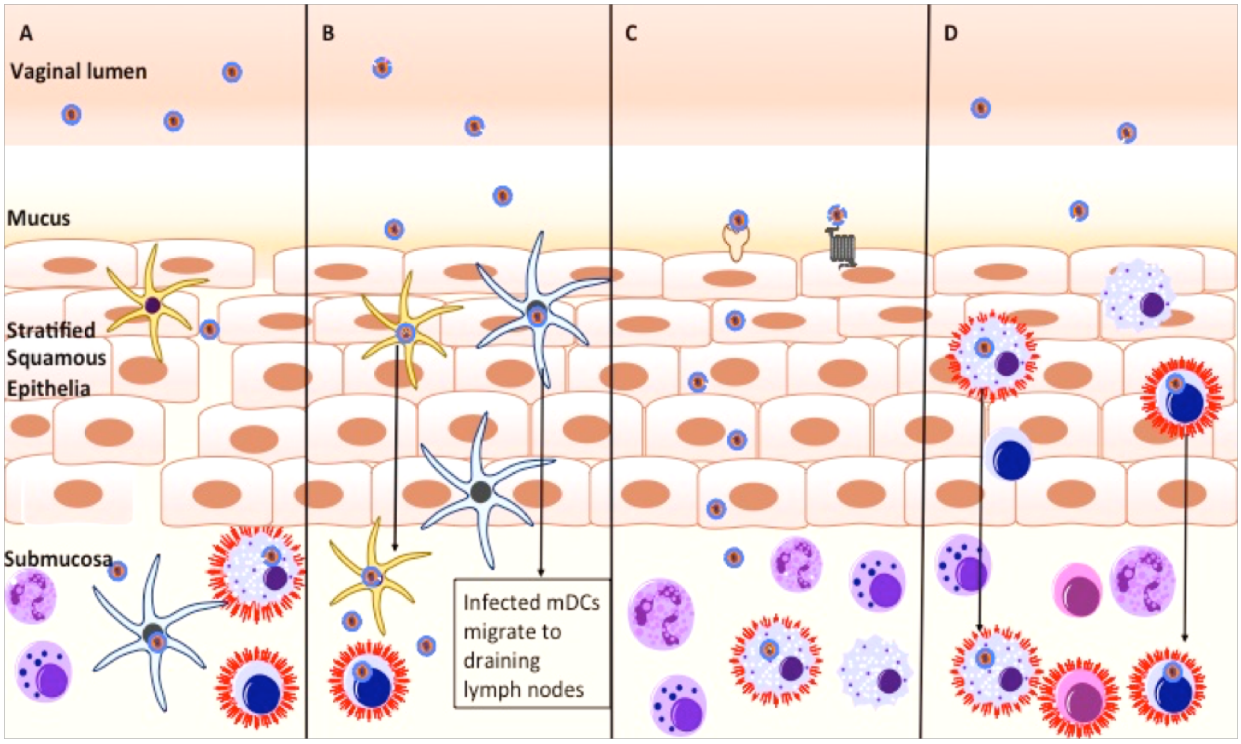
HIV-1 can translocate across the FGT epithelium through various mechanisms. Disruption of the cervicovaginal epithelia through trauma, infection-associated mucosal inflammation, or ulceration and erosions all provide HIV with the necessary access to underlying immune target cells residing within the lamina propria[72,73]. Myeloid dendritic cells and Langerhans cells present at the interface between the vaginal and ectocervical epithelial and lamina propria have been shown to take up HIV-1 and transfer it directly to the lamina propria or draining lymph nodes where they will trans-infect target cells[73,74]. Also, through binding to the epithelial cells naturally expressing the receptor gp340, HIV-1 can cross the intact mucosal epithelial layers, throughout the upper and lower FGT, via transcytosis[75]. Lastly, initial infection can also occur through direct entry into lymphocytes and macrophages present within the epithelial tissue followed by subsequent replication within the infected cells[73,74].

1.4.4 Role of female sex hormones in HIV-1 transmission

The primary female sex hormones estradiol and progesterone are produced cyclically by the ovaries throughout the menstrual cycle and are not only responsible for the regulation of the mucosal immune responses of the FGT but also significantly affect the integrity of the epithelium[76,77]. Within non-human primate models and human studies[78,79], there has been a "window of vulnerability" suggested for HIV that takes place one week before mensturation (during luteal phase), which corresponds to a period with high progesterone levels[80-82]. This data complements the various studies that implicate medroxyprogesterone acetate (MPA), the active ingredient in the DMPA (depot medroxyprogesterone acetate) hormonal contraceptive, as a risk factor for HIV acquisition, increasing an individual's overall risk by up to 40%[147,148]. It has been suggested that medroxyprogesterone acetate exhibits its effect primarily through increasing the rate at which HIV is taken up by and transcytosed across the FGT epithelial tissues, resulting in an increased number of infected T cells[83]. Similarly, increased levels of

estradiol result in decreased integrity of the epithelial barrier through remodeling of occludin within the epithelial tight junctions of the uterus, vagina, and ectocervix[77]. This allows an increase in fluid and molecules from the uterine tissue into the lumen, resulting in optimal transport of sperm[84]. However, it may also lead to an increased vulnerability to HIV-1 infection.

Estradiol and progesterone do not exert their effects equally across FGT cell types[85]. For example within the epithelial cells of the uterus, estradiol stimulates secretion of human β-defensin type 2 (HBD2) as well as other immune modulatory molecules, while in the vagina epithelial cells show decreased production of HBD2[86,87]. Additionally, within the uterus, the effects of estradiol and progesterone during the proliferative phase of the menstrual cycle result in lymphoid aggregates consisting of $CD4^+$ and $CD8^+$ T cell clusters at a concentration of approximately 300 cells/aggregate. However, during the secretory/luteal phase, this number increases to 3000-4000 cells/lymphoid aggregate, providing a greater number of HIV target cells[88]. Similarly, cytotoxic T lymphocyte (CTL) activity is suppressed within the uterus during the secretory phase reducing the number of protective cells at the primary site of infection, an effect not observed in the vagina and assumed to aid in the implantation process of the embryo[88]. Thus, significant evidence has been put forward demonstrating varying degrees of susceptibility to HIV infection throughout the menstrual cycle, with the secretory/luteal phase offering the least amount of protection.



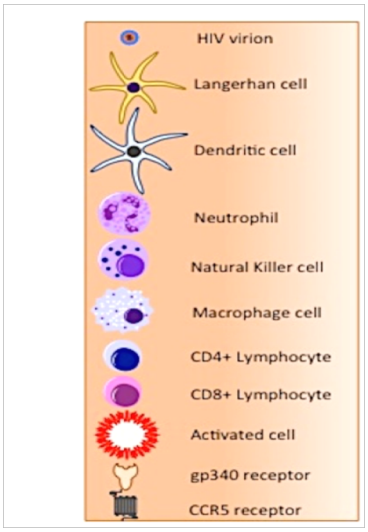


Figure 6: Routes of HIV transmission within the female genital tract

A: Disruption of the epithelial barrier, B: Taken up by Langerhans or myeloid DCs and shuttled to lamina propria or lymph nodes, C: HIV binds to gp340 on specific cells and transcytosis across intact membranes, D: Direct infection of macrophages or lymphocytes within the epithelial layer.

This figure was constructed by author using pre-made cells produced by motifolio.com.

1.5 General Immunology

The immune system is separated into two arms, the innate and adaptive. The primary difference between these two systems involves the inability of the innate system to generate a memory response to previous pathogens while the adaptive response is capable of this function. The adaptive immune response functions through the generation of 10^{14}-10^{18} different somatic immunoglobulin and T cell receptors while the innate immune response relies on germ-line encoded receptors and, therefore, employs more receptors with fewer diversity[89]. The diversity of the adaptive immune response causes this arm of the immune system to function at a slower pace, making the innate system the initial response to invading pathogens[89]. Considering this thesis focused primarily on innate immune responses/proteins, this will be the arm of the immune system that will be the focus from this point forward, with a description of the adaptive arm only as it relates to mucosal compartments.

1.5.1 Innate immune responses

1.5.1.1 Epithelial barriers

The most common portals of entry targeted by microbes are the skin, gastrointestinal tract, respiratory tract and reproductive tract[90]. Aside from providing a physical barrier of continuous epithelium at these sites, epithelial cells also produce peptides with anti-microbial properties capable of killing or slowing the invasion of pathogens[90]. Also, within the epithelial layers of these locations are γδ T lymphocytes which are situated between epithelial tissue layers and express antigen receptors with limited diversity, similar to other innate immune cells. These cells recognize microbial lipids and various other structures shared among microbes and aid in the capture and killing of pathogens as well as of infected cells[90,91].

1.5.1.2 Phagocytes

The three major types of professional phagocytes circulating throughout the immune system are neutrophils, dendritic cells (DCs) and monocytes/macrophages. These cells are

recruited to the site of infection where they recognize pathogens based on interactions between the cells' pattern recognition receptors (PRR) and the pathogen-associated molecular patterns (PAMP) which are uniquely expressed on different classes of pathogens[90]. These interactions can lead to subsequent uptake and digestion of the microbe[90].

Neutrophils are often the first cell responder to sites of bacterial and fungal infection[90]. Although they only survive for a limited number of hours following uptake of microbes, neutrophils are the most abundant leukocyte present in the blood and their production is stimulated by granulocyte colony-stimulating factor (G-CSF), interleukin (IL) -23 and IL-17A[90,92]. Neutrophils have granules that contain proteins capable of killing microbes and digesting tissues[93]. Some of these proteins are the same as those secreted by the liver, including haptoglobin, α_1-acid glycoprotein, and α_1-antitrypsin[94-96]. However, neutrophils also produce a variety of other proteins including lysozyme and α-defensins. Neutrophil granules are categorized into three subsets. Primary (azurophil) granules contain mainly myeloperoxidase and neutrophil elastase, secondary (specific) granules contain lactoferrin, and tertiary (gelatinase) granules contain gelatinase[97].

Tissue neutrophils are more actively phagocytic than are blood neutrophils[94,98]. Within the tissue, neutrophils release a higher level of immune mediators, including neutrophil elastase. A transcriptional cascade becomes activated shortly after neutrophils become activated by their stimulus[99], which stimulates the generation of numerous chemokines including, IL-8 and Groα (CXCL1), both of which lead to immune cell infiltration[98,100]. While the presence of functional neutrophils in tissues is imperative for defense against microbes it is also important that the influx of neutrophils be controlled to prevent neutrophil-mediated tissue damage.

Macrophages originate from monocytes either after they migrate from the blood into tissues (resulting in tissue macrophages) or once monocytes remain in the blood long enough for

maturation to occur, eventually becoming part of the blood-resident macrophage population[101]. Macrophages not only clear invading pathogens from the blood and tissues but also remove cellular debris caused by tissue damage or remodeling, as well as cells that have undergone apoptosis[102-104]. The detection of necrotic tissue debris by macrophages occurs through recognition of danger signals, including heat-shock proteins, nuclear proteins, and detection of histones by TLRs 2, 5 and 6 as well as through intracellular PRRs and the IL-1 receptor[105]. The majority of these homeostatic phagocytic functions are independent of other immune cells and often occur in the absence of macrophage activation[105,106]. Macrophages also have a role in tissue remodeling through the production of soluble factors, including Prostaglandin E2 (PGE2), that induce the stimulation of epithelial progenitor cell proliferation[102,107].

If macrophages are to kill invading pathogens, they are activated to effector status. The combination of interferon gamma (IFN-γ) and tumor necrosis factor (TNF) result in macrophages with increased microbicidal and tumoricidal effects and the production of high levels of proinflammatory cytokines[108,109]. Natural killer (NK) cells are the primary innate immune cell to produce IFN-γ once they experience stress or encounter infections. The presence of IFN-γ leads to stimulation of macrophages to produce cytokines and increased superoxide production to enhance their killing capacity[109]. However, NK cells only produce IFN-γ for a short period, after which antigen-specific T_H1 cells from the adaptive response assume this function[106].

Activated macrophages produce myriad of cytokines including IL-1, IL-6, IL-8, IL-10, IL-12, IL-23, TNF-α, IFN-α, IFN-γ, monocyte chemoattractant protein-1 and -3 (MCP-1, MCP-3), macrophage colony-stimulating factor (M-GSF), G-CSF, granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein-1 (MIP-1) and transforming growth factor beta (TGF-β)[110]. While many of these cytokines are involved in the clearance of

invading pathogens, a select few, namely IL-1, IL-6 and IL-23, can contribute to extensive host tissue damage[106]. It is, therefore, important to regulate the activity of macrophages. This regulation is performed by cytokines, namely, IL-4, IL-10, IL-13, and TGF-β[110].

Two broad classes of macrophages have been described based largely on their activiation state[111]. M1 macrophages are considered to be activated in a classical manner, in the presence of IFN-γ, combined with either Lipopolysaccharide (LPS) or TNF[111]. These activated M1 macrophages secrete reactive oxygen species (ROS), IL-12, IL-23, IL-10, IL-1, TNF and IL-6, and function in Type I inflammation (characterized by strong phagocytic activity), delayed-type hypersensitivity, and killing of intracellular pathogens[111]. Alternatively, M2 macrophages are separated into M2a, M2b and M2c subtypes. M2a cells become activated in the presence of IL-4 and IL-13 [112,113]. Once activated, these cells begin to secrete polyamines, IL-10, IL-1RII, and IL-1ra, which results in an overall type II inflammation (characterized by elevated antibody titers), allergic responses and killing and encapsulation of parasites[112]. Alternatively, M2b cells become activated in the presence of immune complexes and TLR binding as well as the presence of IL-1R. These cells then begin to secrete high levels of IL-10 and low levels of IL-12, TNF, IL-1 and IL-6, resulting in the T_H2 activation and immunoregulation[112,113]. Lastly, M2c cells become stimulated when exposed to IL-10. These activated cells will produce more IL-10, as well as TGF-β and induce immunoregulation, matrix deposition and tissue remodeling[113].

Dendritic cells (DCs) constitute a critical link between the innate and adaptive immune responses. Within the innate system, DCs recognize and kill invading pathogens. DCs become mature and develop into distinct subsets upon sensing danger signals, either from pathogens or from dying host cells, through recognition primarily via TLR receptors[114,115]. Stimulation via TLRs results in an increased uptake of antigens and presentation at lymph nodes, as well as an increase in expression of co-stimulatory molecules, secretion of cytokines and up-regulation of

chemokine receptors[114,116-119]. Conventional DCs within human blood produce IL-12, TNF and IL-6 in response to TLR ligation[120,121]. Conversely plasmacytoid DCs sense viruses through TLR-7 and TLR-8[122], which induce the production of type 1 interferons (IFN-α and IFN-β). Lastly, inflammatory DCs primarily respond to LPS, however, they also contain the repertoire of TLRs found on conventional DCs (TLR-1, 2, 3, 5, 6, 8) and thus sense similar pathogens[123-125].

DCs will also respond to pathogens by secreting TNF-α and nitric oxide (NO). TNF-α binds to the receptors on Gram-negative bacteria targeting them for phagocytosis by macrophages[126]. The IL-12, IL-15, and type I IFNs produced by DCs activate NK cells, which then produce IFN-γ[127,128].

Langerhans cells (LCs) have a similar function to conventional DCs except that they reside in the epidermal layer and can be identified by expression of the antigen Ly6C. These cells express TLR-2, -3, -5, -6 and -10. Therefore, LCs are able to sense peptidoglycan and lipoteichoic acid from Gram-postive bacteria, via TLR-2, as well as dsRNA via TLR-3[129].The binding of one or both of these TLRs results in the production of IL-6, IL-8, IL-10 (TLR-2 only) and TNF-α (TLR-3 only) [129]. Alternatively, LCs are not able to respond to ssRNA (TLR-7/8) or LPS (TLR-4) and do not produce IL-12 or type I IFNs[129]. In addition, LCs function to capture and present antigens from the external environment to naïve T cells within the lymph nodes, causing the T cells to become activated and begin to proliferate[119,130].

1.5.1.3 Natural Killer cells

NK cells develop from common lymphoid progenitors and can be divided into multiple subtypes with different activation states. Two main subtypes are defined based on their density of CD56 expression[131,132]. Those defined as $CD56^{dim}CD16^{+}$, constitute $\geq$95% of the total peripheral blood NKs, contain high levels of perforin and high cytolytic activity. Alternatively,

NKs described as $CD56^{bright}CD16^{-}$, comprise $\leq$ 5% of the total peripheral blood and produce many pro-inflammatory cytokines including IFN-γ and TNF[133,134].

The degree to which an activated NK cell reacts to a foreign antigen is dependent on expression of enhancing and inhibitory receptors present on the NK cell surface. The primary activating receptors are NKG2D and the natural cytotoxicity receptors (NCRs) (NKp30, NKp44, and NKp46)[135]. These receptors recognize and respond to several molecules up-regulated following infection or stress of cells, including hemagglutinin, which is specifically up-regulated in virus infected cells[136,137]. Alternatively, inhibitory receptors, including CD94 and killer cell immunoglobulin-like receptors (KIRs), bind non-classical and classical MHC I molecules on healthy nucleated cells and function to control the activation of NK cells to avoid killing of healthy "self" cells[90,119,138,139]. The balance of activating and inhibitory stimuli determines the resulting NK cell function[140].

If the activating signal is more robust than the inhibitory signal, the cell proliferates and secretes cytokines such as IFN-γ[119]. The connection between NK cells and macrophages is maintained through macrophage production of IL-12, which stimulates NK cells to produce IFN-γ, which in turn stimulates macrophages to produce more IL-12[90]. Once activated, NK cells function primarily to kill virus-infected and tumour cells[141].

1.5.1.4 The complement system

The complement system is made up of both circulating and membrane-bound proteins, many of which are classified as proteolytic enzymes[90]. Activation of one of the three pathways (classical, alternative or lectin) requires sequential activation resulting in an enzyme cascade[90]. The alternative and lectin pathways are considered part of the innate immune system due to the lack of antibody-binding required for initiation[90]. The classical pathway requires binding of

antibodies to the surface of microbes/antigens and is therefore considered to be part of the adaptive immune response[90].

All three pathways employ various effector functions to eliminate pathogens. These functions include opsonization[90,142], or osmotic lysis of microbial cells through activation of the membrane attack complex (MAC)[142]. Furthermore, specific complement peptides, including C3, C4, and C5, produced through proteolysis during the complement cascade, are chemotactic for neutrophils, resulting in the release of inflammatory cytokines/chemokines, enhancing the overall immune response to infection[90]. This latter function is the primary response evoked during infection with HIV.

1.5.2. Apoptosis

Apoptosis is a form of programmed cell death initiated by the cell and is a mechanism for control of the immune system[142,143]. Failure to initiate apoptosis may result in increased levels of activated lymphocytes, infected phagocytic cells, autoimmune disease, or an inability to control immune responses and malignancies[144]. Apoptosis is characterized by an increase in caspase activation, formation of apoptotic bodies, cell shrinking, membrane blebbing caused by the detachment of the cytoskeleton from the cell membrane, condensation of nuclear chromatin, and breakdown of DNA into nucleosomal units[143,145].

Both extrinsic and intrinsic pathways exist to induce apoptosis. The extrinsic pathway is mediated by a superfamily of receptors known as the tumor necrosis factor receptors (TNFR). Binding of TNFR-1 through both the death domain as well as the death effector domain of FAS-associated death domain protein (FADD), pro-caspase 8 or pro-caspase 10 leads to subsequent initiation of the apoptotic signaling cascade[144]. Conversely, binding of FADD-like IL-1β converting enzyme-inhibitory protein (cFLIP), which is a negative regulator of apoptosis, blocks this cascade resulting in cell survival and NF-κB mediated pro-inflammatory responses[144].

The intrinsic (mitochondrial) apoptotic pathway is caused by a collapse of the inner mitochondrial membrane potential and alterations within the ion gradients due to loss or accumulation of metabolites/ions, resulting in the release of cytochrome c[146]. Cytochrome c is a cofactor for activation of caspase-9, which is an activator of the effector caspases, -3, -6 and -7, which initiate cell death similarly to that observed in the extrinsic apoptotic pathways[146,147].

While apoptotic cells maintain their plasma membrane integrity, they begin to express ecto-CRT, phosphatidylserine, heat shock proteins 70 (HSP70) and HSP90, opsonins, as well as other molecules that target them for phagocytosis by macrophages and dendritic cells[148,149].

1.5.3 Mucosal female genital tract immunity

1.5.3.1 Innate mucosal response

Due to the presence of an epithelial barrier at every mucosal surface, cells within this tissue are the first responders to any invading pathogen. PRRs on epithelial cells allow for early recognition of, and differential responses to, myriad of pathogens and foreign molecules. TLR mediated activation of genital epithelial cells results in up-regulation of IL-6, IL-8, TNF-α, stromal derived factor-1, MIP-1α, MIP-1β and RANTES[141,150,151,152,153]. Virus-specific responses have also been observed within these FGT cells through the up-regulation of proinflammatory cytokines and chemokines, including IFN-β. These cytokines have been shown to correlate with antiviral responses via induction of IFN-inducible genes and proteins, including PKR, iNOS and MyxA, all of which have been directly or indirectly linked to inhibition of viral replication in Cytomegalovirus (CMV), Herpes Simplex Virus-2 (HSV-2) and HIV[151,154-156].

Macrophages and dendritic cells are present throughout the FGT, specifically in the ectocervix and endocervix[157,158]. The macrophage population is highest throughout the mid-secretory phase of the menstrual cycle and is most abundant in the cervix and endometrium as well as other uterine tissues as part of the uterine lymphoid aggregates[88]. DCs are present in

much smaller numbers throughout the uterus and are localized primarily throughout the subepithelial stroma of the endometrial layer[157]. Within the vagina and ectocervix, DCs, including $CD1a^+$ Langerhans cells, are present within the squamous epithelial layers and at the interface between the stroma and epithelial layers. However, DCs within tissues, including those of the FGT, differ from those found in circulation. Within the mucosal membranes of the genital tract, DCs have an immature phenotype[141], which changes to mature upon stimulation. Mature DCs express increased levels of MHC II and co-stimulatory molecules and migrate to draining lymph nodes for antigen presentation to adaptive immune cells, in the case of HIV-1, resulting in HIV dissemination and an increased number of HIV-1 specific effector cells[141].

Neutrophils are located throughout the FGT but are most abundant within the fallopian tubes and in lower abundance throughout the upper and lower reproductive tracts[141,159]. In response to the IL-8 produced by FGT epithelial cells, neutrophils migrate across the epithelium into the lumen to phagocytose sperm, microorganisms, and residual cellular debris following trauma or apoptosis[141]. While neutrophil abundance within the lower FGT remains stable throughout the menstrual cycle, an increase in neutrophils is observed within the endometrium of the uterus just before menstruation as part of the natural breakdown and restoration process involved in menses[58,141]. This increase in activated neutrophils may result in enhanced susceptibility to HIV acquisition, due to the increased levels of inflammation and tissue damage that are hallmarks of prolonged neutrophil infiltration[160,161].

NK cells make up 10-30% of the total leukocyte population within the FGT[159]. Within the uterus, the abundance of NK cells increases from the proliferative phase into the secretory phase, wherein the latter stage they comprise as much as 70% of the leukocyte population within the endometrium[162]. Uterine NK (uNK) cells are phenotypically distinct from circulating NK cells. Unlike circulating NK cells, uNKs express CD56 and very minimal levels of CD16, they also express CD9 and CD69[163]. However, similarly to circulating NK cells, uNKs from non-

pregnant women exhibit cytolytic activities and produce IFN-γ, GM-CSF, IL-10, TGF-β, and IL-8[141]. IL-15 produced by endometrial stromal cells is also required for uNK cells to proliferate and survive[158]. Thus, these highly cytolytic uNKs will enhance the level of tissue breakdown within the uterus during the late secretory stage, which may provide enhanced opportunity for HIV acquisition. Additionally, within the ectocervix and endocervix, both $CD56^{+}CD16^{-}$ and $CD56^{-}CD16^{-}$ NK cells have been identified; however, their abundance does not fluctuate throughout the menstrual cycle[158].

1.5.3.2 Adaptive mucosal response

While both $CD4^{+}$ and $CD8^{+}$ T cells are present throughout the upper and lower FGTs, CD8 T lymphocytes are present in higher abundance[88]. Elevated levels of cytotoxic T-lymphoctye (CTL) activity has been observed in the cervix and vagina, independent of menstrual cycle phase[58,88]. Most of the T cells within the lower FGT are located at the stroma-epithelial interface however they are also found in low abundance throughout the ectocervical and vaginal epithelial layers[58]. Within the uterus, the highest level of CTL activity occurs throughout the proliferative phase and minimally within the secretory phase. Also, compared to circulating $CD4^{+}$ T cells, endometrial and endocervical $CD4^{+}$ T cells express higher levels of CCR5 and consist of a large population of activated effector memory T cells, which may increase their susceptibility to infection with HIV[164]. Furthermore, lymphoid aggregates are present, within the uterine endometrium stratum basalis layer, consisting of a B cell core surrounded by $CD8^{+}$ T cells and macrophages. These aggregates are in highest abundance during the secretory phase[88].

Lastly, both IgA and IgG have been identified within cervical secretions, against HIV-1[165]. The endocervix contains the highest levels of both IgG and IgA antibody producing and antibody-expressing B cells. Unlike other mucosal sites throughout the body, the dominant antibody response found in the genital secretions is IgG, although both IgA1 and IgA2 are found within these secretions[165]. Transport of both IgA and IgG to the lumen of the FGT is dependent

on the expression of the transporter proteins polymeric immunoglobulin receptor (pIgR) and neonatal Fc receptor (FcRn) on the epithelial cells, respectively[166]. The expression of pIgR is up-regulated by estradiol in the uterine epithelium, whereas progesterone has the opposite effect on its expression, causing a fluctuation in the level of IgA present within the FGT throughout the menstrual cycle[166].

1.6 HIV specific innate immune response

1.6.1 Overview of mucosal innate HIV response

The epithelial layer of the mucosal surface along with secreted factors (β-defensins, SLPI, trappin-2/elafin, lactoferrin) act as primary barriers to HIV infection either through physical interference with the virus or inhibition of NF-κB activation[167]. Membrane-associated mucins are also expressed on the apical surfaces of epithelial cells throughout the FGT, which trap and eliminate pathogens, including HIV before they can reach the epithelial surface[168].

While all ten TLRs have been identified on various cells throughout the FGT, only TLR3, 4, 7, 8 and 9 can induce antiviral activity through the induction of IFN-α/β and IFN-λ [152,169]. Early production of these type I and III interferons are critical for efficient antiviral defense. Specifcally, IFN-α induces the expression of genes that encode restriction factors including APOBEC3, TRIM5, tetherin, and SAMHD1 that are capable of directly inhibiting HIV replication[204-206]. APOBEC3 and SAMDH1 interfere with reverse transcription, Tetherin interferes with release of virions from the host cell, and TRIM5α recognizes the HIV capsid protein and blocks viral infection shortly after the release of the capsid into the host cell cytoplasm[204-206]. However, while there is a well-established body of evidence suggesting a protective role for IFNs during early exposure to HIV[170-172], during chronic infection, IFN-α/β induce significant levels of immune activation resulting in increased disease pathogenesis[170,173,174]. Furthermore, both type I interferons have been identified as inversely

correlated with $CD4^+$ T cell count and positively correlated with both HIV-1 RNA plasma levels, and the expression of the activation marker, CD38, on $CD8^+$ T cells[173-175]. This may, in part, be attributed to the antagonistic mechanisms that HIV has evolved against specific host restriction factors induced by type 1 interferons. Specifically Vpu interferes with the activity of Tetherin, Vpx with SAMDH1, and Vif with APOBEC3[204].

Following exposure to HIV, one of the first detectable immune responses includes a release of acute phase proteins triggered by proinflammatory cytokines including IL-1, which occurs in two waves. The first of which takes place shortly after exposure and the second involves a massive cytokine storm/release, coinciding with a rapid increase in plasma viremia[176]. With increasing levels of viremia, there is a subsequent increase in plasma cytokines and chemokines. Delayed IL-10 production occurs, along with transiently high levels of IL-15, type I IFNs and CXCL10, while rapid and long-lasting levels of IL-18, TNF, IFN-γ, and IL-22 are also present[177]. Many of these cytokines and chemokines enhance both innate and adaptive immune responses (Appendix, Table 17), however, an intense and prolonged response may drive immune activation, and result in the promotion of viral replication and immunopathology[177].

The number of DCs present at mucosal surfaces during acute HIV infection is significantly reduced, which may be due to activation-induced apoptosis of DCs or to the migration of activated DCs from the tissue and circulation into draining lymph nodes[178]. Once in the lymph nodes, DCs become activated through binding of viral envelope proteins to CD4 resulting in subsequent endocytosis of the virion and triggering of TLR7-induced pathways with increased expression of IFN-α[176,178]. Plasmacytoid DCs (pDC) ($CD123^+$, present in the lymph nodes) are recruited to the FGT within 1-3 days post-infection following increased expression of CCL20 (MIP-3α)[179]. Upon arrival, these pDCs begin to secrete MIP-1α and MIP-1β, which are chemoattractants for $CCR5^+$ $CD4^+$ T cells. These $CD4^+$ T cells become activated following

exposure to HIV and begin to produce IFN-α, which may lead to immune hyperactivation within the FGT during acute infection and thus assisting in the propagation of HIV[176,179].

Conventional DCs are capable of priming virus-specific adaptive $CD4^+$ and $CD8^+$ T cell responses following exposure to HIV. Conversely, HIV-activated pDCs produce indoleamine 2,3-dioxygenase (IDO) resulting in differentiation of $CD4^+$ T cells into Tregs, which may lead to a dampening of HIV-specific immune responses[180].

Langerhans cells within the FGT epithelium express CD1a, the C-type lectin receptor langerin (CD207), CD4 and CCR5 but not CXCR4 or DC-SIGN, making them targets for HIV infection with R5-tropic viruses. In fact within an SIV model, genital tract Langerhans cells have been identified as harboring SIV virions within 24 hours of intravaginal inoculation[181,182].

NK and NKT cells also become activated during acute HIV infection. These cell populations proliferate before the observed peak in viremia[183]. Once activated NK cells affect HIV replication through induction of cytolysis in virus-infected cells, production of antiviral cytokines/chemokines and through interactions with DCs which induce T cell responses[176,183,184]. While NK cells play a significant role in early control of HIV they do not contribute to the development and selection of virus escape mutations before peak viremia (21-28 days after infection), whereas cells of the adaptive immune response, specifically $CD8^+$ T cells, do play a critical role in this process.[176]

1.6.2 Role of immune activation in HIV transmission

Chronic immune activation is one of the most prominent characteristics of HIV disease and is thought to be the driving force behind HIV-1 transmission[55], as well as $CD4^+$ T cell depletion following infection[185]. Activation is also a better predictor of disease outcome in HIV-infected individuals than viral load or CD4 count[186]. Manifestations of immune activation include increased B cell polyclonal activation, increased $CD4^+$ and $CD8^+$ T cell activation as determined by increased expression of activation markers (CD38, CD69, HLA-DR), increased

level of apoptosis within uninfected cells and elevated levels of proinflammatory cytokine and chemokines[185,187]. If activated $CD4^+$ T cells are present at the site of HIV replication they become easily infected by HIV-1 allowing for the establishment of a founder virus and thus acquisition of HIV-1 infection[188]. The presence of subsequent activated $CD4^+$ T cells results in rapid dissemination of the virus. In fact, *in vitro* studies have demonstrated that while quiescent $CD4^+$ T cells are susceptible to infection, limited viral replication occurs within these cells, and thus HIV preferentially establishes infection in activated T cells[188,189].

The mechanism by which HIV induces chronic activation is still under debate, however, multiple factors are likely at play[185]. One of the most prominent causes of activation is the direct innate and adaptive immune responses against the virus[185]. These responses include recognition of viral antigens presented to T cells and B cells, the binding of HIV components to TLR 7 and TLR 9, and induction of signaling pathways through binding of HIV to CD4 as well as to co-receptors (CCR5, CXCR4)[178]. Another well-described cause for chronic activation is the destructive effect that HIV has on mucosal barriers, which allows for continuous sensing of the genital tract lumen, and bacterial translocation into the lamina propria, resulting in immune activation in response to microbes present within these sites[185].

1.6.2.1 Co-infections within the FGT

The presence of sexually transmitted infections (STIs) has been strongly associated with an increase in HIV transmission[190,191]. This may be due to the presence of ulcerations caused by the associated pathologies of many STIs, or it may be attributable to the increase in recruitment of activated immune cells to the site of infection which has the potential to act as target cells for HIV[192,193]. Specifically, HSV-2 infection is associated with a three-fold increase in HIV susceptibility, likely due to the presence of ulcerations during this infection. These ulcers generate an influx of activated $CD4^+$ T cells, which persist in the FGT for months after healing of the ulcer[194,195]. Similarly, bacterial vaginosis (BV), characterized by the change in natural

vaginal flora from one dominated by *Lactobacillus* species to one dominated by *Prevotella bivia, Atopobium vaginae, Gardnerella vaginalis, Bacteroides ureolyticus and Mobiluncus curtisii*[196,197], is associated with a 60% increased risk of HIV-1 acquisition in women and an increase in the abundance of shedding virus within the FGT due to immune activation in FGT cells[198,199].

Immune, and other cells including epithelial, within the genital tract respond to STIs by upregulating proinflammatory cytokines, including TNF-α, IL-6, and IL-1β[199,200] (Table 29, Appendix). Similarly, Ferreira et al. demonstrated that following infection with herpes simplex virus type 1 or 2 or with *Neisseria gonorrhoeae*, endometrial epithelial cells have been shown to produce TNF-α, IL-6, IL-8 and MCP-1 all of which contribute to the indirect induction of HIV-LTR promoter in T cells resulting in an increased level of HIV replication[155].

1.7 Natural protection against HIV

1.7.1 HIV-Exposed Sero-Negative (HESN) phenotype

HIV exposed seronegative (HESN) individuals are defined collectively as those who are highly exposed to HIV yet remain uninfected[201,202]. In some cases, these individuals may have mucosal IgA specific HIV-1 responses, however, remain seronegative systemically for anti-HIV IgG[201] and have no evidence of HIV DNA or RNA.

Multiple cohorts around the world have been identified as containing HESN individuals. These cohorts include HIV-1 serodiscordant couples, injection drug users, hemophiliacs, commercial female sex workers (FSW), men who have sex with men (MSM) and infants breastfeeding from an HIV-infected mother[201,202]. Determining levels of exposure is challenging within these populations. Mathematical models of the frequency of high-risk behavior and the

prevalence of HIV-1 in the community are used to determine approximate exposure and, therefore, the risk of HIV acquisition[201].

Within the Pumwani commercial sex worker cohort in Nairobi, Kenya, a group of HESN women who are epidemiologically defined as relatively resistant to infection have been identified[203]. To meet this definition, the women must be followed within the cohort for at minimum seven years[82]. Women who are HIV seronegative and in the cohort for less than seven years are defined as HIV-susceptible. Several studies have identified genetic and immune correlates of protection that appear to play a role in protecting against HIV acquisition.

1.7.2 Natural correlates of protection

1.7.2.1 Genetic correlates

Humans have multiple host restriction factors that assist in protection against HIV infection, some which HIV has adapted itself to evade while others appear to protect against HIV infection. The most well studied genetic correlate of protection against HIV is the APOBEC family of proteins[204]. Specific members of this protein family, namely APOBEC3D, APOBEC3E, APOBEC3F, APOBEC3G, and APOBEC3H, have protective functions against HIV-1 through hypermutation of the viral genome, inhibition of reverse transcription[85] and inhibition of viral cDNA integration into the cellular host DNA[205]. HIV has evolved to counteract these functions through Vif, which specifically binds to APOBEC proteins leading to their degradation[204,206].

Other genetic correlates of protection include; KIR3DL1 (NK cell receptor) polymorphisms, which have been associated with slow progression to AIDS, as well as resistance to HIV infection[207-209]. Homozygosity for a 32-base pair deletion in the CCR5 allele, identified in Caucasian individuals, results in complete loss of CCR5 expression and subsequent resistance to R5-tropic HIV viruses[210-213]. Additional polymorphisms have been identified as

modestly protective against HIV infection including single nucleotide polymorphisms (SNPs) within the CCR5 promoter region as well as within CCR2 and CCL5[212,214]. Certain groups of class I alleles have also demonstrated strong effects on the outcome of HIV infection. Specifically, HLA-B*27 and HLA-B*57 have consistently been associated with positive disease prognosis[215,216]. Lastly, genetic polymorphisms within the IRF1 gene confer significantly reduced IRF1 expression levels and subsequent reduction in responsiveness to IFN-γ within peripheral blood mononuclear cells (PBMCs) reducing the level of HIV-1 LTR activation[217].

1.7.2.2 Immune correlates

While HIV-specific adaptive immune responses (humoral and T cell-mediated) have been observed within HESN individuals, it is unclear as to whether these responses are protective or merely evidence of exposure to HIV[201]. However, numerous natural, innate immune correlates have been identified in HESN individuals that are believed to contribute to their protection against infection[202,218-222].

Increased numbers, and function, of NK cells and DCs have been shown to associate with the HESN phenotype[201]. Additionally, many secreted factors have been identified as correlates of HIV protection[219,223,224]. Specifically, elevated expression of the CC (β)-chemokine family, including macrophage inflammatory protein (MIP)-1α and MIP-1β as well as RANTES (regulated upon activation normal T-cell expressed and secreted) are thought to be protective by competing with HIV-1 for CCR5 co-receptor binding [223,225].

Several small cationic proteins within mucosal secretions have also associated with reduced HIV susceptibility. Within the saliva of HESN individuals, Secretory leukocyte protease inhibitor (SLPI) has been identified as up-regulated and exhibits anti-HIV activity[220,224,226-228]. Lactoferrin, a component of breast milk and genital tract secretions, has

also been shown to reduce the level of HIV-1 replication and transmission within DCs and T cells[229]. However, within one HESN group of women, lactoferrin, SLPI and RANTES were all found to be associated with vaginal bacterial vaginosis and inflammation within the female genital tract instead of with exposure to HIV-1[226]. This studies suggests that these factors may not be directly related to HIV susceptibility but rather are up-regulated in response to inflammatory changes within the FGT caused by many different pathogens. Additionally, α-defensins and β-defensins are expressed at mucosal surfaces and have been associated with antimicrobial properties against HIV-1 within an HESN cohort of breastfed infants[230]. Both α-defensins and θ-defensins (Retrocyclins, homologues of α-defensins) have also been shown to bind with relatively high efficiency to HIV gp120 and cellular CD4, thereby protecting cells from infection with HIV-1[231-233]. However, the presence of α-defensins within the cervicovaginal fluids of women has been associated with increased HIV-1 acquisition due to their association with bacterial sexually transmitted infections[234]. Elafin/trappin-2 has been associated with protection against HIV-1 transmission and was elevated within the genital sections of HESN women from the Pumwani sex-worker cohort in Nairobi, Kenya[219]. Furthermore, proteomic evaluation of the cervicovaginal lavage (CVL) from HESN women in this cohort compared to HIV-susceptible and HIV-positive women revealed numerous up-regulated proteins in HESN women. The majority of these up-regulated proteins were antiproteases, including members of the serpin family (Serpin A1, A3, C1, B1, B13, C1, G1), type 1 cystatins (Cystatin A and B), and alpha-2 macroglobulin-like protein (A2ML1). Many of these anitproteases have defined anti-inflammatory and anti-HIV-1 specific activity[235,236] and are thus proposed to protect against HIV-1 infection through modulation of immune responses, maintenance of mucosal epithelial barrier integrity and direct antiviral activity[250].

1.8 HIV Prevention

1.8.1 Current preventative strategies

Although antiretroviral drugs have been incredibly effective in slowing the spread of HIV[6,237] and prolonging the life expectancy of individuals living with HIV/AIDS[238,239], there are also numerous disadvantages of their use. Multi-drug resistant HIV strains continue to emerge in persons who have been on ARVs for an extended period, as well as in individuals who are unable to comply with the proper regimen due to inaccessibility of drugs, severe side effects, and improper education on the importance of consistent dosing[240]. In light of these limitations, there has been increased focus placed on developing effective preventative strategies, including treatment of other sexually transmitted infections (STIs)[241]. Condoms are also efficient at reducing the spread of HIV and other sexually transmitted infections, however, the use of them requires the agreement of both partners. This poses significant challenges in situations where women are not equal members of the decision-making process and are forced to participate in risky, unprotected sexual intercourse. Also, circumcision is 70% effective in preventing male acquisition of HIV[242]. However, there is no significant reduction in the rate of transmission from a circumcised male to their female partner[242]. Most recently, Pre-Exposure Prophylaxis (PrEP), consisting of an oral dose of tenofovir disoproxil fumarate and emtricitabine (Truvada) has been recognized as a means of significantly reducing the risk of HIV infection among individuals at high risk for HIV acquisition, including MSM, people who use drugs and serodiscordant couples[243,244]. Furthermore, ARVs have been employed as post-exposure prophylaxis, following suspected exposure to HIV-1 and as a measure to reduce the risk of transmission between an HIV-infected individual and their partner(s)[245,246].

While a vaccine would be the most effecient way to control the spread of HIV, the only one to show efficiency (Thailand RV144) offered approximately 54.5% protection over the first six

months, however, efficacy for this vaccine drops rapidly to approximately 29.2% following 42 months of follow-up[247,248]. It is, therefore, imperative that research continues to explore other preventative options, particularly those that are female-controlled. One such option is a microbicide, which would prevent HIV acquisition and transmission during heterosexual intercourse[249]. It has been predicted that with only a partially effective microbicide, millions of new HIV infections would be avoided worldwide[250].

1.8.2 Hurdles for microbicides

Designing a microbicide to inhibit HIV acquisition must take into account factors unique to targeting HIV replication while also considering the delicate environment of the FGT, which must be kept in balance if infection with HIV is to be avoided.

It is also imperative that any microbicide is able to prevent endocytosis of the virus by LCs and DCs, as well as to be able to access the lymphatic system[251]. This may be possible through the use of nanoparticle technology, which contains small enough particles (similar in size to viruses) to be able to breach the epithelial and endothelial membranes, allowing access to the blood and lymphatic systems[252]. It is also important that chronic use of microbicides not induce irritation or inflammation of the FGT, which would increase the risk of HIV acquisition[253]. On a similar note, any HIV-specific microbicide should also ideally be efficient at preventing secondary genital tract infections, to prevent local immune activation[254]. Lastly, with the emergence of multi-drug resistant HIV strains[255], the design of any novel HIV preventative agent may also incorporate minimal virus-specific proteins and rather target host factors. HIV will develop escape mutations against compounds that target virus-specific proteins, conversely, targeting of host factors makes it more challenging for HIV to be able to develop efficient mutations.

1.8.3 Classification of ARVs/microbicide candidates

1.8.3.1 Entry/binding inhibitors

This class of inhibitors includes gp120 or gp41 blockers as well as CCR5 antagonists, which act by blocking attachment of HIV-1 to the host cell through interfering with gp120 attachment or through blocking of the cellular surface co-receptors (CD4, CCR5, CXCR4)[256]. Alternatively, many of the compounds included in this category are non-specific microbicides as they do not specifically target gp120-CD4 binding but rather prevent non-specific adsorption and attachment caused by positively charged Env with negatively charged proteoglycans on the host cell surface[257,258]. Among these non-specific compounds are dextran sulfate[259], cyclodextrin sulfate, heparin and soluble anionic polymers[256]. However, most of these agents, save for PRO 2000 (a naphthalene sulfonate polymer), have not proven to be successful in clinical trials due to lack of protection against HIV-1, disruption of the natural flora within the FGT, or causing increased inflammation and epithelial toxicity within the FGT[260-263]. Alternatively, PRO 2000, has been incorporated into the Vaginal PRO 2000 topical gel microbicide. This polymer's anti-HIV activity functions through binding to CD4 receptor non-specifically (Appendix, Table 13)[249]. Numerous other specific HIV-binding inhibitors have also been identified (Appendix, Table 13) which have exhibited varied levels of success in clinical trials, with Enfuvirtide and Maraviroc as the only fusion/binding inhibitors currently available for clinical use.

1.8.3.2 Reverse transcriptase inhibitors

This group of compounds includes both nucleoside/nucleotide (NRTIs/NtRTIs) and non-nucleoside (NNRTIs) reverse transcriptase inhibitors. NRTIs/NtRTIs, including tenofovir, are analogs of natural deoxynucleotides, however, they lack a 3'-hydroxyl group on their deoxyribose[264]. Thus, these compounds interfere with virus reverse transcription by preventing proper elongation of the daughter DNA strand[264]. Alternatively, NNRTIs, including both

nevirapine, delavirdine, and dapivirine (full list presented in Table 13 Appendix), interfere directly with reverse transcriptase by binding to an allosteric hydrophobic binding site in close proximity to the enzyme's catalytic site. Binding of the NNTRI causes conformational changes in the binding site, thereby preventing polymerase binding to its target and halting DNA strand transcription[264]. One disadvantage of using NNRTIs is the rapid rate at which HIV-1 can mutate amino acids surrounding the NNRTI binding site, minimizing, its effect on the RT enzyme[265]. To prevent the rapid development of drug resistance, NNRTIs are either given in combination with another NNRTI or are given at high doses from the onset of treatment[265].

1.8.3.3. Integrase inhibitors

These potential microbicide agents prevent efficient integration of the viral DNA into the host genome[264]. One caveat to the use of the most common integrase inhibitor, Raltegravir (full list presented in Table 13, Appendix), is that its permeability is highly dependent on being at its optimatl pH (pH 5)[266]. The vaginal pH typically ranges from 3.8 to 4.5, thus, it is important to ensure microbicides maintain the proper pH for the active agent to perform optimally while not disrupting the natural balance of the FGT.

1.8.3.4 Protease inhibitors

Lastly, protease inhibitors (PI), including ritonavir and indinavir, target the HIV-1 enzyme protease. The protease enzyme cleaves the Gag-Pol polyprotein precursor at nine sites to produce mature HIV proteins[267,264]. While there are numerous PIs being employed in ARV therapy regimens (Appendix, Table 13), current treatment guidelines suggest that all should be combined with at least two other ARVs of differing mechanisms of action to ensure efficacy. This stipulation would also likely be required in a microbicide, due to the fact that these drugs function to inhibit HIV within the late stages of the viral lifecycle, however, to date there have not been any microbicide candidates employing a PI.

1.8.4 Current microbicide trials

Numerous microbicides are in various stages of clinical trials, however, perhaps one of the most informative, although ineffective, recent phase III trials is the Vaginal and Oral Interventions to Control the Epidemic (VOICE). This trial was conducted between 2009 and 2012 with over 5,000 women enrolled from South Africa, Uganda, and Zimbabwe combined[268]. These women were required to either take a daily oral tenofovir (NRTI) tablet or apply vaginal gel with the same active ingredient. However, neither arm of the study proved effective, and further investigation revealed that less than 25% of the participants had detectable levels of tenofovir in their blood, indicating an incredibly low adherence rate for both the oral and vaginal options[268]. These results suggest that a while a daily treatment regimen may be effective if taken regularly, this rarely occurs, and thus, alternative strategies better suited to women's lifestyles must be explored to increase adherence.

The importance of adherence was also illustrated in The Centre for the AIDS Programme of Research in South Africa (CAPRISA) 004 trial, which demonstrated strong dose-dependent responses, with higher levels of adherence resulting in higher levels of protection[269]. This was a phase IIB randomized controlled trial that examined the effectiveness of 1% tenofovir gel in preventing the acquisition of HIV in South African women when applied vaginally before and after sexual intercourse[243]. Women who were high adherers to the regimen (>80%) achieved a 54% increase in protection compared to the placebo gel arm, while a 28% rate of protection was observed in women who had lower levels of adherence (used gel $\leq$50% of the required time)[243].

Considering that daily or coitus-dependent application of a vaginal gel system has yielded inconsistent levels of success within women, certain microbicide trials are beginning to focus on changing the mode of delivery from a gel to an intravaginal ring. Vaginal ring systems are designed to fit comfortably within the vaginal vault and release the active agent over time[270-272]. This system has been adopted as a standard delivery mechanism for hormonal contraception in

numerous countries and is considered to be a safe alternative to the gel application system[270,271,273,274]. Two of the first studies to employ intra-vaginal rings are the ASPIRE and The Ring Study both of which are currently in phase III trials that are examining the effectiveness of vaginal rings containing dapivirine (NNRTI) for one month or two years, respectively[275]. The ASPIRE study has enrolled nearly 3,500 women at sites in Malawi, Uganda, South Africa and Zimbabwe while The Ring Study has enrolled 1,650 women from Uganda and South Africa[274,275].

Lastly, the MTN-013/IPM 026 is a phase I trial that is currently examining the safety and cellular uptake of an intravaginal ring containing dapivirine and maraviroc (CCR5 inhibitor), alone or in combination for 28 days[264]. Employing both an NNRTI and CCR5 inhibitor is predicted to perform with higher efficacy than microbicides containing only a single agent.

All of these studies outline the complexity surrounding the development of an effective female-controlled HIV-preventative strategy. It is not enough to develop compounds that efficiently inhibit HIV infection/replication, rather the system by which these compounds are delivered to women is equally as important, and if done improperly, has the potential to nullify any beneficial effects exhibited by the anti-HIV agent.

1.8.5 Women's perspective on female-controlled HIV prevention

In the wake of numerous unsuccessful microbicide trials, due primarily to lack of adherence to drug regimens, it is imperative to begin involving women at risk of acquiring HIV in the design strategy of future preventative measures. When women from the VOICE trial were asked why they did not adhere to their medications, the most common responses were fear of unknown side-effects of the drugs, or fear of being labeled as HIV-positive by community members once it became known that they were taking medications that are commonly used by HIV-positive individuals (Tenofovir)[276,277]. Furthermore, the highest degree of stigma was feared to come from male sexual partners who often provide financial stability for the women.

Women were concerned that their partners would assume they were engaging in risky behavior outside of their relationship[278]. Hence, evidence suggests that women are most interested in preventative measures that are in their control, do not require daily application, and are in a form different from the medications taken by people infected with HIV. It is also necessary for counseling to be provided for both members of a couple so as to ensure a thorough understanding of the reasons for taking the preventative agent and to foster supportive roles for male partners[278].

1.8.6 The ideal preventative strategy

Similar to the development of any new medication there are a few key aspects that should always be considered including safety, cost, efficacy and acceptability by the target population. In addition, aside from the obvious requirement to prevent the acquisition of HIV infection, female microbicides must not pose a risk to the reproductive health of the individual. Any substance that comes into direct contact with the FGT must be tested extensively for effects on fertility and fetal development[264]. Also, any microbicide should be safe to use during sexual intercourse and not have negative interactions with semen or alter the natural flora of the FGT[264].

The stability of the active agent within a microbicide is not only essential for ensuring adequate uptake into cells and duration of protection but also for the overall cost of the product[264]. If a product is more stable, it will be produced in larger quantities and have a longer shelf-life, making it cheaper to manufacture and thus cheaper for individuals to purchase[264].

Any successful microbicide will likely require the incorporation of a combination of active agents with differing mechanisms of action so as to decrease the likelihood of developing HIV strains resistant to all components of the microbicide. The use of multiple active agents will also provide the opportunity for production of Multipurpose Prevention Technologies (MPTs). MPTs not only protect against HIV infection but also act as a contraceptive or antibacterial

agent, preventing unwanted pregnancy and secondary FGT infections as well as limiting biofilm production on intravaginal rings, which may lead to increased inflammation in the FGT[279].

Lastly, any successful preventative strategy will include not only medical interventions but also structural approaches, including cash transfers, food vouchers, nutritional support and sexual violence counseling, all of which are integral aspects of a truly complete and effective strategy for HIV prevention for all vulnerable populations, but most specifically for women[5].

Cumulatively this information suggests that identifying novel, natural, HIV inhibitory factors, such as antiproteases (Serpins, cystatins), to be used in microbicides, is the starting point for developing a truly effective preventative strategy for HIV. However, before any of these factors can be chosen as a candidate, a clear understanding of their anti-HIV mechanism of action must be defined. Once the mechanism is determined to be beneficial and adequate to prevent productive HIV infection, the stability of that compound within a female accepted delivery system (i.e. vaginal ring system) must also be determined as well as the pharmacokinetics of that agent within the delivery system to ensure adequate levels of protection. However, designing the optimal microbicide will prove to be futile if social/structural systems are not set in place for women to be properly educated and supported in their desire to protect themselves against HIV.

1.9 Antiproteases as potential bioactive compounds for use in microbicides

1.9.1 Serpins

1.9.1.1 Structure and enzymatic mechanism

Serine protease inhibitors (Serpins) are a superfamily of proteins that regulate the activity of proteases. All serpins contain a shared 350 residue core domain, consisting of three β-sheets (A-C) and 8-9 α-helices (hA-hI) [280]. This core may include minor modifications due to small insertions or deletions within the α-helices, however, the primary differences observed between

serpins occurs as a result of N- or C-terminal polypeptide extensions and N- or O-type glycosylation. The majority of known serpins are approximately 40-60 kDa, however, the largest member, Serpin C1 (105 kDa), has a 100 residue glycosylated N-terminal extension[281].

The region of serpins responsible for protease inhibition is the reactive centre loop (RCL)[282]. This loop forms an extended structure above the body of the serpin and undergoes a conformational change from a stressed to a relaxed state, following enzyme-protein complex formation[283,284]. Initial binding of a target serine or cysteine protease with the serpin results in the formation of a non-covalent complex through interactions with the residues flanking the scissile bond on the Serpin (P1-P1'). A covalent ester linkage is then formed between the Ser-195 of the protease and a backbone carbonyl of the P1 residue in the serpin with subsequent cleavage of the peptide bond[285,286]. It is at this point that the amino-terminal portion of the RCL begins to insert itself into the centre of β-sheet A[285,287]. This conformational change alters the environment of the RCL from completely solvent-exposed in the native conformation to mostly buried, in the cleaved form[287]. Not only does the RCL change its structure, the bound protease also undergoes dramatic distortion[288]. Serpins function through irreversible covalent binding, rendering both the serpin and protease inactive. This is, in effect, a suicide mission for the serpin, resulting in permanent enzyme inactivation[282,289]. This process is highly energetically favourable as evidenced by the dramatic increase in unfolding temperatures of serpins following protease binding (un-cleaved 60^{o}C, cleaved >120^{o}C)[290].

Co-factors can regulate the function of particular serpins[282]. An example of this is the relatively weak inhibitory function of Serpin C1 (antithrombin) without the presence of heparin[291]. This attribute of serpins allows for more eloquent control of their function within the immune response. Also, the conformational changes that occur in both the serpin and protease provide a distinct mechanism for cells to recognize and remove inactivated serpin-protease complexes, whereas native serpins are generally not internalized by cells of the immune system[282,292].

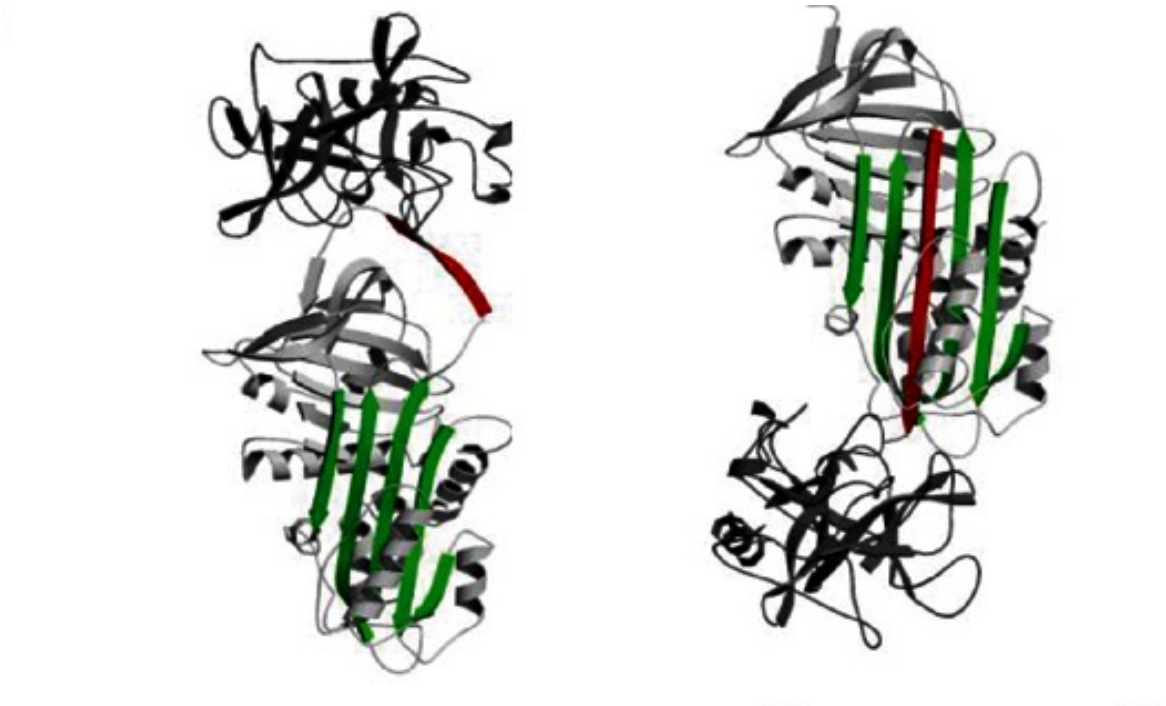


Figure 7: Serpin inhibition of target proteases
Following docking, and the formation of a covalent ester linkage between Ser-195 of the protease (black) and a backbone carbonyl of the Serpin, a peptide bond becomes cleaved. This cleavage allows for a dramatic, irreversible, conformational change to occur in the Serpin as well as to the bound protease, as the RCL (red) inserts itself into the β-sheet A, pulling the protease from a position at the upper pole of the Serpin to one on the lower pole.
This figure was reproduced from Lomas and Carrell with permission[293] *(Appendix 11.8.1)*

1.9.1.2 Classification

While most of the more than 3000 characterized serpins contain some form of serine protease inhibitory function, there are select members, including ovalbumin, which do not[294,295]. Other members exhibit both serine and cysteine protease inhibitory activity. Due to the highly diverse functions present within this superfamily, sixteen clades have been identified[280]. All serpins are named according to their serpin clade with the formula, SERPINXy, with X designating the clade and y the number within the clade. However, nearly all serpins will also have alternative names that are more descriptive of their particular function[282]. While serpins have been identified in nearly all multicellular higher eukaryotes as well as in members of the *Poxviridae* family, 36 distinct serpins have been identified within humans (clades A-I)[286]. However, the two most prominent clades in humans are clade A, consisting of 14 members (A1-A14), and clade B, which consists of 13 members (B1-B13)[286].

1.9.1.3 Biological functions

Serpins' primary functions are to inhibit serine proteases, including trypsin, chymotrypsin, cathepsins, elastase and granzymes[282,285,296-298]. Specific serine proteases (elastase, proteinase 3, cathepsins, granzymes) that are present within cytoplasmic granules of neutrophils, NK cells, CTLs and macrophages, are involved in the direct killing of pathogens within the phagolysosomes of phagocytes or following release from granules into the surrounding tissues. These protesases also function to trigger an inflammatory response through induction of complement activation or secretion of pro-inflammatory cytokines and chemokines[299,300]. Thus, serpin regulated control of proteases' functions to prevent over-activation of these immune processes, decreasing the degree of inflammation, apoptosis, tissue damage as well as other destructive pathologies[282,299,301]. Specific serpins, including Serpin B9, have strong anti-apoptotic functions related to their inhibitory activity against granzyme B as well as against the executioner cathepsins B, L, and D[302]

While the majority of serpins contain anti-protease activity, many members also have alternative functions that are non-inhibitory. Specifically, HSP47/Serpin H1 functions as a chaperone[286], maspin/Serpin B5 as a tumor suppressor[303], angiotensinogen/Serpin A8 as a regulator of blood pressure[304], Serpin A6 binds cortisol[305] and thyroxine-binding globulin/Serpin A7[306] has a known role in transport[286,298].

Members of the clade B serpin family contain shorter N and C-termini compared to other serpins and lack cleavable N-terminal secretory signal peptides[286]. However, specific B serpins, namely Serpin B1, B3, B4, B5 and B7, may also have extracellular functions as they have been shown to be released during certain cellular conditions [286,307]. The release of these proteins is facilitated by an embedded, uncleaved hydrophobic N-terminal signal sequence which is involved in both conventional and non-endoplasmic reticulum-Golgi secretory pathways[307,308]. However, once in the extracellular environment, serpin B clade members that contain either cysteine or methionine residues within their RCL are susceptible to oxidative inactivation and have a limited half-life within this environment[307]. All intracellular human serpins (B clade) function as competitive inhibitors of serine or cysteine proteases with many members of this clade capable of inhibiting more than one protease, with dual reactive sites[308]. As such this group of proteins has an incredibly diverse group of functions including protection of cells such as CTLs and NK cells from auto-destruction by granzyme B and protection of bystander cells from injury due to over-expression of this, and other proteases[286,309]. Additional functions include regulation of cell growth and differentiation, regulation of the invasiveness of tumor cells, and regulation of the level of cellular motility as well as angiogenesis[309-311], all of which may have a role in limiting the number of available target cells present at the portal of HIV entry/site of founder virus acquisition. Furthermore, the ability of intracellular serpins to inhibit multiple proteases and their presence within epithelial cells may suggest that they have a role in barrier protection, or host defense against microbial and viral infection.

1.9.1.4 Potential for control of HIV infection

Specific host cell proteases increase the strength of the overall inflammatory response and, thus, increase HIV infectivity. Serpins function to inhibit these proteases. Numerous studies have been performed on Serpin A1, A3, and C1, to determine their potential role in the control of HIV-1 replication[323]. Serpin A3 has been shown to inhibit Cathepsin G and elastase, which promote the expression of pro-inflammatory cytokines and lead to the recruitment of potential HIV-1 target cells, including T cells and macrophages while enhancing the susceptibility of macrophages to infection by HIV[312,313]. Serpin A1 (α_1-antitrypson) has direct inhibitory activity against HIV. A small C-terminal fragment (C-36) is released, when Serpin A1 binds to a serine protease, which functions as a gp120 competitive binding inhibitor of CD4 and a gp41 fusion inhibitor[314]. Serpin A1 also interferes with gp160 processing into gp120 and gp41 as well as processing of p55 into p24[315]. Furthermore, Serpin A1, along with Serpin C1, have known indirect anti-HIV activity involving interference with activation of the NF-κB pathway as well as interferences with the induction of numerous pro-inflammatory cytokines that HIV relies on for replication and propagation of infection[297,316]. Serpin A1 also inhibits the migration of neutrophils to the site of infection through inhibition of calpain, resulting in reduced immune activation and reduction in the number of potential HIV target cells[316].

These serpins, as well as several others, have been identified as being up-regulated within cervicovaginal secretions of HESN women from the Pumwani, commercial sex worker cohort in Nairobi, Kenya[235]. It is, therefore, reasonable to predict that similar to Serpin A1, A3, and C1 other serpins may also have a role in controlling HIV infection/acquisition.

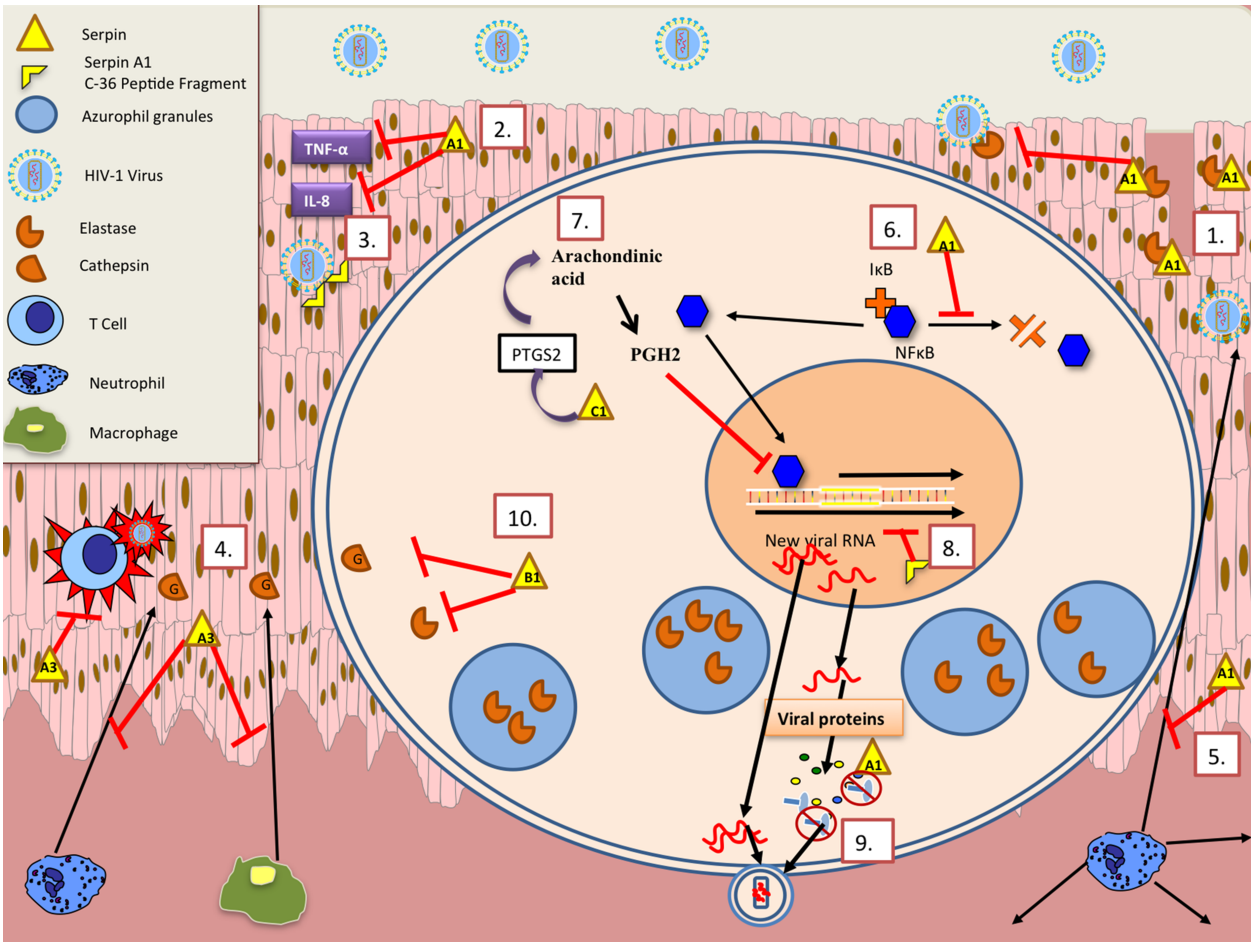


Figure 8: General serpin functions

1. Serpin A1 inhibits elastase, which is a protease that increases HIV infectivity and impairs would repair[317], 2. Serpin A1 inhibits proinflammatory cytokines and chemokines thereby reducing the number of target cells for HIV[315], 3. The C-36 fragment of Serpin A1 inhibits gp120 binding to host cells and thus interferes with virus uptake into cells[314], 4. Serpin A3 inhibits the activity of Cathepsin G, which is a chemoattractant for neutrophils and macrophages[312,313], 5. Serpin A1 inhibits neutrophil migration towards the site of infection[318], 6. Serpin A1 interferes with dissociation of NF-κB from IκB which prevents NF-κB from entering the nucleus where it acts as a transcription factor for HIV-required genes[332], 7. Serpin C1 upregulates prostacyclin expression which causes inhibition of NF-κB-mediated transcription[297], 8. The C-36 terminal fragment of Serpin A1 inhibits HIV-1 LTR-driven transcription[319], 9. Serpin A1 interferes with proper processing of gp160 into gp120 and gp41 and processing of p55 to p24 by HIV protease[315], 10. Serpin B1 protects cells from intracellular proteases released during stress or phagocytosis[314].

This figure was reproduced from Aboud et. al., 2014[316] with permission (Appendix 11.8.2).

1.9.2 Serpin B1

1.9.2.1 Cellular location and target proteins

Serpin B1 was found to be up-regulated in CVL from HESN women within the Pumwani sex worker cohort in Nairobi, Kenya.

Serpin B1 is a 42 kDa antiprotease and has alternative names including Monocyte/Neutrophil Elastase Inhibitor (MNEI) and Leukocyte Elastase Inhibitor (LEI). This protein is encoded on chromosome 6[320] and is most abundant within the cytoplasm of neutrophils, however, it is also present within other myeloid lineage cells including monocytes/macrophages, mast cells, and dendritic cells as well as epithelial cells and NK cells[321-323].

The best characterized function of Serpin B1 is the inhibition of neutrophil serine proteases (NSPs), namely, Cathepsin G, Granzyme H, proteinase-3 and most notably Elastase[324]. Neutrophil Elastase is expressed by neutrophils as well as macrophages and is stored within the azurophilic granules of these cells. Excessive levels of this protease has been implicated in the breakdown of extracellular matrix proteins resulting in emphysema and cystic fibrosis[325]. The mechanism by which Serpin B1 inhibits both elastase-like and chymotrypsin-like proteases relies on two distinct P1 residues within the RCL[326]. Elastase-like proteases including proteinase-3 and Neutrophil Elastase bind the Cys344 within Serpin B1's RCL and chymotrypsin-like proteases including Cathepsin G and mast cell chymase bind the residue before Cys344, at Phe343[324].

1.9.2.2 Biological functions of Serpin B1

Serpin B1 regulates specific serine proteases and is essential in the prevention of numerous disease pathologies. Many of the serine proteases induce cell death of virus-infected or transformed tumor cells and as such their response must be tightly regulated. For instance, if Granzyme H is under-expressed the overall immune responsiveness will be reduced, allowing for

uncontrolled proliferation of tumor cells, thereby resulting in infection and cancer. However, if this protease is overexpressed, cell and tissue damage can result[321].

Aside from its function as an NSP inhibitor, Serpin B1 also interferes with the production of neutrophil extracellular traps (NET), a process referred to collectively as NETosis [327]. NETosis is a programmed cell death pathway that is initiated by mature neutrophils following interactions with pathogens and inflammatory mediators. This pathway acts to sequester pathogens thereby preventing the spread of infection. However, it can also have pathological consequences including inflammatory-induced tissue damage[327]. Although the exact mechanism by which Serpin B1 interferes with this pathway is not clearly defined, it has been reported that Serpin B1 translocates to the nucleus before early stages of NETosis where it appears to have a regulatory role in three separate NET-generating pathways, specifically the PMA (phorbol 12-myristate 13-acetate), MIP (Macrophage Inflammatory Protein) -2, and PAF (Platelet-activating factor) pathways[327]. It has been proposed that Serpin B1's control of Elastase may be the functional link between Serpin B1 and the PMA pathway since Elastase participates in this pathway. However, the exact mechanism of regulation of MIP-2 and PAF pathways remains unclear[327].

Unlike many other serpins, upon cleavage by its target proteases, Serpin B1 develops endonuclease activity when it becomes transformed into LEI-derived DNase II (L-DNase II). This endonculease translocates to the nucleus where it functions to degrade DNA, participating in the caspase-independent apoptosis pathway [328,329]. Within this pathway L-DNase II is the final effector molecule and interacts with other pro-apoptotic proteins including Poly-ADP-Ribose polymerase (PARP) and Apoptosis Inducing Factor (AIF)[328,329]. Together these proteins have a central role in the caspase-dependent pathway. However, BCL-2 and BAX, are both capable of binding to and regulating L-DNase II[329], thereby inhibiting its pro-apoptotic function. L-DNase II has also been identified as protective against apoptosis when in the presence of

etoposide (a cytotoxic anticancer drug) challenge yet pro-apoptotic when in the presence of HMA (Hexamethylene Amiloride) and staurosporine (protein kinase inhibitor), suggesting variable roles for L-DNase II in apoptosis[329].

Alternatively, Serpin B1 has been shown to be associated with protection of neutrophils from apoptosis through regulation of the pro-apoptotic Caspase-3 protease. Caspase-3 is activated by Proteinase-3 and is the primary effector molecule in neutrophil apoptosis and is stored within granules of mature cells, which progressively releases this protein into the cytosol during neutrophil aging. Serpin B1 is also present in the cytosol of neutrophils and counterbalances Proteinase-3 activity, and thus the activity of Caspase-3, resulting in reduced rates of neutrophil killing[330]. Taken together, this data strengthens the argument for a role of Serpin B1 in apoptosis, however, the intricacies of the necessary molecular interactions for L-DNase II-induced apoptosis and Serpin B1 protective functions to occur are far from clearly defined.

1.9.2.3 The role of Serpin B1 in disease pathogenesis

Serpin B1 has been associated with numerous disease pathologies. Excessive inflammatory host responses are common within pulmonary infections characterized by an over-production of neutrophil elastase at the site of infection [331-333]. Elevated levels of Serpin B1 have been strongly associated with reduced pulmonary inflammation, tissue damage and thus improved prognosis in patients suffering from cystic fibrosis, *Pseudomonas aeruginosa,* and influenza infections[331-333]. This protective function is due, primarily, to the role that Serpin B1 has in regulating the function of Neutrophil Elastase, which is a powerful inducer of inflammation and tissue damage.

Overexpression of Serpin B1 has also been associated with suppressed levels of cell invasiveness and reduced migration within lung cancer[334], breast cancer[335,336], hepatocellular carcinoma[335] and Glioma[336]. While the mechanism employed by Serpin B1 within many of these

cancer cells is not understood, within Glioma cells Serpin B1 has been shown to regulate the invasiveness and level of migration of glial cells through continuous suppression of the phosphorylation of focal adhesion kinase (FAK) which is involved in the down-regulated expression of MMP-2[336]. Alternatively, Serpin B1 has also been identified as being over-expressed in invasive oral squamous cell carcinoma and with the promotion of cancer cell motility[337]. It is, therefore, fair to conclude that Serpin B1 likely positively or negatively regulates the expression of specific chemotactic mediators which, depending on the cell type and thus cancer type, determines whether or not the migration of cancer cells is affected.

Serpin B1 has also been associated with decreased pathological manifestations in individuals with Psoriasis[338]. Psoriasis is characterized by the excessive proliferation of keratinocytes and intra-epidermal neutrophil accumulations[339]. Excessive release of proteases from neutrophils results in tissue damage and impaired clearance of apoptotic cells during chronic inflammation[338]. Serpin B1 has not only been identified as a regulator of the neutrophil derived proteases, but also of excessive keratinocyte proliferation[338].

Thus, Serpin B1 has been shown to exhibit immune modulatory activity resulting in reduced inflammation and reduced cellular proliferation. However, within specific tissues Serpin B1 has also induced increased cellular chemotaxis and variable effects on apoptosis. While these functions have been examined in particular cell types (neutrophils, epithelial cells) and within specific disease models (pulmonary-based) none have been studied in the context of HIV acquisition within the FGT.

1.9.3 Cystatins

1.9.3.1 Classification and structure

The cystatin superfamily is made up of three distinct families. The stefins (Stefin A/Cystatin A and Stefin B/Cystatin B) are approximately 11kDa, unglycosylated inhibitors that lack signal sequence disulfide bonds and are largely expressed intracellularly[340]. Family 2

consists of proteins (cystatin C, D, S, SA and SN) with a molecular mass of 13-14 kDa and contain a signal sequence along with disulfide bonds at the carboxyl terminus. The kininogen family (Family 3) consists of proteins that are much larger than the other cystatins with molecular weights of approximately 88-114 kDa. The large size is due to glycosylation and the presence of three cystatin domains two of which contain inhibitory activities[341,342].

Each cystatin has a core made up of five-stranded antiparallel β-sheets around a central α-helix[343]. Among cystatins, three conserved motifs exist which form a wedge-shaped structure that functions to block the active site of C1 cysteine proteases[340]. Interactions between cystatins and cysteine proteases occur through high-affinity non-covalent binding and are therefore reversible. Hence, cystatins do not act as suicide substrates[340].

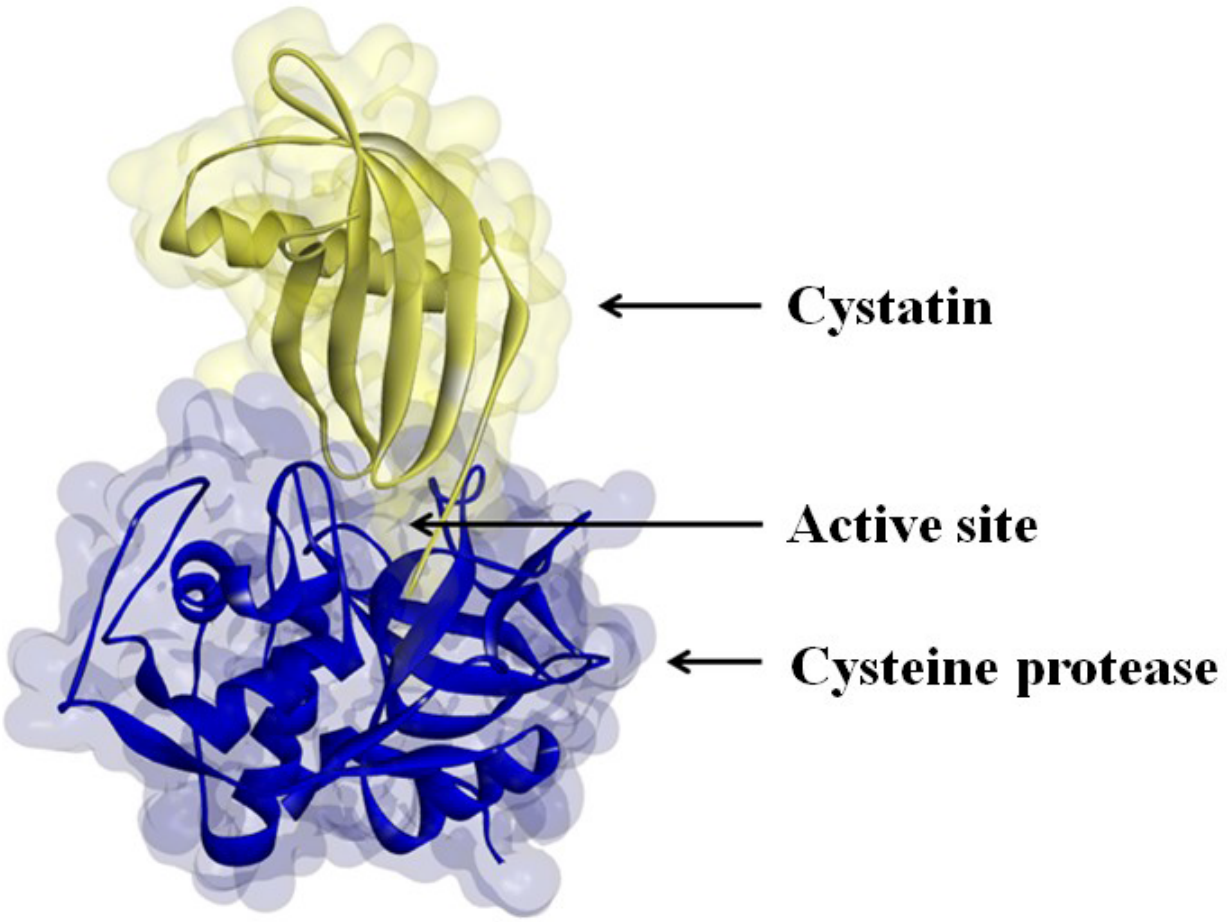


Figure 9: Cystatin inhibition of cysteine proteases
The five core antiparallel β-sheets and one central α-helix that make up Cystatins, function to inhibit cysteine proteases by physically blocking the protease active site through formation of non-covalent, reversible bonds.
This figure was reproduced from Vorster et. al., 2013[344] *with permission (Appendix 11.8.3)*

1.9.3.2. Biological functions

Cystatins regulate the activity of papain-like cysteine proteases and are present in numerous cell types, including epithelial cells, granulocytes, monocytes, macrophages, and DCs. The primary targets of type I cystatins are cathepsins B, L, and S[340,343]. Regulation of these proteases plays a significant role in control of inflammation. The expression of cystatins is up-regulated following activation of TLRs, production of TNF-α, IL-1β, and IFN-β[345], stimulation of monocytes/macrophages by LPS[346] as well as following the activation of transcription factor IRF-8 within macrophages, $CD8^+$ T cells and DCs[345,347].

Cystatins from all three families have also been shown to up-regulate production of nitric oxide (NO) from activated macrophages[348]. This induction of NO is caused by cystatin-induced up-regulation of TNF-α and IL-10, not by the conserved inhibitory domain of the enzyme[348-350]. Overexpression of NO has proven to be lethal to specific pathogens, such as visceral Leishmaniasis[351]. NO induction has also been associated with inhibition of lymphocyte proliferation and modulation of cytokine gene expression[348].

Cystatin C strongly inhibits both Cathepsin S and L. Significant levels of this cystatin have been observed within lysosomes of immature DCs, macrophages and Langerhans cells [352]. Following maturation of DCs, the level of Cystatin C is reduced allowing for up-regulation of Cathepsin S and L[352]. Thus, it has been suggested that Cystatin C assists in the regulation of antigen presentation through the control of Cathepsin S in B cells and DCs. These proteases have known intracellular functions in the generation and presentation of MHC class II molecules[352-354]. Hence, Cystatin C functions to regulate the abundance MHC II molecules on the surface of immature APCs.

1.9.3.3 Potential for control of HIV infection

Both Cystatin A and B demonstrate neutralization capacity against HIV, which may be due to the strong immune-modulatory effects that these proteins have.[355]. Furthermore, cystatins

have a known role in the control of HIV-target cell chemotactic abilities through the regulation of pro-inflammatory proteases at the site of infection and in reducing apoptosis within these cells[356]. While mechanistic studies have not been performed to determine whether there is a direct inhibitory mechanism against HIV, both Cystatin A, and B have been shown to be up-regulated within the CVL of HESN women from the Pumwani commercial sex worker cohort, suggesting a potential role in the protective HESN phenotype[235].

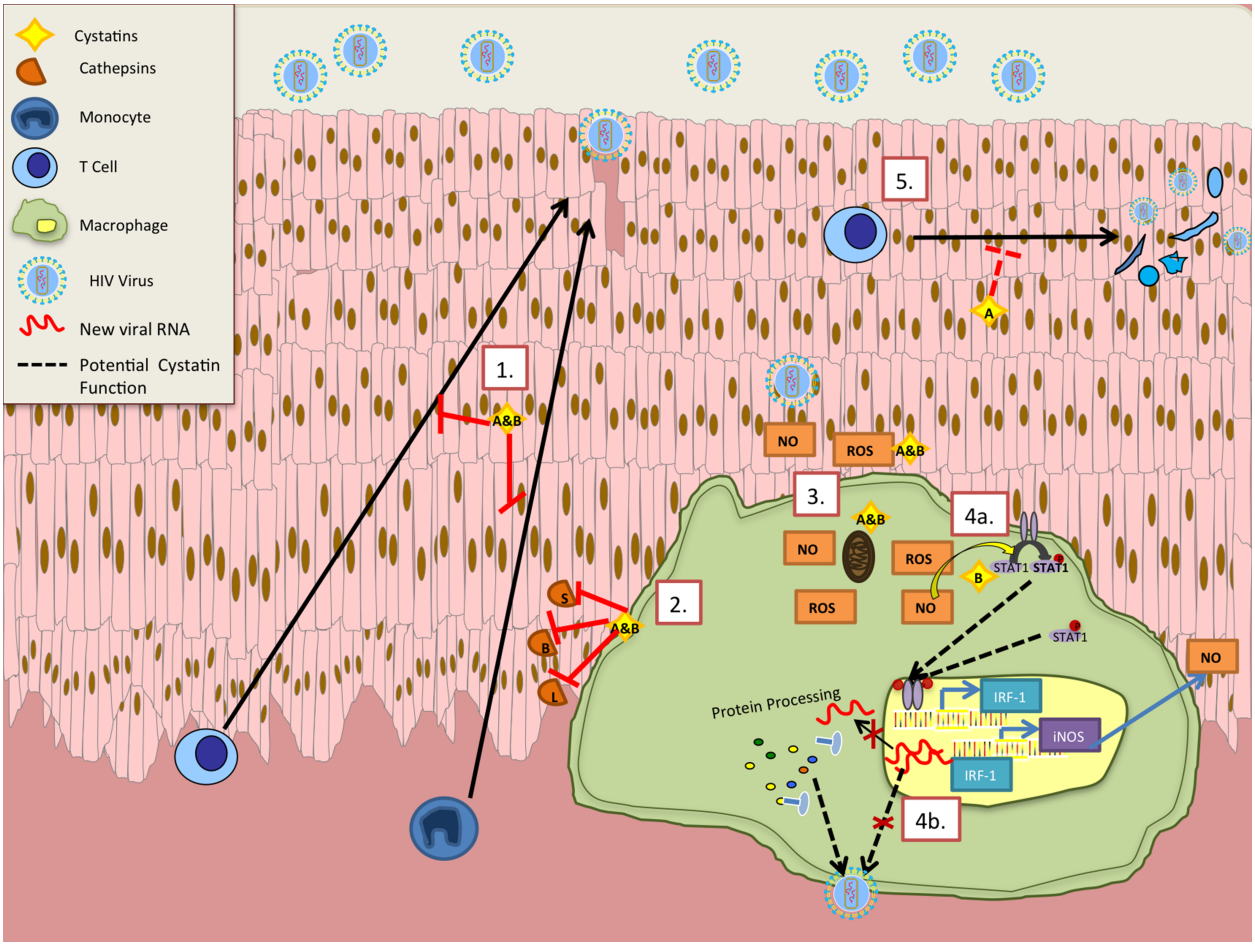


Figure 10: General cystatin functions

1. Cystatin A and B inhibit migration of monocytes and T cells decreasing the number of target cells for HIV[405], 2. Cystatin A and B inhibit pro-inflammatory cathepsins B, L, and S[340,343], 3. Cystatin A and B increase production of reactive oxygen species in macrophages thereby killing infected cells[109], 4a. Cystatin B upregulates NO and is associated with the STAT-1 pathway, 4b. Activation of STAT-1 results in the induction of IRF-1 and decreases HIV-1 replication[348], 5. Cystatin A is anti-apoptotic and reduces programmed cell death[356].

This figure was reproduced from Aboud et. al., 2014[354] with permission (Appendix 11.8.2).

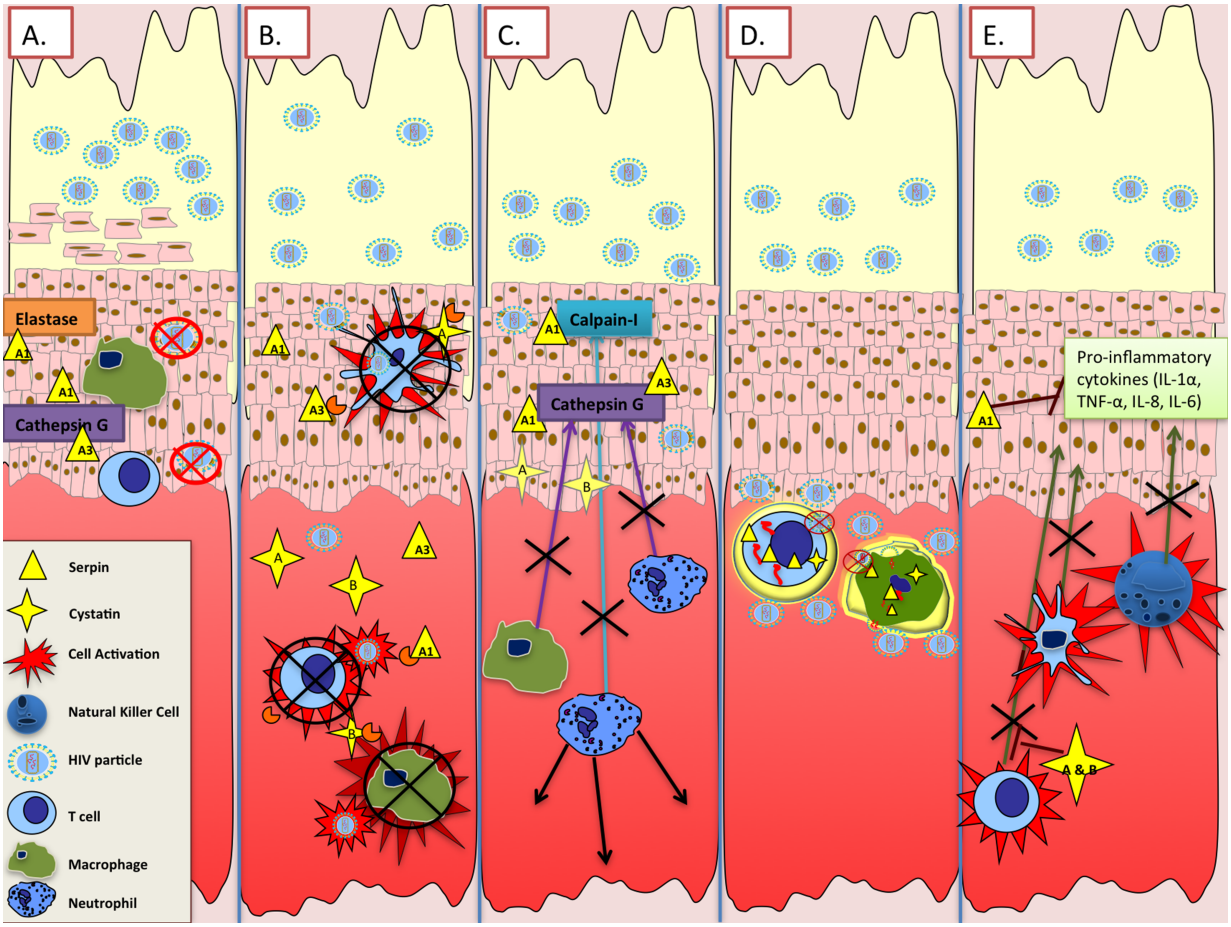


Figure 11: Potential anti-HIV mechanisms for serpins and cystatins
A: Inhibition of HIV gp120 binding to host cells by the C-36 fragment of Serpin A1 would reduce the number infected cells. Also inhibition of proteases that cause cellular activation would decrease the number of activated cell, thereby reducing the efficiency of HIV replication and reducing the number of infected target cells. B: Regulation of protease activity reduces activation of monocytes, T cells, and DCs. C: Inhibition of chemotactic proteases such as Calpain-1 and Cathepsin G will decrease the number of HIV target cells at the site of infection. D: Reducing the level of apoptosis in infected cells that are actively producing virus will reduce the number of virions released. E: Reducing the amount of pro-inflammatory cytokines present at the site of infection will decrease the number of target cells present for HIV.
This figure was reproduced from Aboud et. al., 2014[355] *with permission (Appendix 11.8.2)* .

1.10 Study rationale and global hypothesis

1.10.1 Study Rationale

Women acquiring HIV through heterosexual transmission account for an increasing burden of new annual infections[5]. However, an individual's risk of infection is dependent on numerous host factors, including the composition of cervicovaginal secretions and the integrity of the innate immune response. The cervico-vaginal fluid of women contains numerous pro-viral and anti-viral factors, each of which contributes to the overall susceptibility to HIV. One class of immune modulators up-regulated within the CVL of HESN women of the Pumwani commercial sex worker cohort in Nairobi, Kenya, are antiproteases, specifically members of the serpin and cystatin superfamilies. Only a select few have been studied in the context of HIV infection with many of the up-regulated antiproteases having yet to be examined in this regard.

This project examines the role that physiological concentrations of complete cervico-vaginal fluids, as well as that of individual antiproteases, have on HIV infection. Within the CVL of individual women, the proteins that most affect HIV-1 acquisition were examined. Lastly, the mechanism of action of Serpin B1, which exhibited the highest degree of HIV neutralization, was determined.

1.10.2 Global hypothesis

Antiproteases identified as up-regulated within the CVL of HESN women, exhibit both direct anti-HIV and indirect cellular mechanisms to regulate HIV replication.

“Words may show a man’s wit, but actions his meaning”
~Benjamin Franklin

CHAPTER 2: MATERIALS AND METHODS

2.1 General reagents

2.1.1 Solutions & buffers

Phosphate Buffered Saline (PBS): 48.5g PBS powder (0.138M, 0.0027M KCl, 0.0081M Na_2HPO_4, Sigma Aldrich, Oakville, ON, Canada) dissolved in 1L of ddH_20, pH 7.4.

FACS wash: PBS and 2% sterile filtered fetal bovine serum (FBS, Sigma, Oakville, ON., Canada). FBS was first heat inactivated for 1 hour at 56^oC.

Dulbecco's Modified Eagle Medium (DMEM) 1x: contained 25mM Hepes, 4.5g/L glucose, 4.00mM L-Glutamine (Hyclone GE Healthcare Lifesciences, Utah, USA).

Rosewell Park Memorial Institute (RPMI) -1640 medium: contained 2.05mM L-glutamine (Hyclone GE Healthcare Lifesciences, Utah, USA).

Complete media: RPMI-1640 or DMEM supplemented with 10% heat-inactivated FBS and 1% Penicillin/Streptomycin (Gibco, Burlington, ON, Canada).

Keratinocyte-SFM Media (ATCC, Manassas, VA, USA): contained 0.05mg/mL bovine pituitary extract (BPE), 0.01ng/mL epidermal growth factor (EGF), and 22.05mg/500mL $CaCl_2$. Used in cultures with FGT epithelial cell lines.

PBMC/C8166T/A3R5.7/ACH2 Freezing Media: 10% dimethyl sulfoxide (DMSO, Sigma, Oakville, ON, Canada) and 90% FCS.

TZM-bl Freezing Media: 50% FBS, 40% DMEM, and 10% DMSO.

ELISA wash buffer: PBS, pH 7.4 and 0.05% Tween 20 (Sigma-Aldrich, Oakville, ON, Canada).

ELISA coating buffer: 1.59g Na_2CO_3 (0.015M), and 2.93g (0.035 M) $NaHCO_3$ at pH 9.6 (both reagents were purchased from Sigma Aldrich, Oakville, ON, Canada).

ELISA blocking buffer: PBS pH 7.4, and 1% BSA.

ELISA dilution buffer: PBS pH 7.4, 0.1% BSA, and 0.05% Tween 20.

DEA buffer: 122mg magnesium chloride hexahydrate ($MgCl_2$, Fisher, Ottawa, ON, Canada) was first dissolved in Millipore H_2O, add 117mL Diethanolamine (Fisher, Ottawa, ON, Canada) was added. Adjusted pH to 9.8 using HCl, and enough water was added for a final volume of 1L.

4% Sodium dodecyl sulfate (SDS) Lysis buffer: 50mM HEPES pH 8.8, 100mM DTT, and 4% SDS. The final volume was achieved with autoclave sterilized reverse osmosis (RO) H_2O.

Urea Exchange Buffer: 9.6g Urea (GE Healthcare Lifesciences, Utah, USA), 2mL 0.5M 4-(2-Hydroxyethyl) piperazine-1-ethansulfonic acid (HEPES, Sigma-Aldrich, Oakville, ON, Canada), and enough autoclave sterilized W6-4 H_2O (Fisher, Ottawa, ON, Canada) was added to a final volume of 20mL. Vortexed until all urea was dissolved.

HPLC Buffer A: 20mM ammonium formate (NH_4FA, Fisher, Ottawa, ON, Canada) diluted in W6-4 H_2O (Fisher, Ottawa, ON, Canada). Used to re-suspend peptide fractions before HPLC processing and used within the HPLC runs.

HPLC Buffer B: 90% acetonitrile (C_2H_3N, Fisher, Ottawa, ON, Canada) with 10% ammonium formate (Fisher, Ottawa, ON, Canada). Used in HPLC cycle.

Mass Spectrometry (MS) Buffer A: 2% acetonitrile (C_2H_3N, Fisher, Ottawa, ON, Canada) with 0.1% formic acid (Millipore, Burlington, ON, Canada) diluted in W6-4 H_2O (Fisher, Ottawa, ON, Canada). Used for dilution of samples following HPLC before mass spectrometry analysis. Also used throughout the mass spectrometry runs.

Mass Spectrometry (MS) Buffer B: 98% acetonitrile (Fisher, Ottawa, ON, Canada) with 0.1% formic acid (Millipore, Burlington, ON, Canada). Used for running of samples in the mass spectrometer.

2.1.2 Commercial kits

Neutrophil Elastase Inhibitor Screening Kit (Fluorometric) (BioVision, Milpitas, CA, USA): used in the confirmation of the biological activity of commercially produced Serpin B1.

QiAamp Viral RNA Mini Kit (Qiagen, Toronto, ON, Canada): used for the isolation of mRNA from cell cultures for quantitation of HIV mRNA splice variants

QiAamp DNA Mini Kit (Qiagen, Toronto, ON, Canada): used for the isolation of total DNA from cell cultures for quantification of viral DNA.

Quantitect Reverse Transcription Kit (Qiagen, Toronto, ON, Canada): used in production of DNA from viral mRNA in the quantitation of viral mRNA splice variants

QuantiTect SYBR Green PCR Kit (Qiagen, Toronto, ON, Canada): used in all PCR reactions

Human Cytokine/Chemokine Magnetic Bead Panel (Milliplex Map Kit, HCYTOMAG-60K, Millipore, Burlington, ON, Canada): Analytes included, Fractalkine, GM-CSF, GRO, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17A, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-

1β, RANTES, TNF-α. Used for quantification of cytokine/chemokine levels within Serpin B1 PBMC and epithelial cell line cultures.

Human Cytokine/Chemokine Magnetic Bead Panel III (Milliplex Map Kit, HCYP3MAG-63K, Millipore, Burlington, ON, Canada): Analytes included, CXCL11/I-TAC, CXCL9/MIG, CCL20/MIP-3α. Used for quantification of cytokine/chemokine levels within Serpin B1 PBMC and epithelial cell line cultures.

BCA Protein Assay Kit (Novagen, Etobicoke, ON, Canada): used in the quantification of protein within CVL and supernatant samples, prior to running through liquid chromatography and mass spectrometry to normalize protein concentration across samples.

2D Quant Kit (GE Healthcare, Lifesciences, Utah, USA): used in determining normalizing protein content in samples that contain SDS, prior to liquid chromatography and mass spectrometry analysis.

FluoroProphile Protein Quantification Kit (Sigma, Oakville, ON, Canada): used to normalize peptide quantities following liquid chromatography, prior to mass spectrometry analysis.

Tropix Gal-Screen kit (Applied Biosystems, Burlington, ON, Canada): used to quantify β-galactosidase abundance following TZM-bl neutralization assays

Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA): used in determining the level of cell viability within TZM-bl cell neutralization assays through the detection of ATP levels within each well.

2.1.3 Cell lines

TZM-bl cells: This HeLa human cervical carcinoma adherent cell line was obtained through the NIH AIDS Reagent Program, Germantown, MD, USA, Division of AIDS, NIAID, NIH: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. catalog number 8129.

C8166 $CD4^+$ T cells: This C63/CRII-4 clone was derived from primary umbilical cord blood cells fused with HTLV-1 cells from an adult T cell leukemia lymphoma patient. They fuse to form syncytia in the presence of HIV. These cells were gifted by Dr. Xiaojian Yao's laboratory at the University of Manitoba, Department of Medical Microbiology[357].

A3R5.7 cells: This human T-lymphobastoid suspension cell line, expresses CD4, CXCR4, CCR5, and $\alpha 4\beta 7$. They were obtained through the NIH AIDS Reagent Program, Germantown, MD, USA, Division of AIDS, NIAID, NIH: Catalog number: 12386, from Dr. Robert McLinden.

VK2/E6E7 (ATCC ® CRL-2616, Manassas, VA, USA): This reagent is an adherent vaginal epithelial cell line and was obtained through ATCC. It is an HPV-16 E6/E7 transformed cell line.

End1/E6E7 (ATCC ® CRL-2615, Manassas, VA, USA): This reagent is an adherent endothelial cell line and was obtained through ATCC. It is an HPV-16 E6/E7 transformed cell line.

Ect1/E6E7 (ATCC ® CRL-2614, Manassas, VA, USA): This reagent is an adherent ectocervical cell line and was obtained through ATCC. It is an HPV-16 E6/E7 transformed cell line.

A3.01 cells: This suspension cell line is a derivative of the CEM peripheral T-cell line, and express Leu-3, Leu-8, Leu-1, and transferrin receptor. These cells were gifted by Dr. Xiaojian Yao's laboratory at the University of Manitoba, Department of Medical Microbiology. This cell

line was originally obtained through NIH AIDS Reagent Program, Germantown, MD, USA, Division of AIDS, NIAID, NIH: A3.01 from Dr. Thomas Folks. Catalog number: 166.

ACH-2 cells: These cells are a subclone of the parent cell line, A3.01. This suspension cell line expresses CD5, transferrin receptor, and Leu-1, however lack CD4. They were gifted by Dr. Xiaojian Yao's laboratory at the University of Manitoba, department of Medical Microbiology. This cell line was originally obtained through the NIH AIDS Reagent Program, Germantown, MD, USA, Division of AIDS, NIAID, NIH: ACH-2 from Dr. Thomas Folks, Catalog number: 349

2.1.4 Stimulants

Dynabeads® Human T-activator CD3/CD28 beads (Gibco by Life Technologies, Burlington, ON, Canada): used as a positive control for T cell activation assays

Phytohaemagglutinin (PHA, Sigma, Oakville, ON, Canada): This reagent was diluted in PBS. Used at a final concentration of 5ug/mL for stimulation of PBMCs before infections

Phorbol myristate acetate (PMA, Sigma, Oakville, ON, Canada): used as a positive control for T cell activation assays, and as a stimulant for ACH2 cells to produce infectious HIV particles.

Ionomycin (Sigma, Oakville, ON, Canada): used in conjunction with PMA as a positive control for T cell activation

IL-2 (Hoffman-La Roche, Mississauga, ON, Canada): Used at a concentration of 20IU/mL for stimulation of PBMCs in neutralization assays that were six days or longer.

3'-Azido-3'-deoxythymidine (AZT, A2169-Sigma, Oakville, ON, Canada): Reverse Transcriptase Inhibitor. Used at a concentration of 50mM/mL as a positive control in HIV-1 neutralization assays.

ENF (Bachem, Bubendorf, Switzerland): gp41 fusion inhibitor. Used at a concentration of 50mM/mL as a positive control in HIV-1 neutralization assays.

2.1.5 Virus strains

Bal: R5-tropic (preferentially infects cells expressing the CCR5 co-receptor), clade B, lab strain HIV-1 virus.

IIIB: X4-tropic (preferentially infects cells expressing the CXCR4 co-receptor), clade B, lab strain HIV-1 virus.

HIV_{1956} : Duo-tropic (capable of infecting cells that express either CCR5 or CXCR4 co-receptors), clade A1, human primary isolate, HIV-1 virus isolated from a woman in the Pumwani sex worker cohort in Nairobi, Kenya.

VSV-G-pseudotyped single-cycle-replicating pNL-Bru-ΔBgl/Luc^{+}/R^{-} HIV-1 virus: Lacks HIV-1 nef, vpu, and env and instead contains a VSV envelope, thus is not R5-tropic or X4-tropic as it is taken up through endocytosis rather than through receptor binding. This virus was constructed by, and gifted from Dr. Xiaojian Yao's laboratory at the University of Manitoba, Department of Medical Microbiology

2.1.6 Antiproteases

Serpin A1 (alpha-1-antitrypsin), Serpin A3 (alpha-1-antichymotrypsin), Serpin C1 (antithrombin III): Exogenous proteins were purchased from Athens Research and Technology. The purity of proteins was determined to be >95%.

Serpin B1: Exogenous Serpin B1 was produced by GenScript (Piscataway, NJ, USA) via transient transfection of 293-6E cells with a recombinant plasmid encoding Serpin B1. Purity of protein was determined to be 80-85% by GenScript. Functional analysis of the protein was performed using the Neutrophil Elastase Inhibitor Screening Kit from Biovision according to manufacturer's instructions, and plates were read using BioTek Synergy H1 Hybrid Reader.

Serpin B13: Exogenous protein was produced by GenScript (Piscataway, NJ, USA) via transient transfection of 293-6E cells with a recombinant plasmid encoding Serpin B13. Purity of protein was determined to be ~80% by GenScript.

Cystatin A: Exogenous protein was produced by GenScript (Piscataway, NJ, USA) via transient transfection of 293-6E cells with a recombinant plasmid encoding Cystatin A. Purity of protein was determined to be >85% by GenScript.

Cystatin B: Exogenous protein was produced by GenScript (Piscataway, NJ, USA) via transient transfection of 293-6E cells with a recombinant plasmid encoding Cystatin B. Purity of protein was determined to be >95% by GenScript.

A2ML1: Exogenous protein was produced by GenScript (Piscataway, NJ, USA) via transient transfection of 293-6E cells with a recombinant plasmid encoding A2ML1. Purity of protein was determined to be ~80% by GenScript.

2.2 General methods

2.2.1 Cohorts

2.2.1.1 Pumwani sex worker cohort in Nairobi, Kenya

The Pumwani sex worker cohort was initiated in 1985 in a collaboration between the University of Manitoba and the University of Nairobi[203]. All commercial sex workers that visit the clinic, including those that agree to have biological samples collected for research purposes, are offered free general health care for themselves and their children. These day-to-day services were provided by numerous Kenyan doctors, nurses and laboratory technicians. HIV status for each woman was determined through serology, consisting of Recombigen (Trinity Biotech) ELISA, and confirmed via HIV-1 PCR Detect HIV-1/2 immunoassay (Adaltis), both of which had to test positive for the woman to be considered HIV-infected. Women who were determined to be HIV-negative were classified as either HESN or HIV-susceptible based on the amount of time they had been involved in active, transactional sex work. Those women who had been engaged in sex work for longer than seven years and remained HIV-negative were considered to be HESN while women who were involved in sex work for less than seven years were classified as HIV-susceptible. The HIV-susceptible women have been shown to be exposed to the same HIV strains and to be engaged in similar levels of daily sexual activity as HESN women, however, are expected to seroconvert.

2.2.1.2 Women's health clinic cohort in Winnipeg, Manitoba

Lindsay Aboud of the University of Manitoba established this cohort in collaboration with healthcare professionals at the Health Sciences Centre Women's Clinic in Winnipeg, Manitoba, Canada. All women who were approached for the study were previously scheduled for a pelvic exam and agreed to have a CVL sample obtained. Samples were collected by Dr. Helen Pymar (MD), Dr. Jennifer Chan (MD), or Mary Driedger (nurse practitioner). Participants

were classified into either the non-postpartum, pregnant or postpartum category based on information provided by the healthcare providers and the women.. A woman was considered to be post-partum if she had delivered a baby within the past three months regardless of whether she was currently breastfeeding, however, approximately 80% were identified as breastfeeding at the time of sample collection.

2.2.1.3 Local Winnipeg Donors

Healthy HIV-negative volunteers from either the University of Manitoba or the J.C. Wilt Infectious Disease Research Centre served as blood donors in accordance with the standards required by the University of Manitoba, and the Public Health Agency of Canada Research Ethics Boards.

2.2.2 Ethics

Participants were enrolled from the Pumwani Commercial Sex Worker Cohort in Nairobi, Kenya, the Health Sciences Centre Women's Clinic, or the University of Manitoba/J.C. Wilt Infectious Disease Research Centre, in Winnipeg, Manitoba, Canada. Informed consent was obtained from all participants prior to collection of blood or CVL samples, in compliance with the University of Manitoba, Public Health Agency of Canada, and the University of Nairobi Research Ethics Board standards and requirements (Appendix 11.7). All participants had the right to withdraw from the study at any point up to the publication of research data.

2.2.3 Sample collection & processing

2.2.3.1 Collection & processing of cervicovaginal lavage

Cervicovaginal lavage (CVL) samples were collected during speculum examinations. Following insertion of the speculum, two milliliters of PBS was inserted into the vaginal vault using a sterile syringe. The PBS effectively washed the walls of the vagina as well as the

endocervix. After pooling at the base of the vagina, the PBS was collected using a sterile Pasteur pipette and stored in a 15mL falcon tube on ice. Samples were transferred on ice to the laboratory, centrifuged at 1,000rpm for 10 minutes to remove cellular debris and stored at -80^{o}C. Samples were shipped from Nairobi to the University of Manitoba on dry ice.

2.2.3.2 Collection & processing of blood

Approximately 4 mL of whole blood was collected per heparinized tube through venous-puncture. Human peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy local, Winnipeg donors through the use of gradient centrifugation of Ficoll-Hypaque (Lymphoprep, Cedarlane). Briefly, whole blood was spun at 1500rpm for seven minutes, at which point the serum was removed, approximately seven mL FACS wash was added and mixed with the cells of each tube. This blood/PBS mixture was then carefully layered onto 12mL Ficoll-Hypaque and centrifuged at 1400rpm for 25 minutes, without the brake. The layer of PBMCs was collected at the intercept of the Ficoll and PBS/serum, transferred to a fresh tube and washed with FACs wash at a final volume of 40mL for 10 minutes at 1600rpm. Supernatants were discarded and cells were washed a final time at 1200rpm for 10 minutes with 25mL RPMI. Following final discard of the wash solution, complete media was added to cell pellets, gently resuspended and stored at 37^{o}C in complete RPMI media at a concentration of 2 x 10^{6} cells/mL.

2.2.4 Cell Cultures

2.2.4.1 TZM-bl

TZM-bl cells were stored in liquid nitrogen prior to use in assays. Five million cells were thawed in a 37^{o}C water bath and washed with 10mL DMEM, centrifuged at 1300rpm for eight minutes with 5mL fresh, complete DMEM added. These cells were then transferred to a T25

flask and incubated at 37°C (5% CO_2) until cells had grown to 80-90% confluence, at which point cells were moved to a T75 flask and allowed to grow again to 80-90% confluence before being used in assays. TZM-bl cells were transferred between flasks through first washing with room temperature PBS, followed by addition of TrypLE (Life Technologies, Burlington, ON, Canada) and incubation at 37°C (5% CO_2) for ten minutes. The flask was then manually tapped to loosen the cells followed by addition of DMEM to stop the action of the trypsin within TrypLE. These cells were centrifuged at 1300rpm for eight minutes and the media/TrypLE was discarded before fresh complete media is added to the cells.

2.2.4.2 PBMCs

Following isolation of PBMCs from whole blood they were incubated at 37°C (5% CO_2) for three days at a concentration of 2 x 10^6 cells per mL of media. For all assays (excluding flow cytometry and cytokine/chemokine analysis) IL-2 (20IU/mL) and PHA (5ug/mL) was added to the complete RPMI for the three-day stimulations.

2.2.4.3 C8166 T-cell and A3R5.7 cell lines

Both C8166T and A3R5.7 cells were stored in liquid nitrogen until they were thawed in a 37°C water bath for use in assays. After thawing, these cells were washed with RPMI at 1500rpm for eight minutes followed by discarding of the resulting supernatants. Five million cells were stored in separate T25 flasks overnight in 10mL complete RPMI media, at which point they were transferred to a T75 flask with fresh complete media. C8166T and A3R5.7 cells were allowed to culture at 37°C (5% CO_2) until the media took on an orange colour (approximately 3-5 days) before washing and use in assays.

2.2.4.4 Epithelial cell lines

All three of the FGT epithelial cells lines (VK2, End1, Ect1) were stored in liquid nitrogen prior to use in cultures. These cells were thawed in a 37°C water bath and washed in DMEM at 1300rpm for eight minutes followed by discarding of all supernatants. Five milliliters

of Keratinocyte – SFM media was then added and cells were transferred to a T25 flask until they reached 80-90% confluence. The cells were then removed using TrypLE (as described for TZM-bl cultures) and transferred to a T75 flask in 20mL Keratinocyte – SFM media and incubated at 37^{o}C (5% CO_2) until 80-90% confluence was reached before use in assays.

2.2.5 BCA assay

BCA assays were performed according to manufacturer's instructions. Briefly, standards were created using 2000µg/mL BSA standard to final concentrations of 2000µg/mL, 1000µg/mL, 500µg/mL, 250µg/mL, 125µg/mL, 50µg/mL, 25µg/mL, 5µg/mL, 0µg/mL. Twenty-five µl of each standard was added in duplicate to each plate, while only 12.5µl sample combined with 12.5µl PBS was added to each of the experimental wells. Per well 200µl BCA solution was combined with 4µl cupric solution and mixed well prior to addition to wells. Two-hundred µl of this BCA/cupric solution was added to each well and plates were placed on a plate shaker for 30 seconds at 4^{o}C, followed by incubation at 37^{o}C (5% CO_2) for 30 minutes. Plates were read using BioTek Synergy H1 Hybrid Reader at 562nm and final concentrations were multiplied by two to account for the dilution factor.

2.2.6 Virus production

PBMCs were isolated from 8 x 4mL blood collection tubes per virus, as previously outlined. Following 72 hours incubation at 37^{o}C (5% CO_2) and stimulation with 5ug/mL PHA, cells were centrifuged at 1500rpm for ten minutes and washed two times with 10mL complete media followed by counting via trypan blue staining. Thirty million cells were taken into the enhanced biocontainment Level 2 laboratory in 1mL of complete media. The remaining cells that were isolated were left in complete media (no PHA) until added as feeder cells at later time

points. Two mL of feeder virus, with a minimum $TCID_{50}$ of $5.0x10^6$, was added to each 50mL canonical tube containing $30x10^6$ PBMCs in each, and incubated at 37°C (5% CO_2) for four hours. Following this incubation, the final volume in each tube was brought up to 10mL with complete media and transferred to a T25 flask, and incubated overnight at 37°C (5% CO_2). Cells were then washed with complete media twice and resuspended in 10mL fresh complete media, 5mL of which was placed into each of two T25 flasks and incubated for an additional three days at 37°C (5% CO_2). On day four of this protocol, an additional 20 x 10^6 feeder PBMCs were washed and added to each flask in 10mL complete media and all the contents were then transferred to a T75 flask. On day seven 1mL of supernatants was collected, and 50%-tissue culture infective dose ($TCID_{50}$) calculations were performed on this sample within 24 hours of collection. At this point half of the supernatants were collected, centrifuged at 1500rpm for 10 minutes and frozen at -80°C. The remaining cultures were fed with an additional $20x10^6$ PBMCs in 5mL of complete media and incubated for five days, at which point 1mL was again collected for $TCID_{50}$ analysis while the remaining culture was collected, centrifuged at 1500rpm for 10 minutes, and aliquoted for storage at -80°C.

2.2.7 $TCID_{50}$ titrations

PBMCs were isolated from local donors and stimulated for three days with PHA and IL-2. Cells were brought to a final concentration of 2 x 10^6/mL. In a round-bottom 96-well plate (Costar, Fisher Scientific, Pittsburgh, PA., USA), viral supernatants collected from the virus production protocol were diluted 1:4 by adding 50µl of media to subsequent wells containing 150µl fresh complete media, this was performed a total of eleven times (1:4, 1:16, 1:64, etc.) in replicates of six. In the twelfth column of the plate uninfected media was added as a negative control. Plates were incubated overnight at 37°C (5% CO_2) at which point media was changed to remove initial viral inoculum, followed by addition of 150µl fresh complete media and

incubation for an additional two days. At this point, 100µl supernatant was removed, and 120µl fresh media was added back into each well. On day six of the assay 120µl supernatants were harvested from each well and transferred into a non-sterile round-bottom 96 well plate (Sarstedt, Germany) containing 12µl Triton-X100 (Sigma, Oakville, ON., Canada), effectively inactivating the virus through disruption of the viral envelope. Plates were sealed and frozen at -80^{o}C for 24 hours at which point p24 ELISA analysis was performed. The $TCID_{50}$ was calculated using the 50% endpoint dilution which determines the concentration of infectious viral particles present in each supernatant using the algorithm presented by Reed and Muench[358].

2.2.8 p24 ELISA

ELISAs were developed by our laboratory and included the use of a primary, coating antibody, cultured from an HIV-1 p24 Hybridoma (183-H12-5C) obtained through the NIH AIDS Reagent Program Division of AIDS, NIAID, from Dr. Bruce Chesebro. Plates were coated with this primary antibody at 4µg/mL in 100µl/well for 16 hours followed by blocking of secondary binding through the use of a blocking buffer for two hours at 37^{o}C (5% CO_2) before addition of standards and supernatants. Standard recombinant proteins, diluted in dilution buffer, from Abcam (ab43037, Cambridge, MA., USA) were used for the creation of a standard curve ranging from 200ng/mL – 0.05ng/mL. One hundred µl of each standard was added in duplicate to each plate along with 100µl of each sample supernatant followed by incubation at 37^{o}C (5% CO_2) overnight. The biotinylated, secondary detection antibody, (Abcam ab20774, Cambridge, MA., USA), in dilution buffer, was added at a final concentration of 0.75µg/mL in 100µl to each well for one hour following washing of plates with wash buffer. Streptavidin-Alkaline Phosphatase (SAAP, Cedarlane, Burlington, ON., Canada), was diluted 1:13,200 in dilution buffer, 100µl of which was then added to each well for an additional hour before addition of the

substrate, in DEA buffer (2 substrate tablets per 10mL DEA, Sigma, Oakville, ON., Canada). Plates were read using BioTek Synergy H1 Hybrid Reader following 15, 30 and 45 minutes of incubation, in the dark, at room temperature.

2.3 Specific methods

2.3.1. Confirming biological activity of commercially produced Serpin B1

Neutrophil elastase was reconstituted into 220µl of assay buffer. Each well (save for the wells designated as the negative control and blanks) of a 96-well, round-bottomed plate had 48µl of assay buffer added to it along with 2µl Neutrophil elastase solution. Serpin B1 was serially diluted 1:2 (50µg/mL-0.78µg/mL) in assay buffer and 1µl of 3mM MeOSuc-Ala-Ala-Pro-Val-chloromethylketone (SPCK, inhibitor control) was diluted in 24µl of assay buffer. The SPCK mixture was incubated for 5 minutes at 37^{o}C (5% CO_2). Serpin B1 dilutions and SPCK solution were added to the appropriate wells in duplicate and the plate was read immediately using the fluorescence setting of the BioTek Synergy H1 Hybrid Reader at an excitation/emission of 400/505nm and a set temperature of 37^{o}C. Readings were obtained every five minutes until saturation point was achieved (approximately 25 minutes).

2.3.2 HIV neutralization assays

2.3.2.1 TZM-bl, PBMC, C8166 T cells

Sixteen hours before infection of cells, 10,000 TZM-bl cells and 50,000 PBMCs or C8166 T cells were seeded in 96 well plates in appropriate media. Final concentrations of Serpin B1 (50µg/mL), BSA (50µg/mL, protein control), CVL (50µg/mL), azidothymidine (AZT, 50mM, positive reverse transcriptase inhibitor control), and Enfuvirtide (ENF, 50mM, positive binding/fusion inhibitor control) (Bachem, Torrance, CA., USA) were achieved through dilution

in phosphate buffered saline (PBS) prior to addition to culture wells. To the appropriate wells, 50μl of Serpin B1 or BSA was added to experimental wells, 50μl of 50mM AZT or ENF were added to positive control wells, and 50μl of PBS was added to negative control wells, immediately followed by addition of virus. HIV-1 Bal, HIV_{1956} , and HIV-1 IIIB were used at 0.2 MOI for PBMCs, and C8166 T cell line, however, IIIB was added at an MOI of 1.0 to TZM-bl cells due to the low level of CXCL4 co-receptors expressed on this cell line. Cells were incubated for three hours at 37°C (5% CO_2), at which point the cells were washed with PBS 2x and fresh complete media was added along with 50ul of SB1/BSA/ENF/AZT/PBS for a final volume of 200μl. Three days post infection (D.P.I.) β-galactosidase activity was determined in the TZM-bl assay using the Gal-Screen system (Applied Biosystems, Burlington, ON., Canada) according to manufacturer's instructions, with the following amendments: 25μl of β-gal buffer/substrate was diluted 1:1 with PBS prior to addition to each well, which, once added to plates was left to incubate at room temperature for one hour. TZM-bl cells were transferred to white opaque Greiner CELLSTAR® 96-well, polystyrene, flat-bottom plates (Sigma-Aldrich, Oakville, ON., Canada) immediately before reading on the BioTek Synergy H1 Hybrid Reader. PBMCS and C8166 T cell supernatants were transferred into non-sterile, round-bottom, 96 well plates (Sarstedt, Germany). Addition of 10% Triton-X100 (Sigma, Oakville, ON., Canada), to each well ensured inactivation of virus before removal from the enhanced laboratory. PBMC and C8166 T cell culture supernatants were analyzed for p24 activity via ELISA. At a minimum, each assay was performed with three technical replicates at three separate time points.

2.3.2.2 C8166 T cell Infection with VSV-G-pseudotyped HIV-1

C8166 T cells were seeded at 200,000 cells per well in a round-bottom 96-well plate. Cells were treated with serially diluted concentrations (1:2 with highest concentration of 50μg/mL) of Serpin B1, AZT (50mM), ENF (50mM) or PBS and infected at an MOI of 0.2 with

a VSV (vesicular stomatitis virus)-G-pseudotyped single-cycle-replicating pNL-Bru-ΔBgl/Luc$^+$/R$^-$ virus, which was produced in 293T cells as described previously[357]. C8166 T cells were cultured in complete RPMI-1640 medium at 37^{o}C (5% CO_2). Three D.P.I. cells were washed with PBS and lysed with 1X lysis buffer (provided in luciferase kit), at which point luciferase activity was detected using Dual-Glo® Luciferase Assay System (Fisher Scientific, Burlington, ON., Canada) according to manufacturer's instructions. Plates were read using Biotek Synergy H1 Hybrid Reader. This assay was performed three times with three technical replicates per experiment.

2.3.2.3 ACH2 cell assays

ACH2 cells were seeded at 500,000 into 12-well plates, in complete RPMI. Serpin B1 was added to cells at a concentration of 50 μg/mL. PBS was added in place of Serpin B1, to negative control wells. Virus production was induced from the latent provirus through the addition of 5ng phorbol myristate acetate (PMA) to experimental wells containing Serpin B1 as well as to virus control wells. Cells were cultured at 37^{o}C (5% CO_2) in a final volume of 1mL for 24, 48 or 72 hours. At each time point, supernatants were collected and centrifuged at 1500rpm to remove cells, and frozen at -80^{o}C until p24 ELISAs were performed as previously described. This assay was performed three times with three technical replicates per experiment.

2.3.3 Cytotoxicity assays

2.3.3.1 Cell-glo luminescent assays

All reagents (CellTiter-Glo® Substrate, CellTiter-Glo® Buffer) from the commercial kit were brought to room temperature and mixed before addition to cells. Fifty μl of the mixed solution was added to each well and plates were placed on a shaker for two minutes before incubation at room temperature for 10 minutes. Immediately prior to reading on the luminescence setting of the Biotek Synergy H1 Hybrid Reader, reagents were transferred to

Greiner CELLSTAR® 96-well, white, polystyrene, flat-bottom plates (Sigma-Aldrich, Oakville, ON., Canada).

2.3.3.2 Trypan blue staining

Cell suspensions were mixed thoroughly via vortexing, 30µl was then removed and added to a fresh well. Thirty microliters of trypan blue (ThermoFisher Scientific, Burlington, ON., Canada) was added and mixed with the 30µl cell suspension. This staining/cell solution was applied to a hemocytometer and cells were counted. Dead cells stained blue, while live cells were colourless. Viability was determined by dividing the total number of live cells by the total number of cells (live + dead).

2.3.3.3 Flow cytometric live/dead staining

One million PBMCs or ACH2 cells were spun in 5mL polypropylene tubes at 1500rpm for 10 minutes, and supernatants were discarded. Cells were washed twice more with PBS prior to staining. LIVE/DEAD Fixable Read Dead Cell Stain (for 488nm excitation, Life Technologies, Burlington, ON., Canada) was diluted 1:100 in PBS prior to addition to cells. 6.5µl of the diluted staining solution was added to each tube and incubated for 30 minutes at 4^{o}C in the dark. Following incubation, cells were washed two times with FACS wash at 1500rpm for 10 minutes. Two-hundred µl FACS wash and 100µl PFA(1%) was added to each tube prior to analysis on the LSR II flow cytometer. Compensation was accomplished for Live/Dead staining through the use of Arc™ reactive and negative beads (BD, Franklin Lakes, NJ., USA) and consisted of the addition of one drop reactive beads to a fresh 5mL polypropylene tube with 6.5µl (1:100) Live/Dead stain. This solution was incubated at 4^{o}C for 25 minutes and washed twice with FACS wash at 1600rpm for five minutes followed by addition of 300µl fresh FACS wash. One drop of negative Arc™ beads was added immediately prior to reading on the LSR II (BD Biosciences).

2.3.3.4 Statistical analysis

All neutralization assays, and viability assays were analyzed using Microsoft Excel and Prism 6 for Mac OS X. Microsoft Excel was employed for the initial conversion of raw data into fold change or $_{LOG}2$ fold change which was then entered into Prism 6. Prior to calculating fold changes, background cell control was used to normalize all data. Prism 6 column statistics (D'Agostino and Pearson Omnibus K2 Normality Test) was used to confirm Gaussian distribution of data prior to running individual unpaired, student t-tests between the experimental samples and the negative virus control (neutralization) or cell culture (cell viability). These t-tests were Welch-corrected for unequal SDs, and an alpha of 0.05 was applied. Longitudinal analysis of CVL samples were analysed using one-way Anovas rather than multiple t-test so as to maintain a 5% chance of type I statistical error. VSV-G-pseudotyped HIV infections were analyzed using Mann-Whitney U-tests since it was determined that this dataset did not follow Gaussian distribution.

2.3.4 Protein digestion and mass spectrometry

2.3.4.1 Protein digestion

CVL samples were thawed on ice, and total protein concentration was determined using a Bicinchoninic acid (BCA) assay according to manufacturer's instructions and as previously described in section 2.2.5. Alternatively, PBMCs treated with Serpin B1, BSA or PBS were washed three times with PBS and lysed in 100µl 4% SDS lysis buffer. Cells were heated at 95^{o}C for five minutes and applied to a Qiashredder (Qiagen, Toronto, ON., Canada). The tube in which the sample was in was washed with an additional 100µl SDS buffer and added to the shredder, which was centrifuged for two minutes at 15,000rpm. Proteins were quantified using a 2D Quant kit, a method capable of quantitating proteins that have been subjected to SDS treatment during processing, according to manufacturer's instructions. All cell lysates were

frozen at -80°C until processed for mass spectrometry. From all samples, including CVL and PBMC cell lysates, 100μg of protein was digested and analyzed via label-free mass spectrometry according to the methods described previously[125]. Briefly, 10kDa cartridges were employed for digestion of proteins. Running of W6-4 H_2O (Fisher, Burlington, ON., Canada), and urea exchange buffer separately, and centrifuging at 10,000xg for three minutes, equilibrated these cartridges. Protein samples were then centrifuged at 10,000xg for five minutes until all sample had passed through the cartridge. Cartridges were washed three times with urea exchange buffer prior to the addition of 100μl 25mM DL-Dithiothreitol (DTT, Sigma-Aldrich, Oakville, ON. Canada) to each cartridge, and incubation at room temperature for 20 minutes. Tubes were centrifuged at 15,000rpm for 3 minutes to remove DTT, and 100μl 50mM Iodocetamide (IAA, Sigma, Oakville ON., Canada) was added to each sample, which was incubated at room temperature, in the dark for 20 minutes. Cartridges were then washed twice with urea exchange buffer and twice with 50mM HEPES solution. Trypsin, diluted in HEPES, was added to each cartridge and incubated at 37°C (5% CO_2) overnight in a canonical tube containing W6-4 H_2O (Fisher, Burlington, ON., Canada). Samples were transferred into clean tubes, and cartridges were washed three times with 50mM HEPES solution. Samples were dried using the Centrivap centrifuge cold trap. All PBMC cell lysates had their peptides quantified using the FluoroProphile Protein Quantification Kit (Sigma, Oakville, ON., Canada) according to manufacturer's instructions so as to ensure normalized protein concentrations prior to mass spectrometry analysis.

2.3.4.2 High-Performance Liquid Chromatography (HPLC)

All runs performed on the HPLC, and mass spectrometer were conducted by the J.C. Wilt Infectious Disease Research Centre Mass Spectrometry core (Winnipeg, Canada). All peptide fractions were resuspended in HPLC buffer A and approximately 1.5μg from each sample was

injected into the C18, 3.5µm pre-column via the Agilent Technologies 1200 Infinity series LC system. Through the use of a 105-minute gradient at 150µl/minute, the peptides were resolved using a 100mm analytical column (XBridge: 3.5µm, 2.1 x 100mm, Waters). Specifically for the first seven minutes, 99% HPLC Buffer A and 1% HPLC Buffer B were run through the column. Over the next 66 minutes, the proportions of buffers adjusted to 40% Buffer A and 60% Buffer B. Following 73.10 minutes, at which point the peptide was primarily washed from the column, 100% Buffer B was run through the column which was sustained until 78.0 minutes. The final 27 minutes had 99% Buffer A and 1% Buffer B run through to clean out the column.

2.3.4.3 LTQ Orbitrap XL label-free mass spectrometry

Mass spectrometry analysis was performed on each peptide fraction using a nano-flow nLC I connected inline to an LTQ Orbitrap XL mass spectrometer, containing a nano electronspray ion source at 2.1 kV (Thermo Fisher Scientific, Burlington, ON., Canada). A total of 5µl (0.1µg/µl) was loaded onto a 3cm long, 100µm diameter, C_{18}-reversed phase trap column (5µm particles) with 30µl of 100% MS Buffer A at 5µl/minute. The fractions were then separated on a 15cm long, 75µm diameter, C_{18}-reversed phase column (2.4 µm particles). A 60-minute linear gradient consisting of 2-35% MS Buffer B, at a flow rate of 250nl/minute was employed to elute the peptides.

This spectrometer was set to a data dependent acquisition mode, which chose the top five abundant precursor ions from each scan for isolation in the LTQ (2.0 *m/z* isolation width) and fragmentation by Collision-induced dissociation (CID) (35% normalized collision energy, with 30 ms activation time). Within the Orbitrap, scans were acquired over m/z 300-1700 (target resolution was 60,000 at an m/z of 400) and resulting fragment ion scans were obtained within the LTQ ion trap. Exclusion criteria were as follows: a list size of 500 features, an m/z tolerance of 15ppm, repeat count of 1, repeat duration of 30 seconds and exclusion period of 60 seconds.

2.3.4.4 Data analysis

All raw data files obtained from the Orbitrap XL mass spectrometer (Thermo Fisher, Burlington, ON., Canada), were processed using Progenesis LC-MS software (Nonlinear Dynamics, New Castle, UK). Feature detection, normalization, and quantification were all performed using default settings from the software. Retention time alignment was performed using peptide mixes that consisted of a pool of all samples included in the study. Each mix was injected between every ten samples throughout the experiment's run time on the mass spectrometer. Alignment was manually reviewed for correctness. Only charge states between 2+ and 7+ were included to exclude contaminations from the analysis. All features detected before 10 minutes and during the last 15 minutes in retention time were discarded. Database searching was performed with Mascot Daemon (v2.4 Matrix Science, London, UK). These mascot-generated files were compared against the UniProtKB/SwissProt database (2013) restricting the taxonomy to Human and Bacteria only. A decoy database was also searched to determine the false discovery rate of peptides identified. The search parameters were the following: carbamidomethylation was tolerated as a fixed modification, oxidation was tolerated as a variable modification, MS/MS tolerance of 0.5Da, monoisotopic precursor (MS) tolerance of 10 ppm, and enzyme specificity was set to trypsin with a maximum tolerance of one missed cleavage. Scaffold (Proteome Software, OR, USA) v4.0 was then employed to determine the confidence in which peptides and proteins were identified. Within Scaffold, the following parameters were set; minimum peptide probability 80%, minimum protein probability 95%, and a minimum of two unique peptides identified per protein; resulting in a 0.1% protein and 1.5% peptide false discovery rate. The scaffold data was then imported back into Progenesis LC-MS software to perform label-free differential protein expression analysis based on MS peak intensities. Protein abundances were normalized to the total ion current. Every protein that was identified via mass spectrometry was annotated by function through the UniProtKB database

(www.uniprot.com). Average abundance of each protein was determined from all samples analyzed and each sample's protein concentration was then compared to this average, resulting in relative expressions, which were then converted into $_{LOG}2$ relative expressions. Data was analyzed by column statistics using D'Agostino and Pearson Omnibus NK test for normality to ensure Gaussian distribution of relative expessions prior to performing unpaired (data in adjacent rows are not matched), two-tailed, student t-tests between data sets.

2.3.4.4.1 Protein differential expression analysis

Percent covariance (CV), which detects the amount of experimental technical error, was set to 0.25, thus, all proteins falling above this were discarded. Percent covariance is determined by obtaining the standard deviation (SD) in individual protein concentrations across the control mixes (performed for each mass spectrometry run) and dividing by the mean concentration of each protein across the control mixes. $_{LOG}2$ relative expressions were determined through comparison of each sample's individual protein abundance to the average abundance of each protein across all samples. These $_{LOG}2$ relative expressions were compared between cohorts using multiple t-tests, without assuming consistent SD and with an alpha of 0.05. Student t-tests or non-parametric Pearson correlations were performed using these $_{LOG}2$ relative expressions followed by Benjamini Hochberg analysis for multiple comparisons, with a false discovery rate of 5% compared to the more stringent Bonferroni and Holm-Sidak methods commonly used. However, many of the studies were underpowered and thus did not perform well following multiple comparison analysis. For this reason, p value frequency distributions were examined for each analysis to ensure that data followed a non-uniform pattern, suggesting results based on true effects rather than data caused by random chance thereby proving null hypotheses correct. In order to avoid type II statistical error all protein differential expression analyzes were performed using an alpha of 0.05 rather than the more stringent q value of 0.0002 which was determined following multiple comparison analysis. P values for each t-test were log

transformed and plotted against the difference between $_{LOG}2$ relative expression values to create a volcano plot.

2.3.4.4.2 Cluster analysis

Cluster analysis was performed using Gene Cluster 3.0. The program conducted uncentered correlations, and complete linkage clustering was depicted. These analyzes were completed using $_{LOG}2$ relative expression values for the proteins within individual samples compared to the average abundance of each protein in all samples analyzed. Clusters were displayed using the Java Treeview software program.

2.3.4.4.3 Correlations

Two-tailed Pearson correlations, with 95% confidence intervals were performed to determine correlations between the abundance of antiproteases within the CVL from women of both the Pumwani and the Winnipeg cohorts, and the HIV-1-neutralization capacity of these women's CVL samples. Identical correlations were performed when determining which proteins were positively or negatively correlated with Serpin B1 expression within the CVL of these women. Proteins that were significantly correlated with Serpin B1 were determined through the use of a two-tailed, independent t-test (α=0.05 corrected for multiple comparisons using the Benjamini-Hochberg method). All statistical analysis was performed using Graph Pad Prism 6.0.

2.3.5 Real-time polymerase chain reaction (RT-PCR)

2.3.5.1 Amplification of HIV-1 DNA

One million C8166 T cells, or A3R5.7 cells were cultured with either 50ug/mL Serpin B1, 50uM AZT or ENF, or PBS with either HIV-1 IIIB (C8166 T cells) or HIV-1 Bal (A3R5.7), both at an MOI of 0.2. Cells were incubated with virus for three hours at 37^{o}C before being washed with PBS two times and fresh, complete RPMI was added back. Cells were cultured for

an additional 5, 12 and 24 hours. DNA was extracted and purified from cell cultures using QIAamp DNA mini kit (Qiagen, Toronto, ON., Canada). The quantity of DNA was determined using Nanodrop technology and 200ng of DNA was used in each subsequent PCR reaction. Primers were designed according to Yamamoto et al[359] (Appendix 11.3, Table 14), and synthesized by the Genomic Core at the National Microbiology Laboratory in Winnipeg, Canada. All of the primers were diluted to 10μM concentration and 1ul of each forward, and reverse primer was added to the appropriate reaction tubes. Ten microliters of Sybr Green (Quanti Tect SYBR Green PCR Kit from Qiagen) was added to each tube along with enough DNAse/RNase-free water to reach a final volume of 20μl. Real-Time PCR settings were determined according to previously described protocols[359,360], and were performed using Light Cycler 96 according to the following conditions: denature 1 cycle at 95^{o}C for 15 minutes; amplification for 50 cycles at 94^{o}C for 15 seconds, 58^{o}C for 1 minute, and 72^{o}C for 30 seconds; 1 cycle melting at 95^{o}C for 5 seconds, 65^{o}C for 1 minute, and 97^{o}C continuous; cooling 1 cycle with a target temperature of 40^{o}C for 10 seconds. The ratio of gene expression change (R) was calculated with 18S (Life Technologies, Burlington, ON., Canada), and GAPDH as normalizing controls, and uninfected cells as the negative control.

2.3.5.2 Amplification of HIV mRNA splice variants

ACH2 culture supernatants from the 48-hour Serpin B1 treatment assay were collected and stored for use in determining the effect of Serpin B1 on HIV RNA splice variants. PBMCs were isolated from healthy, local donors as previously described. Five-hundred thousand PBMCs were seeded into 5mL polypropylene tubes and treated with 50μg/mL Serpin B1, 50μM ENF or PBS. HIV_{1956} virus was added at an M.O.I. of 0.2. Cells were incubated for 3 hours at 37^{o}C (5% CO_2) prior to washing with PBS two times and the addition of fresh complete RPMI. Cells were left to incubate for 48 hours at which point viral RNA was isolated using QIAmp

UltraSens Virus Kit (Qiagen, Toronto, ON., Canada) according to manufacturer's instructions. RNA was quantified using Nanodrop technology and cDNA was synthesized from 500ng RNA using QuantiTect Reverse Transcription Kit (Qiagen, Toronto, ON., Canada), and an Applied Biosystems Veriti 96 well Thermal Cycler. Each rt-PCR reaction well contained 1μl cDNA, 10μl Syber Green (Qiagen, Toronto, ON., Canada), 7μl RNase/DNase free water and 1μl of both the forward and reverse primers (10μM stock concentrations). All primers used in these studies were designed according to Kauchic et al.[83](Appendix 11.3, Table 15) and synthesized by the Genomic Core of the National Microbiology Laboratory (Winnipeg, Canada). Reaction protocols were adapted from previously described protocols[38,83] and were run on the Light Cycler 96. The amplification cycle used for 18S, Gag and single-spliced mRNA was: 50 cycles at 94^{o}C for 15 seconds, 58^{o}C for 60 seconds, and 72^{o}C for 30 seconds. Unspliced mRNA conditions were: 50 cycles at 95^{o}C for 15 seconds, 60^{o}C for 15 seconds, and 72^{o}C for 15 seconds. Multi-spliced mRNA conditions were: 50 cycles at 94^{o}C for 15 seconds, 55^{o}C for 60 seconds, and 72^{o}C for 30 seconds. Relative quantification was performed with 18S as the housekeeping gene. The ratio of gene expression change (R) was determined. Gag primers were employed to determine the overall level of mRNA within each sample.

2.3.5.3 Statistical analysis

Data files were exported from the Light Cycler 96 software into Microsoft Excel where relative abundance, represented as ratio of gene expression change (R), was determined for experimental wells containing the primers of interest, compared to wells treated in the same manner yet containing 18S primers as an rRNA control. This was accomplished by retrieving the C_q value for each reaction (containing experimental primers or 18S primers) from the Light Cycler 96 software. The mean C_q was determined for duplicate samples and differences in C_q values for the gene of interest (experimental splice variant, or HIV DNA primers) compared to

the endogenous control (18S primers) were calculated (ΔC_q). The control ΔC_q (virus or cell control) was then subtracted from the experimental/treated ΔC_q to yield the $\Delta\Delta C_q$ for each reaction, and thus, it was this value that was entered into the equation $R=2^{-\Delta\Delta Cq}$. R is, therefore, equal to the corrected number of cycles to reach the threshold, 2 is used as the base of this equation since the assumption is that the PCR product will double in each cycle. For ACH2 cell cultures, the R value depicted represents that ratio of Serpin B1 treated ACH2 cells that had been stimulated with PMA compared to the PMA treated ACH2 cells that had not been treated with Serpin B1. For the PBMC cultures and C8166 T/A3R5.7 cell cultures, the R value represents the ratio of gene present within Serpin B1, or BSA treated, infected cells compared to untreated, infected cells. These R values were tested for Gaussian distribution using D'Agostino and Pearson Omnibus K2 Normality Test and experimental results were compared to the controls by either student t-tests (ACH2 mRNA experiment) or by Mann Whitney U-tests (PBMC mRNA splice variants and C8166 T cell /A3R5.7 DNA quantitation).

2.3.6 Flow cytometry

2.3.6.1 Surface staining

Following culturing, cells were washed twice with PBS before staining. Supernatants were discarded, and 1µl Human TruStain FcX (Biolegend, San Diego, CA., USA) was added to each tube, and incubated for ten minutes at room temperature. A master mix of all surface antibodies, as well as the Live/Dead stain, was created and added to each tube. Cells were incubated for 30 minutes at 4^{o}C in the dark, followed by washing with FACS wash. Lastly, 200µl FACS wash and 100µl PFA (1%) was added to each tube prior to data acquisition on the BD LSRII flow cytometer and using BD FACS Diva. Fifty-thousand lymphocyte events were collected for each sample. FlowJo version 10.0.8 was employed to analyze data.

2.3.6.2 Intracellular p24 staining

PBMCs from three Winnipeg donors were infected with HIV_{1956} for three hours, and incubated with either Serpin B1 (50μg/mL), BSA (50μg/mL), or PBS for five days. Cells were then stained for surface markers and washed with FACS wash. Supernatants were discarded, and 150μl FIX-PERM solution (BD Bioscience, San Jose, CA., USA) was added. Cells were incubated for 20 minutes in the dark at 4°C and washed with 3mL of PERM WASH (1x) buffer (BD Bioscience, San Jose, CA., USA). Tubes were centrifuged at 1600rpm for 10 minutes and supernatants discarded. Intracellular KC-57 antibody (FITC, Beckman Coulter, Mississauga, ON., Canada), which detects p24 core antigen, was added to cells and incubated for 30 minutes in the dark at 4°C followed by washing with 3mL PERM-WASH 1x buffer as well as with FACS wash. Cells were then resuspended in 300ul FACS wash and analyzed in the same manner as cells that were stained for surface markers.

2.3.6.3 Compensation

Negative anti-mouse or anti-rat (depending on the antibody specificity) CompBeads (BD, Franklin Lakes, NJ., USA) and Reactive CompBeads (BD, Franklin Lakes, NJ., USA) were mixed together and diluted in FACS wash and added to empty tubes. Each antibody (1μl) to be used in the staining protocol was added separately to one of the tubes containing negative compensation beads. Tubes were vortexed briefly and incubated in the dark at 4°C for 20 minutes. Following incubation 300μl FACS wash was added to each tube and used for compensation set-up on the BD LSRII flow cytometer.

2.3.6.4 eFluor proliferation staining

PBMCs from six separate healthy local donors were isolated and stimulated as previously outlined. Unstimulated cells were used in parallel to those that were stimulated. Also, both ACH2 and A3.01 cells were analyzed for proliferation following treatment with Serpin B1.

Cells were seeded at 5.0 x 10^5 per 5mL polypropylene tube, followed by staining with 5μM Cell Proliferation Dye efluor670 (Bioscience, San Jose, CA., USA) according to manufacturer's instructions. Briefly, cells were washed twice with PBS and supernatants were discarded before the addition of 5μM cell proliferation dye (diluted in PBS), while vortexing cells. Tubes were incubated for ten minutes at 37°C (5% CO_2) in the dark followed by incubation on ice for five minutes while covered in foil. Two milliliters of cold complete RPMI was added to each tube to stop the staining reaction and cells were washed three times with complete media prior to culturing with Serpin B1. Cells were grown in complete RPMI with 50μg/mL Serpin B1, 50ug/mL BSA as a protein control, or PBS, for six days. Cells were then stained for 20 minutes at 4°C for surface markers, namely PBMCs were stained with CD3 (V500, BD, Franklin Lakes, NJ., USA), CD4 (BB515, BD, Franklin Lakes, NJ., USA) and CD8 (APC/H7, BD, Franklin Lakes, NJ., USA). ACH2 cells were stained for CD5 (APC-Cy7, Biolegend, San Diego, CA., USA), and CD71 (PE/Cy7, Biolegend, San Diego, CA., USA). A3.01 cells were stained with CD71 (PE/Cy7, Biolegend, San Diego, CA., USA). All cells were then re-suspensed in 1% paraformaldehyde solution.

2.3.6.5 Apoptotic marker staining

PBMCs were isolated from six healthy local donors and cultured with PHA and IL-2 for three days before either being treated with HIV_{1956}, or not, and being incubated with Serpin B1 (50μg/mL), BSA (50μg/mL, protein control) or PBS (negative control). ACH2 cells were not pre-cultured with anything prior to treatment, however, they were treated with 5ng/mL PMA at the time of treatment to induce viral output. Once treated with the appropriate stimulus, cells were incubated for six days prior to staining for flow cytometric analysis. Cells were stained for surface markers, namely CD3 (V500, BD, Franklin Lakes, NJ., USA), CD4 (PE-Cy5, Biolegend, San Diego, CA., USA), and CD8 (APC-Cy7, BD, Franklin Lakes, NJ., USA) for PBMCs, or

CD5 (APC-Cy7, Biolegend, San Diego, CA., USA), and CD71 (PE-Cy7, Biolegend, San Diego, CA., USA) for ACH2/A3.01, and live/dead staining of both cell types. All cells were then washed twice with FACS wash and once with 1x Binding Buffer from the Annexin V Apoptosis Detection kit (eBioscience, San Diego, CA., USA). Cells were resuspended in 100µl of this binding buffer. Fluorochrome-conjugated Annexin V (PE) was added to each cell suspension. Tubes were incubated in the dark for 15 minutes at room temperature, and washed with binding buffer. Supernatants were discarded and 200µl FACS wash, along with 100µl 1% PFA was added to each tube. Cells were analyzed via flow cytometry.

2.3.6.6 PBMC activation

PBMCs (500,000) isolated from three healthy local donors in Winnipeg, Canada were stimulated with either 2ng/mL PMA (Sigma, Oakville, ON., Canada), and 200ng/mL ionomycin (Positive control, Sigma, Oakville, ON., Canada), 12.5µl CD3/CD28 Dynabeads (Positive control, Gibco by Life Technologies, Burlington, ON., Canada), 50µg/mL Serpin B1 (Genscript, Piscataway, NJ., USA), 50µg/mL BSA (protein control, Bio-RAD, Mississauga, ON., Canada) or PBS (negative control). Cells were incubated for 14 hours at 37^{o}C (5% CO_2) at which point cells were washed to remove PMA/Ionomycin, Serpin B1, BSA, or PBS. For those treated with CD3/CD28 Dynabeads, cells were placed in a DynaMag-2 magnet (ThermoFisher Scientific, Burlington, ON., Canada) to remove the beads. Cells were all washed twice with PBS and stained for surface markers, CD3 (V500, BD), CD4 (BB515, BD, Franklin Lakes, NJ., USA), CD8 (APC-H7, BD, Franklin Lakes, NJ., USA), CD62L (BV605™, Biolegend, San Diego, CA., USA), CCR7 (PE/Cy7, Biolegend, San Diego, CA., USA), CCR5 (AlexaFluor®700, Biolegend, San Diego, CA., USA), CD27 (BV 785™, Biolegend, San Diego, CA., USA), CD69 (PE, Biolegend, San Diego, CA., USA), CD45RA (BV421™, Biolegend, San Diego, CA., USA),

HLA-DR (APC, Biolegend, San Diego, CA., USA), and CD38 (PE-Cy5, BD, Franklin Lakes, NJ., USA). All samples were analyzed via flow cytometry.

2.3.6.7 Statistical analysis

FlowJo version 10.0.8 was employed for analysis of all data sets. This software was used to identify individual cell populations which were then applied to all other samples within the same study. Percent population statistics were exported to Prism 6 for Mac OS X where all further statistical analysis was performed. Following testing for Gaussian distribution via D'Agostino and Pearson Omnibus K2 Normality Testing, either multiple student t-tests, with one unpaired t-test performed per row, were carried out (intracellular p24 data, ACH2 and PBMC proliferationon analysis, and cellular activation analysis) or Mann-Whitney U-tests (ACH2/A3.01 and PBMC apoptosis). Both statistical methods were performed with an alpha of 0.05.

2.3.7 Milliplex cytokine and chemokine analysis

2.3.7.1 Cell cultures

PBMCs were isolated from two healthy Winnipeg donors and cultured in complete media (no IL-2) with PHA for three days before treatment. VK2, End1, and Ect1 cells were cultured as previously described. All epithelial cells were seeded at 10,000 per well, and PBMCs at 50,000 per well, in a 96-well plate. Cells were treated with either 50μg/mL Serpin B1 (Genscript, Piscataway, NJ., USA), 50μg/mL BSA (protein control, Bio-Rad, Mississauga, ON., Canada) or PBS (negative control). Immediately afterward, cells were either infected at an MOI of 0.2 with HIV_{1956} or left uninfected and rather treated with equal volumes of complete media. All plates were incubated for three hours at 37^{o}C (5% CO_2), at which point all media/stimulants were removed, and cells were washed with PBS followed by addition of fresh complete media and

fresh Serpin B1, BSA or PBS at equal concentrations as that initially added. Supernatants were collected and stored at -80^{o}C for 3, 6, 12, 18, 24, 72 or 120 hours, however, only the 72 hour (epithelial data) and 120 hour (PBMC data) data is presented in this thesis, as these were the time points that exhibited the most significant effects.

2.3.7.2 Cytokine/chemokine Milliplex analysis

Cytokines and chemokines were quantified according to the instructions outlined in the Human Cytokine/Chemokine Magnetic Bead Panel kits by Millipore. Specifically the analytes examined included; GM-CSF, GRO, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17A, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-1β, RANTES, TNF-α, I-TAC, MIG, and MIP-3α. Briefly, this two-day protocol included sonicating each analyte antibody tube for 30 seconds and vortexing for one minute. These antibodies were then combined into one mixing bottle and diluted with bead dilution buffer. Both the quality control and the kit standard were reconstituted in milliplex H_2O, 25µl of which were added individually to each well of each plate along with 25µl of the culture media used in the experiments. Additionally, 25µl of assay buffer was added to each of the sample wells followed by addition of 25µl supernatants to each well. Lastly, 25µl of the antibody mixture was added to each well (samples, quality control, and standard wells), sealed in aluminum foil and placed on a shaker at 4^{o}C overnight. The following day the plates were washed on the Bioplex Pro II washer with wash buffer and 25µl of the secondary detection antibody was added, which was then incubated at room temperature for one hour on the shaker. Without removing the secondary antibody, 25µl of Streptavidin-phycoerythrin was added to each well and incubated on the shaker at room temperature for 30 minutes followed by washing of each plate twice with wash buffer, and the addition of 150µl sheath fluid to each well. Following shaking for a final five minutes, each plate was analyzed on the Bioplex 200 machine using Bioplex manager software.

2.3.7.3 Statistical analysis

Observed concentrations were exported from Bioplex manager software and plotted in Prism 6 software. Results obtained for each analyte at time point 0 hour, was used to normalize data. Experimental samples, treated with either Serpin B1 or BSA, were compared to the negative virus/cell controls (treated with PBS) through performing Mann-Whitney U tests since this data did not follow Gaussian distribution according to D'Agostino and Pearson Omnibus K2 test for Normality. An alpha of 0.5 was used.

2.3.8 Confocal microscopic imaging of Serpin B1

2.3.8.1 Staining protocol

PBMCs were seeded at 300,000 per 5mL polypropylene tube in complete RPMI. Cells were centrifuged to pellet cells, at which point media was discarded, and Serpin B1 added exogenously at a final concentration of 50μg/mL. Equal volumes of PBS were added to control wells as Serpin B1 was added to experimental wells. HIV_{1956} was added at an MOI of 0.2. Cells were incubated at 37^{o}C for three hours at which point the first time point was terminated. The remaining tubes were washed twice with PBS to remove any remaining virus. Fresh complete RPMI was added to cells along with Serpin B1 or PBS. Following 12 and 24 hour incubations at 37^{o}C, the remaining cells were processed and stained. At each time point cell suspensions were centrifuged at 1500rpm for 10 minutes and media was discarded. Cells were then incubated for 20 minutes in CytoFix/CytoPerm solution (BD Bioscience, San Diego, CA., USA) followed by washing with 1x Perm-Wash Buffer (BD Bioscience, San Diego, CA., USA). Samples were stored in PBS at 4 ^{o}C until all time points were collected. Nonspecific binding sites were blocked through 30 minutes incubation with PBS containing 10% goat serum prior to addition of the staining antibody. Serpin B1 poly rabbit IgG antibody (Abcam, Toronto, ON., Canada) was fluorescently labeled through the use of Zenon Alexa Flour 488 rabbit IgG (Life Technologies, Burlington, ON., Canada) according to manufacturer's instructions. The labeled antibody was

added at a concentration of 0.4μg/300,000 cells. Samples were incubated with the antibody mixture for 45 minutes at room temperature in the dark. Following washing with PBS, the cells were once again permeabilized and fixed through incubation with Cytofix/Cytoperm solution for 20 minutes at 4°C. Cells were washed with Perm/Wash buffer 1x and stored in PBS at 4 °C.

2.3.8.2 Mounting and imaging protocol

The concentrated fluorescently-stained PBMCs were mounted (10μl) onto microscope slides and allowed to dry, protected from light. Once dried, one drop of Platinum ProLong Diamond Antifade Mountant with DAPI (Life Technologies, Burlington, ON., Canada) was added to each slide and a high performance, 1.5H thickness cover glass was applied immediately (Zeiss). Samples were then allowed to cure overnight at room temperature, again protected from the light, at which point these multi-channel fluorescent samples were imaged using an LSM 700 Zeiss confocal microscope with Zen software (Zeiss, North York, ON., Canada).

2.3.8.3 Statistical analysis

Integrated Density (area multiplied by the mean pixel for the area) was determined after background pixel intensity was controlled for. PBMCs that had been treated with Serpin B1 for 0 hours and for 24 hours and had been stained with a fluorescently labeled antibody, were used to establish the dynamic range for pixel intensity against which all other samples were measured. After integrated density was determined, column statistics consisting of D'Agostino and Pearson Omnibus K2 Normality test was performed to confirm Gaussian distribution of data. Two-way, unpaired student t-tests with Welch's correction for unequal SDs, and an alpha of 0.05 were then performed for each sample integrated density compared to control PBMCs that had not been treated with Serpin B1 at the same time point.

“Unexpected results and problems are part of life. Never lose hope in any condition because darkness of night always finishes with light.” ~Anonymous

CHAPTER 3: EFFECT OF COMPLETE CVL ON HIV INFECTION

3.1 Rationale

CVL from HESN women within the Pumwani sex worker cohort demonstrates increased expression of various anti-HIV factors, including specific antiproteases[219,235,361], which may contribute to the protective phenotype of these women. With high levels of innate and adaptive antiviral factors present, CVL alone, in theory, should be capable of inducing an HIV-neutralizaing effect more dramatically within HESN women compared to HIV-susceptible women, the CVL of whom does not possess the same concentration of factors. Furthermore, the immune system of women regularly exposed (HIV-infected) and thus primed to the virus, exhibit adaptive, HIV-specific $CD8^+$, and $CD4^+$ T-cell responses as well as IgG and IgA antibodies specific for HIV epitopes. Additionally, while it has not yet been demonstrated, women that are highly-exposed to HIV may have elevated levels of specific innate antimicrobial proteins that would not be present in women from HIV-low-exposure settings (Winnipeg cohort). Hence, these highly-exposed women may exhibit an increase in antiviral factors both in the genital tract epithelium and the blood compared to women that experience low exposure to HIV. Women from Winnipeg, Canada are considered to not be regularly exposed to HIV. Therefore, these women are not expected to exhibit strong anti-HIV responses within their CVL, and thus will not be capable of neutralizing HIV to the same degree as CVL from women who are regularly exposed to the virus, such as those women in the Pumwani sex worker cohort. Conversely, women who are HIV-positive should exhibit strong anti-HIV responses, comparable to or more robustly than that observed in HESN women due to the increased concentrations of IgG and IgA adaptive immune response[362,363].

3.2 Hypothesis

CVL fluids from HESN women will inhibit HIV-1 infection more effectively than CVL from susceptible high-exposure commercial sex workers in Nairobi, Kenya or low-exposure women from Winnipeg, Canada.

3.3 Objectives

- Compare the HIV inhibitory effect of CVL from HESN women to that of CVL from HIV-susceptible and HIV-infected women within the Pumwani commercial sex worker in Nairobi, Kenya.
- Determine the variability/stability of the HIV-inhibitory capacity of CVL samples from individual women within the Pumwani cohort over multiple time points.
- Ascertain the difference in HIV-inhibitory capacity of CVL between HIV-highly exposed women in the Pumwani cohort and low-exposure women from Winnipeg.

3.4 Results

3.4.1 Study population description

3.4.1.1. Pumwani sex worker cohort in Nairobi, Kenya (high-exposure)

Frozen CVL samples from Nairobi were shipped to Winnipeg on dry ice and kept at -80^{o}C until use in assays. A minimum of forty samples for each group of women was evaluated in the HIV-inhibition assays, HESN (n=41), HIV-Susceptible (n=51), HIV-Positive (n=76). Women who had been followed in this cohort for longer than seven years and remained seronegative were classified as HESN. Women were identified as HIV-susceptible based on participation in sex work for less than seven years as epidemiological data from this cohort shows that most of these women will seroconvert within the first three years in the cohort[203 364]. HIV-positive women were identified as infected based on serologic testing. HIV-positive women were not on ARVs at the time of sample collection, and had CD4 counts above 400 cells/mm^3. Age, stage of menstrual cycle, and mode of contraception were all determined based on self-reporting. Women who were reported to have a current genital tract infection were excluded from the study.

3.4.1.2. Winnipeg women's clinic cohort (low-exposure)

Fresh CVL samples were collected from the Health Sciences Centre Women's Clinic in Winnipeg, Manitoba. Upon processing, they were kept at -80^{o}C until assays were performed. All women were 18 years of age or older and provided informed consent. Women were separated into two groups: post-partum, consisting of women who had delivered a baby within the past three months, the majority of whom were breastfeeding (80%). All other non-pregnant nor post-partum women comprised the second group of non-post-partum, low-risk women. There were 26 women enrolled in the postpartum group and 74 within the non-post-partum group. Any participants that were positive for STIs were excluded from the study. Age, contraceptive method, stage of the menstrual cycle as well as ethnicity were all self-reported.

3.4.2 Comparison of HIV neutralizing activity between HESN, high-exposure HIV-susceptible, and HIV-infected women

To determine the difference in HIV-inhibitory capacity between CVL samples of HESN, HIV-susceptible, and HIV-infected women, HIV neutralzing assays were performed using β-Galactosidase-expressing TZM-bl cells in a three-day assay and infected with R5-tropic HIV-1 Bal. Initial experiments included testing pooled CVL from women from each of the three groups. CVL from each study group demonstrated no significant effects on HIV replication, compared to the virus control (100% infectivity, Figure 12).

Pooling of CVL samples may mask any of the individual anti- or pro-HIV capabilities of individual CVL samples, and thus, HIV-inhibitory assays were repeated using both HIV-1 Bal and X4-tropic HIV-1 IIIB together with individual participants' CVL specimens (Figure 13). Similar to the results obtained in the pooled CVL assays, when a groupwise analysis was performed on infection data from both R5-topic (Bal) and X4-tropic (IIIB) HIV assays, no significant difference was observed in HIV-inhibitory capacity (Figure 13). However, when the

inhibitory activity of each woman was compared to that of other women, it became apparent that CVL from specific women within each group exhibited the ability to either enhance or inhibit HIV infection. These effects were much more prominent and variable in X4-tropic HIV-1 IIIB infections compared to that observed with R5-tropic, HIV-1 Bal (Figure 13). Significant differences in infection between these two viruses was observed in HIV-susceptible and HIV-infected women. CVL from susceptible women neutralized HIV-1 Bal by 13.3% and exhibited no inhibition of HIV-1 IIIB (101.7% infection, $p = 0.0006$), while the CVL from HIV-1 infected women inhibited HIV-1 Bal by 8.6% and enhanced HIV-1 IIIB infection by a cumulative 5% ($p = 0.0064$).

This data demonstrates clearly that CVL from HESN women overall does not have a stronger HIV-inhibitory effect in TZM-bl cell assays compared to susceptible or infected women in HIV-high-exposure settings. Rather, there is a considerable degree of variability in the HIV-inhibitory activity shown by individual CVL samples from women within both study groups. Samples, irrespective of study group, that exhibit the strongest anti-HIV activity may contain valuable information on potential natural anti-HIV factors, which may inform development of future novel microbicides.

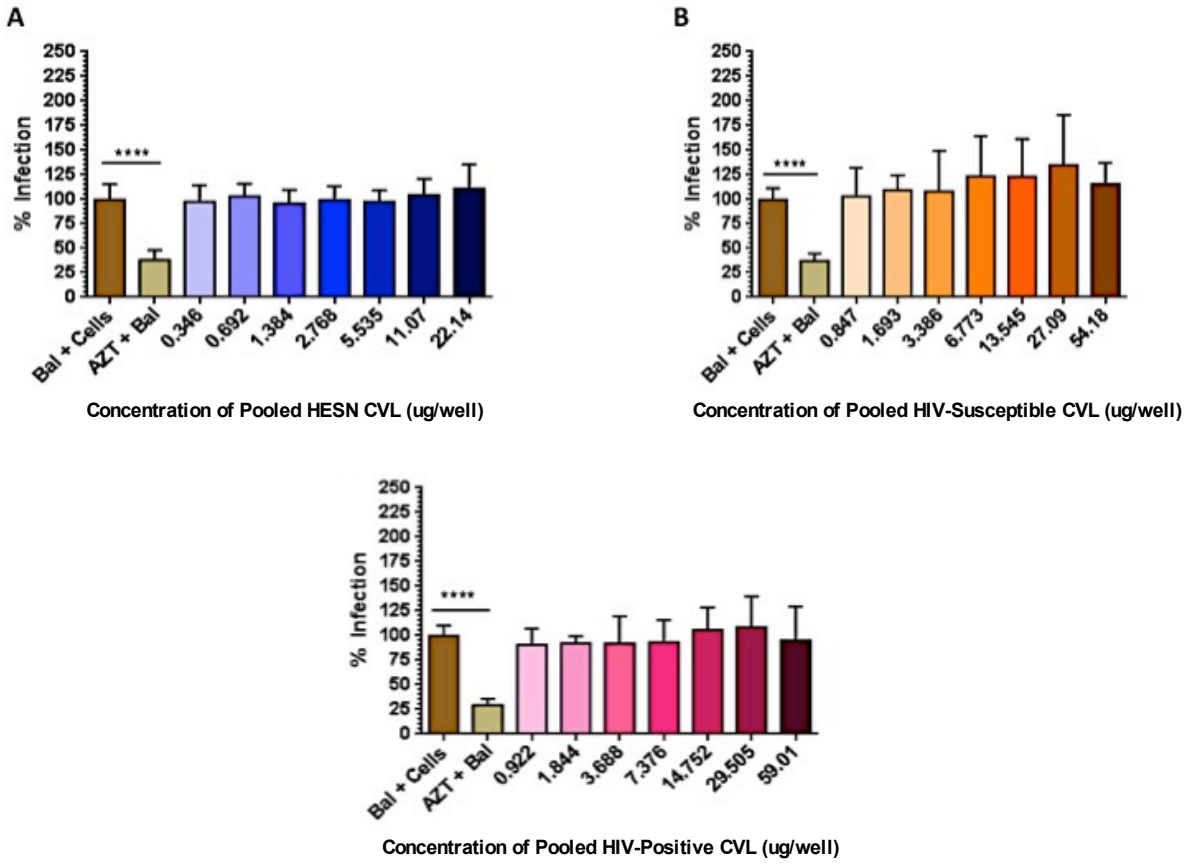


Figure 12: Pooled CVL from HESN, HIV-susceptible and HIV-infected women demonstrated no neutralization of HIV-1 Bal

Each bar represents three biological replicates performed in triplicate. D'Agostino and Pearson Omnibus K2 Normality testing was performed followed by unpaired student t-tests with Welch's correction (did not assume equal SDs) and an alpha of 0.05 were conducted between virus control and experimental conditions. TZM-bl cells were treated with PBS (virus control), AZT (RT positive control) or pooled CVL from either, A: 41 HESN, B: 51 susceptible or, C: 76 HIV-positive women, at serially diluted protein concentrations and infected with HIV-1 Bal.

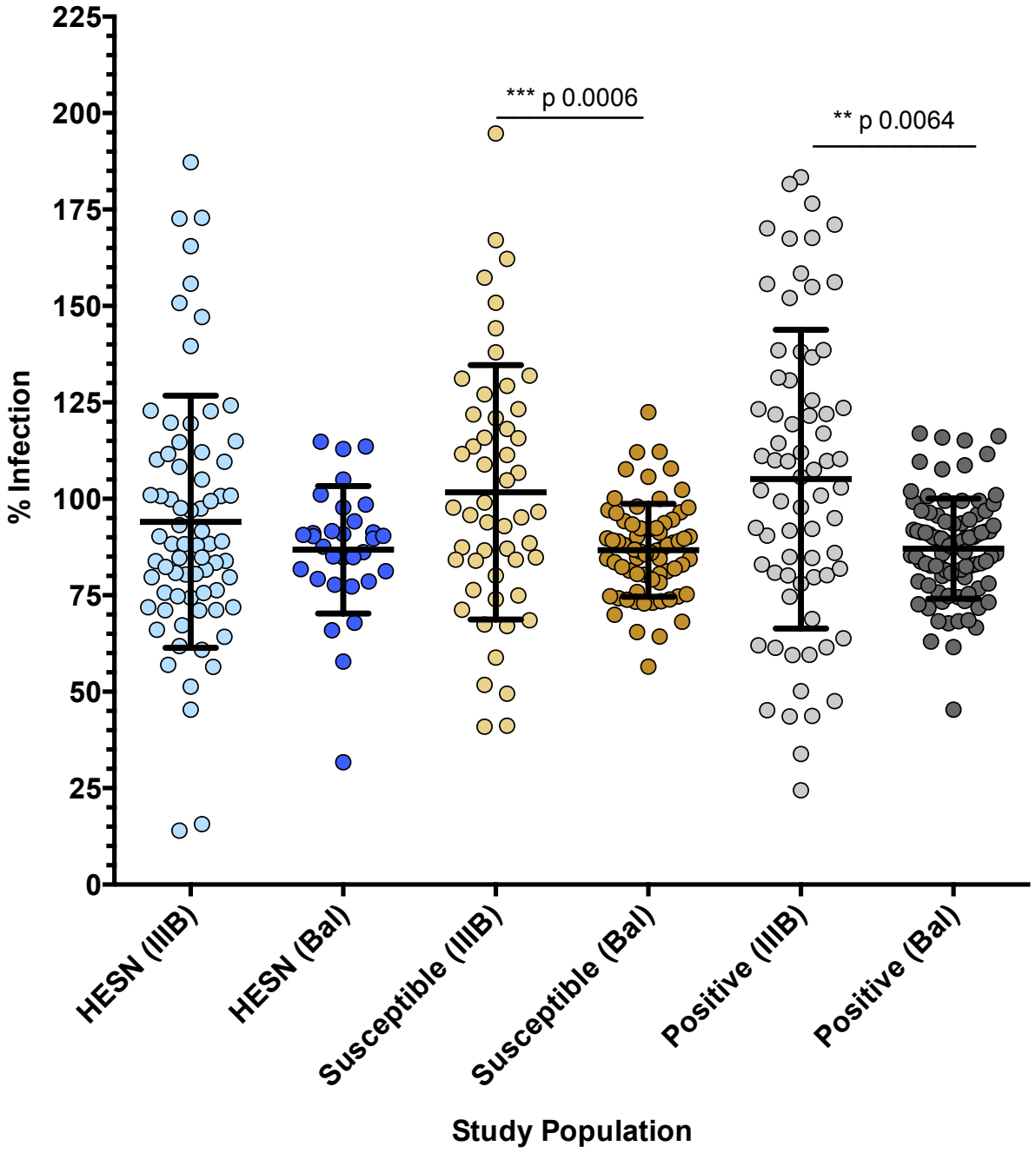


Figure 13: Individual CVL samples vary greatly in their neutralizing capacity of HIV-1 IIIB and Bal in TZM-bl cell cultures

Each data point represents the mean of three biological replicates per individual. D'Agostino and Pearson Omnibus K2 Normality testing was performed, followed by unpaired student t-tests with Welch's correction (did not assume equal SD) and alpha of 0.05. TZM-bl cells were treated with individual CVL samples, at a concentration of 50μg/mL of total protein, from the HESN, HIV-suscepible, or HIV-infected study groups. These cells were then infected with either HIV-1 IIIB or Bal. Percent infection was determined as compared to a virus control which was set at 100% infection.

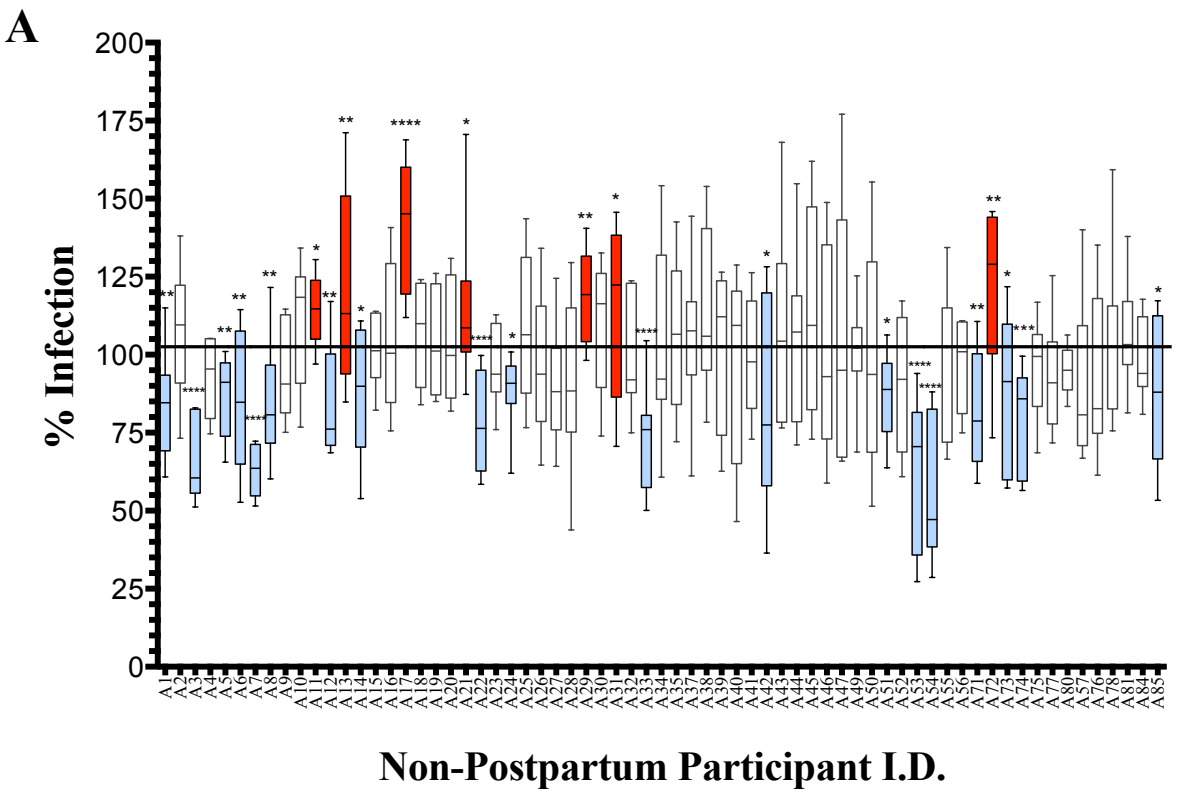
3.4.3 Comparison of HIV neutralizing activity between HIV-low-exposure Winnipeg women and high-exposure women from the Nairobi, Pumwani sex worker cohort

CVL from women attending a women's health clinic in Winnipeg was collected to provide an HIV low-exposure population. These Winnipeg women likely vary in their FGT microbiomes and have a differing composition of their CVL / genital fluids, compared to women from Nairobi[197]. This low-exposure population of women provided a broader spectrum of samples to provide a more diverse understanding of how the components that make up CVL affect HIV replication. CVL samples from these women were tested in HIV-1 Bal neutralization assays and seperated into two study groups: post-partum (within three months of delivery, n = 26) and non-post-partum (n=74). A third group, comprised of pregnant women (n=2), was omitted from analysis. No significant HIV-1 neutralizing effect was observed in the CVL from either of the study groups compared to the control (Figure 15). However, as Figure 14 illustrates, CVL from women within the non-post-partum group exhibited significantly more variable enhancing or inhibitory effects on HIV infection compared to the post-partum group ($p = 0.0238$).

No differences were seen in HIV-1 inhibition when comparing CVL from low-exposure non-post-partum women to low-exposure post-partum women. However, CVL from both high-exposure groups exhibited significantly higher levels of inhibition (11-13%) compared to either group consisting of CVL from low-exposure women, which showed less than 3% inhibition (Figure 15).

Taken together these results confirm that CVL from women in HIV low-exposure settings, such as Winnipeg, Canada exhibit milder inhibitory effects on HIV compared to the CVL from women in high-exposure settings, such as the Pumwani cohort in Nairobi, Kenya.

Also, the overall impact on HIV was not significantly different between post-partum and non-post-partum women, however, there was a higher proportion of non-post-partum women who demonstrated either HIV inhibitory or enhancing effects compared to that of the post-partum women whose CVL largely did not exhibit any effect on HIV neutralization, suggesting that epidemiological factors, other than gravidity, may be involved in affecting an individual's ability to neutralize the activity of HIV-1.


A
% Infection
Non-Postpartum Participant I.D.

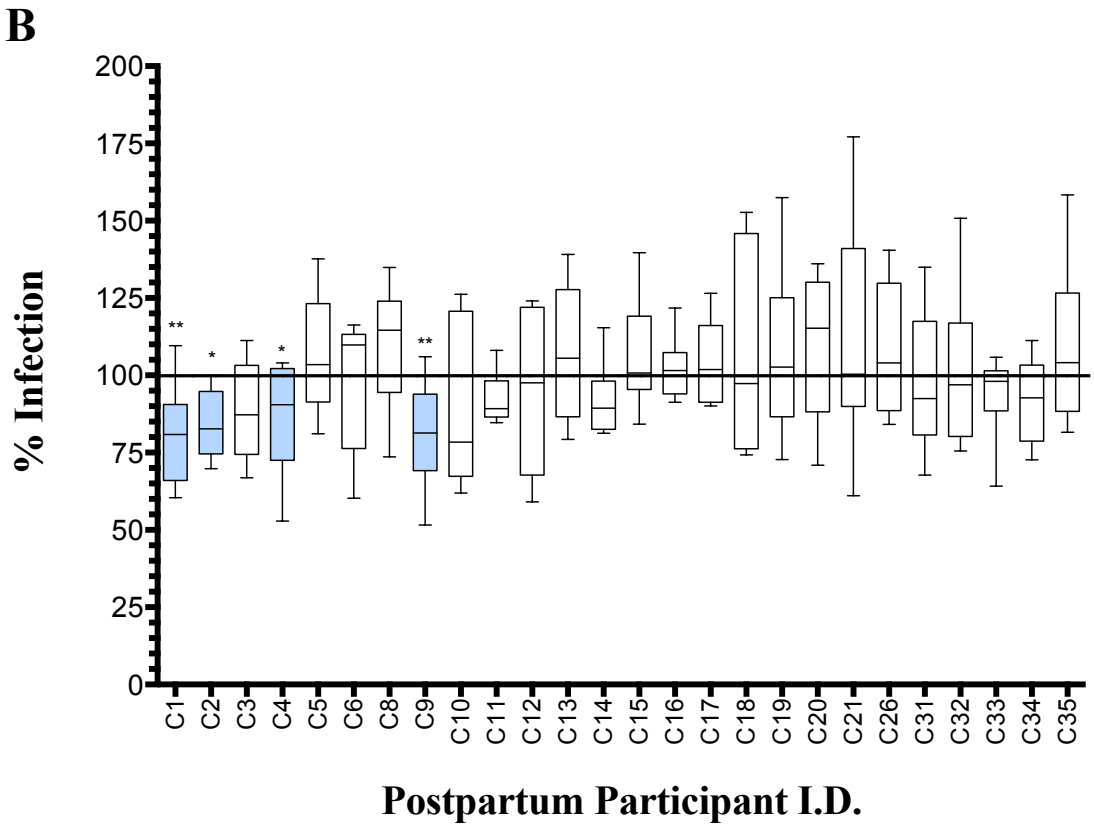

B
% Infection
C1
C2
C3
C4
C5
C6
C8
C9
C10
C11
C12
C13
C14
C15
C16
C17
C18
C19
C20
C21
C26
C31
C32
C33
C34
C35
Postpartum Participant I.D.

Figure 14: Variability in neutralization capacity of CVL from HIV low-exposure Winnipeg women

TZM-bl cells infected with HIV-1 Bal treated with 50μg/mL of individual CVL samples from either post-partum women or non-post-partum women in a three-day assay. Each bar represents results from three assays performed in triplicate. Blue bars indicate significant inhibition of HIV infection. Red bars indicate significant enhancement of infection. D'Agostino and Pearson Omnibus K2 Normality testing was performed followed by unpaired student t-tests between virus control and individual samples. A: Mean with SEM for the effect of CVL from non-post-partum Winnipeg women on HIV infection. B: Mean and SEM for the effect of CVL from individual post-partum women on HIV infection.

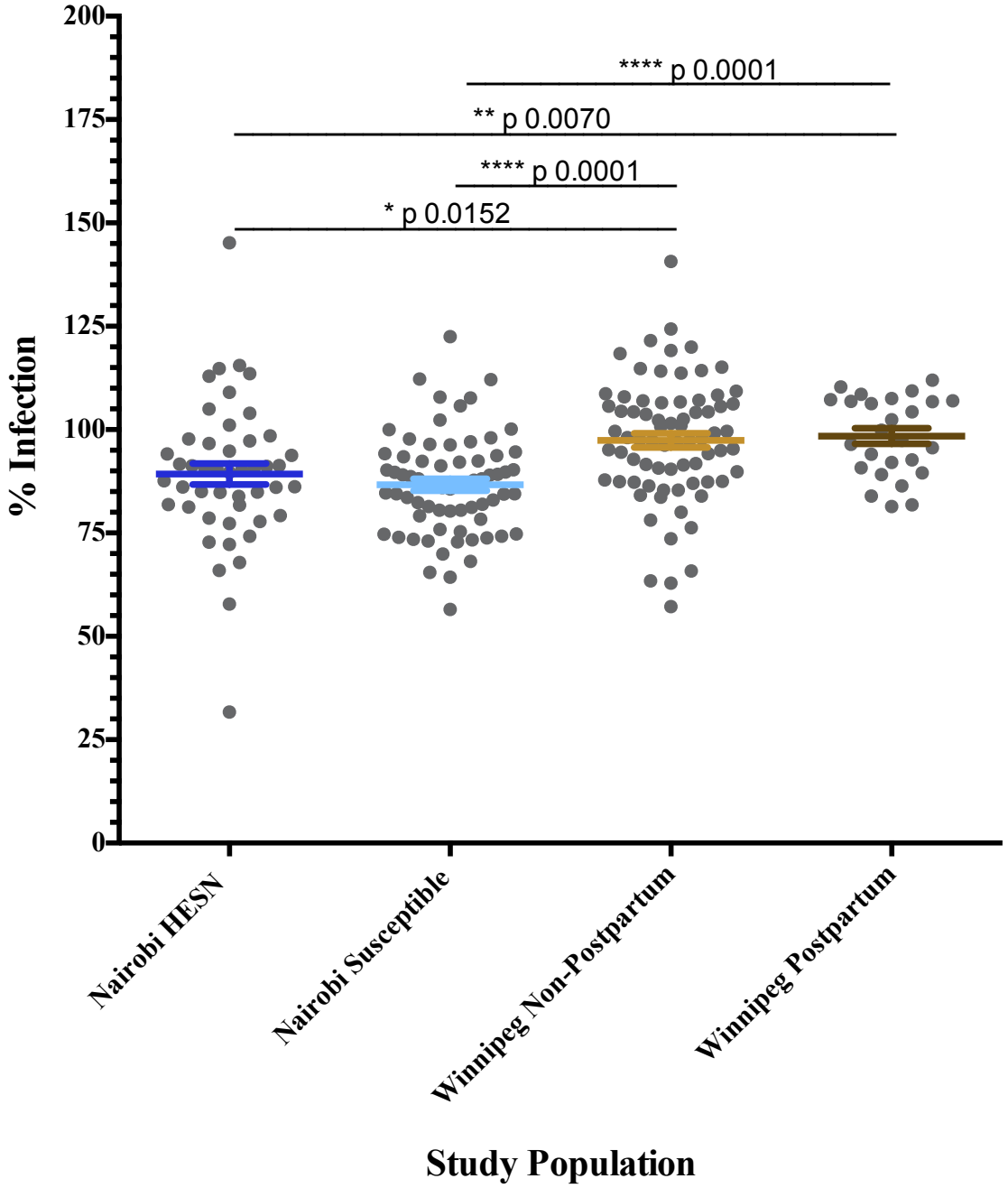


Figure 15: CVL from HIV low-exposure women exhibits lower neutralization capacity compared to CVL from high-exposure commercial sex workers

TZM-bl cells treated with CVL from HIV low-exposure non-post-partum women or post-partum women from the Winnipeg cohort or with CVL from high-exposure HESN women, or HIV-susceptible women from the Nairobi, Pumwani cohort, followed by infection with HIV-1 Bal. Each data point represents results from one individual participant consisting of three assays, each performed in triplicate. Error bars represent mean with SEM. D'Agostino and Pearson Omnibus K2 Normality testing was performed followed by unpaired student t-tests, not assuming equal SD (Welch's correction) with an alpha of 0.05.

3.4.4 Longitudinal analysis of the HIV-neutralization effects exhibited by CVL samples from individual women in the Pumwani cohort

To determine if the inhibitory or enhancing effects exhibited by individual women in the Pumwani cohort remains consistent over time, TZM-bl cell neutralization assays, infected with HIV-1 Bal were performed with CVL samples from various time points for individual women within the three study groups: HESN, HIV-susceptible, and HIV-infected. Analysis was not performed on CVL from low-exposure, Winnipeg women due to limited numbers of samples from multiple time points. Limited variability in HIV neutralizing activity, between time points, was observed in samples from HESN and HIV-susceptible women, with only three of the 17 (Figure 16A, Table 1) and three of the 22 (Figure 16B, Table 1) women having demonstrated significant variability in their HIV neutralizaing capacity, respectively. CVL samples from HIV-infected women exhibited higher variability with eight of the 24 women, having significant variation in neutralization capacity over the examined time points (Figure 16C, Table 1).

The number of individuals exhibiting significant inhibitory activity between these three study groups was highly variable. Ten HIV-infected women and four HESN women and only one HIV-susceptible woman exhibited significant HIV inhibitory activity at various time points. Conversely, only one woman from the HIV-positive, and HESN study groups exhibited enhancing effects on HIV infectivity, with the HESN participant (ML2771) having this effect at just one of the two measured time points. Conversely, three of the HIV-susceptible women had CVL that exhibited a consistent enhancing effect at all measured time points.

This data suggests that within HESN and HIV-susceptible women the factors present in CVL capable of inhibiting or enhancing HIV infection remain relatively stable in their effectiveness, in an *in vitro* assay, over time. Determining whether or not this stability in anti-HIV activity is reflected in the proteomic composition of CVL would potentially allow for prediction of an individual's risk of acquiring HIV.

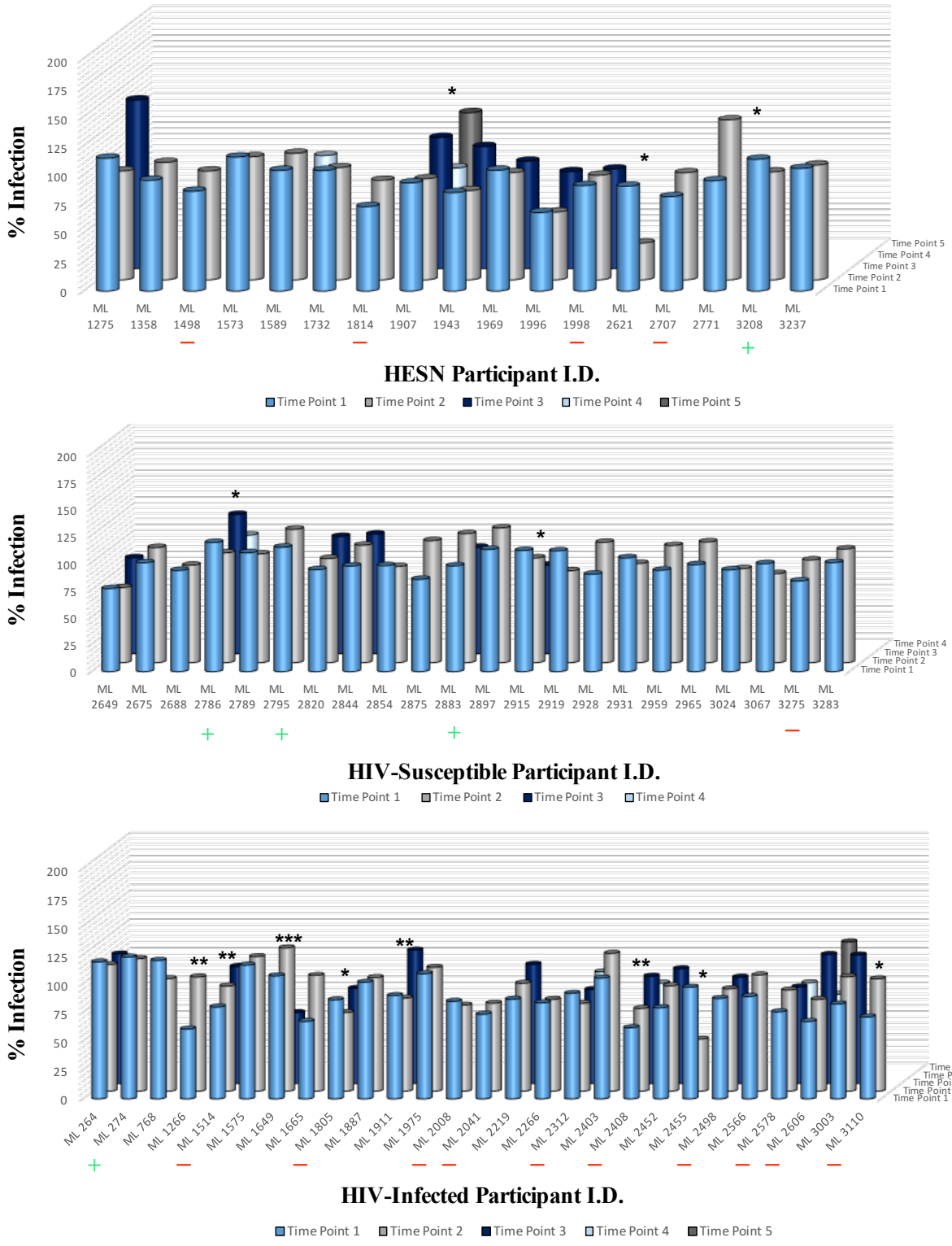

% Infection
200
175
150
125
100
75
50
25
0
ML 1275
ML 1358
ML 1498
ML 1573
ML 1589
ML 1732
ML 1814
ML 1907
ML 1943
ML 1969
ML 1996
ML 1998
ML 2621
ML 2707
ML 2771
ML 3208
ML 3237
Time Point 5
Time Point 4
Time Point 3
Time Point 2
Time Point 1
HESN Participant I.D.
Time Point 1
Time Point 2
Time Point 3
Time Point 4
Time Point 5
% Infection
ML 2649
ML 2675
ML 2688
ML 2786
ML 2789
ML 2795
ML 2820
ML 2844
ML 2854
ML 2875
ML 2883
ML 2897
ML 2915
ML 2919
ML 2928
ML 2931
ML 2959
ML 2965
ML 3024
ML 3067
ML 3275
ML 3283
Time Point 4
Time Point 3
Time Point 2
Time Point 1
HIV-Susceptible Participant I.D.
Time Point 1
Time Point 2
Time Point 3
Time Point 4
% Infection
ML 264
ML 274
ML 768
ML 1266
ML 1514
ML 1575
ML 1649
ML 1665
ML 1805
ML 1887
ML 1911
ML 1975
ML 2008
ML 2041
ML 2219
ML 2266
ML 2312
ML 2403
ML 2408
ML 2452
ML 2455
ML 2498
ML 2566
ML 2578
ML 2606
ML 3003
ML 3110
Time Point 5
Time Point 4
Time Point 3
Time Point 2
Time Point 1
HIV-Infected Participant I.D.
Time Point 1
Time Point 2
Time Point 3
Time Point 4
Time Point 5

Figure 16: CVL from HESN, HIV-susceptible, and HIV-infected women exhibit limited variability in HIV-neutralizing capacity

Each bar represents the mean and SEM of three assays performed in triplicate for each participant at a specific time point. D'Agostino and Pearson Omnibus K2 Normality testing was performed followed by unpaired, two-tailed student t-tests on individual sample points compared to the viral control sample (set at 100% infection) to determine if a significant inhibitory or enhancing effect was observed for each CVL time point. Indiviudal one-way Anovas were performed between time points to determine if there was a significant different in the means resulting in a p-value < 0.05. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

A: Longitudinal effect of CVL from HESN women on HIV replication. B: Longitudinal effect of CVL on HIV replication in Susceptible women from the Pumwani cohort. C: Longitudinal effect of CVL from HIV-infected women on HIV replication.

Table 1: Statistical results for CVL longitudinal analysis

		Time Point 1			*Time Point 2*			*Time Point 3*			*Time Point 4*			*Time Point 5*		
	LD	**Mean**	**SD**	**SEM**	**Mean**	**SD**	**SEM**	**Mean**	**SD**	**SEM**	**Mean**	**SD**	**SEM**	**Mean**	**SD**	**SEM**
HESN	**ML 1275**	114.49	24.66	14.24	93.78	17.15	9.90	145.20	43.71	25.24						
	ML 1358	95.41	19.39	11.20	101.17	4.76	2.75									
	ML 1498	86.04	8.12	4.69	93.76	12.61	7.28									
	ML 1573	115.49	12.07	8.54	106.03	12.72	7.34									
	ML 1589	103.97	34.37	19.84	108.99	23.04	13.30									
	ML 1732	103.88	33.08	19.10	96.66	8.22	4.74									
	ML 1814	72.80	2.12	1.23	85.72	19.49	11.25									
	ML 1907	93.22	24.89	14.37	87.10	16.94	9.78	112.91	20.39	11.77	87.54	25.05	14.46	114.77	8.33	4.81
	ML 1943	84.88	16.87	9.74	76.84	23.13	13.35	105.00	15.93	9.20						
	ML 1969	104.10	7.77	4.48	92.26	5.87	4.15	92.46	17.40	10.05						
	ML 1996	67.68	0.35	0.25	58.34	16.57	11.72	83.43	12.19	8.62	76.81	3.55	2.51			
	ML 1998	90.85	1.30	0.92	90.09	22.27	15.75	85.68	15.12	8.73						
	ML 2621	90.32	20.93	14.80	31.72	7.90	4.56									
	ML 2707	81.38	28.44	20.11	92.22	7.48	5.29									
	ML 2771	95.04	6.70	3.87	137.81	16.43	11.62									
	ML 3208	113.57	10.76	6.21	92.95	14.19	8.19									
	ML 3237	105.68	17.82	10.29	98.83	17.54	10.12				90.25	17.42	10.06			
HIV-Susceptible	**ML 2649**	76.27	10.24	5.91	68.99	1.19	0.84	88.11	18.50	13.08						
	ML 2675	100.02	17.77	12.56	105.72	4.55	2.62									
	ML 2688	92.98	8.86	6.27	90.77	1.83	1.30									
	ML 2786	118.57	4.56	3.22	100.85	27.87	16.09	127.90	7.85	4.53	100.65	14.22	8.21			
	ML 2789	109.15	34.37	19.84	99.84	13.47	7.78									
	ML 2795	114.20	9.47	5.47	122.47	6.21	4.39									
	ML 2820	93.59	7.75	5.48	95.71	11.38	8.05	107.72	0.51	0.36						
	ML 2844	96.97	21.13	14.94	107.88	11.63	8.23	109.89	12.99	7.50						
	ML 2854	97.32	9.76	5.63	88.33	6.31	3.64									
	ML 2875	84.79	9.07	5.24	112.08	37.34	21.56									
	ML 2883	97.09	12.30	7.10	118.47	10.26	7.25	97.73	8.53	4.93						
	ML 2897	112.23	13.53	7.81	123.76	9.66	6.83									
	ML 2915	111.26	8.48	4.90	96.22	8.33	4.81	81.23	13.20	7.62						
	ML 2919	110.87	12.75	9.01	84.39	3.61	2.55									
	ML 2928	89.40	11.83	8.36	110.53	14.91	8.61									
	ML 2931	104.48	7.99	5.65	91.11	18.00	10.39									

	ML2959	93.13	13.63	7.87	107.65	33.11	19.12									
	ML2965	98.15	12.73	7.35	110.89	15.78	9.11									
	ML3024	93.41	16.51	9.53	86.28	23.14	13.36									
	ML3067	99.15	31.31	18.08	81.76	11.24	6.49									
	ML3275	83.18	6.28	3.62	94.28	13.50	7.79									
	ML3283	100.14	15.32	8.84	104.46	18.58	10.73									
HIV-Infected	ML264	118.98	33.24	19.19	109.97	6.59	4.66									
	ML274	123.13	7.27	4.20	115.13	3.80	2.19									
	ML768	120.28	23.02	13.29	97.79	29.81	17.21									
	ML1266	60.62	6.07	3.50	99.26	12.44	7.18									
	ML1514	79.84	7.08	4.09	91.31	2.70	1.91	101.77	13.22	7.63	66.62	0.50	0.29			
	ML1575	116.22	15.75	9.09	116.94	11.38	6.57									
	ML1649	106.89	3.92	2.26	124.42	10.13	5.85	61.55	9.06	5.23						
	ML1665	67.30	19.19	11.08	100.60	45.82	26.45									
	ML1805	81.87	5.80	4.10	68.28	0.83	0.59	82.65	6.86	3.96						
	ML1887	101.27	28.63	16.53	98.69	17.67	10.20									
	ML1911	89.72	6.67	3.85	80.84	7.20	4.16	115.90	0.90	0.63	52.78	14.39	8.31			
	ML1975	108.69	19.38	11.19	107.61	6.28	4.44									
	ML2008	84.77	12.28	7.09	74.68	5.80	3.35									
	ML2041	73.56	16.15	9.32	76.44	8.67	5.01									
	ML2219	86.52	25.86	14.93	93.72	4.79	2.77	103.49	16.91	9.76						
	ML2266	83.44	14.01	8.09	79.68	19.82	11.45									
	ML2312	91.65	9.51	5.49	76.33	4.23	2.44	81.64	7.18	4.14	90.25	17.42	10.06			
	ML2403	104.98	15.74	9.09	119.76	10.28	5.93									
	ML2408	61.82	2.63	1.86	71.90	1.25	0.89	93.21	6.38	4.51	80.52	1.75	1.24			
	ML2452	79.11	8.82	6.24	91.53	21.73	12.55	99.75	12.59	7.27						
	ML2455	96.93	18.76	10.83	45.31	16.88	9.74									
	ML2498	87.26	13.08	7.55	88.92	11.42	6.59	92.36	1.64	0.95						
	ML2566	88.96	9.21	5.32	100.96	18.08	10.44									
	ML2578	75.62	13.21	7.63	87.91	33.43	19.30	83.84	23.69	13.68	80.77	17.04	9.84			
	ML2606	67.23	5.13	2.96	79.68	13.57	7.83	112.11	10.77	7.62	71.02	11.46	6.62	109.61	35.80	20.67
	ML3003	82.38	5.88	3.40	99.42	27.53	15.90	111.61	18.02	10.41						
	ML3110	71.14	13.30	7.68	97.60	6.23	3.59									

3.5 Summary

The HESN phenotype is clearly not solely dependant on the neutralizing capacity of CVL. The CVL from HESN women did not exhibit a stronger cumulative inhibitory effect on HIV-1 replication compared to HIV-susceptible women or HIV-infected women. All three of these groups exhibited similar levels of HIV-1 neutralizing capacity. However, within each of these groups, individual women were identified whose CVL is capable of exhibiting distinctly different inhibiting or enhancing effects on the replication of HIV-1. A similar spectrum was observed with the CVL from Winnipeg-based women. While both the post-partum and non-post-partum women exhibited an overall weaker neutralizing effect than that seen in Nairobi-based women, there was again a wide range of effects observed. Some of these differences may be explained by specific epidemiological factors such as age and contraceptive method for both groups, or by factors such as the number of clients per week for commercial sex workers in Nairobi. However, overall an individual woman's CVL is relatively stable in HIV neutralizing ability across time points, except in women who are HIV-infected.

The differences observed in HIV neutralzing capacity of CVL from women in low-exposure settings compared to women at high-exposure to HIV, may be due to distinctly different protein composition. Women from the Pumwani cohort may express higher levels of antiviral factors thus offering them enhanced protection. This will be the focus of chapter four.

CHAPTER 4: PROTEOMIC ANALYSIS OF CVL

4.1 Rationale

Key determinants of HIV transmission across mucosal surfaces include the integrity of the mucosal barrier, the number of target cells present at the site of infection and the presence of either antiviral or proviral factors at the time of viral contact [235,236]. Specific factors, including antiproteases, have been shown to be up-regulated within the FGT of HESN women, whether or not these proteins contribute to the protective phenotype of these women is not known. The previous chapter revealed that there was no statistically significant difference between the HIV inhibitory activity exhibited by CVL from HESN, and HIV-susceptible or HIV-infected controls. However, individual differences in HIV inhibitory activity was observed amoung women, and thus, it is likely that individual protein levels may associate with reduced susceptibility at the individual level. Therefore, it is important to determine the functional role of specific factors in inhibiting HIV at the individual level.

One other gap in knowledge is a comparison of proteomic analysis of CVL samples from women that are exposed to HIV at low levels compared to women who are highly exposed, to determine the basal levels of antiviral proteins in women who are not regularly exposed to HIV. The significantly increased HIV inhibition exhibited by women from the Pumwani cohort, compared to women in the Winnipeg cohort, suggests that the women from Nairobi may express higher levels of antiviral factors within their FGT. An unbiased analysis of proteomic profiles of women from these two cohorts may provide insight into this enhanced inhibitory capacity of the CVL from women in the Pumwani cohort. Thus, unbiased label-free mass spectrometry-based proteomic analysis of CVL was performed on samples from individuals within the Winnipeg and Nairobi cohorts.

4.2 Hypothesis

CVL from women in HIV low-endemic areas (Winnipeg cohort) contain distinct protein profiles compared to women in high-endemic areas (Pumwani cohort). Low-exposure women will express a lower abundance of known antiviral proteins, including antiproteases.

4.3 Objectives

- Compare the proteomic profiles of CVL from women of a low-exposure Winnipeg cohort to CVL from women in an HIV-high exposure Pumwani commercial sex worker cohort.
- Correlate the expression of antiproteases detected in CVL samples by label-free mass spectrometry with HIV inhibitory and non-inhibitory capacity as determined in Chapter three.

4.4 Results

4.4.1 Comparison between protein expression in CVL from Nairobi, Pumwani cohort women and Winnipeg cohort women

4.4.1.1 Differential protein expression analysis of Pumwani and Winnipeg women's CVL

To determine if there are any significant differences in the protein profiles of CVL from HIV low-exposure women (n = 56), and HIV high-exposure women (n = 58), label-free mass spectrometry was conducted. Two-hundred and thirteen proteins, that met the 25% covariance criterion, were identified. The relative expression was compared between these two groups of women using multiple student t-tests. Benjamini-Hochberg multiple comparison analysis, with a false discovery rate (FDR) of $q<0.0002$, was then performed. Due to the stringency of this analysis, only five proteins were identified to be statistically significantly expressed between the two study groups (Table 2). Three of these proteins, in particular, Glycodelin ($p = 3.83 \times 10^{-16}$, $q = 0.0002$), Ig Gamma-4 chain C region ($p = 0.0006$, $q = 0.0012$), and Prostate-specific antigen($p = 0.0004$, $q = 0.0007$) were determined to be upregulated within the CVL of women from the Pumwani cohort. The remaining two proteins were upregulated within the CVL of women from

the Winnipeg cohort and consisted of Ras-related protein Rab-1A ($p = 0.0006$, $q = 0.0009$) and Eukaryotic translation initiation factor-6 ($p = 0.0003$, $q = 0.0005$). In an effort to expand the number of proteins used for subsequent analysis, p-value frequency distributions were constructed (Figure 17B). Since the allocation of these p-values was not uniformly distributed, as would be expected if the results were due to random chance, which would prove the null hypothesis of no effect to be correct, the stringency for statistical significance was reduced to a p-value of 0.05 or lower. With this new criterion, a total of 32 proteins were significantly differentially expressed between the two cohorts. Seventeen of these proteins were upregulated within the Pumwani cohort, and the remaining 15 were upregulated within CVL from women in the Winnipeg cohort (Figure 17A). IPA and Uniprot analysis was conducted to determine the individual biological functions for each of these differentially expressed proteins (Table 2). Analysis demonstrated that the most striking differences in protein expression occurred in proteins associated with immune responses. These included factors associated with responses to invading pathogens, namely apoptosis (Glycodelin $p = 3.83 \times 10^{-16}$, 14-3-3 protein $p = 0.0025$, Purine nucleoside phosphorylase $p = 0.0054$, Clusterin $p = 0.0322$) and complement activation (Ig gamma-4 chain C region $p = 0.0006$, Clusterin $p = 0.0322$), as well as structural proteins responsible for cellular integrity (Filamin-B $p = 0.0061$, Envoplakin $p = 0.0120$, Keratin type I cytoskeletal 16 $p = 0.0141$) were found to be over-expressed within the Pumwani cohort (Table 2). Within the Winnipeg cohort increased levels of proteins associated with neutrophil recruitment and function, including Ras-related protein -1A (RAB1A, Winnipeg relative expression -0.397, Pumwani relative expression -1.328, $p = 0.0006$), which stimulates the production of IL-8, a neutrophil chemotactic chemokine. CVL from Winnipeg women also contained proteins highly associated with CTL and NK cell release of cytotoxic granules. These proteins included Neutrophil gelatinase-associated lipocalin (Winnipeg relative expression -0.393, Pumwani relative expression -1.190, $p = 0.0207$) and Apolipoprotein AI-1 (Winnipeg relative expression -1.244, Pumwani relative expression -2.369, $p = 0.0057$), both of which

induce IL-1β production from macrophages. Furthermore, both Angiotensinogen (Winnipeg relative expression -0.420, Pumwani relative expression -1.195, p = 0.0051) and Rho-GDP dissociation inhibitor 2 (Winnipeg relative expression -0.523, Pumwani relative expression -1.328, p = 0.215) were up-regulated within the women from the Winnipeg cohort. Both of these proteins control reactive oxygen species and nitric oxide production following stimulation of T cells and macrophages (Ingenuity pathways analysis software and Uniprot.org).

IPA pathway analysis of these 32 proteins also revealed associations with specific canonical pathways. Specifically, the up-regulated proteins within the CVL from Pumwani women, were associated with Atherosclerosis signaling (3/124 proteins, $p = 1.36 \times 10^{-4}$), the Intrinsic Prothrombin activation pathway (2/29 proteins, $p = 2.56 \times 10^{-4}$) as well as with various purine nucleobase salvaging pathways (Xanthine and Xanthosine $p = 8.24 \times 10^{-4}$, Guanine and Guanosine $p = 1.65 \times 10^{-3}$, Adenine and Adenosine $p = 1.65 \times 10^{-3}$) (Figure 17C). Additionally, these 17 proteins were found to be associated with cellular functions involving lipid (p-value range: $4.99 \times 10^{-2} – 4.42 \times 10^{-4}$) and carbohydrate (p-value range: $3.25 \times 10^{-2} – 8.63 \times 10^{-5}$) metabolism (Figure 17E). Alternatively, the upregulated proteins within the CVL of Winnipeg women were found to be associated with the LXR/RXR (4/121 proteins, $p = 7.73 \times 10^{-7}$) and FXR/RXR (4/126 proteins, $p = 9.09 \times 10^{-7}$) activation pathways as well as with acute phase response signaling (3/169 proteins, $p = 1.45 \times 10^{-4}$), intrinsic prothrombin activation pathway (2/29 proteins, $p = 1.47 \times 10^{-4}$) and the coagulation system (2/35 proteins, $p = 2.16 \times 10^{-4}$) (Figure 17D). These 15 up-regulated proteins were also found to be associated with functions related to cellular growth and proliferation ($2.99 \times 10^{-3} – 2.30 \times 10^{-6}$) as well as with basic cellular maintenance (p-value range: $3.59 \times 10^{-3} – 2.30 \times 10^{-6}$) and development (p-value range: $3.15 \times 10^{-3} – 2.30 \times 10^{-6}$), compared to those found to be up-regulated in the CVL of women from the Pumwani cohort (Figure 17F). These results suggest that the expression of numerous proteins differ significantly between women in these two cohorts. The primary differences include proteins associated with specific immune responses. CVL from Nairobi women

contained higher levels of proteins associated with apoptosis and general inflammation while women from Winnipeg expressed proteins associated with neutrophil, macrophage and T cell functioning as well as acute phase response proteins.

In order to evaluate the differential levels of antiproteases between the two cohorts, $_{LOG}2$ relative expressions were compared for all antiproteases identified through label-free mass spectrometry analysis. Many of the antiproteases examined were expressed at similar levels between the two cohorts, however, four of them were significantly differentially expressed. CVL from women in the Pumwani cohort over-expressed SLPI (Pumwani relative expression 0.3504, Winnipeg relative expression 0.1383, $p = 0.00180$) and Serpin C1 (Pumwani relative expression 0.6566, Winnipeg relative expression 0.4443, $p = 0.0495$) while Winnipeg women expressed higher levels of Serpin B5 (Winnipeg relative expression 0.3398, Pumwani relative expression 0.159, $p = 0.00673$) and angiotensinogen (Winnipeg relative expression 0.7494, Pumwani relative expression 0.4745, $p = 0.0173$) as illustrated in Figure 18. Of the antiproteases identified there were a select few that were unique to each cohort. Specifically, elafin and Serpin B12 were only detected within the CVL of women from the Pumwani cohort whereas Serpin B10 and Serpin F2 were observed only within the CVL of women from the Winnipeg cohort. Other proteins that are not generally categorized as antiproteases or proteases, yet exhibit functions similar to proteins within these groups were also expressed within the CVL of women from both cohorts as can be observed in Table 2. One such protein is Prostate-specific antigen (PSA) which is generally only found within semen and exhibits peptidase activity. Considering this protein is secreted by cells of the prostate gland, it is logical to conclude that with higher abundance of this protein found within the CVL of women from the Pumwani cohort, these women likely also had higher levels of semen mixed with their CVL compared to women from the Winnipeg cohort.

Overall these results suggest that women from areas of HIV low-exposure and high-exposure settings express unique proteins.

Table 2: Differentially expressed proteins in the CVL of women from the Pumwani and Winnipeg cohorts

Accession Number	Protein Name	Pumwani Average Log2 Rel. Exp.	Winnipeg Average Log2 Rel. Exp.	Difference	P value	Log Trans. P value	q value	Biological Function
OVER-EXPRESSED IN PUMWANI COHORT								
PAEP_HUMAN	Glycodelin	-0.6361	-4.5279	-3.8918	$3.83x10^{-16}$	15.4167	0.0002	Apoptotic process; Positive regulation of GM-CSF production; Positive regulation of IL-13 secretion; Positive regulation of IL-6 secretion; Transport
IGHG4_HUMAN	Ig gamma-4 chain C region	-0.5288	-1.9646	-1.4358	0.0006	3.2091	0.0012	Classical complement activation; Fc-gamma receptor signaling pathway involved in phagocytosis
KLK3_HUMAN	Prostate-specific antigen	-2.0338	-3.4150	-1.3813	0.0004	3.3688	0.0007	Tissue kallikrein-kinin cascade; Zymogen activation, peptidase activity
SLPI_HUMAN	Antileukoproteinase	-1.5675	-2.9350	-1.3675	0.0026	2.5777	0.0016	Enzyme binding; Peptidase inhibitor activity; Protein binding; Serine-type endopeptidase inhibitor activity
1433E_HUMAN	14-3-3 protein epsilon	-1.2898	-2.5593	-1.2695	0.0025	2.6082	0.0014	Intrinsic apoptotic signaling pathway; Regulation of cysteine-type endopeptidase activity involved in apoptotic process; Transcription initiation from RNA polymerase II promoter; Viral reproduction; MHC class II protein complex binding
PON1_HUMAN	Serum paraoxonase/ arylesterase 1	-1.1809	-2.3988	-1.2179	0.0141	1.8499	0.0038	Blood circulation; Cholesterol metabolic process
TPM3_HUMAN	Tropomyosin alpha-3 chain	-1.1441	-2.1971	-1.0531	0.0316	1.4999	0.0059	Cellular component movement; Muscle contraction; Muscle filament sliding
FLNB_HUMAN	Filamin-B	-0.5839	-1.6155	-1.0316	0.0061	2.2165	0.0028	Actin cytoskeleton organization; Cell differentiation; Cytokine-mediated signaling pathway;
PNPH_HUMAN	Purine nucleoside phosphorylase	-0.7959	-1.8025	-1.0066	0.0054	2.2704	0.0023	Apoptotic mitochondrial changes; IL-2 secretion; Negative regulation of T cell apoptotic process; Positive regulation of alpha-beta T cell differentiation; Positive regulation of B cell differentiation; Positive regulation of DNA repair; Positive regulation of T cell mediated cytotoxicity; Positive regulation of T cell proliferation
CO1A2_HUMAN	Collagen alpha-2(I) chain	-1.4323	-2.3198	-0.8875	0.0174	1.7584	0.0040	Blood coagulation; Blood vessel development; Leukocyte migration; Platelet activation; Receptor-mediated endocytosis; TGF-B receptor signaling pathway
EVPL_HUMAN	Envoplakin	-0.5498	-1.3638	-0.8139	0.0120	1.9210	0.0033	Keratinization; Keratinocyte differentiation; Peptide cross-linking
K1C16_HUMAN	Keratin, type I cytoskeletal 16	-0.3158	-1.0671	-0.7513	0.0141	1.8516	0.0035	Cell proliferation; Cytoskeleton organization; Establishment of skin barrier; inflammatory response; innate immune response;
CLCA4_HUMAN	Calcium-activated chloride channel regulator 4	-0.8027	-1.4694	-0.6666	0.0389	1.4105	0.0070	Cysteine-type peptidase activity; Metallopeptidase activity; Transmembrane transport

RAB2A_HUMAN	Ras-related protein Rab-2A	-0.7422	-1.4025	-0.6603	0.0486	1.3131	0.0075	ER to Golgi vesicle-mediated transport; Mitotic cell cycle; Rab protein signal transduction
GGCT_HUMAN	Gamma-glutamylcyclotransferase	-0.2637	-0.9124	-0.6486	0.0195	1.7099	0.0042	Release of cytochrome c from mitochondria; Small molecule metabolic process
PEPL_HUMAN	Periplakin	-0.5273	-1.1135	-0.5862	0.0262	1.5824	0.0052	keratinization
CLUS_HUMAN	Clusterin	-0.4482	-1.0145	-0.5663	0.0322	1.4926	0.0061	Blood coagulation; Classical complement pathway activation; Regulation of apoptotic process; Positive regulation of cell proliferation, NF-kB transcription factor activity, NO biosynthetic process and TNF production; Response to virus
OVER-EXPRESSED IN WINNIPEG COHORT								
KPYM_HUMAN	Keratin, type II cytoskeletal 6C	-0.7168	-0.2762	0.4406	0.0257	1.5902	0.0049	ATP biosynthetic process; Carbohydrate metabolic process; Glucose metabolic process; Programmed cell death
PPIA_HUMAN	Kallikrein-11	-0.9445	-0.4286	0.5159	0.0474	1.3241	0.0073	Blood coagulation; Entry into host cell; Leukocyte migration; Positive regulation of viral genome replication; Protein folding; Release of virus from host; RNA-dependent DNA replication; Uncoating of virus; Viral infectious cycle; Viral reproduction; Virion assembly
FIBA_HUMAN	Neutrophil gelatinase-associated lipocalin	-1.2619	-0.6321	0.6299	0.0271	1.5672	0.0054	Blood coagulation
RINI_HUMAN	Rho GDP-dissociation inhibitor 3	-1.2375	-0.6071	0.6304	0.0283	1.5489	0.0056	mRNA catabolic process; Negative regulation of catalytic activity
HSP7C_HUMAN	Kininogen-2	-0.9820	-0.3253	0.6566	0.0362	1.4417	0.0068	mRNA processing; Negative regulation of transcription, DNA-dependent; Positive regulation of nuclear mRNA splicing, via spliceosome; Viral reproduction
HBG1_HUMAN; HBG2_HUMAN	Ras-related protein Rab-1A	-1.4838	-0.7206	0.7632	0.0050	2.3007	0.0019	Reversibly binds oxygen and transports from the lungs to the peripheral tissues; Transports CO2 from tissues to lungs
ANGT_HUMAN	Apolipoprotein A-I	-1.1948	-0.4204	0.7744	0.0051	2.2907	0.0021	Serine-type endopeptidase inhibitor, Activation of NF-kB-inducing kinase activity; Cytokine secretion; ERK1 and ERK2 cascade; cell growth and cell proliferation; NO and ROS mediated signal transduction; Activation of JAK2 kinase activity, endothelial cell migration, extrinsic apoptotic signaling pathway, inflammatory response, MAPK cascade and DNA-dependent transcription
KLK10_HUMAN	Kallikrein-10	-1.4686	-0.6797	0.7888	0.0332	1.4784	0.0063	Serine-type endopeptidase activity
NGAL_HUMAN	Neutrophil gelatinase-associated lipocalin	-1.1904	-0.3926	0.7978	0.0207	1.6844	0.0045	Apoptotic process; Cellular response to IL-1; Cellular response to LPS; Cellular response to TNF; Extrinsic apoptotic signaling pathway in absence of ligand; Response to bacterium; Response to oxidative stress; Response to virus
GDIR2_HUMAN	Rho GDP-dissociation inhibitor 2	-1.3284	-0.5235	0.8049	0.0215	1.6677	0.0047	Actin cytoskeleton organization; Cellular response to redox state; immune response Rho protein signal transduction
KNG1_HUMAN	Kininogen-1	-1.3738	-0.5315	0.8422	0.0072	2.1438	0.0031	Cysteine-type endopeptidase inhibitor activity, negative

								regulation of intrinsic blood coagulation; Platelet activation; Platelet degranulation; Positive regulation of apoptotic process
RAB1A_HUMAN	Ras-related protein Rab-1A	-1.4680	-0.3968	1.0712	0.0006	3.2581	0.0009	Cell migration; Defense response to bacterium; Endocytosis; IL-8 secretion;Transport; Virion assembly
APOA1_HUMAN	Apolipoprotein A-I	-2.3694	-1.2441	1.1254	0.0057	2.2479	0.0026	Blood coagulation; endothelial cell migration and proliferation; Cholesterol metabolic process; ERK1 and ERK2 cascade; inflammatory response and IL-1B secretion; Protein oxidation; Protein stabilization; Receptor-mediated endocytosis; Vitamin transport
IF6_HUMAN	Eukaryotic translation initiation factor 6	-2.0866	-0.6017	1.4849	0.0003	3.5953	0.0005	Mature ribosome assembly; Translational initiation
K2C6C_HUMAN	Keratin, type II cytoskeletal 6C	-1.4442	0.1142	1.5584	0.0351	1.4548	0.0066	Intermediate filament cytoskeleton organization

Antiprotease activity

Protease activity

All biological functions were determined using IPA software (http://www.ingenuity.com) and Uniprot.org

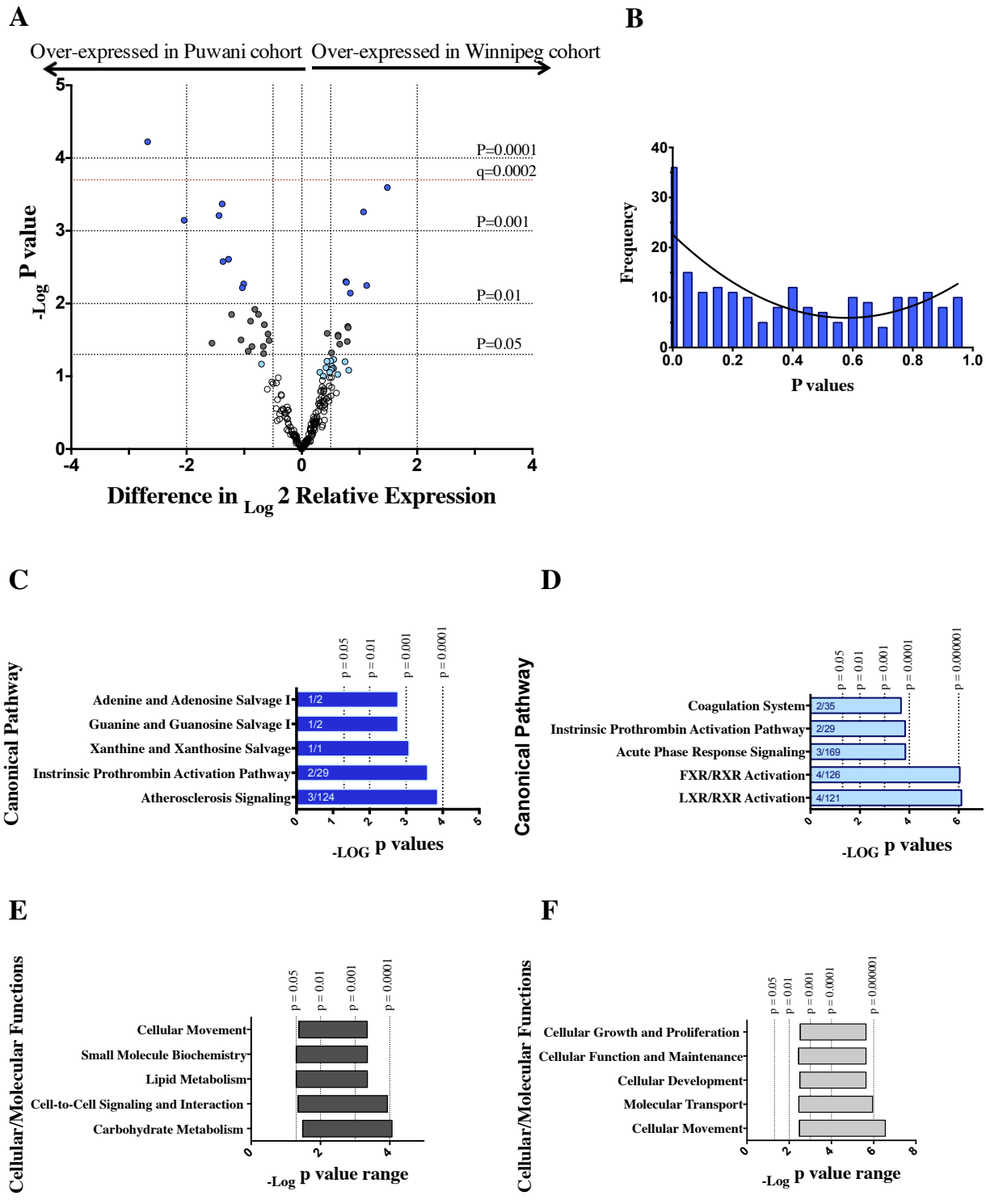

A
Over-expressed in Puwani cohort
Over-expressed in Winnipeg cohort
P=0.0001
q=0.0002
P=0.001
P=0.01
P=0.05
-Log P value
Difference in Log 2 Relative Expression
B
Frequency
P values
C
Canonical Pathway
Adenine and Adenosine Salvage I
Guanine and Guanosine Salvage I
Xanthine and Xanthosine Salvage
Instrinsic Prothrombin Activation Pathway
Atherosclerosis Signaling
1/2
1/2
1/1
2/29
3/124
p = 0.05
p = 0.01
p = 0.001
p = 0.0001
-LOG p values
D
Canonical Pathway
Coagulation System
Instrinsic Prothrombin Activation Pathway
Acute Phase Response Signaling
FXR/RXR Activation
LXR/RXR Activation
2/35
2/29
3/169
4/126
4/121
p = 0.05
p = 0.01
p = 0.001
p = 0.0001
p = 0.000001
-LOG p values
E
Cellular/Molecular Functions
Cellular Movement
Small Molecule Biochemistry
Lipid Metabolism
Cell-to-Cell Signaling and Interaction
Carbohydrate Metabolism
p = 0.05
p = 0.01
p = 0.001
p = 0.0001
-Log p value range
F
Cellular/Molecular Functions
Cellular Growth and Proliferation
Cellular Function and Maintenance
Cellular Development
Molecular Transport
Cellular Movement
p = 0.05
p = 0.01
p = 0.001
p = 0.0001
p = 0.000001
-Log p value range

Figure 17: CVL from women in the Winnipeg and Pumwani cohorts differentially express specific proteins

A: From the proteins identified through label-free mass spectrometry 213 were confidently identified to have a maximum covariance of 0.25. $_{LOG}2$ relative expression was determined through comparison of each sample's individual protein abundance to the average protein abundance across all samples. These $_{LOG}2$ relative expression values were compared between cohorts using multiple t-tests, without assuming consistent SD, alpha = 0.05. P values for each t-test were log transformed and plotted against the difference between $_{LOG}2$ relative expression values to create the volcano plot. B: Distribution of p-value frequencies for the 213 proteins. C: IPA canonical pathway analysis for the 17 up-regulated proteins identified within the CVL of women from the Pumwani cohort. D: IPA canonical pathway analysis for the 15 up-regulated proteins identified within the CVL of women from the Winnipeg cohort. E: Summary of the cellular and molecular functions, as determined by IPA software analysis, for the 17 up-regulated proteins identified within the CVL of women from the Pumwani cohort. F: Summary of the cellular and molecular functions, as determined by IPA software, for the 15 up-regulated proteins identified within the CVL of women from the Winnipeg cohort.

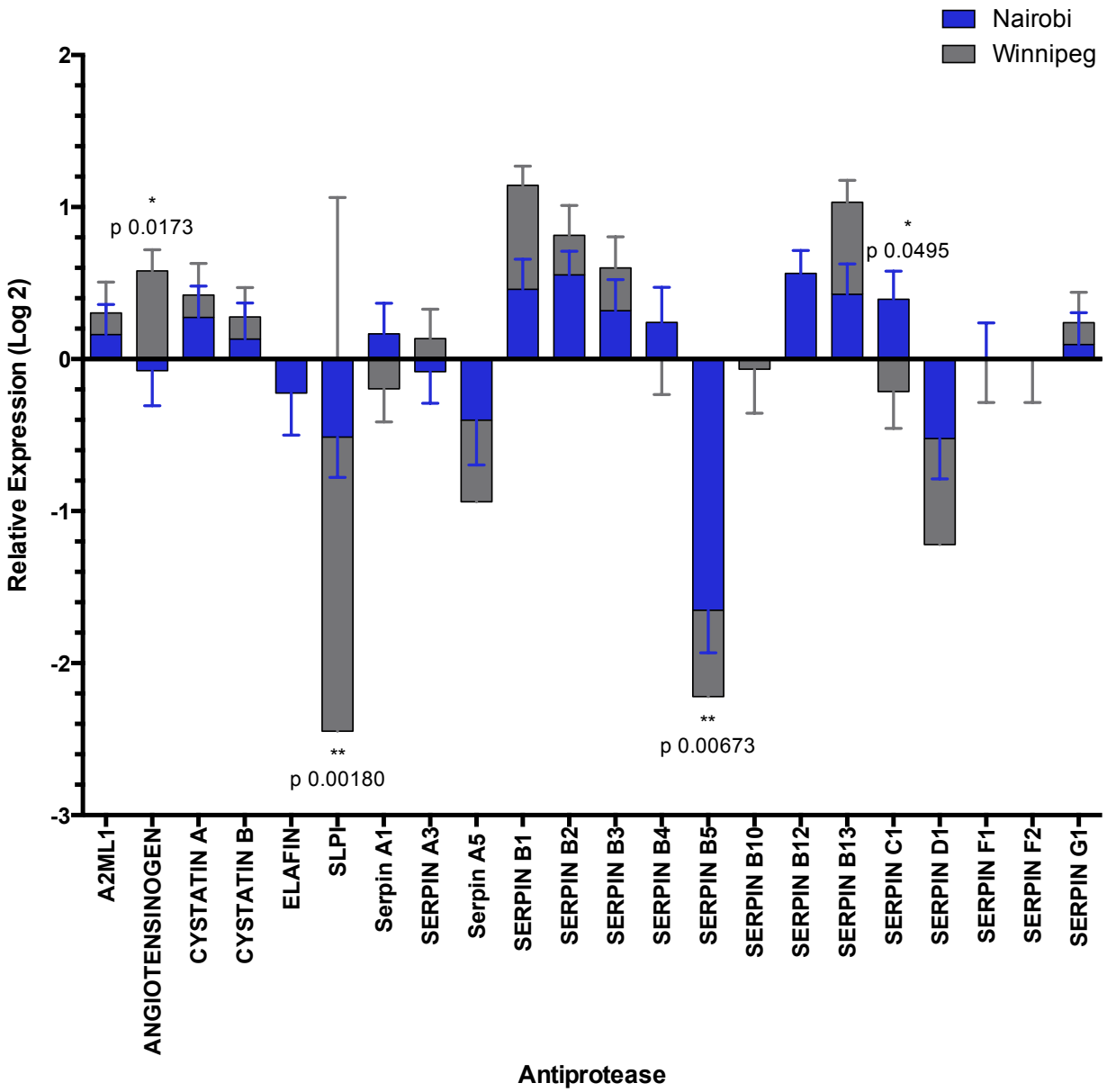


Figure 18: Differentially expressed antiproteases within CVL from women in the Pumwani and Winnipeg cohort

Antiproteases that were detected via label-free mass spectrometry were analyzed by comparison of $_{LOG}2$ relative expression between cohorts using multiple t-tests with an alpha of 0.05.

4.4.1.2 *Cluster analysis*

To determine the relatedness of the proteomes between CVL from Winnipeg and Pumwani women, Cluster analysis was performed on the 114 samples (56 Winnipeg and 58 Pumwani). The analysis was conducted using $_{LOG}2$ relative expressions for each protein within each sample. The resulting dendrogram (Figure 19) illustrates five primary clusters, each of which, save for Cluster 5, is made up of two sub-clusters. Of the 114 samples, approximately 61% (70/114) formed clusters based primarily on cohort (Cluster one Winnipeg, Cluster two Pumwani and Cluster five Pumwani). Interestingly, Clusters three and four contained nearly equal numbers of Winnipeg and Pumwani samples. However, the sub-clusters of cluster three were separated into two distinct groups based on cohort. Alternatively, Cluster four consisted of 24 samples, made up of CVL samples from both cohorts in approximately equal proportions (14 Winnipeg, 10 Pumwani) without any apparent sub-clustering based on cohort designation (Figure 19).

Aside from the specific cohort that each woman was a part of, numerous other factors may play a role in determining the clustering of women into descrete groups (Table 3). While multivariante analysis would be the optimal method for analyzing the effect that these additional epidemiological factors have on cluster patterns, general inferences can also be made by examining the distribution of each factor across the different clusters. For instance, the median age (48) for women in Cluster five, was much higher than that of women in any of the four other clusters (Median age: 32, 34, 34, 34.5, respectively). Thus, age may have played a role in determining the composition of the FGT proteome and thus in the distinct clustering of these ten samples. Also, menstrual cycle phase and contraceptive usage between Cluster two and five varied significantly, which may have contributed to the distinct clustering of these two groups,

both of which contained only samples from the Pumwani cohort. Within the women that comprised Cluster two a large proportion was either in the luteal phase (28.1%, 9/32) of their menstrual cycle or the actual phase was not able to be determined (62.5%, 20/32) due to lack of menstruation (11/20) caused by specific hormonal contraceptive methods (Depo-Provera, combination pill, or Mirena IUD), or due to lack of disclosure for LMP on the provided questionnaire (9/20). Conversely, within Cluster five, 50% (5/10) of the women had undergone menopause, 30% (3/10) were in the follicular phase of their cycle, and only one woman was in the luteal phase. Also, within the women whose samples comprised Cluster two, 15.6% (5/32) were taking an oral combination hormone contraceptive pill, 18.8% (6/32) were receiving Depo-Provera injections, 31.3% (10/32) were not using any hormone contraceptive but rather used condoms or no contraception, and 3.1% (1/32) either had a Mirena IUD or a tubal ligation procedure. Conversely, 70% (7/10) of the women, whose samples made up Cluster five, were using condoms or no form of contraception while the remaining three women either had a copper IUD, were on Depo-Provera or had a tubal ligation.

Clusters three and four were comprised of approximately equal numbers of Winnipeg and Pumwani women. The primary epidemiological factors that were noted to be significantly different between these two clusters consisted of the menstrual cycle and contraceptive methods. Specifically, 30% (6/20) of the women whose samples comprised Cluster three, were determined to be in the follicular phase of their menstrual cycle, while a similar proportion (37.5%, 9/24) of the women who made up Cluster four, were found to be in the luteal phase. Conversely, only 10% (2/20) of women from Cluster three were in luteal phase, and 12.5% (3/24) from Cluster four were in follicular. Additionally, a total of 35% of the women from Cluster three were taking some form of hormonal/invasive contraception (Mirena IUD copper IUD or Depo-

Provera) while 40% (8/20) were using condoms or no contraception. Within cluster four, 70.8% of the women were using condoms as their sole contraceptive method or no contraception, while only 12.5% were using any form of IUD (Table 3). Also, the overall inhibitory effect that the CVL samples had was significantly stronger (14.3% inhibition) within cluster four compared to cluster three (5.4% inhibition). This suggests that the proteins that caused the deviation in cluster determination between these two groups may be due to proteins that have specific anti-HIV activity. Since there were approximately equal proportions of Winnipeg and Pumwani women, the difference in inhibition is not likely due to the presence of more women within the cluster from Nairobi whose CVL samples were previously identified as having stronger anti-HIV activity.

The local environment (Winnipeg vs. Nairobi), the number of sex partners (sex worker vs. non-sex worker), exposure to HIV (high-prevalence vs. low-prevalence), age, menstrual cycle, contraceptive method, or genetic markers caused by ethnic ancestry, may all contribute to the distinct protein composition of the fluids within the FGT. However, there are also countless other factors that may be at play in determining how related the protein make-up of these fluids is amoung women in Winnipeg, Canada versus women in Nairobi, Kenya. Nevertheless, these results do suggest that women from HIV-low exposure settings, such as Winnipeg, Canada, have distinct protein profiles compared to women in high-exposure settings, such as commercial sex workers in Nairobi, Kenya. However, since these conclusions are largely based on qualitative analysis a more in-depth multivariant statistical analysis must be performed to confirm these findings.

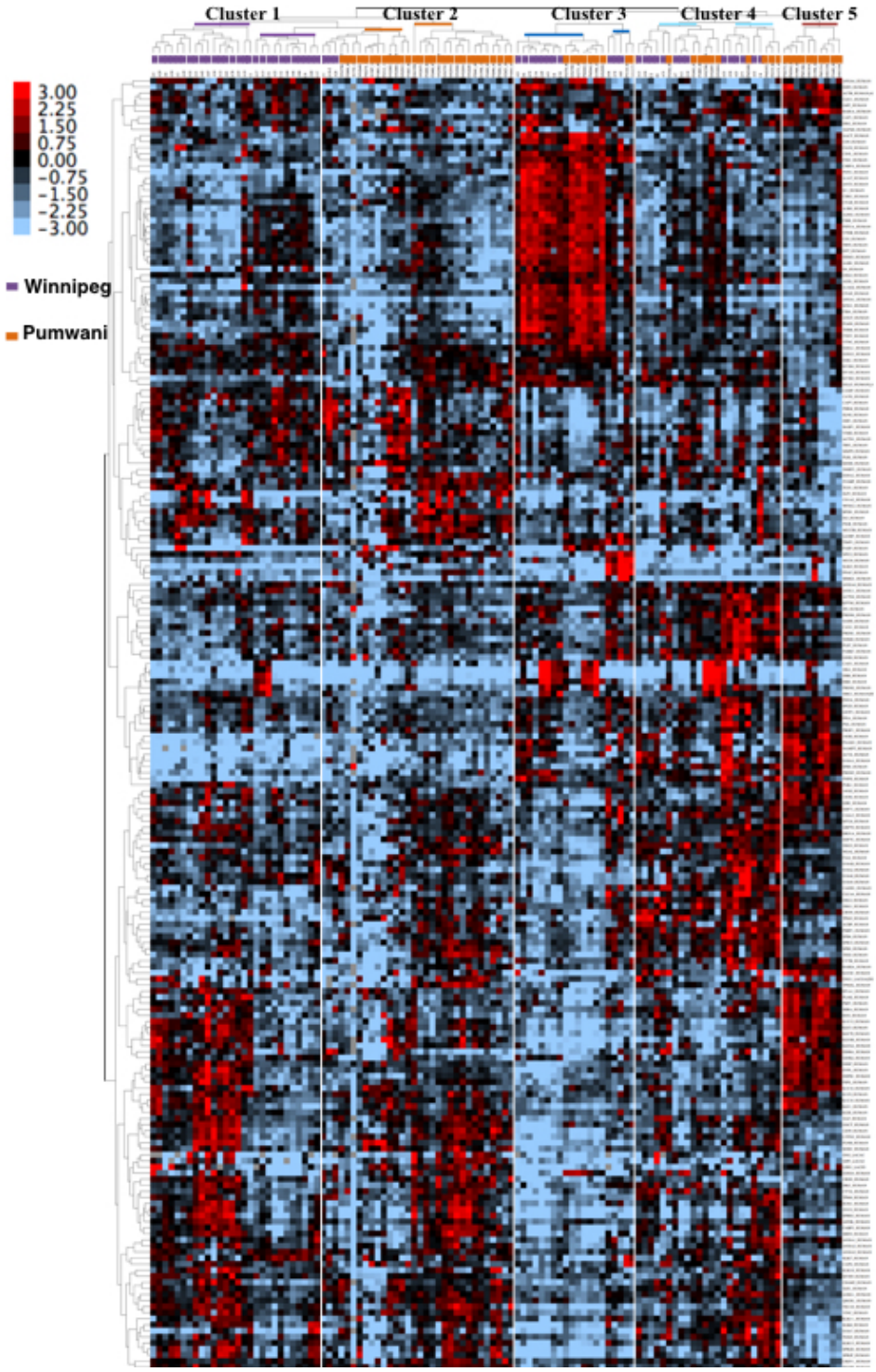


Figure 19: Dendrogram depicting protein cluster analysis of Winnipeg and Pumwani CVL samples

The relative expression of 213 proteins identified in the CVL of 114 women (58 from the Pumwani cohort, 56 from the Winnipeg cohort), via label-free mass spectrometry, were $_{LOG}2$ transformed and compared across samples for distinct cluster patterns.

Table 3: Epidemiological factors possibly affecting Winnipeg and Pumwani CVL cluster analysis

		Cluster 1			Cluster 2			Cluster 3			Cluster 4			Cluster 5
		Sub. 1	*Sub. 2*	*Total (%)*	*Sub. 1*	*Sub. 2*	*Total (%)*	*Sub. 1*	*Sub. 2*	*Total (%)*	*Sub. 1*	*Sub. 2*	*Total (%)*	*Sub. 1 (%)*
Cohort	***Winnipeg***	15	13	**28 (100)**	3	0	**3 (9.4)**	8	3	**11 (55.0)**	8	6	**14 (58.3)**	**0**
	Pumwani	0	0	**0**	8	21	**29 (90.6)**	7	2	**9 (45.0)**	6	4	**10 (41.7)**	**10 (100.0)**
Age	***1st Quartile***	25	22.5	**24.5**	30	30.5	**30**	26	30	**30**	23	27	**24**	**42**
	Median	35	32	**32**	35	33	**34**	34	31	**34**	34	39	**34.5**	**48**
	3rd Quartile	44	34	**37**	45	36.5	**38**	41	36.5	**40.5**	36	42	**40**	**52**
Menstrual Phase	***Pregnant***	0	0	**0**	0	1 (4.5)	**1 (3.1)**	0	0	**0**	2 (14.3)	0	**2 (8.3)**	**0**
	Post-Partum	0	5 (38.5)	**5 (17.9)**	0	0	**0**	3 (20.0)	0	**3 (15.0)**	1 (7.1)	1 (10.0)	**2 (8.3)**	**0**
	Follicular	3 (20.0)	2 (15.4)	**5 (17.9)**	1 (9.1)	0	**1 (3.1)**	5 (33.3)	1 (20)	**6 (30.0)**	3 (21.4)	0	**3 (12.5)**	**3 (30.0)**
	Ovulation	1 (6.7)	2 (15.4)	**3 (10.7)**	0	0	**0**	1 (6.7)	0	**1 (5.0)**	1 (7.1)	0	**1 (4.2)**	**1 (10.0)**
	Luteal	6 (40.0)	2 (15.4)	**8 (28.6)**	3 (27.3)	6 (28.6)	**9 (28.1)**	2 (13.3)	0	**2 (10.0)**	4 (28.6)	5 (50.0)	**9 (37.5)**	**1 (10.0)**
	Menopause	1 (6.7)	0	**1 (3.6)**	1 (9.1)	0	**1 (3.1)**	0	0	**0**	0	0	**0**	**5 (50.0)**
	Unknown	4 (26.7)	2 (15.4)	**6 (21.4)**	6 (54.5)	14 (66.7)	**20 (62.5)**	4 (26.7)	4 (80)	**8 (40.0)**	3 (21.4)	4 (40.0)	**7 (29.2)**	**0**
Contracep -tion	***Mirena***	8 (53.3)	0	**8 (28.6)**	1 (9.1)	0	**1 (3.1)**	1 (6.7)	3 (60)	**4 (20.0)**	0	2 (20.0)	**2 (8.3)**	**0**
	Copper IUD	1 (6.7)	2 (15.4)	**3 (10.7)**	0	0	**0**	2 (13.3)	0	**2 (10.0)**	1 (7.1)	0	**1 (4.2)**	**1 (10.0)**
	Combination Pill	1 (6.7)	1 (7.7)	**2 (7.1)**	2 (18.2)	3 (14.3)	**5 (15.6)**	0	0	**0**	0	0	**0**	**0**
	Depo-Provera	0	0	**0**	3 (27.3)	3 (14.3)	**6 (18.8)**	1 (6.7)	0	**1 (5.0)**	0	0	**0**	**1 (10.0)**
	Tubal Ligation	0	0	**0**	1 (9.1)	0	**1 (3.1)**	0	1 (20)	**1 (5.0)**	0	0	**0**	**1 (10.0)**
	None/ Condoms	4 (26.7)	10 (76.9)	**14 (50)**	4 (36.4)	6 (28.6)	**10 (31.3)**	8 (53.3)	0	**8 (40.0)**	11 (78.6)	6 (60.0)	**17 (70.8)**	**7 (70.0)**
	Unknown	0	0	**0**	0	9 (42.9)	**9 (28.1)**	3 (20.0)	1 (20)	**4 (20.0)**	2 (14.3)	2 (20.0)	**4 (16.7)**	**0**
% HIV Inhibition	***1st Quartile***	13.6	10.87	**13.4**	17.3	26.1	**24.7**	12.4	20	**13.68**	16	26.4	**22.6**	**25.5**
	Median	0.80	0.40	**0.07**	13.4	18.3	**16.3**	4.35	19.5	**5.35**	13.3	18.05	**14.3**	**18.285**
	3rd Quartile	-4.2	-3.40	**-3.9**	3.0	8.95	**3.60**	-0.80	12.6	**0.65**	7.6	13.6	**9.2**	**10.7**
Ethnic Ancestry	***European***	9 (60.0)	8 (61.5)	**17 (60.7)**	1 (9.1)	0	**1 (3.1)**	2 (13.3)	3 (60)	**5 (25.0)**	4 (28.6)	1 (10.0)	**5 (20.8)**	**0**
	Canadian First Nations	1 (6.7)	2 (15.4)	**3 (10.7)**	1 (9.1)	0	**1 (3.1)**	4 (26.7)	0	**4 (20.0)**	3 (21.4)	1 (10.0)	**4 (16.7)**	**0**
	African	1 (6.7)	1 (7.7)	**2 (7.1)**	8 (72.7)	21 (100)	**29 (90.6)**	9 (60.0)	2 (40)	**11 (55.0)**	7 (50.0)	6 (60.0)	**13 (54.2)**	**10 (100.0)**
	Asian	4 (26.7)	2 (15.4)	**6 (21.4)**	1 (9.1)	0	**1 (3.1)**	0	0	**0**	0	2 (20,0)	**2 (8.3)**	**0**

4.4.2 Proteins within CVL correlated with HIV infection

To determine if any of the proteins identified in the mass spectrometry analysis were associated with HIV inhibitory activity, Pearson correlation analysis was performed comparing percent inhibition and relative protein expression for the 114 CVL samples from both the Winnipeg and Pumwani cohorts. A total of 47 unique proteins were identified as being positively correlated with increased HIV neutralizing activity (Table 4), indicating that as their concentration increases, the degree of HIV inhibition level would also increase. Of these 47 proteins that were correlated with an increase in HIV neutralization, 39 were identified within the CVL of Winnipeg women and eight were identified from women within the Pumwani sex worker cohort. Alternatively, 28 proteins positively correlated with enhancement of HIV infection (Table 5), which suggests that as the concentration of these proteins increases within CVL the level of HIV replication would also increase in TZM-bl cell cultures. Of these 28 proteins, 17 were identified within the CVL of women from the Winnipeg cohort, and 11 from the Pumwani cohort.

No common correlated proteins were identified between the two cohorts. However, there were a select few proteins that had opposite effects on infection depending on the cohort in which it was identified. For example, neutrophil elastase and lysozyme C are both correlated with increased neutralization when present in CVL from women of the Pumwani cohort. However, these same proteins correlated with enhancement of infection in the CVL of women from the Winnipeg cohort indicating that neutralization is clearly multifactorial and that the combination of individual proteins in CVL must be considered.

Numerous antiproteases were correlated with inhibition of HIV infection, the majority of which were only identified in the CVL of Winnipeg women. Leukocyte elastase inhibitor (Serpin B1) was the only antiprotease identified as associated with HIV neutralization within the Pumwani cohort. Alternatively, Serpin A5, B2, B3, B4, B5, B13, Cystatin B and A2ML1 were

all postively correlated with HIV neutralization within the CVL from women in the Winnipeg cohort. Serpin B2 was the most strongly correlated antiprotease with an r value of -0.4222. Thus, antiproteases appear to offer varying degress of protection against HIV depending on the geographical location, and level of exposure to HIV that individual women are exposed to.

Identifying anti-HIV factors within the FGT secretions of a wide-range of women provides the opportunity to discover potential novel candidates for future microbicides. This analysis provides a comprehensive list of potential anti-HIV candidates, however, it also, importantly, provides information on the proteins that associate with enhanced HIV infection and as such need to be considered when designing novel microbicides for the prevention of HIV acquisition in women.

Table 4: Proteins within CVL positively correlated with increased HIV inhibition

Accession Number	Protein Name	Biological Function	r value	p value	Cohort	# unique peptides
EFTU_LACAC	Elongation factor Tu OS=Lactobacillus acidophilus	Promotes GTP-dependent binding of aminoacyl-tRNA to A-site of ribosomes	-0.4952	0.0001	Winnipeg	7
PAI2_HUMAN	Plasminogen activator inhibitor 2 (Serpin B2)	Peptidase inhibitor activity, serine-type endopeptidase inhibitor	-0.4222	0.0012	Winnipeg	4
IL1RA_HUMAN	Interleukin-1 receptor antagonist protein	Acute phase response, cytokine activity, negative regulator of apoptosis, blood coagulation	-0.4011	0.0022	Winnipeg	6
PEBP1_HUMAN	Phosphatidylethanolamine-binding protein 1	ATP binding, MAPK cascade, kinase binding	-0.3954	0.0026	Winnipeg	10
SPB13_HUMAN	Serpin B13	Cysteine-type endopeptidase inhibitor activity	-0.3932	0.0027	Winnipeg	20
LYSC_HUMAN	Lysozyme C	Catalytic activity, apoptosis	-0.3929	0.0025	Pumwani	8
SPB4_HUMAN	Serpin B4	Serine-type endopeptidase inhibitor, protection from NK cell cytotoxicity	-0.3856	0.0033	Winnipeg	21
AATC_HUMAN	Aspartate aminotransferase, cytoplasmic	Catalytic activity, aminotransferase activity, decarboxylase activity	-0.381	0.0038	Winnipeg	5
A2ML1_HUMAN	Alpha-2-macroglobulin-like protein 1	Peptidase inhibitor activity, serine-type endopeptidase inhibitor	-0.3792	0.0039	Winnipeg	48
CH60_LACGA	60 kDa chaperonin OS=Lactobacillus gasseri	ATP binding, protein refolding	-0.3721	0.0044	Pumwani	2
PERM_HUMAN	Myeloperoxidase	Negative regulation of apoptosis, response to oxidative stress,	-0.356	0.0066	Pumwani	22
ICAL_HUMAN	Calpastatin	Calcium-dependent cysteine-type endopeptidase	-0.3548	0.0073	Winnipeg	12
PRDX6_HUMAN	Peroxiredoxin-6	H2O2 catabolic process, catalytic activity, antioxidant activity	-0.3545	0.0073	Winnipeg	12
SPB5_HUMAN	Serpin B5	Serine-type endopeptidase inhibitor activity, regulation of epothelial cell proliferation, extracellular matrix organization	-0.3527	0.0077	Winnipeg	12
CALL5_HUMAN	Calmodulin-like protein 5	Calcium ion binding, metal ion binding	-0.352	0.0078	Winnipeg	12
LEG3_HUMAN	Galectin-3	Cell differentiation, Macrophage/monocyte chemotaxis, negative regulation of apoptosis	-0.3514	0.0079	Winnipeg	3
SPB3_HUMAN	Serpin B3	Cysteine-type and serine-type endopeptidase inhibitor activity, viral receptor activity/regulate viral entry into host cell, regulation of cell migration	-0.3496	0.0083	Winnipeg	34
ACBP_HUMAN	Acyl-CoA-binding protein	Acyl-CoA metabolic binding	-0.3448	0.0093	Winnipeg	6
TERA_HUMAN	Transitional endoplasmic reticulum ATPase	Activation of cystein-type endopeptidase activity involved in apoptotic process, DNA repair	-0.3432	0.0096	Winnipeg	5
CYTB_HUMAN	Cystatin-B	Cysteine-type endopeptidase inhibitor activity	-0.3401	0.0103	Winnipeg	9
G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	Apoptotic process, cellular response to IFN-γ, gluconeogenesis	-0.3337	0.0119	Winnipeg	13
ELNE_HUMAN	Neutrophil elastase	Acute inflammatory response, endopeptidase	-0.3274	0.0129	Pumwani	6
K2C6C_HUMAN	Keratin, type II cytoskeletal 6C	Protein binding, structural molecule	-0.3255	0.0144	Winnipeg	31
RL12_HUMAN	60S ribosomal protein L12	RNA binding, protein binding, structural component of ribosomes	-0.3251	0.0145	Winnipeg	2
EZRI_HUMAN	Ezrin	Actin binding/reorganization	-0.3238	0.0149	Winnipeg	8
TAGL2_HUMAN	Transgelin-2	Actin filament binding, protein binding, epithelial cell differentiation	-0.3192	0.0165	Winnipeg	3

Accession Number	Protein Name	Biological Function	r value	p value	Cohort	# unique peptides
RAB1A_HUMAN	Ras-related protein Rab-2A f	GTPase activity, IL-8 secretion	-0.3147	0.0182	Winnipeg	2
UB2V1_HUMAN	Ubiquitin-conjugating enzyme E2 variant 1	Transcription regulation, MyD88-dependent toll-like receptor signaling pathway, positive regulation of NF-kB transcription factor activity	-0.3071	0.0213	Winnipeg	3
CAN1_HUMAN	Calpain-1 catalytic subunit	Calcium-dependent cysteine-type endopeptidase	-0.3034	0.023	Winnipeg	8
CAZA1_HUMAN	F-actin-capping protein subunit alpha-1	Actin filament capping	-0.2972	0.0261	Winnipeg	3
TBB4B_HUMAN	Tubulin beta-4B chain	GTPase activity, GTP binding, de novo posttranslational protein folding, mitotic cell division, NK cell mediated cytotoxicity, double-stranded RNA binding, MHC I protein binding	-0.2897	0.0303	Winnipeg	5
PEPC_HUMAN	Gastricsin	Aspartic-type endopeptidase activity, hydrolase activity	-0.2888	0.0309	Winnipeg	2
IPSP_HUMAN	Plasma serine protease inhibitor (Serpin A5)	Serine-type endopeptidase inhibitor, peptidase inhibitor, heparin binding/regulation of blood coagulation	-0.2856	0.0328	Winnipeg	3
LYSC_HUMAN	Macrophage migration inhibitory factor	Lysozyme C precursor, catalytic activity	-0.2848	0.0334	Winnipeg	5
COF1_HUMAN	Cofilin-1	Actin binding	-0.2793	0.0371	Winnipeg	4
PRDX1_HUMAN	Peroxiredoxin-1	Antioxidant activity, heme binding, oxidoreductase activity	-0.2787	0.0376	Winnipeg	9
THIO_HUMAN	Thioredoxin	Oxidoreductase activity, antioxidant action on Vitamin C	-0.275	0.0402	Winnipeg	5
PGRP2_HUMAN	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	Hydrolase activity, metal ion binding	-0.2733	0.0415	Winnipeg	2
ILEU_HUMAN	Leukocyte elastase inhibitor (Serpin B1)	Peptidase inhibitor activity, serine-type endopeptidase inhibitor	-0.2729	0.0382	Pumwani	18
PSA6_HUMAN	Proteasome subunit alpha type-6	Endopeptidase activity, NK-kB binding, RNA binding	-0.2707	0.0436	Winnipeg	3
DSC2_HUMAN	Desmocollin-2	Calcium ion binding, metal ion binding	-0.2707	0.0436	Winnipeg	5
TBA1A_HUMAN	Tubulin alpha-1A chain OS=Homo sapiens	GTPase activity, GTP binding, de novo posttranslational protein folding, mitotic cell division	-0.2706	0.0437	Winnipeg	2
CAPG_HUMAN	Malate dehydrogenase, cytoplasmic	Macrophage-capping protein	-0.267	0.0466	Winnipeg	3
TALDO_HUMAN	Transaldolase	Carbohydrate binding, catalytic activity	-0.2663	0.0452	Pumwani	11
CAP7_HUMAN	Azurocidin	Heparin binding, serine-type endopeptidase activity, toxin binding	-0.266	0.0455	Pumwani	3
HOP_HUMAN	Homeodomain-only protein	DNA binding	-0.2651	0.0483	Winnipeg	2
TPRG1_HUMAN	Tumor protein p63-regulated gene 1 protein	Unknown	-0.265	0.0464	Pumwani	2

*Parametric Pearson correlations performed, two-tailed, with a 95% confidence interval
All biological functions were determined using IPA software

Table 5: Proteins within CVL correlated with decreased HIV inhibition

Accession Number	Protein Name	r value	p value	Biological Function	Cohort	# unique peptides
BASP1_HUMAN	Brain acid soluble protein 1	0.3913	0.0029	Transciption regulator	Winnipeg	7
PP2AA_HUMAN	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform	0.3758	0.004	Enzyme binding, regulation of apoptotic pathways	Pumwani	3
1433B_HUMAN	14-3-3 protein beta/alpha	0.3754	0.0044	Adapter protein, binds phosphoserine and phosphothreonine motifs	Winnipeg	5
COR1A_HUMAN	Coronin-1A	0.3747	0.0044	Actin organization, cell migration	Winnipeg	6
SH3L3_HUMAN	SH3 domain-binding glutamic acid-rich-like protein 3	0.3733	0.0042	GTPase activator, regulation of cellular redox response	Pumwani	5
AMBP_HUMAN	Protein AMBP	0.3656	0.0052	Acute phase response, serine protease inhibitor	Pumwani	4
RET4_HUMAN	Rho GDP-dissociation inhibitor 2	0.3588	0.0066	Transporter	Winnipeg	3
ELNE_HUMAN	Neutrophil elastase	0.3584	0.0067	Acute inflammatory response	Winnipeg	4
MMP8_HUMAN	Neutrophil defensin 1	0.3512	0.008	Metalloendopeptidase activity, serine-type endopeptidase activity	Winnipeg	6
GSTM1_HUMAN	Glutathione S-transferase Mu 1	0.3505	0.0075	Cell detox. of nitrogen compounds, cellular metabolism	Pumwani	2
AFAM_HUMAN	Afamin	0.3329	0.0122	Vitamin transport	Winnipeg	6
LV106_HUMAN	Ig lambda chain V-I region WAH	0.3279	0.0128	Antigen binding	Pumwani	3
BPI_HUMAN	Bactericidal permeability-increasing protein	0.3199	0.0163	Lipid and LPS binding/transporter	Winnipeg	2
FCGBP_HUMAN	IgGFc-binding protein	0.3137	0.0186	Protein binding	Winnipeg	65
CO5_HUMAN	Complement C5	0.3114	0.0195	Acute phase response	Winnipeg	6
K1C16_HUMAN	Keratin, type I cytoskeletal 16	0.3091	0.0193	Protein binding, structural molecule	Pumwani	50
K1C12_HUMAN	Keratin, type I cytoskeletal 12	0.3061	0.0206	Structural molecule	Pumwani	26
GPX3_HUMAN	Glutathione peroxidase 3	0.3012	0.0228	Response to oxidative stress	Pumwani	3
APOA4_HUMAN	Apolipoprotein A-IV	0.2983	0.0256	Lipid/cholesterol transporter, antioxidant enzyme	Winnipeg	16
HRG_HUMAN	Histidine-rich glycoprotein	0.2982	0.0256	Acute phase response, cysteine-type endopeptidase inhibitor activity	Winnipeg	4
LYNX1_HUMAN	Ly-6/neurotoxin-like protein 1	0.2902	0.0286	Acetylcholine receptor inhibitor	Pumwani	2
CAMP_HUMAN	Cathelicidin antimicrobial peptide	0.2855	0.0329	Mucosal innate immune response	Winnipeg	5
TTHY_HUMAN	Transthyretin	0.2831	0.0345	Acute phase response, extracellular matrix organization	Winnipeg	3
VIME_HUMAN	Vimentin	0.279	0.0373	Double-stranded RNA binding, glycoprotein binding, apoptosis regulation	Winnipeg	13
PGK1_HUMAN	Phosphoglycerate kinase 1	0.2774	0.0367	ATP binding, carbohydrate metabolism	Pumwani	14
APOH_HUMAN	Beta-2-glycoprotein 1	0.2754	0.0381	Acute phase response, glycoprotein binding	Pumwani	6
ACTN1_HUMAN	Alpha-actinin-1	0.2676	0.0461	Actin crosslink formation, blood coagulation	Winnipeg	12
LYSC_HUMAN	Lysozyme C	0.2626	0.0506	Catalytic activity, apoptosis	Winnipeg	5

*Two-tailed, parametric Pearson correlations were performed with a 95% confidence interval
All biological functions were determined using IPA software

4.5 Summary

Several proteins in CVL were differentially expressed between women from the Pumwani commercial sex worker cohort, and women from the HIV low-exposure Winnipeg cohort, specifically those related to immune response. CVL from Pumwani women contained a higher abundance of proteins that are associated with increased levels of apoptosis and complement activation, while women from Winnipeg expressed higher levels of proteins associated with neutrophil recruitment and release of cytotoxic granules from CTLs and NK cells, as well as an increase in proteins associated with the acute phase response.

The expression of antiproteases between these two cohorts was relatively similar. However, women from the Pumwani cohort expressed higher levels of SLPI and Serpin C1 while women from the Winnipeg cohort expressed higher levels of angiotensinogen and Serpin B5. However, antiproteases were identified within CVL that were unique to each study group: Serpin B12 and Elafin were only detected within the CVL of Pumwani cohort participants while Serpin B10 and Serpin F2 were only detected within CVL of Winnipeg cohort women. This suggests that women from different geographical locations express partially unique antiprotease profiles. Furthermore, following cluster analysis of the common proteins identified within these two groups, through label-free mass spectrometry, five distinct clusters were observed. Nearly two—thirds of the 114 samples clustered based on cohort alone while the remaining cluster patterns may have been affected by specific epidemiological factors including age, contraceptive method, and menstrual phase.

Numerous CVL proteins, were determined to be correlated with HIV neutralization. Of the proteins determined to be correlated with inhibition or enhancement of HIV infection, 83%, and 61%, respectively, were identified only within the CVL of women from the Winnipeg cohort. Also, within the CVL of Winnipeg women all antiproteases, save for one, correlated with decreased levels of HIV infection. Conversely, while these same antiproteases were

detected within the CVL of Pumwani women they did not correlate with inhibition. From the antiproteases that correlated with inhibition of HIV, Serpin B2, B13 and B4 exhibited the strongest correlations suggesting that any of these antiproteases may be important in HIV neutralizing activity.

Determining CVL proteins that are capable of exhibiting inhibitory effects on HIV is a novel approach for identifying potential candidates for future microbicides. Work in this chapter identified prospective candidates while also providing insight into the mucosal FGT proteomic profiles of women who reside in HIV low-prevalence areas (Winnipeg) compared to women in high-endemic areas (Nairobi).

CHAPTER 5: SCREENING ANTIPROTEASES FOR HIV-NEUTRALIZATION CAPACITY

5.1 Rationale

Specific antiproteases have been identified as up-regulated within the CVL of HESN women compared to HIV-susceptible women[219,235]. Members of the serpin and cystatin families were among these overabundant antiproteases[235]. These proteins exhibit highly immunomodulatory mechanisms, specifically through regulation of their primary targets (serine and cysteine proteases), which function to protect from overstimulation of immune cells and harmful tissue damage[297,298,315,323,327,340,343,348]. Aside from regulating the overall immune response at sites of infection, specific members of the serpin family, namely Serpin A1, A3, and C1 have been studied in the context of HIV-1 infection and have demonstrated neutralizing effects *in vitro*. These neutralizing effects include mitigation of HIV-1 target cell migration, down-regulation of pro-inflammatory cytokines, as well as through direct anti-HIV-1 mechanisms including blocking of HIV binding and fusion[236,297,314,315,365].

With the described immune regulatory and HIV-inhibitory roles of Serpin A1, A3, and C1, and Cystatins A and B it is reasonable to postulate that other members of these families are capable of successfully inhibiting HIV-1 infection, either through indirect cellular mechanisms or through direct interference with particular stages of the HIV-1 lifecycle.

5.2 Hypothesis

Serpin A1, A3, B1, B13, C1, Cystatin A, and B and A2ML1, all which been shown to be over-expressed within the CVL of HESN women, are capable of neutralizing HIV-1 infection of TZM-bl cells and PBMCs.

5.3 Objectives

- Determine the level of cytotoxicity exhibited by physiological levels of antiproteases known to be up-regulated in the CVL of HESN women (Serpin A1, A3, C1, B1, B13, Cystatin A, and B and A2ML1).

- Determine if any of the antiproteases above exhibit anti-HIV activity when added exogenously to a TZM-bl and PBMC cell culture infected with R5-tropic, HIV-1 Bal or X4-tropic, HIV-1 IIIB.

5.4 Results

5.4.1 Cytotoxicity assays

It was important to determine the level of cytotoxic effects caused by each antiprotease; this insured that any observed inhibitory effects in the neutralization assays were in fact due to inhibition of the virus and not merely cell death. Following three and five-day incubation of Serpin A1, A3, B1, C1, B13, Cystatin A, Cystatin B, or A2ML1 individually with TZM-bl or PBMCs respectively, percent viability of cells was determined through the use of Cell Titer-Glo Luminescent Cell Viability Assay (measuring ATP production in culture) or via Trypan blue staining, respectively. No cytotoxicity was observed for Serpin A1, A3, C1, Cystatin A, Cystatin B, or A2ML1 in any of the cell cultures (cytotoxicity < 10%, p-value > 0.05). However, Serpin B13 and Serpin B1 were shown to cause significant cytotoxicity to TZM-bl cells. At the highest concentration, 30μg/mL, Serpin B13 exhibited approximately 24% cytotoxicity to TZM-bl cells ($p = 0.0166$). High concentrations (30μg/mL) of Serpin B1 also exhibited significant cytotoxic effects (14%) within this cell line, ($p = 0.0326$) (Figure 20A).

Cytotoxicity assays were performed in PBMCs using lower concentrations of each antiprotease, with 10μg/mL as the highest concentration. At these concentrations, there were no cytotoxic effects exhibited by any of the antiproteases (Figure 20B), with 13% as the highest level exhibited by Serpin B13 and Cystatin A, however, this did not reach statistical significance.

It can, therefore, be concluded that treatment with Serpin A1, A3, C1, Cystatin B and A2ML1 did not cause any significant cytotoxic effects in TZM-bl cells nor PBMCs. The

concentrations used in these assays can, thus, be employed in testing for the HIV neutralzing capacity of these antiproteases. However in PBMC cultures, Cystatin A and Serpin B13 would need to elicit a neutralizing effect greater than 13% for significance to be achieved. Similarly, within TZM-bl cell cultures inhibitory levels greater than 24% (Serpin B13) and 14% (Serpin B1) would have to be achieved before it could be concluded that HIV neutralization occurred.

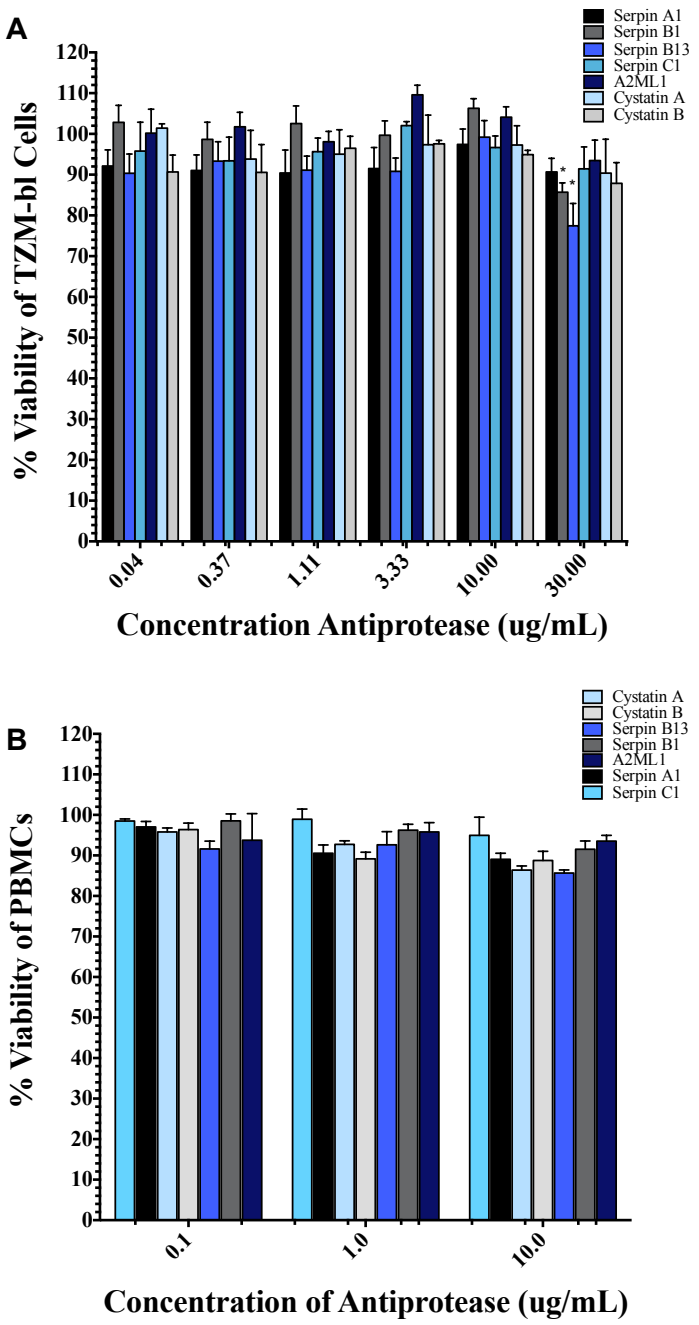


Figure 20: Determination of Serpin A1, B1, C1, B13, Cystatin A, Cystatin B, and A2ML1 cytotoxicity in TZM-bl cells and PBMCs

A: Cell Titer-Glo Luminescent Cell Viability assays were performed on TZM-bl cells with 30μg/mL as the highest tested concentration. Three independent assays were performed with three technical replicates each. Following testing for normality (D'Agostino and Pearson Omnibus K2 test), student t-tests with an alpha of 0.05 were performed comparing experimental cytotoxicity to cell controls. B: Trypan blue cytotoxicity staining was carried out in PBMC cultures with 10μg/mL as the highest tested concentration. Assays were performed twice with two technical replicates each.

5.4.2 Determining the neutralization capacity of antiproteases

The ability of antiproteases to inhibit HIV-1 was evaluated in TZM-bl cell culture assays. Those antiproteases that most actively neutralized HIV-1 within the TZM-bl assays were further validated in PBMC assays.

5.4.2.1 TZM-bl cell cultures

Serpin A1, A3, B1, C1, B13, Cystatin A, Cystatin B and A2ML1, were all screened at high concentrations varying from 30-50μg/mL in three day TZM-bl HIV-1 neutralization assays. For those that were used at concentrations above 30μg/mL, as was presented in the previous cytotoxic assays, additional assays were performed at the increased concentrations, however, no increased cytotoxicity was observed and, thus, results are not presented again here. These proteins were commercially produced by Genscript (New Jersey, USA) and were 80-95% pure. Analysis of the results from these neutralization assays revealed that three of the antiproteases, namely Cystatin A, Serpin B1 and Serpin C1, exhibited significant neutralization capacity of both the HIV-1 Bal and IIIB viruses.

Cystatin A was shown to significantly inhibit HIV at concentrations of 40μg/mL with both HIV-1 Bal (49%, $p = 0.0142$) and IIIB (62.1%, $p = 0.0094$) (Figure 21A & B). In addition, Cystatin A inhibited HIV-1 Bal at 13.33ug/mL by 35% ($p = 0.0093$) (Figure 21A). However, inhibition was not achieved with HIV-1 IIIB at concentrations of 0.055μg/mL to 13.33μg/mL.

At concentrations as low as 3.33μg/mL, Serpin C1 significantly inhibited HIV-1 Bal by 33.8% ($p = 0.0406$). Similarly, at 10μg/mL Bal was inhibited by 52.3% ($p = 0.0069$), which remained relatively constant up to a concentration of 30μg/mL (52.5%, $p = 0.0061$) (Figure 21C). Treatment with Serpin C1 also inhibited HIV-1 IIIB at a concentration of 30μg/mL (72.1%, $p = 0.001$), however, at concentrations of 0.412μg/mL to 10μg/mL no significant inhibition of IIIB was observed (Figure 21D).

Serpin B1 neutralized HIV-1 Bal by approximately 36.6% (35-38.3%) at concentrations of 15.63μg/mL ($p = 0.0002$), 31.35μg/mL ($p = 0.0006$) and 50μg/mL ($p = 0.0001$) (Figure 21E). This antiprotease's effect in culture with IIIB was more modest with neutralization reaching, and remaining steady at, approximately 15% when added at concentrations between 15-50μg/mL. However, significant inhibition was only achieved at concentrations of 15.63μg/mL ($p = 0.0193$) and 31.35μg/mL ($p = 0.0189$) (Figure 21F).

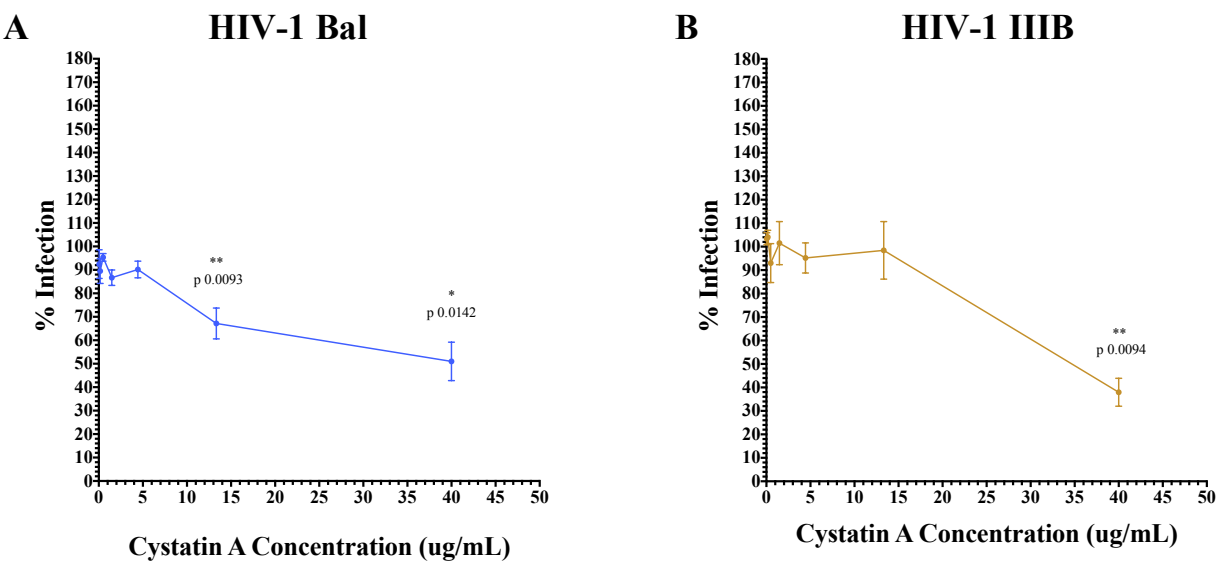
Three antiproteases (A2ML1, Cystatin B, and Serpin B13) inhibited only one of the two viruses. A2ML1 neutralized HIV-1 Bal by 39% ($p = 0.0316$) at the highest concentration tested (30μg/mL), yet did not significantly inhibit infection at any concentration ranging from 0.412μg/mL to 10μg/mL. Furthermore, A2ML1 did not inhibit IIIB at any concentration examined (Figure 22A & B).

Cystatin B exhibited significant anti-HIV activity against HIV-1 Bal at 40μg/mL (45%, $p = 0.0168$), however, did not demonstrate any inhibitory capacity with this virus at concentrations of 0.055μg/mL to 13.33μg/mL nor at any of the tested concentrations for HIV-1 IIIB (Figure 22C & D).

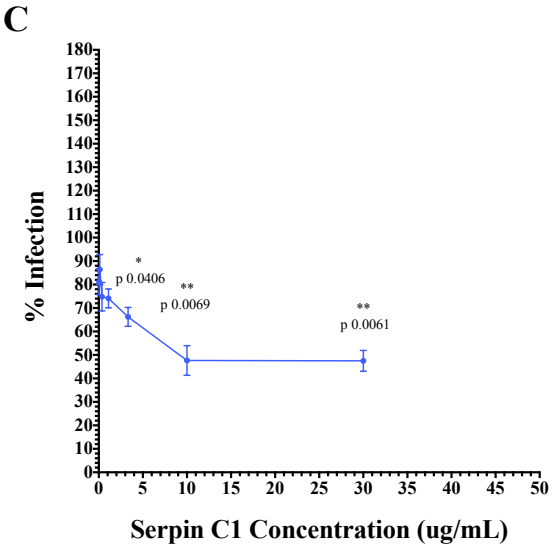
Serpin B13 did not significantly inhibit HIV-1 Bal at any of the concentrations examined (0.412μg/mL to 30μg/mL) yet only inhibited HIV-1 IIIB by 27% ($p = 0.0447$) at a concentration of 30 μg/mL (Figure 22E & F).

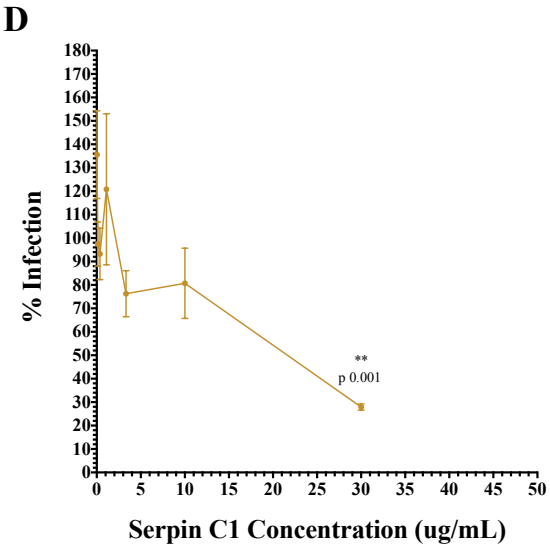
Serpin A1 and Serpin A3 did not significantly neutralize HIV-1 Bal or IIIB infections at any concentration (Figure 23A, B, C, D).

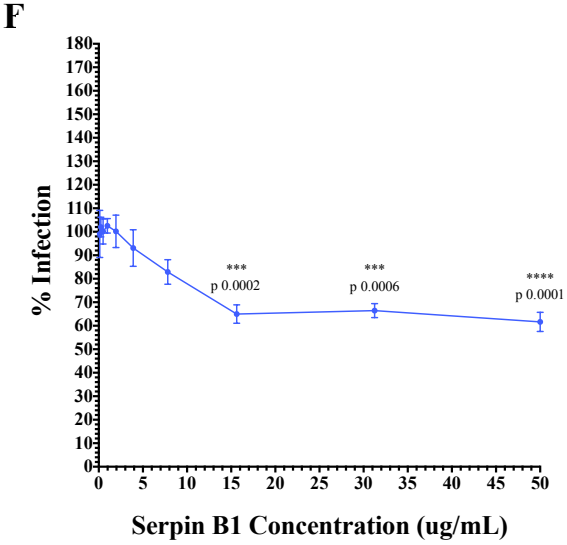
These results suggest that Serpin B1, C1, and Cystatin A had the strongest neutralization profiles in TZM-bl cell assays, and thus are the most promising potential candidates to move forward with for mechanistic studies, however, these results must first be confirmed in another cell system (PBMC).


A
HIV-1 Bal
% Infection
Cystatin A Concentration (ug/mL)
** p 0.0093
* p 0.0142

B
HIV-1 IIIB
% Infection
Cystatin A Concentration (ug/mL)
** p 0.0094


C
% Infection
Serpin C1 Concentration (ug/mL)
* p 0.0406
** p 0.0069
** p 0.0061


D
% Infection
Serpin C1 Concentration (ug/mL)
** p 0.001


F
% Infection
Serpin B1 Concentration (ug/mL)
*** p 0.0002
*** p 0.0006
**** p 0.0001

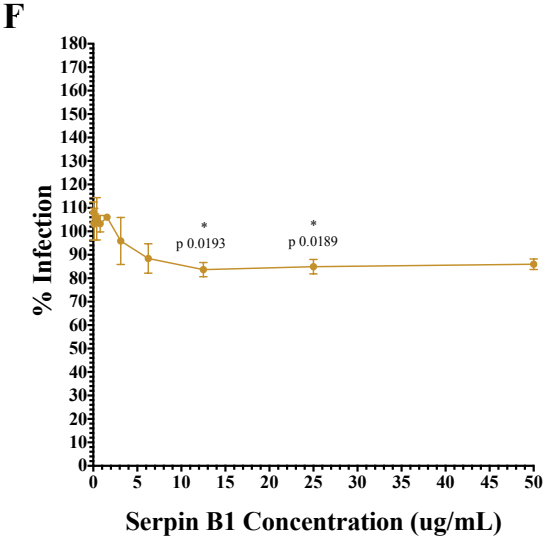

F
% Infection
Serpin B1 Concentration (ug/mL)
* p 0.0193
* p 0.0189

Figure 21: TZM-bl HIV-1 neutralization assays with Cystatin A, Serpin C1, and Serpin B1
Concentrations, ranging from 0.04μg/mL - 50μg/mL, of Cystatin A, Serpin C1 and Serpin B1 were tested in a TZM-bl neutralization assay. Each point represents the mean ± the SEM of three replicate experiments. D'Agostino and Pearson Omnibus K2 Normality test was performed to confirm Gaussian distribution, followed by unpaired student t-tests with Welch's correction (did not assume equal SDs) with an alpha of 0.05, between virus control and experimental conditions. A: Cystatin A (0.055μg/mL - 40μg/mL) and HIV-1 Bal. B: Cystatin A (0.055μg/mL - 40μg/mL) and HIV-1 IIIB. C: Serpin C1 (0.04μg/mL - 30μg/mL) and HIV-1 Bal. D: Serpin C1 (0.04μg/mL - 30μg/mL) and HIV-1 IIIB. E: Serpin B1 (0.012μg/mL - 50μg/mL) and HIV-1 Bal. F: Serpin B1 (0.012μg/mL - 50μg/mL) and HIV-1 IIIB.

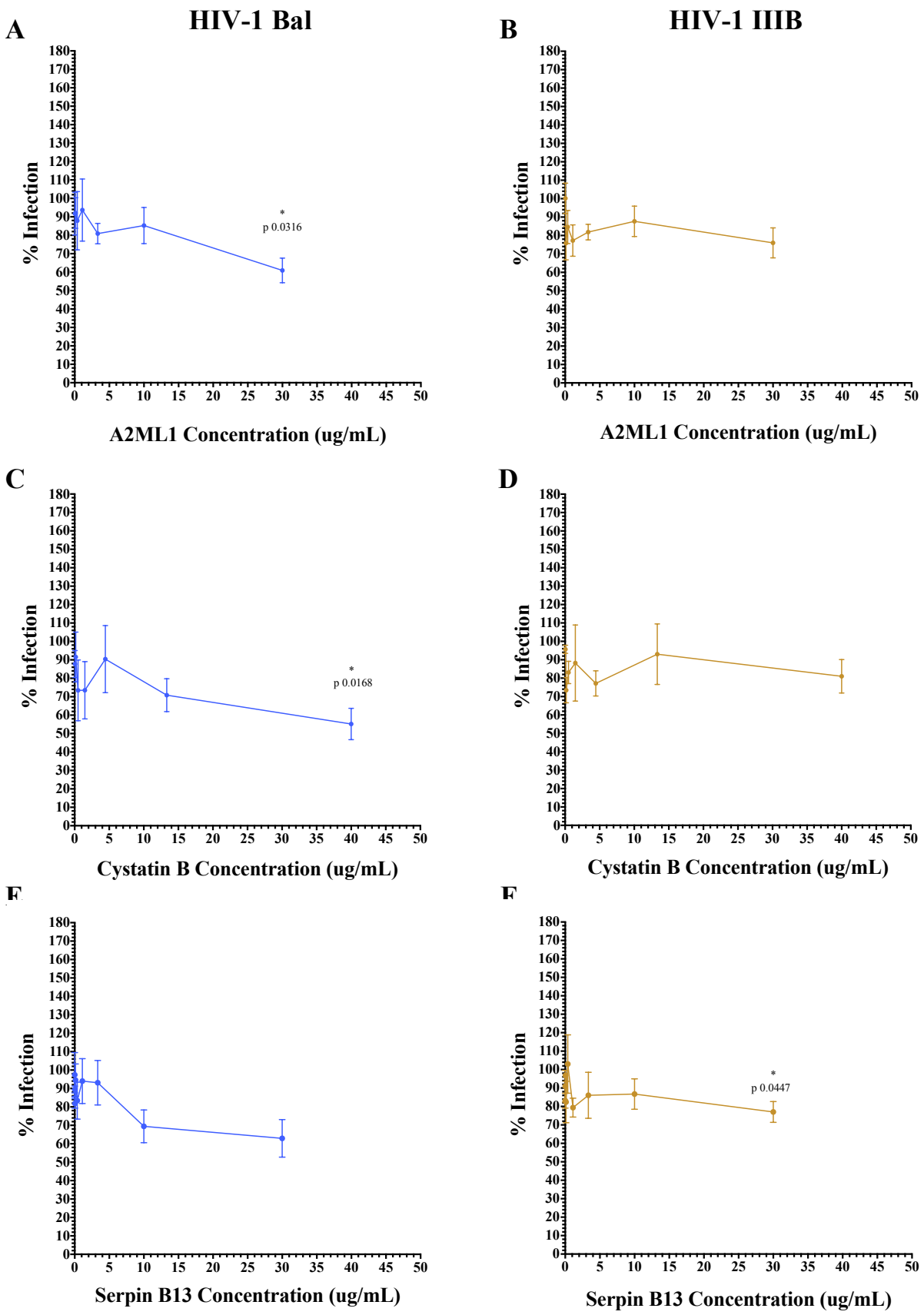

A
HIV-1 Bal
% Infection
A2ML1 Concentration (ug/mL)
* p 0.0316
B
HIV-1 IIIB
% Infection
A2ML1 Concentration (ug/mL)
C
% Infection
Cystatin B Concentration (ug/mL)
* p 0.0168
D
% Infection
Cystatin B Concentration (ug/mL)
E
% Infection
Serpin B13 Concentration (ug/mL)
F
% Infection
Serpin B13 Concentration (ug/mL)
* p 0.0447

Figure 22: TZM-bl HIV-1 neutralization assays with A2ML1, Cystatin B, and Serpin B13
Concentrations, ranging from 0.04μg/mL - 40μg/mL, of A2ML1, Cystatin B, and Serpin B13 were tested in a TZM-bl neutralization assay. Each point represents the mean ± the SEM of three replicate experiments. D'Agostino and Pearson Omnibus K2 Normality test was performed to confirm Gaussian distribution, followed by unpaired student t-tests with Welch's correction (did not assume equal SDs) with an alpha of 0.05, between virus control and experimental conditions. A: A2ML1 (0.04μg/mL - 30μg/mL) and HIV-1 Bal. B: A2ML1 (0.04μg/mL - 30μg/mL) and HIV-1 IIIB. C: Cystatin B (0.055μg/mL - 40μg/mL) and HIV-1 Bal. D: Cystatin B (0.055μg/mL - 40μg/mL) and HIV-1 IIIB. E: Serpin B13 (0.04μg/mL - 30μg/mL) and HIV-1 Bal. F: Serpin B13 (0.04μg/mL - 30μg/mL) and HIV-1 IIIB.

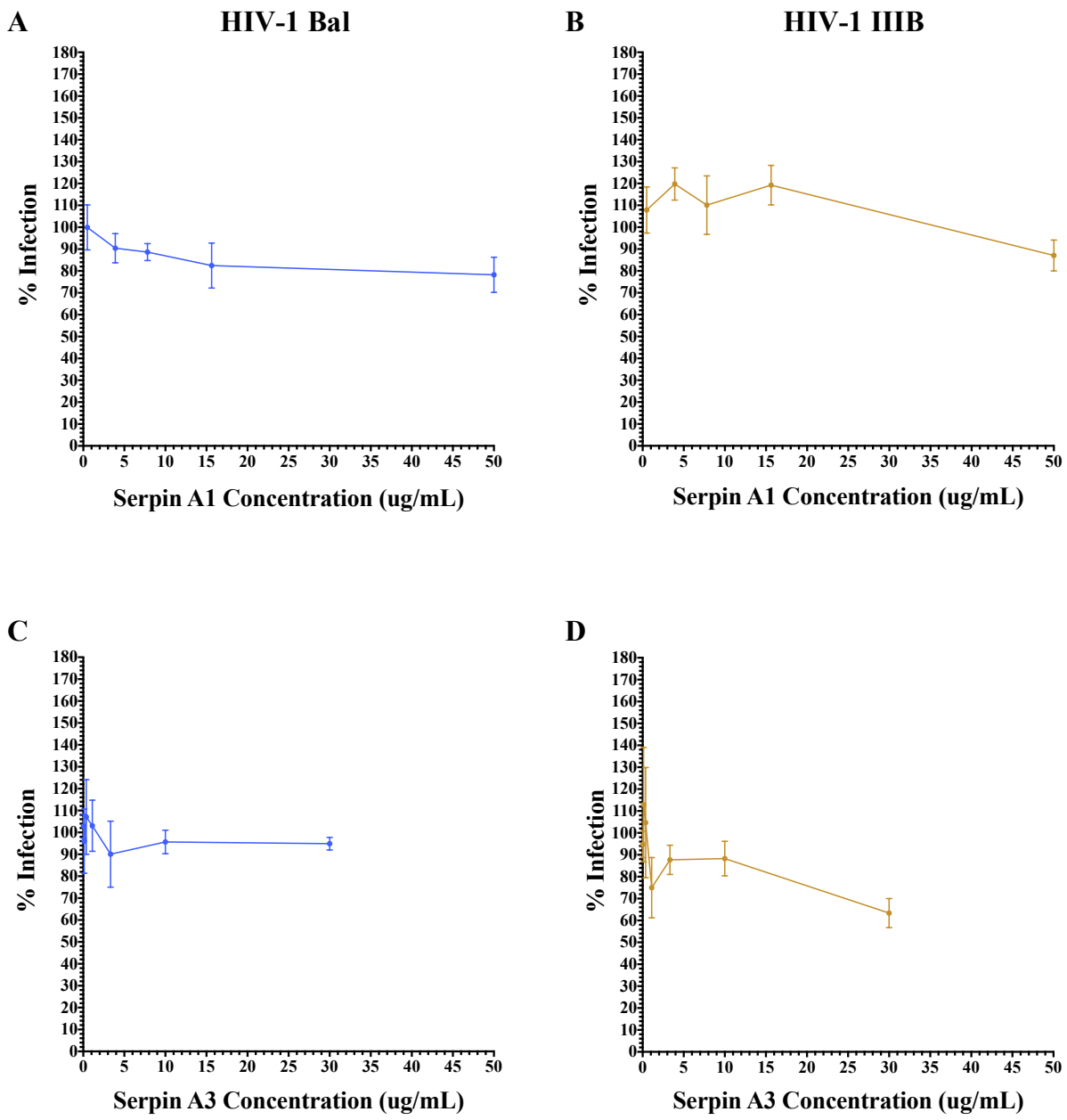


Figure 23: TZM-bl HIV-1 neutralization assays with Serpin A1, and Serpin A3
Concentrations, ranging from 0.04µg/mL - 50µg/mL, of Serpin A1 and Serpin A3 were tested in a TZM-bl neutralization assay. Each point represents the mean ± the SEM of three replicate experiments. D'Agostino and Pearson Omnibus K2 Normality test was performed to confirm Gaussian distribution, followed by unpaired student t-tests with Welch's correction (did not assume equal SDs) with an alpha of 0.05, between virus control and experimental conditions. A: Serpin A1 (0.488µg/mL - 50µg/mL) and HIV-1 Bal. B: Serpin A1 (0.488µg/mL - 50µg/mL) and HIV-1 IIIB. C: Serpin A3 (0.04µg/mL - 30µg/mL) and HIV-1 Bal. D: Serpin A3 (0.04µg/mL - 30µg/mL) and HIV-1 IIIB.

5.4.2.2. PBMC cell cultures

A2ML1, Cystatin A, Cytsatin B, Serpin B1 and Serpin B13 were tested for their ability to neutralize HIV in a PBMC assay. Antiproteases that did not exhibit significant neutralizing capacity in the previous experiments (Serpin A1 and Serpin A3) or that had already been shown to inhibit HIV-1 in a PBMC culture (Serpin C1)[33] were not included. A2ML1, Cystatin A, Cystatin B, Serpin B1 and Serpin B13 were initially screened at concentrations of 10μg/mL in an effort to conserve reagent use. At these concentrations none of the antiproteases, save for Serpin B1, exhibited significant inhibition with either HIV-1 Bal or HIV-1 IIIB (A2ML1, Cystatin A, and Cystatin B, Figure 24. Serpin B13, and Serpin B1 Figure 25). Serpin B1 inhibited HIV-1 Bal by 34.48% at a concentration of 10μg/mL (Figure 25A). Conversely, at this concentration no significant inhibition was observed in HIV-1 IIIB assays (Figure 25B). Once it was determined that Serpin B1 inhibits HIV-1 at concentrations as low as 10μg/mL, increased concentrations were added to determine if a dose-dependent response was observed, eventually resulting in complete (100%) inhibition. First, however, to confirm that any observed neutralizing effects were not due to increased cell death, cytotoxicity assays were repeated with PBMCs at these eleveated concentrations (Figure 26). Significant cytotoxic effects were observed following treatment with Serpin B1 at concentrations of 125μg/mL (15.31% cytotoxicity, $p = 0.0460$) and 250μg/mL (16.47 % cytotoxicity, $p = 0.0332$). While Serpin B1 concentrations of up to 250μg/mL were employed, inhibition levels of HIV-1 Bal plateaued at approximately 40% for all concentrations equal to or greater than 15.6μg/mL (Figure 26A). Specifically, at concentrations of 15.6μg/mL 35.6% ($p = 0.0002$) inhibition was achieved, at 31.25μg/mL 38.35% ($p = 0.0006$) inhibition, at 62.5μg/mL 33.82% ($p = 0.0012$) inhibition, 125μg/mL Serpin B1 exhibited 33.01% ($p = 0.0088$) inhibition, and at 250μg/mL Serpin B1 inhibited Bal by 33.08% ($p = 0.0313$) within PBMC cultures (Figure 26A). Similarly, in PBMCs infected with HIV-1 IIIB, at 15.6μg/mL Serpin B1 inhibited HIV infection by 35.6% (p =

0.0193), at 31.25μg/mL 41.1% (p = 0.0127) inhibition was achieved, at 62.5μg/mL 24.1% (p = 0.0205) inhibition of HIV occurred, at 125μg/mL there was 24.9% (p = 0.0501) inhibition and at 250μg/mL Serpin B1 inhibited HIV-1 IIIB infection by 32% (p = 0.0374) (Figure 26B).

Since Serpin B1 exhibited an inhibitory effect on HIV-1 Bal and IIIB that was stronger than the observed cytotoxicity effects, it is likely that a large portion of the observed inhibitory effects in these assays were due to true anti-HIV activity. Thus, Serpin B1 was chosen as the most suitable candidate to move forward with in mechanistic studies in order to determine whether this antiprotease would make an appropriate candidate for use in a novel microbicide.

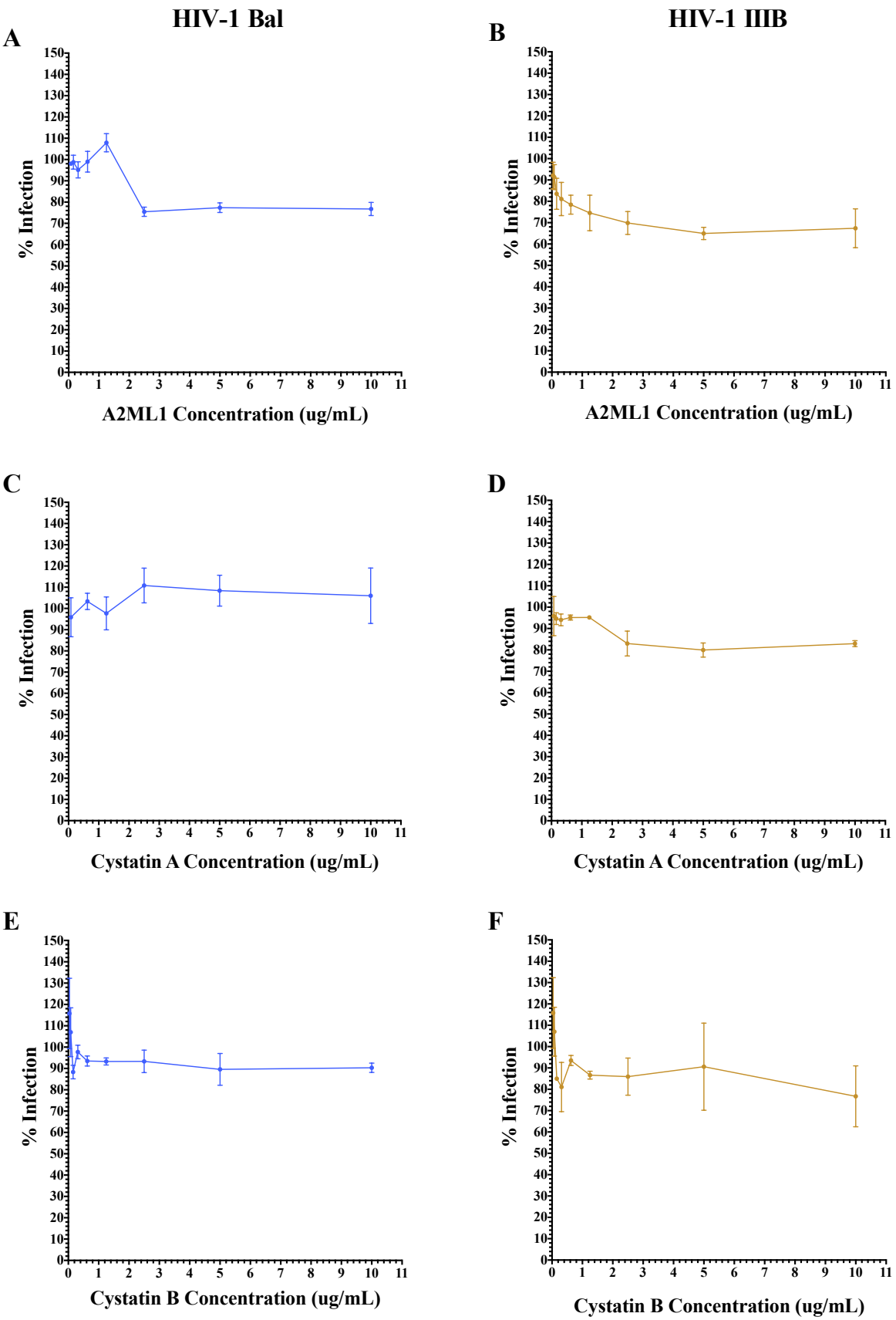

HIV-1 Bal
HIV-1 IIIB
A
B
C
D
E
F
% Infection
A2ML1 Concentration (ug/mL)
Cystatin A Concentration (ug/mL)
Cystatin B Concentration (ug/mL)

Figure 24: PBMC HIV-1 neutralization assays with A2ML1, Cystatin A, and Cystatin B
Concentrations, ranging from 0.078μg/mL - 10μg/mL, of A2ML1, Cystatin A, and Cystatin B were tested in a PBMC neutralization assay. Each point represents the mean ± the SEM of three replicate experiments. D'Agostino and Pearson Omnibus K2 Normality test was performed to confirm Gaussian distribution, followed by unpaired student t-tests with Welch's correction (did not assume equal SDs) with an alpha of 0.05, between virus control and experimental conditions. A: A2ML1 and HIV-1 Bal. B: A2ML1 and HIV-1 IIIB. C: Cystatin A and HIV-1 Bal. D: Cystatin A and HIV-1 IIIB. E: Cystatin B and HIV-1 Bal. F: Cystatin B and HIV-1 IIIB.

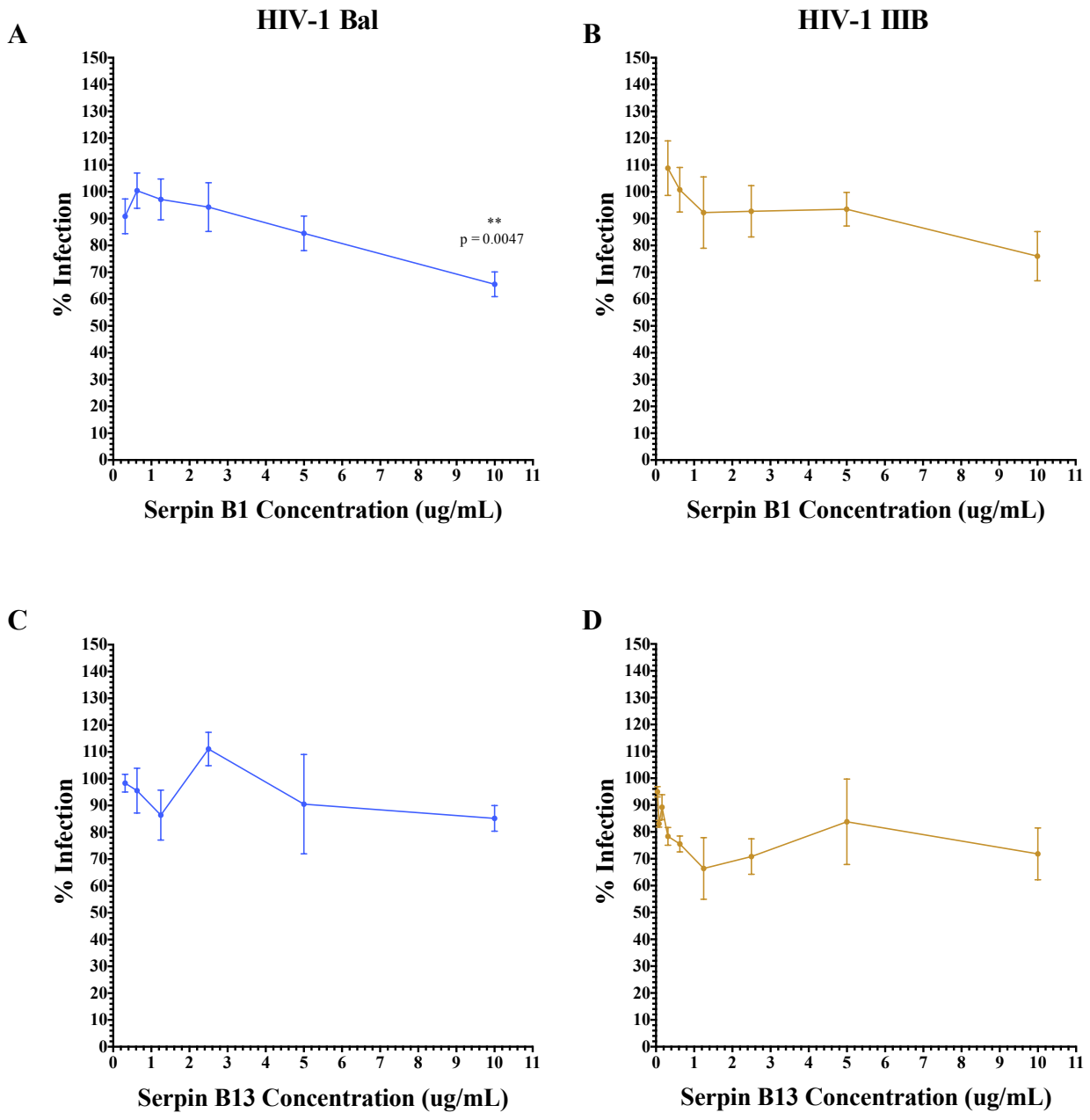


Figure 25: PBMC HIV-1 neutralization assays with Serpin B1 and Serpin B13
Concentrations, ranging from 0.078µg/mL - 10µg/mL, of Serpin B1, and Serpin B13 were tested in a PBMC neutralization assay. Each point represents the mean ± the SEM of three replicate experiments. D'Agostino and Pearson Omnibus K2 Normality test was performed to confirm Gaussian distribution, followed by unpaired student t-tests with Welch's correction (did not assume equal SDs) with an alpha of 0.05, between virus control and experimental conditions. A: Serpin B1 and HIV-1 Bal. B: Serpin B1 and HIV-1 IIIB. C: Serpin B13 and HIV-1 Bal. D: Serpin B13 and HIV-1 IIIB.

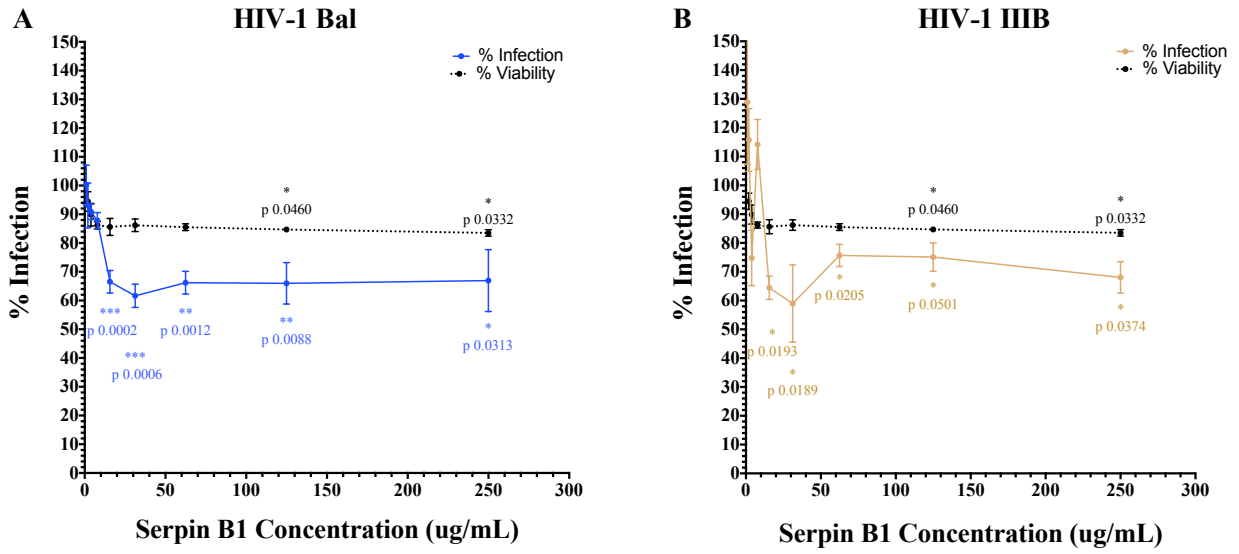


Figure 26: PBMC HIV-1 neutralization and cytotoxicity assays with high concentrations of Serpin B1

Concentrations, ranging from 0.977µg/mL - 250µg/mL, of Serpin B1 were tested in a PBMC neutralization assay and cytotoxicity assay. Each point represents the mean ± the SEM of three replicate experiments. D'Agostino and Pearson Omnibus K2 Normality test was performed to confirm Gaussian distribution, followed by unpaired student t-tests with Welch's correction (did not assume equal SDs) with an alpha of 0.05, between virus control and experimental conditions. A: Serpin B1 and HIV-1 Bal. B: Serpin B1 and HIV-1 IIIB.

5.5 Summary

Testing the inhibitory capacity of specific antiproteases found to be up-regulated within the CVL fluids of HESN women allows for the identification of potential novel candidates for the development of future microbicides. Cytotoxicity assays in both TZM-bl cells and PBMC culture assays displayed minimal effects with the strongest cytotoxicity exhibited by Serpin B13 and Serpin B1 within the TZM-bl cell line only. There were no significant cytotoxic effects observed with the PBMC cell cultures.

Neutralization assays performed in TZM-bl cells revealed that six of the eight antiproteases tested, exhibited significant inhibition of at least one of the two HIV-1 strains employed. However, Cystatin A, Serpin C1 and Serpin B1 were capable of significantly inhibiting both virus strains. Conversely, A2ML1, Cystatin B, and Serpin B13 only significantly neutralized one of the HIV-1 strains. Serpin A1 and Serpin A3 were not found to inhibit either HIV-1 Bal nor IIIB. When these findings were validated in a PBMC neutralization assay, Serpin B1 was the only antiprotease shown to significantly neutralize HIV-1 Bal at concentrations as low as 10μg/mL. However, for an effect to be observed with HIV-1 IIIB, higher concentrations were required. Interestingly, in assays with both HIV-1 Bal and IIIB, there appeared to be a plateau in inhibitory activity of 30-40% regardless of how concentrated the Serpin B1 was.

Due to the consistent inhibitory effects observed with Serpin B1 in both TZM-bl and PBMC cell cultures, and against both R5-tropic, and X4-tropic HIV viruses with limited cytotoxicity, this antiprotease appears to the most promising candidate to move forward with to determine its potential as a novel microbicide agent.

CHAPTER 6: HIV SPECIFIC MECHANISMS OF SERPIN B1

6.1 Rationale

As observed in Chapter Five, Serpin B1 exhibited the most consistent inhibition of HIV-1 compared to the other antiproteases examined, and thus represents a promising natural candidate for use in future microbicide development. However, it is imperative first to determine the exact mechanism(s) of action that Serpin B1 employs to neutralize HIV-1 to confirm that it will inhibit HIV without causing subsequent harm to the surrounding cells/tissue. Determining the mechanism of action may also provide the information necessary to be able to develop a small molecule agonist to target novel molecular pathways of interest.

Serpin A1 and Serpin C1, have been shown to exhibit specific anti-HIV functions by blocking virus entry, or by preventing processing of HIV-1 proteins before viral assembly and budding[281,297,315]. Alternatively, little is known about the overall function of Serpin B1 aside from its role as a potent inhibitor of leukocyte elastase, cathepsin G, proteinase-3, chymase, chymotrypsin and kallikrein-3[336,366] and its well-documented roles in the control of pathophysiologies associated with pulmonary bacterial infections and autoimmune conditions [327,336,366]. It is therefore not clearly understood what role this antiprotease may play in protection against viruses, specifically HIV-1, outside of its general immune-regulatory functions. Thus, this chapter aims to determine Serpin B1's HIV-1 specific inhibitory mechanism.

6.2 Hypothesis

Serpin B1 inhibits HIV-1 through interference with virus binding to host cells or through inhibiting HIV-1 enzymes required for virus replication and assembly.

6.3 Objectives

- Determine if commercially made Serpin B1 is biologically active and inhibits neutrophil elastase and can enter the cytoplasm of cells.

- Confirm the HIV-1 inhibitory capability of Serpin B1 in additional cells lines with an additional strain of HIV-1 (HIV_{1956}).
- Determine if Serpin B1 inhibits HIV-1 binding and entry into target cells through the use of VSV-G pseudotyped single-cycle-replicating pNL-Bru-ΔBgl/Luc^+/R^- HIV.
- Determine if Serpin B1 interferes with HIV-1 reverse transcriptase, nuclear import, and integrase by quantifying viral early, late, and 2-LTR DNA through the use of real-time PCR.
- Determine the effect that Serpin B1 has on post-integration late stages of replication, through the use of provirus-containing ACH2 cells
- Quantify the amount of HIV-1 mRNA splice variants following the treatment of PBMCs, and ACH2 cells with Serpin B1 so as to determine the effect of this antiprotease on HIV transcription.
- Determine if Serpin B1 interferes with the budding of HIV-1 from infected PBMCs through comparison of intracellular and extracellular p24 levels.

6.4 Results

6.4.1 Confirmation of commercial Serpin B1 functionality

To determine whether commercially produced Serpin B1 is biologically active a neutrophil elastase kit (BioVision, Milpitas, CA, USA) was utilized to determine the functional activity of Serpin B1 used in the mechanistic studies described in this chapter. This kit included neutrophil elastase as a negative control and SPCK (3mM), which is an irreversible neutrophil elastase inhibitor, as a positive control. Fluorescence levels were detected following the addition of serially diluted Serpin B1 to the substrate, which was then directly related to the abundance of functional neutrophil elastase in solution. Following analysis of the resulting data, it was

determined that Serpin B1 inhibited neutrophil elastase in a dose-dependent manner with nearly 100% inhibition achieved at concentrations of 25μg/mL (97.99%) and 50μg/mL (99.42%) (Figure 27). At concentrations of 12.5μg/mL there was 93.86% inhibition of neutrophil elastase activity observed, at 6.25μg/mL 85.5%, at 3.125μg/mL 70.27%, at 1.56μg/mL 51.38% inhibition occurred, and at the lowest concentration of 0.78μg/mL neutrophil elastase activity was inhibited by 35.54% (p values for all < 0.0001) (Figure 27). The positive control, SPCK, inhibited NE by 99.93%.

Serpin B1 is naturally produced within the cytoplasm of neutrophils, NK cells, monocytes/macrophages, T cells and epithelial cells and can be exported from the cell as well as trafficked into the nucleus under specific circumstances[327]. It is, therefore, important to confirm that exogenously added Serpin B1 is also capable of entering into the cytoplasm of cells, and the nucleus so as to mimic its natural function. To determine the cellular localization of exogenously added Serpin B1, PBMCs were treated with 50μg/mL of Serpin B1 for 3, 12 or 24 hours followed by addition of a fluorescently labeled Serpin B1-specific antibody and visualized by confocal microscopy. Figure 28 depicts a confocal image of Serpin B1 treated and untreated PBMCs, stained with a nuclear (DAPI) stain and with fluorescently labeled Serpin B1 antibodies (Alexa fluor 488). Alternatively, Figure 29 represents the integrated density values for the fluorescent staining of Serpin B1 in the nucleus, and the cytoplasm. A slight, however, significant increase in Serpin B1 within both the cytoplasm ($p = 0.0148$) and nucleus ($p = 0.0186$) was observed following three hours of incubation compared to untreated controls. In untreated PBMCs, the confocal integrated density was 14,490μm^2 within the nucleus and 25,344μm^2 within the cytoplasm, compared to 18,759μm^2 (fold increase: 1.29, $p = 0.0186$) and 34,422μm^2 (Fold increase:1.36, $p = 0.0148$) within Serpin B1 treated PBMCs, respectively. At 12 hours incubation the increase in Serpin B1 was still detected within the nucleus (21,375μm^2,

fold increase: 1.18) and cytoplasm (36,654μm^2, fold increase: 1.26) compared to untreated, with integrated densities of 18,045μm^2 in the nucleus, and 29,198μm^2 in the cytoplasm. The differences, however, were not significant. The largest increase in Serpin B1 following treatment was observed following 24 hours incubation (Figure 28 & 29). The integrated density within the nuclear (17,918μm^2), and cytoplasmic portions (29,089μm^2) remained similar to that observed after 12 hours in untreated PBMCs. Whereas, in the Serpin B1 treated cultures, the integrated density increased to 26,498μm^2 (fold increase: 1.48, $p = 0.0035$), and 39,681μm^2 (fold increase: 1.36, $p = 0.0385$) within the nucleus, and cytoplasm, respectively. It is also apparent (Figure 28 & 29) that untreated PBMCs express significant endogenous levels of Serpin B1 both within the cytoplasm and the nucleus, which remain relatively constant over the 24 hours of cell culture. It can, therefore, be concluded that the exogenously added Serpin B1 was capable of entering the cytoplasm and nucleus, in the cellular locations that this antiprotease naturally functions.

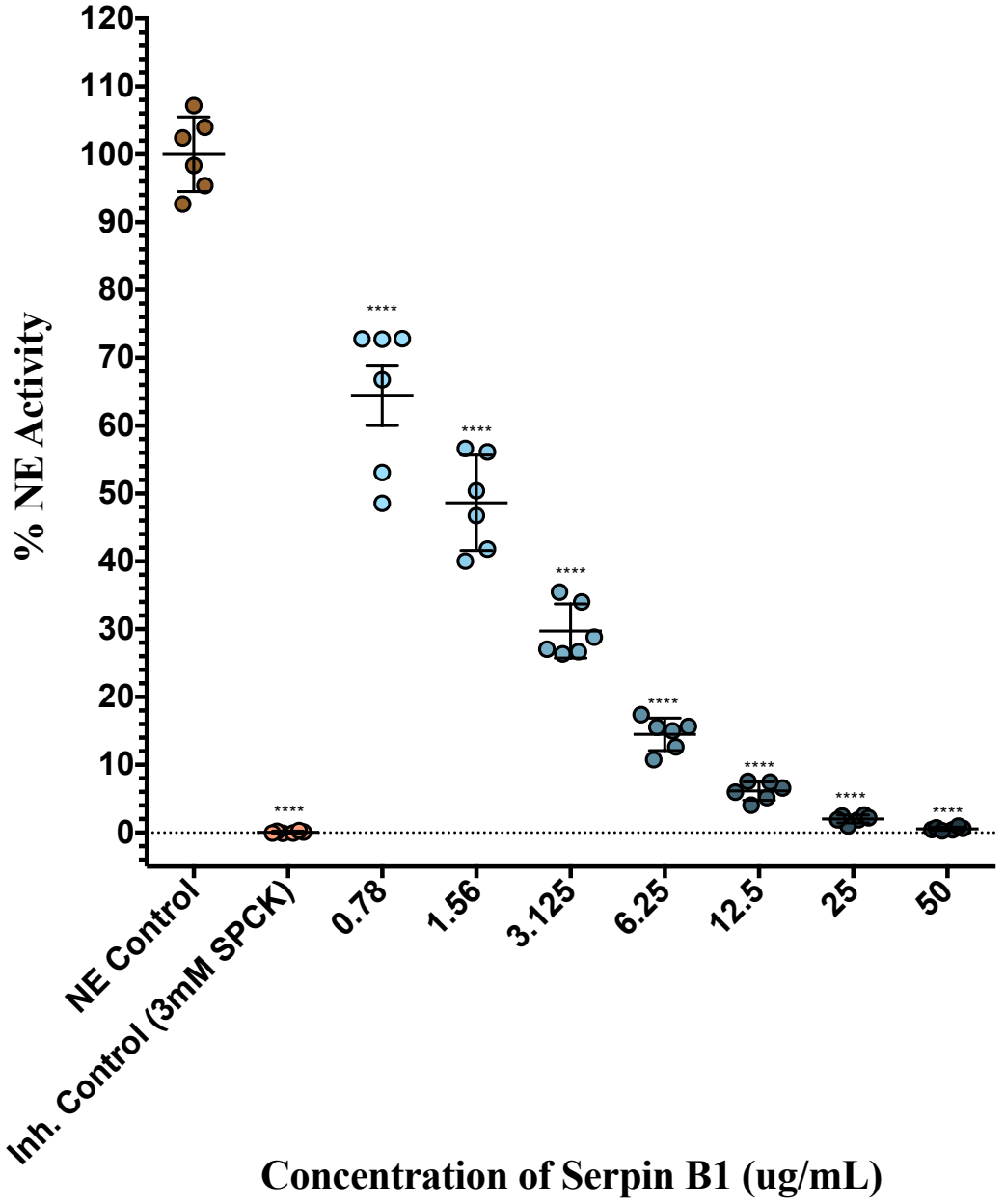


Figure 27: Commercially produced Serpin B1 exhibited Neutrophil Elastase functional activity

Neutrophil Elastase cleaves a synthetic substrate and releases a fluorphore which is detectable at Ex/Em = 400/505nm. Uninhibited Neutrophil Elastase alone was used as a 100% NE control. SPCK was employed as a positive control for NE inhibition. Each point represents one of six independent experiments, with three technical replicates in each, and bars indicate mean ± SEM. Significant inhibition of NE activity was detected by adding Serpin B1 at concentrations of 0.78μg/mL - 50μg/mL to the substrate and meansuring fluorescent activity compared to the controls.

D'Agostino and Pearson Omnibus K2 Normality test was performed followed by unpaired Mann Whitney U-tests with an alpha of 0.05.

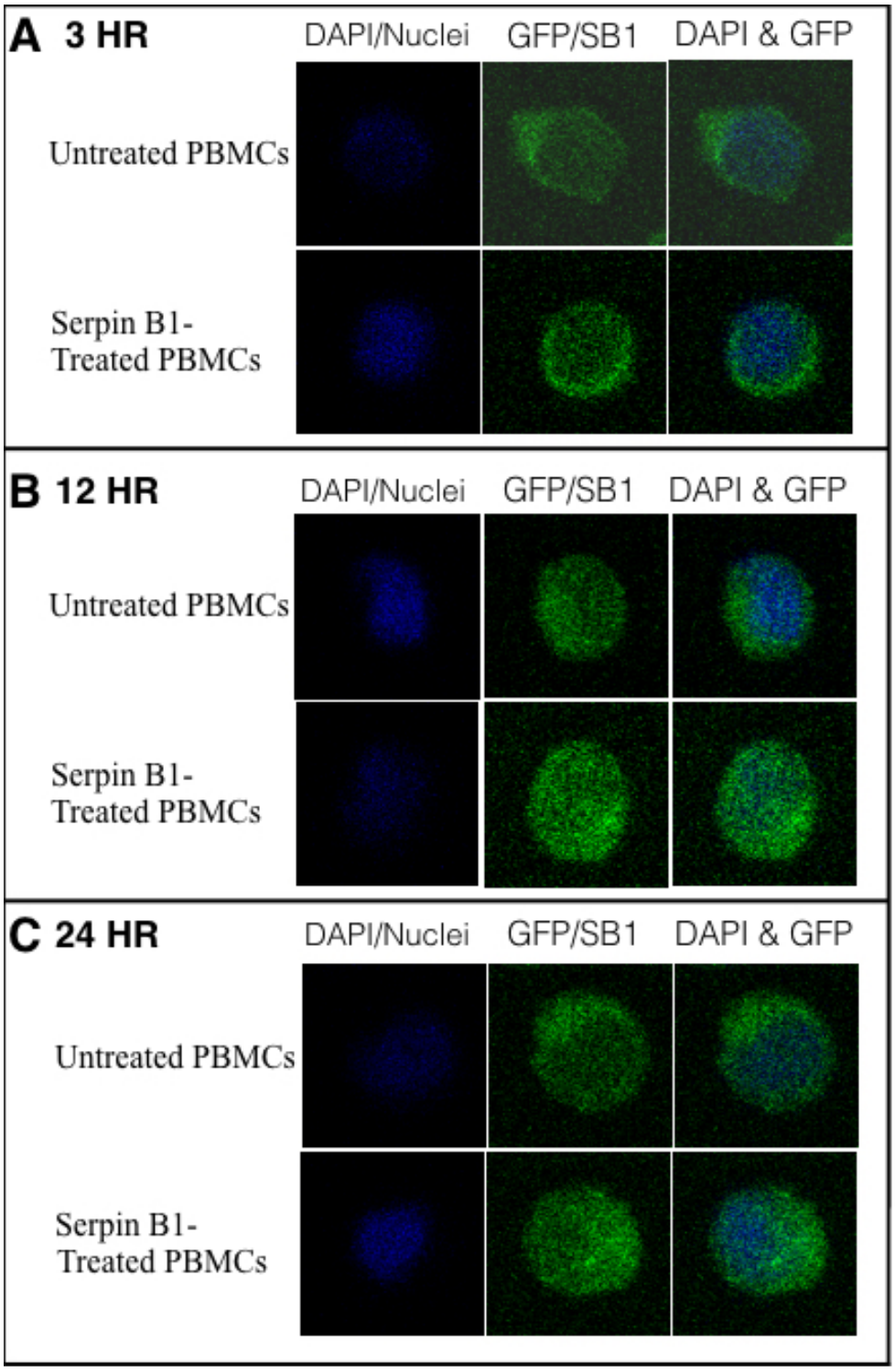


Figure 28: Fluorescent staining in PBMC cultures before and after the addition of exogenous Serpin B1

Confocal microscope image of PBMCs stained with DAPI (blue, nucleus) and Alexa Fluor488 (green, Serpin B1) following treatment with exogenous Serpin B1 for A: 3 hours, B: 12 hours, C: 24 hours.

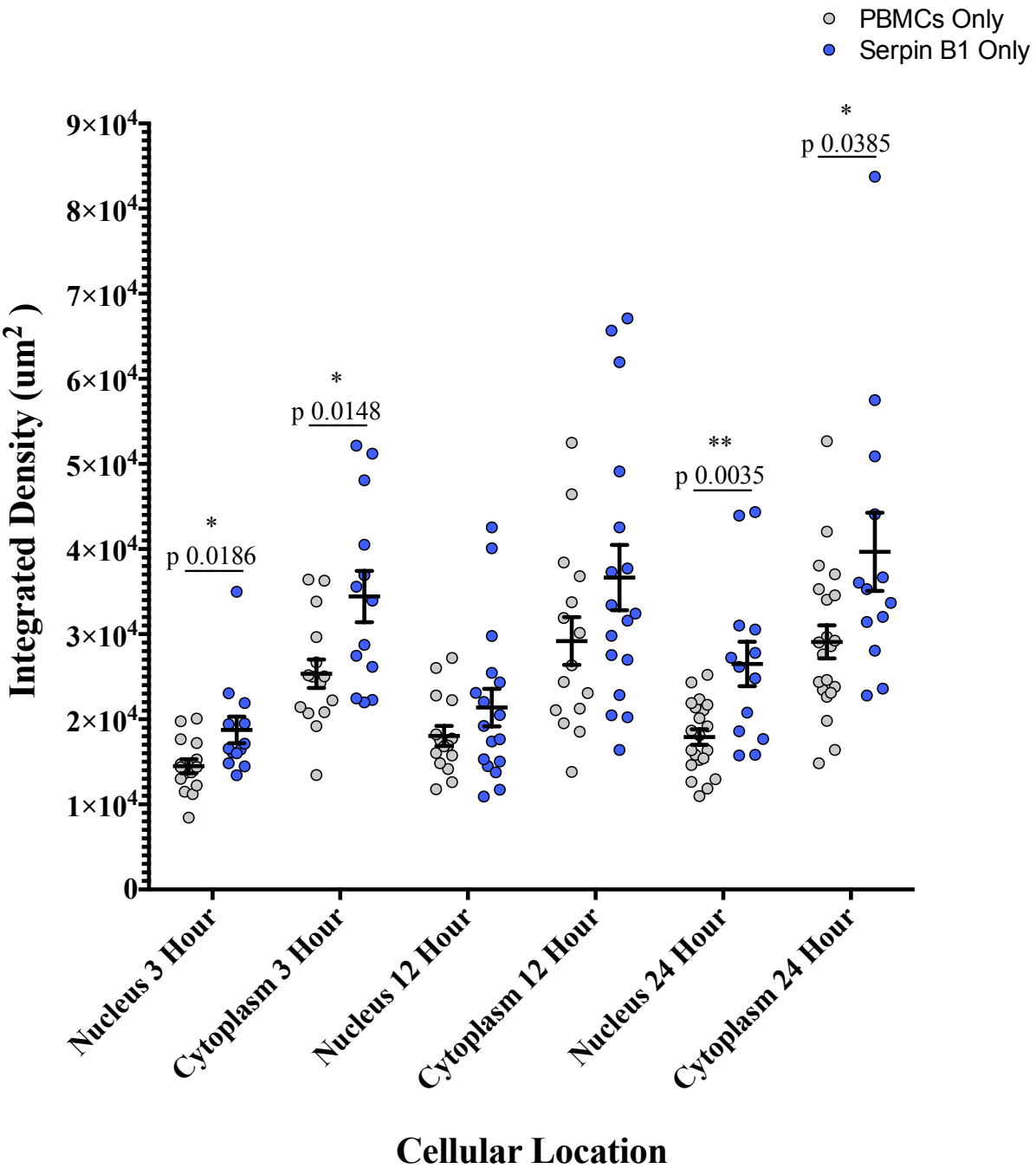


Figure 29: Quantification of Serpin B1 levels within the cytoplasm and nucleus of PBMCs following exogenous Serpin B1 treatment

Quantitative analysis of exogenously added Serpin B1 in the nucleus and cytoplasm of PBMCs following staining and confocal imaging. Confocal images were quantified using Zen software by controlling for background pixel intensity followed by calculating the integrated densities (area of the mean intensity and area measured). Each point represents integrated density for one cell. Each bar represents mean ± SEM. D'Agostino and Pearson Omnibus K2 Normality test was performed followed by unpaired Mann Whitney U-tests with an alpha of 0.05.

6.4.2 Serpin B1 inhibits multiple strains of HIV-1 in multiple cells types

To determine if Serpin B1 inhibits HIV in cell lines commonly used in mechanistic studies, and within a biologically relevant tissue model, assays in addition to those performed in Chapter Five (Figure 21, 25, and 26) were performed using C8166 $CD4^+$ T cells, and an ectocervical explant model (performed by collaborator Carolina Herrera, Imperial College, United Kingdom). These neutralization assays were also repeated with a duo-tropic, clade A1, human primary HIV-1 isolate (HIV_{1956}) to determine the effect that Serpin B1 has on one of the more prevalent HIV clades in Sub-Saharan Africa, compared to the lab adapted clade B viruses (Bal and IIIB) employed to this point[23,51,367]. Results from these additional assays revealed that significant inhibitory activity was also observed within the C8166 $CD4^+$ T cell line as well as in the ectocervical explant model. Furthermore, inhibitory activity was observed against the additional HIV primary isolate, HIV_{1956}.

In a five-day C8166 T cell line neutralization assay, Serpin B1 inhibited HIV_{1956} by 57% at 25μg/mL ($p = 0.0191$), and by 65.7% at 50 ug/mL ($p = 0.0037$) compared to the virus control (Figure 30A). During infection of C8166 T cells with HIV-1 IIIB, Serpin B1 again exhibited significant inhibition by 35.6% at 25μg/mL ($p = 0.0306$), and 39.3% at 50μg/mL ($p = 0.0226$) (Figure 30B). Similar results were obtained when PBMCs were infected with $HIV_{1956,}$ with significant neutralization exhibited at 25μg/mL and 50μg/mL with 42.5% ($p = 0.0054$) and 57.7% ($p = 0.0001$) inhibition observed, respectively (Figure 30C). Lastly, within a cervical explant model Serpin B1 exhibited significant neutralization of HIV-1 Bal infection at a concentration of 25μg/mL (53.5% inhibition, $p = 0.0165$), while 61.7% ($p = 0.0024$) inhibition was reached when 100μg/mL Serpin B1 was used in this culture (Figure 30D).

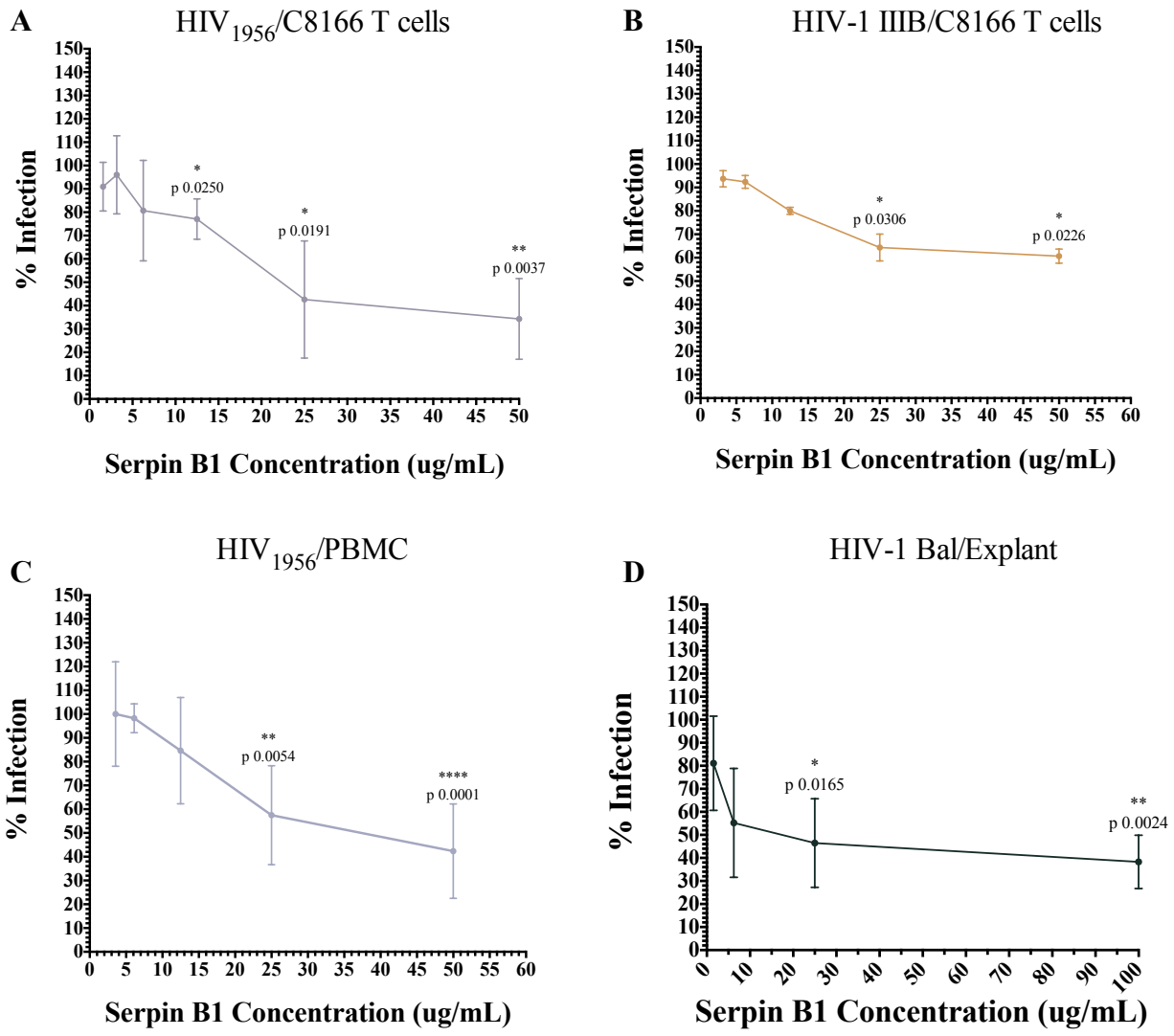


Figure 30: HIV-1 neutralization by Serpin B1 in C8166 T cell, PBMC, and ectocervical tissue explant cultures

Concentrations, ranging from 1.56µg/mL - 100µg/mL, of Serpin B1 were tested in neutralization assays. Each point represents the mean ± the SEM of three replicate experiments. D'Agostino and Pearson Omnibus K2 Normality test was performed to confirm Gaussian distribution, followed by unpaired student t-tests with Welch's correction (did not assume equal SDs) with an alpha of 0.05, between virus control and experimental conditions. A: Serpin B1 (1.56µg/mL - 50µg/mL) and HIV_{1956} in a C8166 T cell culture. B: Serpin B1 (1.56µg/mL - 50µg/mL) and HIV-1 IIIB in a C8166 T cell culture. C: Serpin B1 (1.56µg/mL - 50µg/mL) and HIV_{1956} in a PBMC cell culture D: Serpin B1 (1.56µg/mL - 100µg/mL) and HIV-1 Bal in an ectocervical tissue explant culture.

6.4.3 Effect of Serpin B1 on early stages of HIV-1 replication cycle, from cell binding/entry to integration

A critical component to deciphering the HIV inhibitory mechanism of Serpin B1 includes determining if Serpin B1 interferes with any of the stages in the HIV lifecycle. Thus, to examine its effects on the earliest stages of the replication cycle (binding/fusion) a VSV-G pseudotyped single-cycle-replicating pNL-Bru- ΔBgl/Luc^{+}/R^{-} HIV virus was used to infect Serpin B1 treated C8166 CD4^{+} T cells. This VSV-G pseudotyped virus, does not rely on HIV co-receptors for cell entry, but rather interacts with the plasma membrane and is taken up through clathrin-mediated endocytosis[40]. Once inside the cell this virus' RNA can be reverse transcribed and integrated, resulting in expression of luciferase upon initiation of transcription/translation. Measurement of luciferase activity provides a readout of viral transcription. Therefore, if inhibition was observed it would suggest that Serpin B1 does not interfere with HIV gp120/gp41 binding but rather with early stages of viral replication (up to transcription). These stages would be common between this VSV-G pseudotyped HIV virus and the native form. Conversely if inhibition was not observed, it could be concluded that Serpin B1may have a role in inhibiting gp120 binding to CD4/co-receptors on the host cell or in interfering with the later stages of the life cycle following translation including, viral assembly, budding, and processing of the viral proteins.

Azidothymidine (AZT), a reverse transcriptase inhibitor, was used as a positive control and Enfuvirtide (ENF), a gp41 fusion inhibitor was employed as a negative control. As such, treatment with AZT (50mM) resulted in 99.5% ($p = 0.0001$) inhibition whereas ENF did not effect the level of luciferase activity following infection by VSV-G HIV. When the effect of exogenous Serpin B1 was evaluated, no significant inhibitory activity was observed at any of the concentrations employed (Figure 31). This suggests Serpin B1 may act in preventing binding of gp120 to host cells, or it may exert its effects in the late stages of the HIV lifecycle (post-

transcriptional). Thus, it could be concluded that Serpin B1 does not appear to interfere with RT, nuclear import or integrase stages of the HIV lifecycle.

To verify that Serpin B1 did not affect early post-entry stages of the HIV replication cycle, the ability of HIV-1 IIIB and Bal to infect C8166 T (which overexpress CXCR4), and A3R5.7 (which overexpress CCR5) cell lines was assessed by quantifying HIV early, late and 2-LTR DNA through qRT-PCR. Should Serpin B1 interfere with reverse transcription it was expected that the levels of early and late viral DNA would be reduced compared to normal virus controls. However, if nuclear import were to be impeded there would have been a lower abundance of 2-LTR DNA, which is viral DNA that is circularized by host-DNA repair enzymes following import into the nucleus[432]. Conversely, if there was an over-abundance of 2-LTR compared to virus controls, it could be concluded that integration was being inhibited resulting in over-circularization of DNA that would otherwise typically integrate into the host genome. AZT and ENF were used as positive controls for interference with reverse transcription and viral cell entry, respectively. Untreated cells acted as negative controls. After assessing the levels of early, late and 2-LTR DNA by qRT-PCR no significant differences were observed between Serpin B1 treated and untreated cells. However, as expected, a significant reduction in early, late, and 2-LTR DNA was seen in cells treated with either AZT or ENF (Figure 32). These results confirm that Serpin B1 does not interfere with reverse transcription, nuclear import, or integration of viral DNA.

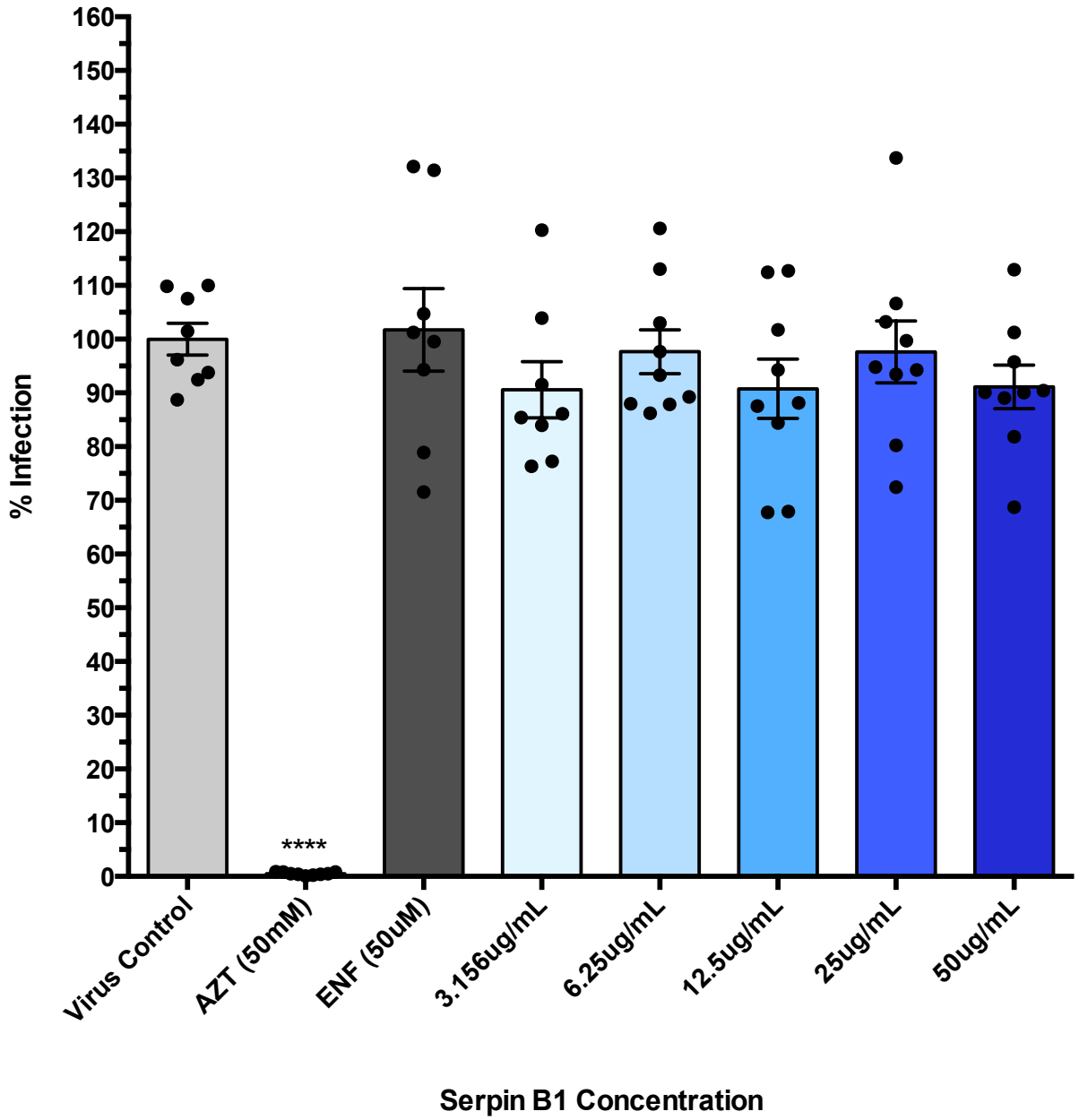
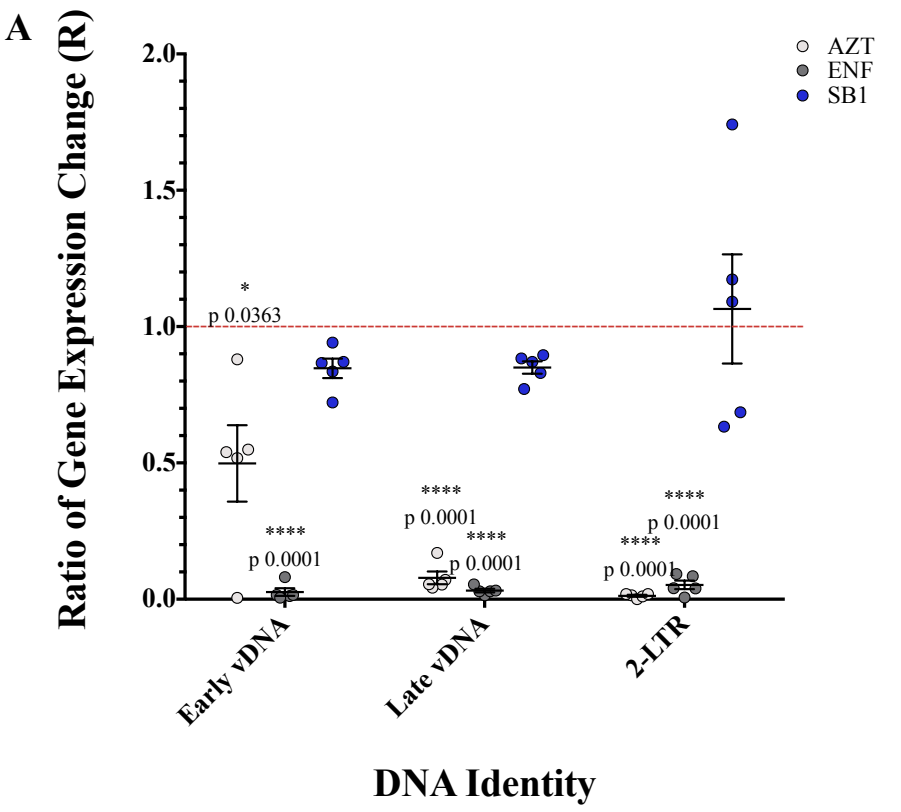


Figure 31: Serpin B1 does not inhibit the replication of a VSV-G pseudotyped HIV virus within a C8166 T cell line

AZT was a positive inhibitory control, ENF a negative inhibitory control, and untreated cells acted as a normal control (set at 100% infection). Each data point represents a technical replicate. Three biological replicates were performed with three technical replicates each. Each bar represents mean $\pm$ SEM. Percent infection was determined by measuring the ability of a pNL-Bru-ΔBg/Luc^{+}/R^{-} VSV-pseudotyped HIV to infect C8166 T cells. Luciferase activity was used as a readout for infection. AZT inhibited the pseudotyped virus by 99.5% (p-value 0.0001). D'Agostino and Pearson Omnibus K2 Normality test was performed, followed by unpaired Mann Whitney U-tests with an alpha of 0.05, between experimental culture results and the virus control.


A
Ratio of Gene Expression Change (R)
2.0
1.5
1.0
0.5
0.0
AZT
ENF
SB1
*
p 0.0363
****
p 0.0001
****
p 0.0001
****
p 0.0001
****
p 0.0001
****
p 0.0001
Early vDNA
Late vDNA
2-LTR
DNA Identity

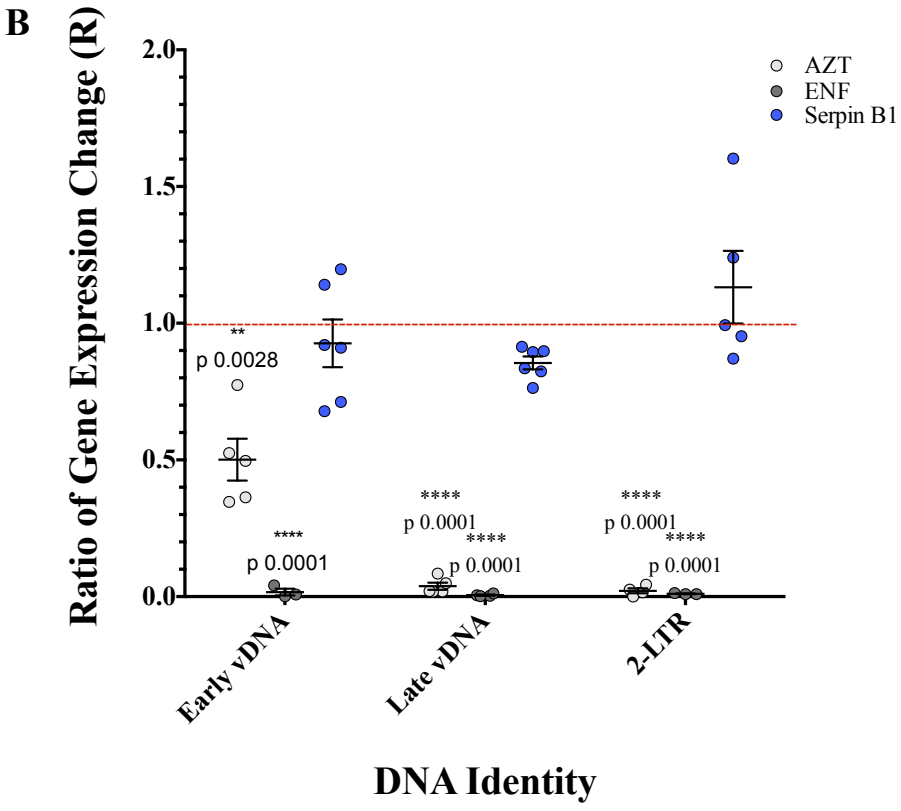

B
Ratio of Gene Expression Change (R)
2.0
1.5
1.0
0.5
0.0
AZT
ENF
Serpin B1
**
p 0.0028
****
p 0.0001
****
p 0.0001
****
p 0.0001
****
p 0.0001
****
p 0.0001
Early vDNA
Late vDNA
2-LTR
DNA Identity

Figure 32: Viral DNA quantitation following Serpin B1 treatment of A3R5.7 and C8166 T cells

Vira DNA was quantified by qRT-PCR. Virus alone was depicted as the red dashed line which is set to 1 as the base-point to compare all quantitative measures to. Each data point represents one technical replicate. Three biological replicates were performed with two technical replicates each. Each bar represents mean $\pm$ SEM. The ΔC_q for the virus controls were subtracted from the ΔC_q for each experimental condition resulting in the $\Delta\Delta C_q$ for each reaction. This value was then used to determine the ratio of gene expression change using the equation $R=2^{-\Delta\Delta Cq}$. AZT and ENF acted as positive controls for inhibition. Each data point represents one reaction technical replicate. Three biological replicates were performed with two technical replicates per assay. D'Agostino and Pearson Omnibus K2 Normality test was performed, followed by unpaired Mann Whitney U-tests with an alpha of 0.05.

A: A3R5.7 cells, infected with HIV-1 Bal, and treated for 24 hours with AZT, ENF or Serpin B1.

B: C8166 T cells, infected with HIV-1 IIIB, and treated for 24 hours with AZT, ENF or Serpin B1.

6.4.4. Effect of Serpin B1 on late stages of HIV-1 replication cycle, from transcription to budding

Since Serpin B1 did not inhibit HIV-1 replication during the early stages of replication, its affects on those stages following integration were tested using an ACH2 cell system. ACH2 cells contain an integrated HIV provirus that upon PMA stimulation produce infectious HIV-1 virions. Thus, any reduction in p24 levels following Serpin B1 treatment would suggest interference within post-transcriptional stages of replication. The Level of p24 in the supernatants was detected using an ELISA.

PMA was used to stimulate ACH2 cells in the presence and absence or 50μg/mL Serpin B1. Following collection of the culture supernatants and quantitation of p24 levels via ELISA, as expected, the cells treated with only PMA (5ng) had a significant increase in p24 expression. Compared to these PMA controls, Serpin B1 treated cells did not show a significant reduction in p24 at 24 hours. However, a significant reduction was observed at 48 hours (35%, $p = 0.004$) and 72 hours (20%, $p = 0.0269$) incubation (Figure 33).

These results suggest that Serpin B1 may have a role in inhibiting HIV-1 replication post-integration. To determine if transcription was being affected by Serpin B1 treatment, either PMA-stimulated ACH2 cells, or PBMCs infected with HIV_{1956} were lysed and the various mRNA splice variants were quantified. HIV-1 transcribes one large pre-mRNA strand, which is then processed and spliced into three forms, unspliced, single-spliced or multi-spliced[38,40]. Each of these splice variants is used to translate different HIV-1 proteins, and thus the proper proportions of each variant need to be present to produce a mature virion. Determining the quantity of each variant will determine if adequate levels of transcription are occurring, which should result in equivalent levels of all three splice variants compared to untreated controls. Any significant discrepancies between the level of splice variants in Serpin B1 treated cells versus controls would result in an over abundance of specific proteins and an under-abundance of others, resulting in limited viral replication dependant on the lowest abundant protein available.

Following quantification of unspliced, single-spliced and multi-spliced HIV-1 mRNA in both ACH2 cells, and PBMCs infected with HIV_{1956}, no significant differences were observed between Serpin B1 (50μg/mL) treated cells and virus control cells (Figure 34). These results suggest that Serpin B1 is not affecting the overall level of transcription nor the mRNA splicing process. Therefore, Serpin B1 may be inhibiting the stages of replication post- translation, such as viral budding.

To determine if Serpin B1 treatment was in fact interfering with the budding of the virus from the host cell, PBMCs from three Winnipeg donors were infected with HIV_{1956} and treated with either Serpin B1 (50μg/mL), BSA (50μg/mL) or PBS for five days. The resulting intracellular p24 levels were quantified via intracellular (p24 FITC) flow cytometry staining of live $CD3^+$ lymphocytes (Figure 35 & 36) and compared to extracellular p24 levels (Figure 30C). Should Serpin B1 be interfering primarily with the budding of the immature virions from the host cell the amount of intracellular p24 should not differ in the Serpin B1 treated cultures compared to the untreated cells. However, if Serpin B1 is affecting the production of viral proteins (translation), not the budding of the virion, there would be an approximately equal decrease in intracellular and extracellular p24 levels.

Results indicated that 19.98% of the total lymphocyte population within the virus control contained intracellular p24. Treatment of cells with the protein control, BSA (50μg/mL), did not significantly impact the proportion of lymphocytes expressing intracellular p24. However, there was a significant reduction in total intracellular p24 levels between PBMCs treated with 50μg/mL ($p = 0.0372$, Figure 36) Serpin B1 and the virus control with only 8.68% of the treated lymphocytes containing intracellular p24 ($p = 0.0372$), accounting for a 56.56% reduction. Comparing this reduction in intracellular p24 to that of extracellular p24, as previously detected via ELISA (Figure 30C), it is evident that the level of detectable p24 both intracellularly, and

extracellularly are very similar. Extracellular levels of p24 were reduced by 57.5% compared to virus controls and intracellular levels were reduced by 56.56% compared to controls. Hence, it can be concluded that Serpin B1 is not interfering with HIV budding since similar levels of p24 are both inside and outside the cell following treatment within this antiprotease, and instead Serpin B1 may be acting post-transcriptionally, before viral budding.

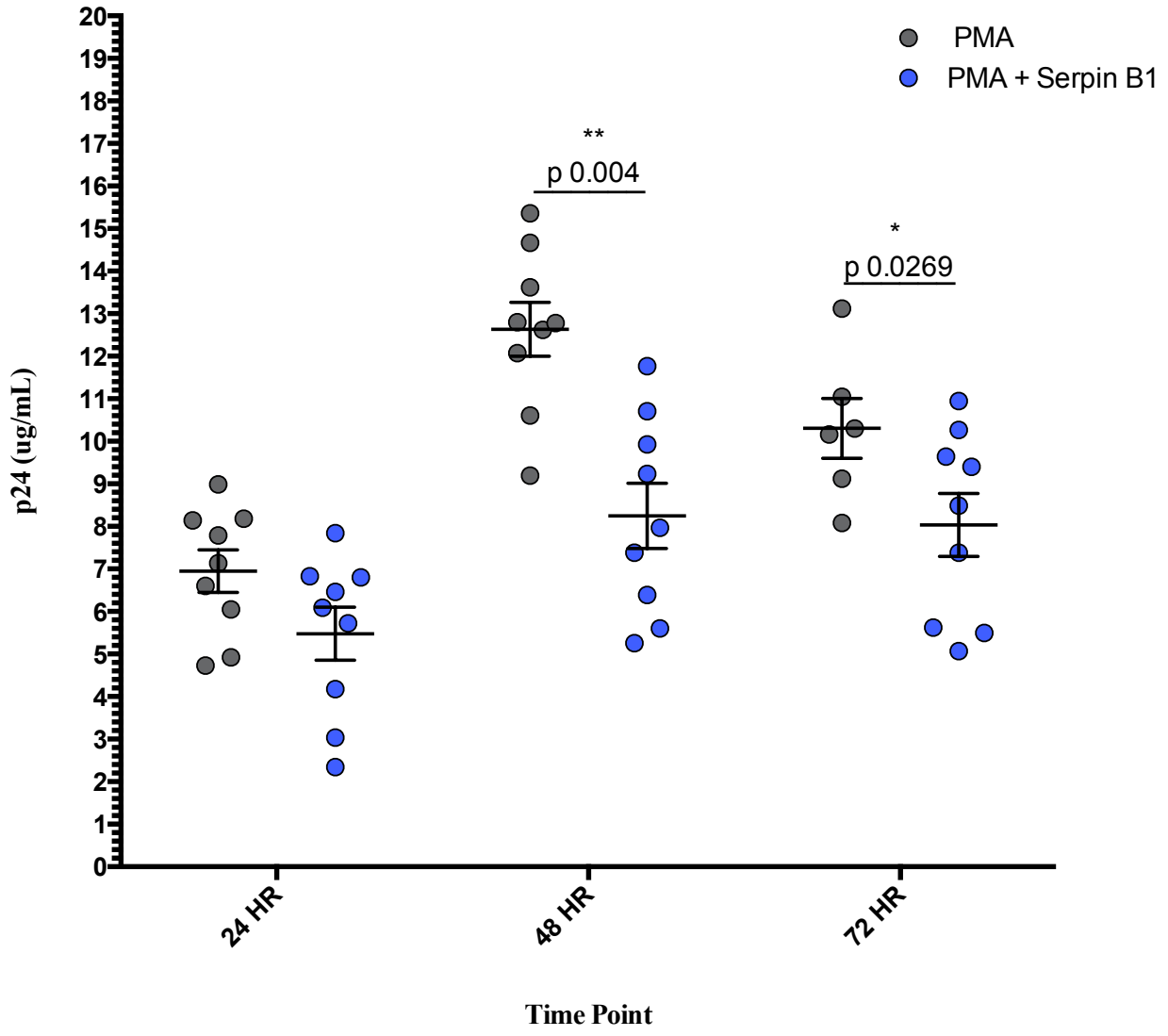


Figure 33: Effect of Serpin B1 on extracellular p24 production by ACH2 cells
Extracellular p24 within ACH2 cell supernatants, quantified with p24 ELISA. Serpin B1 (50 μg/mL) treated ACH2 cells produced significantly lower levels of p24 following 48 ($p = 0.004$) and 72 hours ($p = 0.0269$) incubation. No significant differences were observed between Serpin B1 treated cells and untreated cells following 24-hour incubation. Each data point represents one technical replicate. Three biological replicates were performed with three technical replicates in each. D'Agostino and Pearson Omnibus K2 Normality test was performed to confirm Gaussian distribution, followed by student unpaired t-tests (alpha = 0.05), between ACH2 supernatants treated only with PMA and those treated with Serpin B1 + PMA.

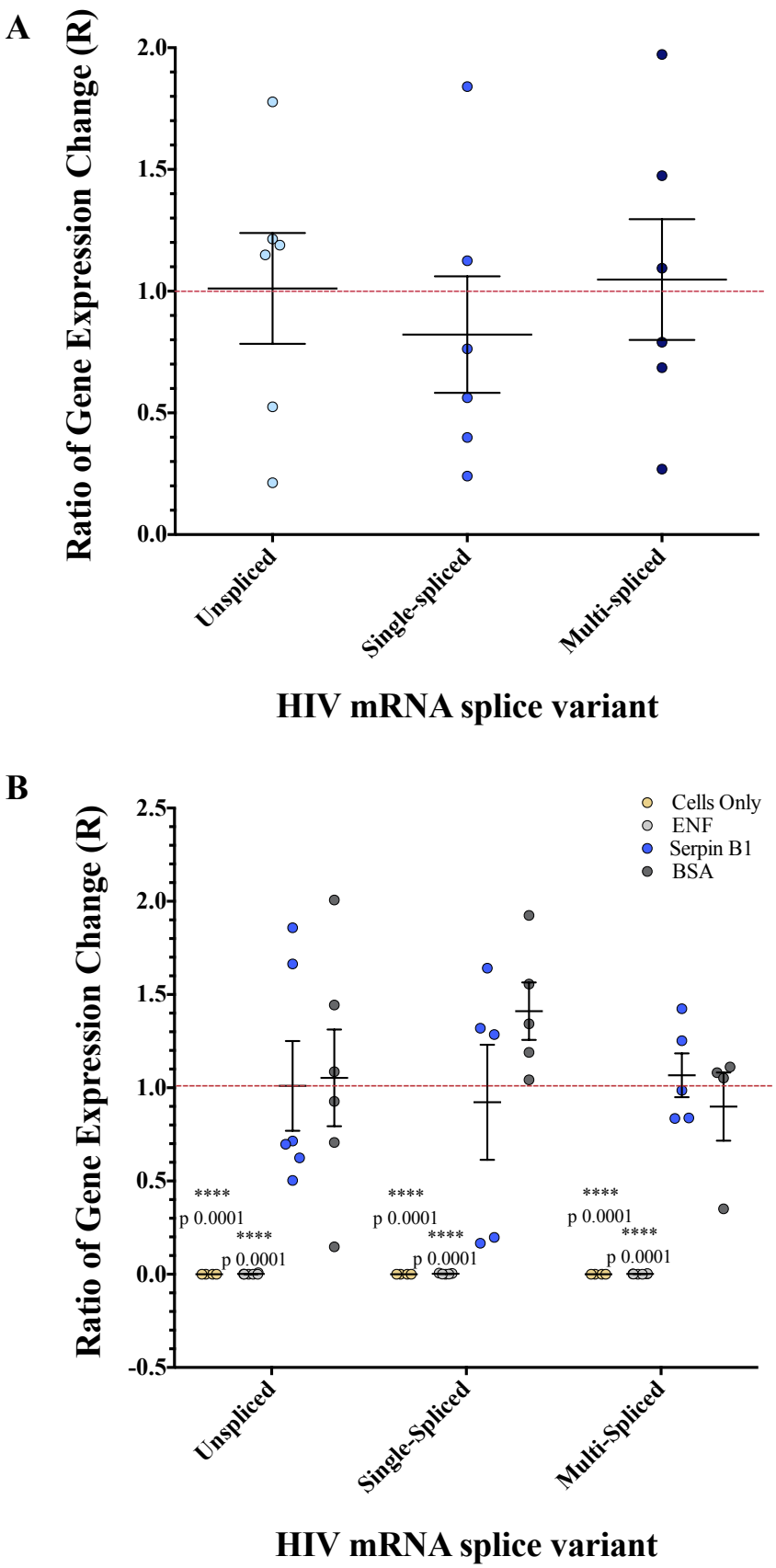

A
Ratio of Gene Expression Change (R)
2.0
1.5
1.0
0.5
0.0
Unspliced
Single-spliced
Multi-spliced
HIV mRNA splice variant
B
Ratio of Gene Expression Change (R)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
Cells Only
ENF
Serpin B1
BSA
****
p 0.0001
****
p 0.0001
****
p 0.0001
****
p 0.0001
****
p 0.0001
****
p 0.0001
Unspliced
Single-Spliced
Multi-Spliced
HIV mRNA splice variant

Figure 34: Real Time PCR quantification of HIV mRNA splice variants following Serpin B1 treatment of PBMCs and ACH2 cells

Viral mRNA splice variants were quantified using qRT-PCR. Virus alone was depicted as a red dashed line, which was set to 1 as the base-point to compare all quantitative measures to. ENF and uninfected cells were used as negative controls in the PBMC cultures. Untreated HIV_{1956} infected PBMCs, as well as ACH2 cells stimulated with only PMA were used as virus controls. Each point represents a technical replicate. Three biological replicates were performed with two technical replicates each. Bars represent mean $\pm$ SEM. The ΔC_q for the virus controls were subtracted from the ΔC_q for each experimental condition resulting in the $\Delta\Delta C_q$ for each reaction. This value was then used to determine the ratio of gene expression change using the equation $R=2^{-\Delta\Delta Cq}$. D'Agostino and Pearson Omnibus K2 Normality test was performed on all R values. Data obtained from ACH2 mRNA quantification were further analyzed using unpaired student t-tests, while results from quantification of mRNA from PBMC cultures were assessed using Mann-Whitney U-tests. All analyses were performed with an alpha of 0.05.

A: Unspliced, single-spliced, multi-spliced HIV mRNA within ACH2 cell cultures treated with Serpin B1 compared to ACH2 cells untreated with Serpin B1. Both treated and untreated cells were stimulated with PMA during culturing. B: Unspliced, single-spliced and multi-spliced HIV_{1956} mRNA within PBMCs treated with Serpin B1, BSA or ENF compared to PBMCs treated with PBS only.

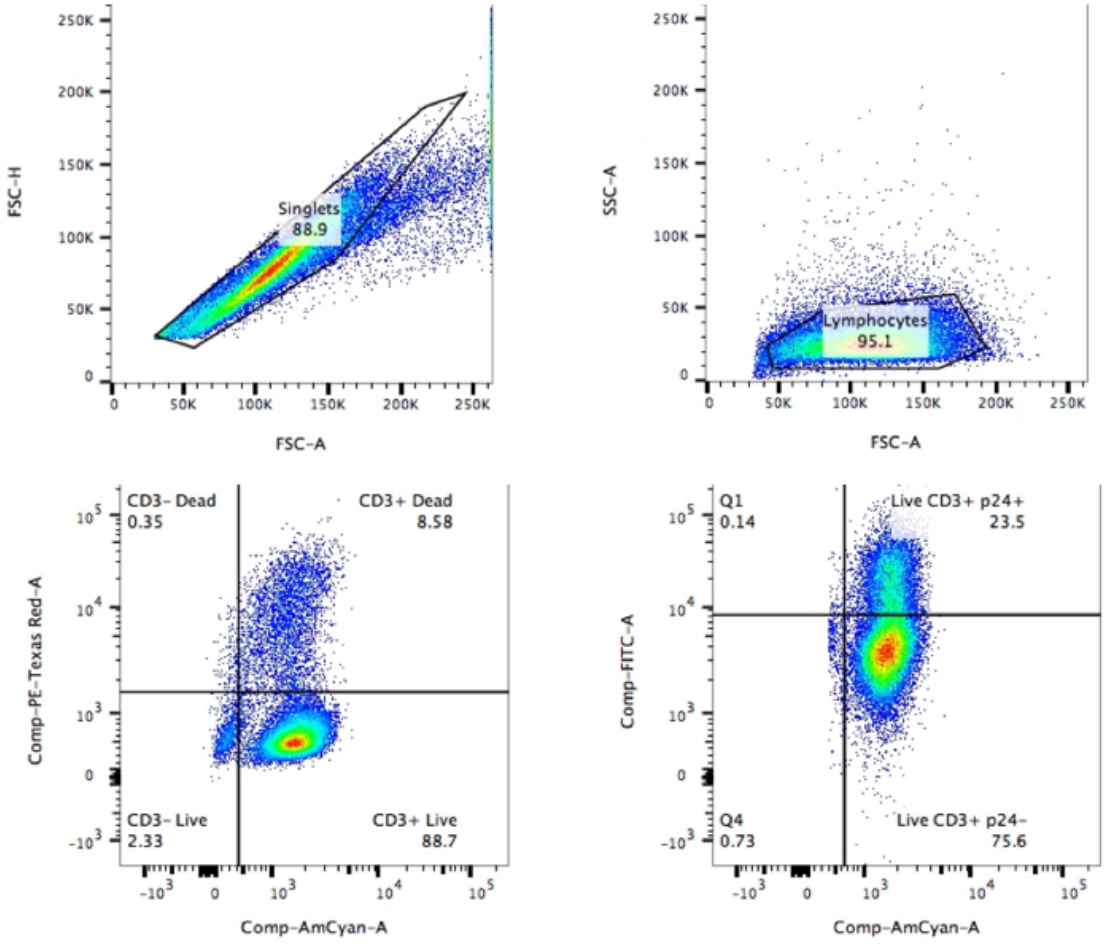


Figure 35: Gating strategy for intracellular p24 PBMC flow cytometry analysis
PBMCs were infected with HIV_{1956} for five days followed by staining with surface and intracellular Abs. Cells were analyzed using the BD LSR II. All cells were first gated on the singlet populations, which were then gated for lymphocytes. These lymphocytes were broken down into live (PE-Texas Red negative), CD3+ (Amcyan+), which were then gated for p24 (FITC+). AZT was used as a negative control (reverse transcriptase inhibitor), and BSA as a protein control. Untreated/uninfected PBMCs were used as a negative control while HIV_{1956} infected PBMCs were present as a cell control.

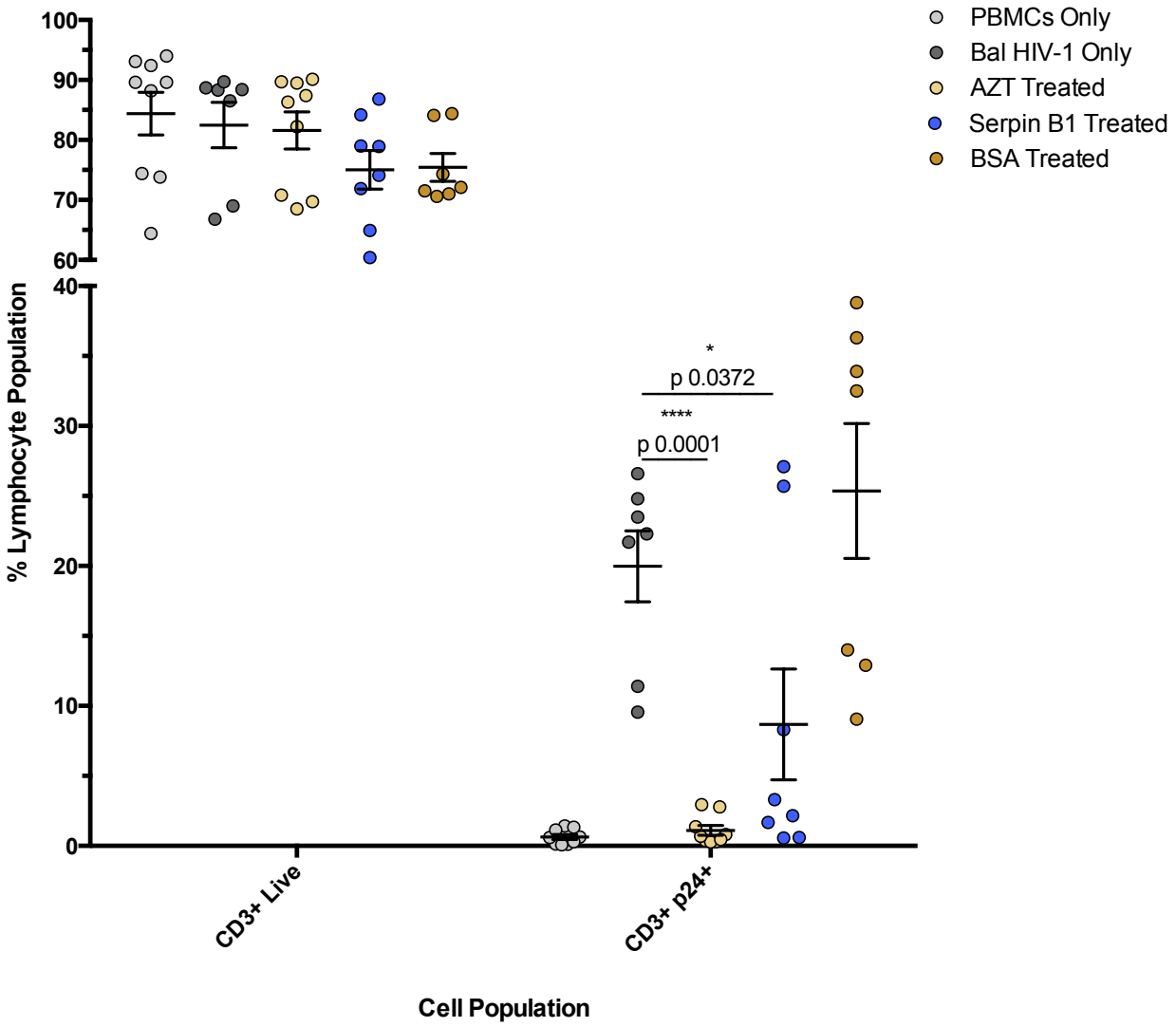


Figure 36: Serpin B1 treatment decreases intracellular p24 levels in PBMCs
PBMCs from three Winnipeg donors were treated with PBS, AZT, BSA (50µg/mL) or Serpin B1 (50µg/mL), and infected with HIV_{1956} for five days. These cells were then stained with surface $CD3^{+}$ Ab or intracellular p24 Ab (KC-57) and quanitified via flow cytometry using the BD LSR II. Each point represents one technical replicate. There were three technical replicates per biological replicate. Bars represent mean $\pm$ SEM. AZT served as a positive control for inhibition, and cells only (no infection/treatment) acted as cellular controls for normalizing data. BSA treated cells acted as a protein control. HIV_{1956} infected PBMCs treated with Serpin B1, or BSA were compared to untreated, infected PBMCs. D'Agostino and Pearson Omnibus K2 Normality test was performed, followed by unpaired student t-tests with an alpha of 0.05.

6.5 Summary

Commercially obtained Serpin B1 was shown to maintain its natural physiological function of inhibiting neutrophil elastase, as well as its ability to traffic to the cellular cytoplasm and nucleus. When this protein was exogenously added to cell cultures containing TZM-bl cells, C8166 T cells, PBMCs or an ectocervical explant, regardless of the HIV-1 strain, significant inhibition of infection was exhibited at concentrations of 25μg/mL or higher.

To determine the mechanism of inhibition for Serpin B1 work in this chapter focused on identifying the specific mechanisms employed by Serpin B1 to inhibit HIV directly.

These results demonstrated that the early stages of HIV-1 replication (reverse transcriptase, nuclear import, and integration) were not affected by Serpin B1 treatment. However, Serpin B1 was capable of inhibiting p24 production by the provirus-containing ACH2 cells, suggesting a role for Serpin B1 in inhibiting later stages of the HIV-1 lifecycle. Experiments examining HIV-1 transcription and budding clearly demonstrated that Serpin B1 did not affect these functions. The only remaining primary stages in HIV replication that could be affected are translation and viral assembly. However, translation-based and viral assembly experiments are beyond the scope of our laboratory and as such would be better executed in a laboratory with capabilities to adequately examine translational dynamics and individual protein assembly at the host cell membrane. Furthermore, the data from mass spectrometry-based proteomic analysis (Chapter 8), which was performed concurrently to these studies, suggests that rather than an HIV-1 specific mechanism, Serpin B1 may be acting by altering host pathways that affect host cellular responses to infection and cellular proliferation, which will be investigated further in Chapter 7.

CHAPTER 7: CELLULAR IMMUNOLOGICAL RESPONSE TO SERPIN B1

7.1 Rationale

Serpin B1 has demonstrated consistent neutralizing capacity against infection in multiple cells types, and with multiple HIV-1 strains, however, its mechanism remains unclear. Serpin B1 has also been shown to exhibit potent immune modulatory effects, and protects against infection by *Pseudomonas aeruginosa* as well as in diseases with pathologies caused by excessive immune activation, including cystic fibrosis[298,327,332,368,369]. Whether its immune modulatory function is due to its antiprotease activity, or whether there are other mechanisms involved is not entirely understood. Interestingly, Serpin A1 and A3 have been identified as modulators of the expression of neutrophil and macrophage chemotactic chemokines (IL-8, MCP-1) [282,298,315,365,370], as well as pro-inflammatory cytokines (TNF-a, IL-1α)[235,282,365,370] through inhibition of specific proteases, including Cathepsin G. Reduced expression of these factors has been proposed to reduce the number of target cells for HIV-1 and limit the amount of tissue damage at the site of infection. Serpin A1 and Serpin C1 have also been identified as potent inhibitors of the NF-κB pathway, resulting in subsequent inhibition of HIV-1 gene transcription[297,316]. Serpin B1 has also been shown to exhibit similar powerful regulatory effects on neutrophils, and the proteases they produce. Serpin B1 may exhibit inhibitory activity against HIV, similar to what has been observed with Serpin A1, and A3, however, this has not yet been examined.

Additionally, Serpin B1 can be cleaved by its target proteases to form L-DNase II which is a protein that contains pro-apoptotic activity[327-329]. Serpin B1 may be inducing an early apoptotic-like state (autophagic) whereby the activity of cell replication machinery is being limited. Furthermore, as the data in Chapter 6 demonstrated, Serpin B1 does not alter HIV replication prior to gene transcription. While Serpin B1 may interfere with proper post-translational steps in replication, it is unclear whether its inhibitory effects may also be due to

regulation of inflammatory responses, or due to indirect cellular mechanisms as has been demonstrated for Serpin A1 and C1.

7.2 Hypotheses

1. Serpin B1 regulates the expression of factors, including proteases and anti-viral cytokines/chemokines, that have previously been shown to result in reduced HIV replication
2. Serpin B1 reduces the level of Immune activation and proliferative capacity of cells, thereby, reducing the amount of HIV virions produced by infected cells.

7.3 Objectives

- Determine the effect of Serpin B1 on cellular activation through the use of flow cytometry staining of extracellular activation markers (CD69, CD38, HLA-DR).
- Determine the effect of Serpin B1 on cell proliferation through cell enumeration, and eFluor cell proliferation staining for flow cytometry.
- Ascertain the impact of Serpin B1 on the level of apoptosis in infected and uninfected cells, through flow cytometry staining of early and late apoptotic markers (Annexin V, Live/Dead stain).
- Determine if Serpin B1 inhibits HIV by decreasing the level of pro-inflammatory cytokine/chemokines, or by increasing the expression of anti-inflammatory cytokines/chemokines present within cell cultures.

7.4 Results

7.4.1 Serpin B1 does not affect cellular activation

To determine whether Serpin B1 is reducing the level of cellular activation required for HIV replication, flow cytometry was performed measuring specific activation markers. PBMCs from three local Winnipeg donors were isolated and treated with either PBS (unstimulated cell control), anti-CD3/CD28 (positive control), PMA (2ng/mL)/Ionomycin (200ng/mL, positive control), BSA (50µg/mL, protein control) or Serpin B1 (50µg/mL). After six and 14 hours of incubation, cells were stained for extracellular markers of activation as well as with a live/dead stain, and analyzed using a BD LSR II. During analysis, cells were gated on singlets, then lymphocytes, followed by live CD3 lymphocytes, which were gated into cell subsets ($CD3^+/CD69^+$, $CD8^+/HLA\text{-}DR^+$, $CD4^+/HLA\text{-}DR^+$) presented in Figure 37. Both anti-CD3/CD28 beads ($p = 0.0000461$) and PMA/Ionomycin ($p = 0.0000194$) induced significant levels of the early activation marker, CD69, and anti-CD3/CD28 beads also caused a significant increase in expression of HLA-DR on both $CD4^+$ ($p = 0.0498$) and $CD8^+$ ($p = 0.0206$) lymphocytes compared to the unstimulated controls. Serpin B1, however, did not show any significant effects on any of the activation markers at either time point compared to the unstimulated control cells (Figure 37, 6-hour time point data not shown). Furthermore, CD38 was not induced on any of the cells, regardless of stimulant condition (data not shown).

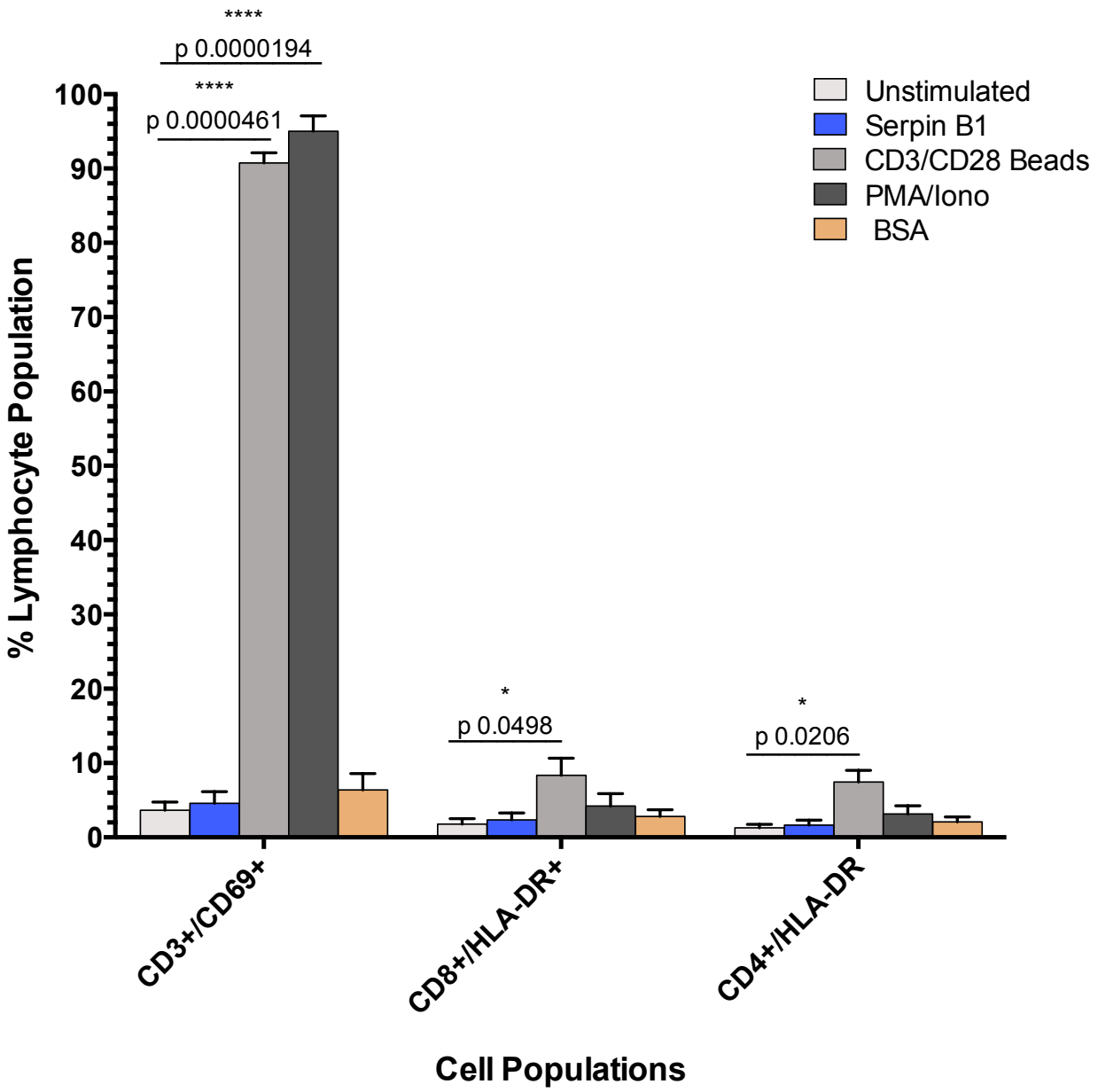


Figure 37: Serpin B1 does not affect PBMC immune activation
Activation markers in T cells treated with PBS (unstimulated), Serpin B1, CD3/CD28 beads, PMA/Ionomycin or BSA for 14 hours.
Each bar represents data from three biological replicates, represented as mean with standard error of the mean (SEM). D'Agostino and Pearson Omnibus K2 Normality test was performed to confirm Gaussian distribution, followed by multiple Student t-tests, each row was analyzed individually, did not assume consistent SD (Welch's correction), an alpha of 0.05 was applied to all analyzes.

7.4.2 Serpin B1 reduces the level of cellular proliferation

To determine whether the observed reduction in HIV replication following Serpin B1 treatment may have been caused, in part, by reduced cellular proliferation, cell numbers and cell viability, via Trypan Blue staining, were evalulated in Serpin B1 treated and untreated cells. PBMCs were incubated for three days, in the presence or absence of PHA. Unstimulated cells served as negative controls, and the PHA-stimulated cells mimicked conditions used in the previous neutralization assays. Cells were then incubated for a further five days with either Serpin B1 (50ug/mL) or PBS (cell control) (Figure 38). The total number of live cells were enumerated following Trypan Blue staining (Figure 38A) and % viability was determined by comparing the ratio of dead to living cells (Figure 38B). Analysis of cell numbers indicated a significant reduction in cells following treatment with Serpin B1 (32.03% reduction, $p = 0.0083$) in PHA-stimulated PBMCs. However, no reduction was noted in unstimulated cells. Furthermore, there was no difference in % viability between control cells and Serpin B1 treated cells, regardless of PHA stimulation (Figure 38B).

The effect of Serpin B1 on cellular proliferation was confirmed via flow cytometry. PBMCs collected from six separate individual donors were PHA-stimulated for three days before being stained with eFlour proliferation dye and incubated for five days with either Serpin B1 (50μg/mL), BSA (50μg/mL, protein control) or PBS (cell control). Following incubation, these cells were stained for extracellular CD3, CD4, CD8 and analyzed using a BD LSR II flow cytometer (Figure 39 and 40). Analysis indicated that significantly lower proportions of lymphocytes were proliferating after Serpin B1 treatment compared to protein or negative controls, ($p = 0.0097$ and 0.0266, respectively). Approximately 45% of the parent $CD3^+$ lymphocyte population was proliferating in the PBS or BSA treated cell cultures while only 33% were actively proliferating in the Serpin B1 treated cultures (Figure 40).

These results were confirmed in ACH2/A3.01 cell lines, in which Serpin B1 treatment was shown to previously inhibit HIV-1 (Chapter 6). A3.01 is the parental cell line for ACH2 and as such does not contain an integrated provirus. Hence, comparing the effect that Serpin B1 had on the proliferative capacity of both these cell lines determined whether Serpin B1 had a stronger effect on cells latently infected with HIV compared to those that were not. Both cell lines were stained with eFlour proliferation dye prior to simulation with 5ng PMA (induces transcription of HIV genes from ACH2 cells), and incubated for six days with either Serpin B1 (50μg/mL) or PBS (cell control). Cells were stained for CD5 (APC/Cy7) on ACH2 cells and CD71 (Transferrin receptor, PE/CY7) on both ACH2 and A3.01 cells, and analyzed by flow cytometry. Both CD5 and CD71 are known surface markers for these cell subsets. The A3.01 parental cell line (18.16% reduction) and ACH2 cells (54.49% reduction), both exhibited significantly reduced proportions of proliferating cells following treatment with Serpin B1 compared to untreated cells ($p = 0.0508$, and 0.0213, respectively, Figure 41). Taken together these results provide evidence of a role for Serpin B1 in the interference of cellular proliferation, with a potentially enhanced effect observed in cells infected with HIV-1.

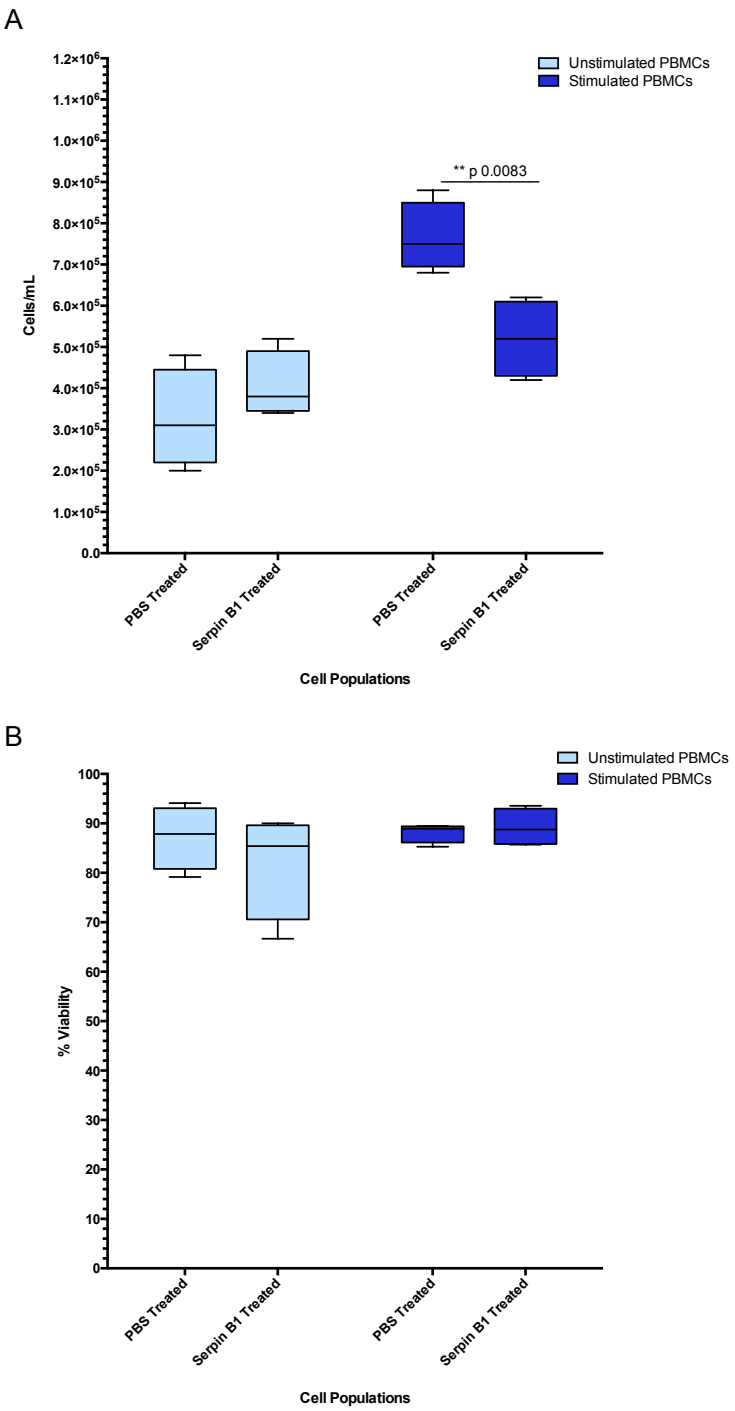


Figure 38: PBMC Trypan blue staining following Serpin B1 treatment

A: Cell counts of viable, unstimulated and PHA-stimulated PBMCs, following five-day incubation with PBS or Serpin B1. B: Cell viability for unstimulated and PHA-stimulated PBMCs following five-day incubation with PBS or Serpin B1. An n of 4 for each cell condition was included in the analysis. Bars represent the mean $\pm$ SEM for four independent experiments. D'Agostino and Pearson Omnibus K2 Normality test was performed to confirm Gaussian distribution followed by student t-tests, did not assume consistent SD, alpha 0.05 (Welch's correction).

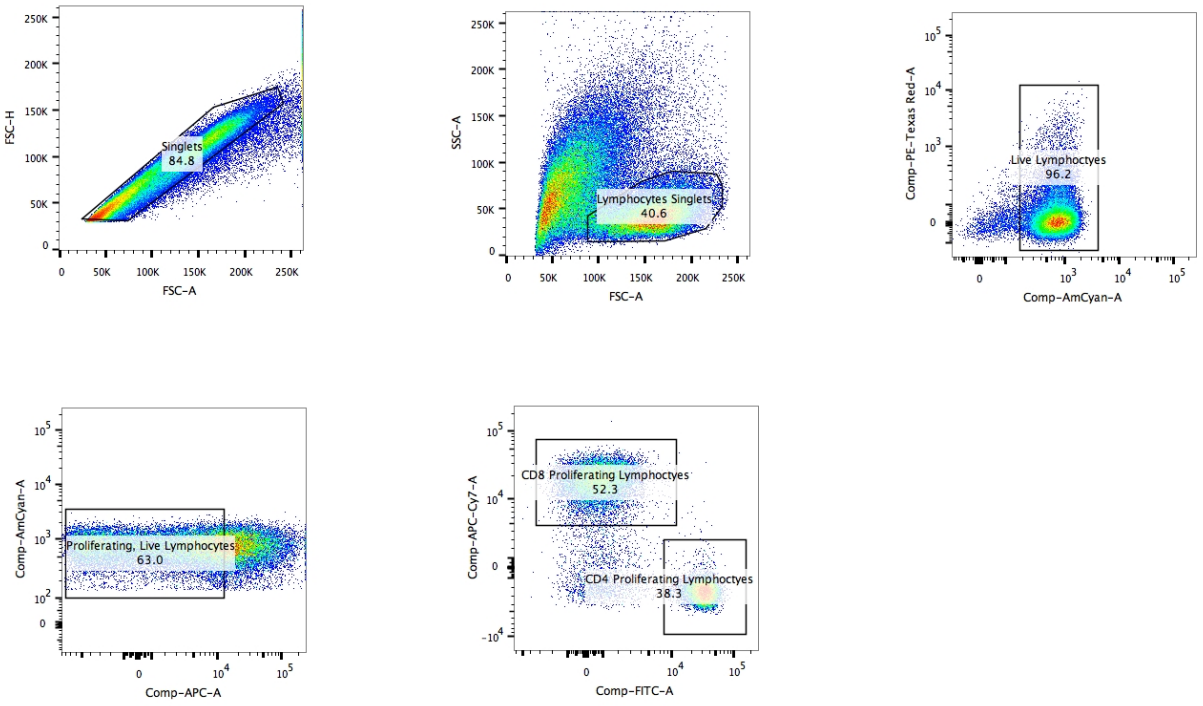


Figure 39: Gating strategy for Flow Cytometric detection of proliferating PBMCs following Serpin B1 treatment

PBMCs were PHA-stimulated for three days and stained with eFluor proliferation dye prior to treatment with either Serpin B1 or PBS for five days. Cells were then stained for surfce markers (CD3, CD4, CD8). Analysis of cell poplulations included first gating on singlets, lymphocytes, live (PE-texas red) $CD3^+$ (AmCyan) lymphocytes, proliferating (APC). The proportion of proliferating $CD4^+$ (FITC) and $CD8^+$ (APC-Cy7) were then determined from the total proliferating live $CD3^+$ lymphocyte population.

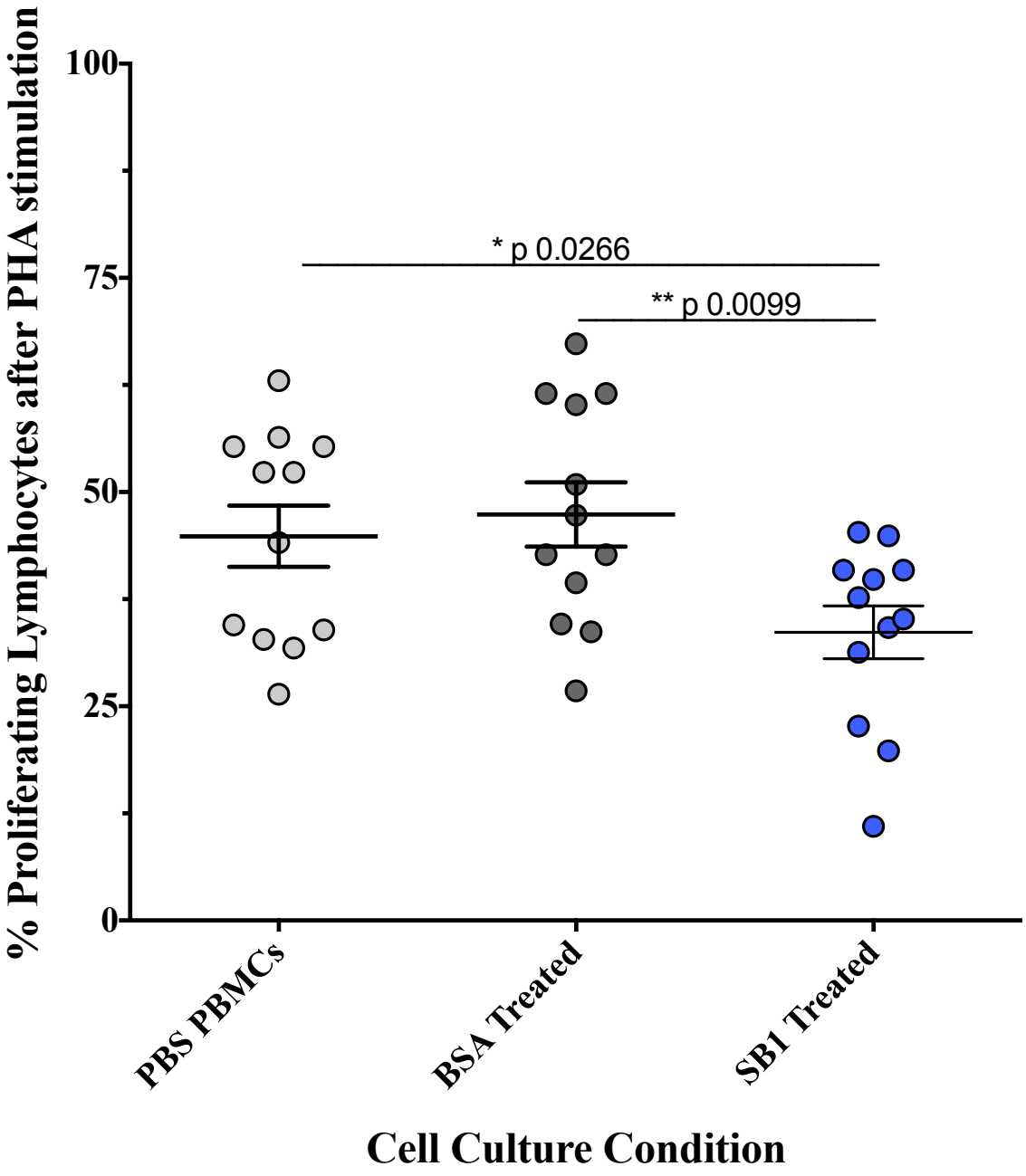


Figure 40: Serpin B1 treatment of PBMCs reduces the proportion of proliferating lymphocytes

Difference in proportion of proliferating $CD3^+$ live lymphocytes in Serpin B1 treated, compared to untreated, and BSA treated PBMCs from six separate donors, performed in duplicate. All cells were pre-stimulated with PHA before treatment with PBS, BSA (50µg/mL), or Serpin B1 (50µg/mL). Each data point represents a technical replicate. Six biological replicates were included with two technical replicates each. Each bar represents mean ± SEM.

D'Agostino and Pearson Omnibus K2 Normality test was performed to confirm Gaussian distribution followed by student t-tests, each row was analyzed individually, and consistent SD was not assumed (Welch's correction). An alpha of 0.05 was used.

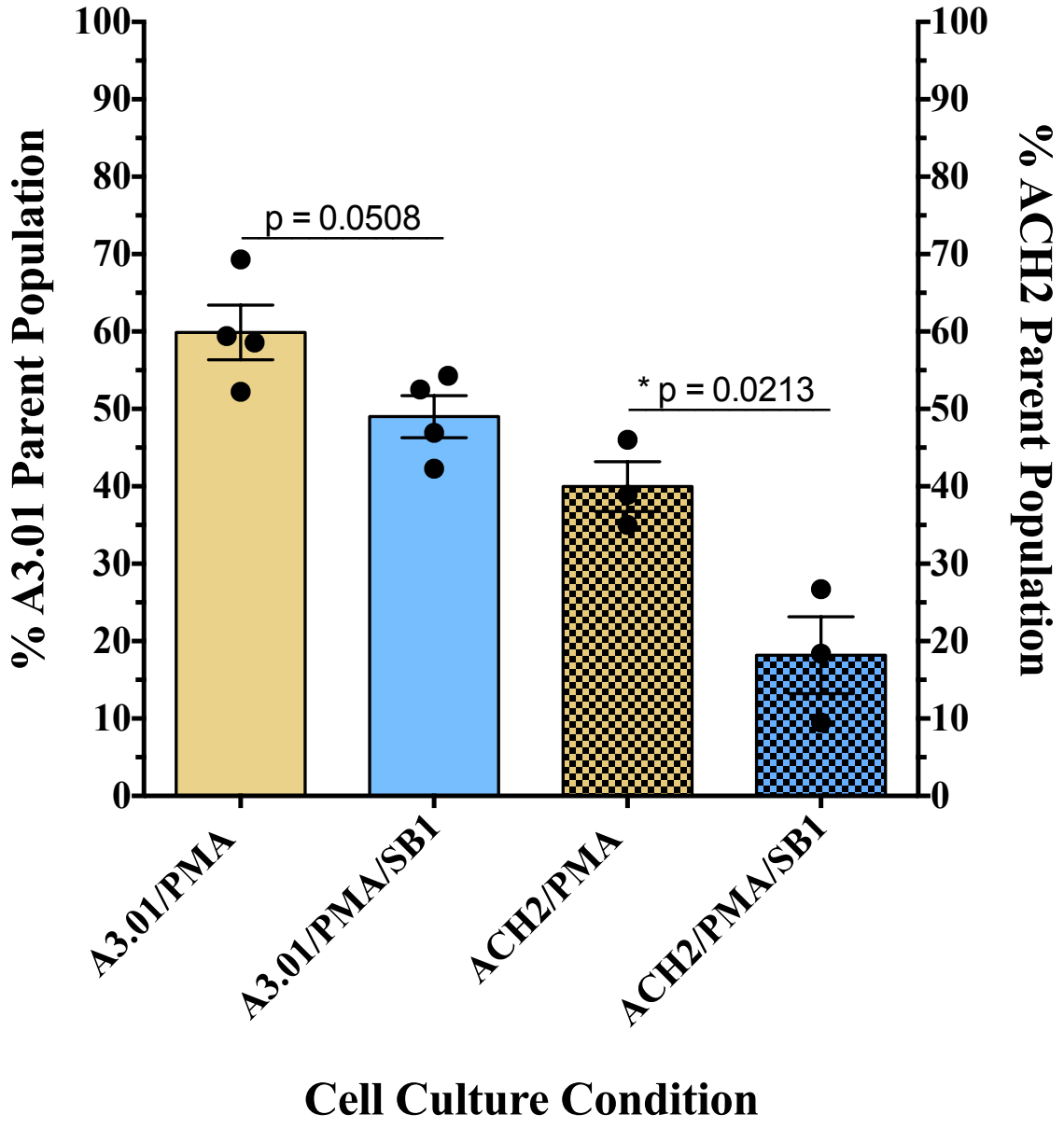


Figure 41: Serpin B1 treatment reduces the proportion of proliferating A3.01 and ACH2 cells

A: Flow cytometry staining (eFluor) for proliferating A3.01, and ACH2 cells following treatment with either PBS, just PMA, or PMA and Serpin B1 after six-day incubation. Parent population for ACH2 were detected by gating on the $CD5^{+}/CD71^{+}$ lymphocytes. The A3.01 parent population was determined by gating on $CD5^{+}$ lymphocytes. Each data point represents one biological replicate, which consisted of three technical replicates each. Bars represent mean $\pm$ SEM.

D'Agostino and Pearson Omnibus K2 Normality test was performed to confirm Gaussian distribution followed by unpaired student t-tests with Welch's correction (did not assume equal SDs), and an alpha of 0.05 between cell controls and experimental cultures, as well as between PMA treated controls and Serpin B1 treated cells.

7.4.3 Serpin B1 induces early markers of cellular apoptosis

To determine the effect that Serpin B1 has on apoptosis, flow cytometry was used to measure the proportion of active (Annexin V^+/Dead,) and early (Annexin V^+/Live) apoptotic cells within PBMCs and ACH2/A3.01 cultures (Figure 42 and 43).

PBMCs from six local Winnipeg donors were stimulated for three days with PHA prior to incubation for five days with Serpin B1 (50μg/mL), BSA (protein control, 50μg/mL) or PBS (cell control). PBMCs were then stained for Annexin V, CD3, CD4, CD8, and live/dead staining before analysis on a BD LSR II flow cytometer (Figure 42). Analysis of these results revealed that a significant increase in early apoptotic (Annexin V^+/Live $Dead^-$) $CD4^+$ lymphocytes were observed in Serpin B1 treated cells compared to PBS (17.65% vs. 5.77%, respectively) treated cells ($p = 0.0001$). Similarly, 18.13% of the $CD8^+$ lymphocytes treated with Serpin B1 were in an early apoptotic state, compared to only 1.04% of PBS treated cells and 2.98% of BSA treated protein control cells ($p = 0.0001$ and 0.0034, respectively) (Figure 43A). When actively apoptotic cells were examined, both $CD4^+$ and $CD8^+$ cells were found to stain for Annexin V+/Dead in Serpin B1 treated cultures compared to the PBS treated cells, statistical significance ($p = 0.0211$) was only reached in CD8+ lymphocytes. Similar proportions of actively apoptotic cells were found in cultures treated with the protein control, BSA, as were observed in Serpin B1 treated cells (Figure 43A), suggesting that treatment with any protein at concentrations of 50μg/mL results in limited levels of active apoptosis.

A similar experiment was performed to test Serpin B1's effect on apoptosis in HIV-infected cells. PHA-stimulated PBMCs from three local Winnipeg donors were infected with HIV_{1956} and being treated with PBS, BSA (50μg/mL) or Serpin B1 (50μg/mL) for five days. Cells were then stained with the same apoptotic and cell identification markers as previously described and analyzed on a BD LSR II flow cytometer (Figure 43B). Similar results were

obtained in this experiment, with increased proportions of $CD4^+/CD3^+$ and $CD8^+/CD3^+$ lymphocytes expressing early apoptotic (Annexin V^+/Live $Dead^-$) markers in Serpin B1 treated cells (12.66% and 10.31%, respectively), compared to either PBS treated (4.84% and 1.98%) or BSA treated (5.19% and 2.5%) cells, although statistical significance was not reached. The proportion of actively apoptotic $CD4^+$ and $CD8^+$ lymphocytes (Annexin V+/ Live $Dead^+$) were significantly increased within the Serpin B1 ($p = 0.0211$ and 0.0416, respectively), and BSA treated cells ($p = 0.0575$ and 0.0540, respectively) compared to PBS treated PBMCs (Figure 43B), again suggesting that treatment with any protein induces minimal levels of cell death.

To determine whether Serpin B1 was preferentially causing induction of apoptosis in HIV-infected cells, both ACH2, and A3.01 cells were stimulated with PMA either in the presence or absence of Serpin B1 for 48 hours and stained with anti-Annexin V antibodies and live/dead stain. Analysis of Flow cytometric results revealed that in both the A3.01 (Figure 44A) and ACH2 (Figure 44B) cell cultures, Serpin B1 significantly increased the proportion of early apoptotic cells (Annexin V+/Live) compared to PMA only treated cells. In A3.01 cells early apoptotic markers were increased by 26.6% and in ACH2 cells by 11.65% following treatment with Serpin B1 ($p = 0.0075$ and 0.0454, respectively). However, within these cell lines, the proportion of actively apoptotic cells (Annexin +/ Live $Dead^+$) was not increased in the presence of Serpin B1 compared to controls.

These results indicate that Serpin B1 may induce an early apoptotic state within both HIV-infected and uninfected cell cultures. This was apparent due to the induction of the early apoptotic marker Annexin V in the absence of significant active apoptosis within both ACH2, and A3.01 cells.

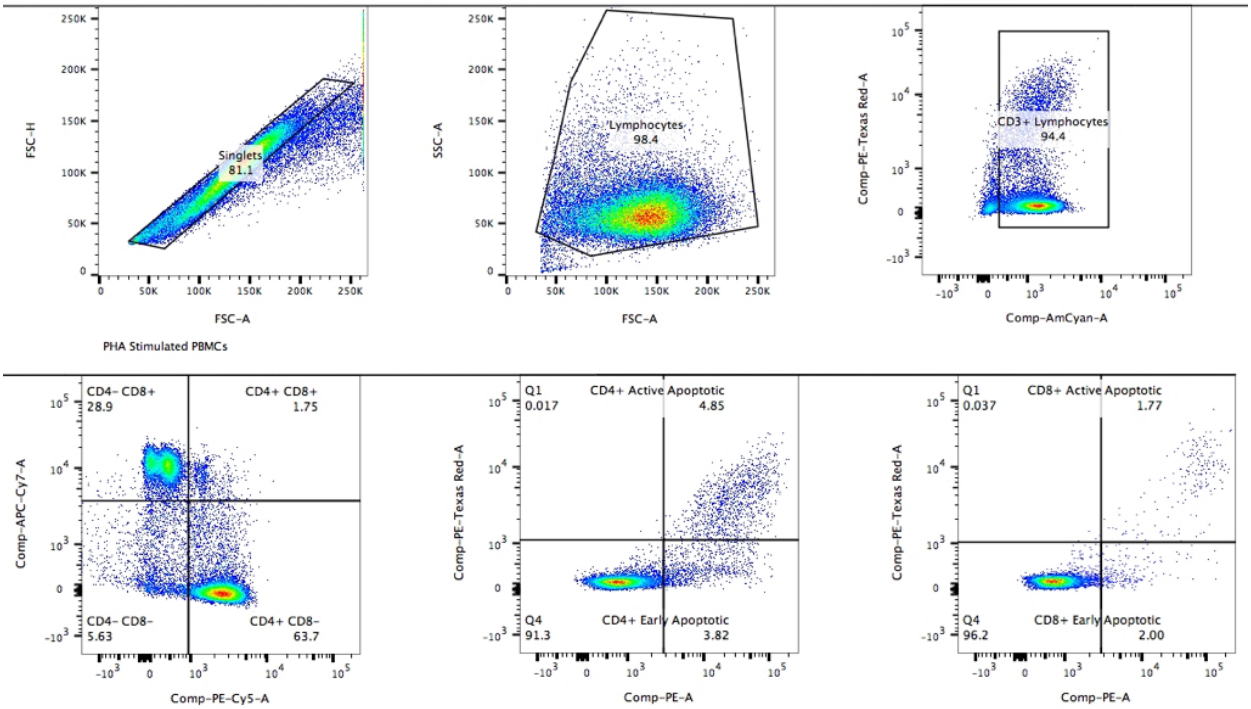


Figure 42: Gating strategy for Flow Cytometric analysis of PBMC apoptosis staining
PBMCs were stimulated for three days with PHA and were either infected with HIV_{1956} or remained unifected. Cells were then treated for five days with either Serpin B1 (50µg/mL), BSA (50µg/mL), or PBS and stained for flow cytometric analysis using a BD LSR II. All samples were first gated on singlets, lymphocytes, $CD3^+$ (AmCyan) lymphocytes, which were then divided into CD4/CD8 subsets with $CD4^+$ (PE-Cy5) and $CD8^+$ (APC-Cy7), and lastly were gated for Annexin V (PE) and Live/Dead (PE-Texas Red).

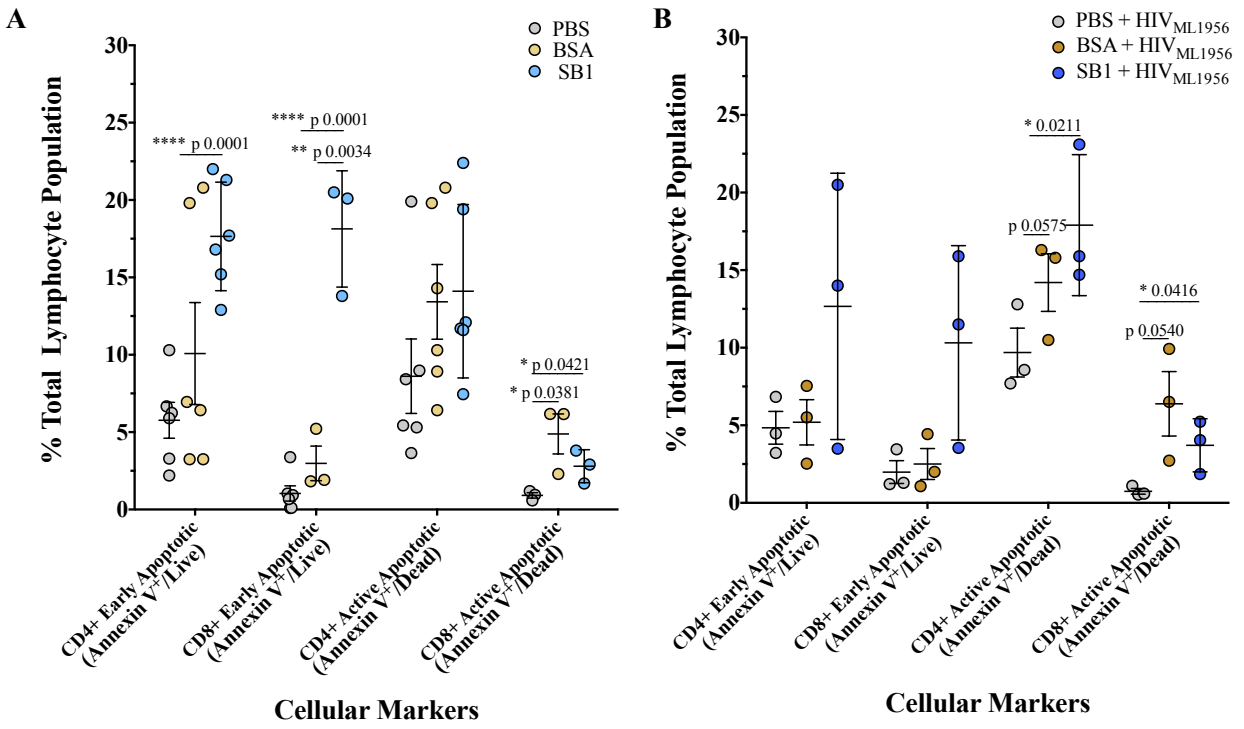


Figure 43: Proportion of early and actively apoptotic PBMCs present within uninfected and HIV_{1956}-infected cell cultures treated with Serpin B1

A: Apoptotic markers in uninfected, PHA-stimulated PBMCs from six donors (each data point represents one donor), treated with either PBS, Serpin B1 or BSA. B: Apoptotic markers identified in infected, PHA-stimulated PBMCs, from three donors, treated with either PBS, Serpin B1 or BSA.

Each data point represents one technical replicate. Each bar represents mean $\pm$ SEM. D'Agostino and Pearson Omnibus K2 Normality test was performed followed by unpaired Mann Whitney U-tests with an alpha of 0.05 between cell controls and experimental cultures.

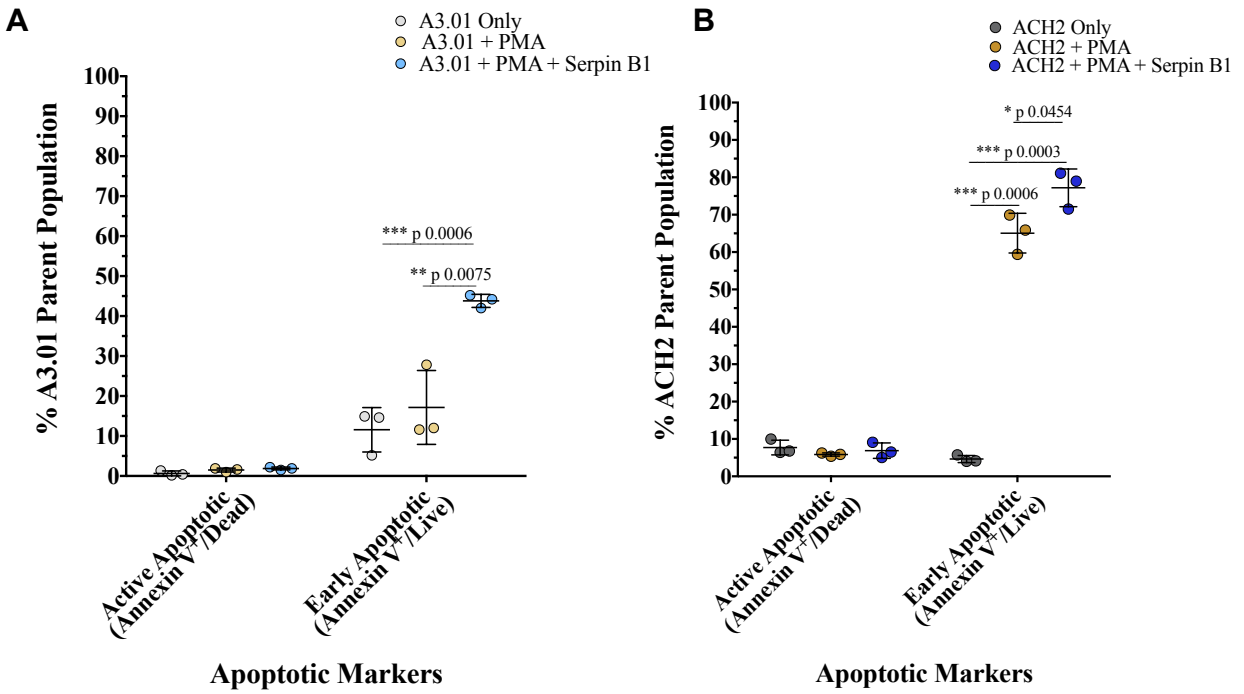


Figure 44: Proportion of early and actively apoptotic A3.01 and ACH2 cells present within cell cultures treated with Serpin B1

A: Apoptotic markers (Annexin V and live/dead stain) on A3.01 cells untreated or treated with Serpin B1 + PMA, or with PMA alone for 48-hours. B: Apoptotic markers on ACH2 cells untreated or treated with Serpin B1 + PMA, or with PMA alone for 48-hours.

Each data point represents one biological replicate with three technical replicates each. Each bar represents mean $\pm$ SEM. Parent population for ACH2 were detected by gating on the $CD5^+/CD71^+$ lymphocytes. A3.01 parent population was determined by gating on $CD5^+$ lymphocytes. D'Agostino and Pearson Omnibus K2 Normality test was performed followed by unpaired Mann Whitney U-tests with an alpha of 0.05 between cell controls and experimental cultures.

7.4.4. Serpin B1 induces the expression of multiple cytokines and chemokines in PBMCs and FGT epithelial cell line cultures

Determining the effect that Serpin B1 has on the expression of specific anti-viral cytokines and chemokines may provide information on how this antiprotease is inhibiting HIV infection. Therefore, PHA-stimulated PBMCs were cultured for 3 hours, 12 hours, 18 hours, 24 hours, 3 days and 5 days with PBS (cell control), BSA (protein control, 50μg/mL) or Serpin B1 (50μg/mL). The highest levels of all cytokines were detected following five-day incubation and hence this is the data presented (Figure 45). Analysis of the supernatants revealed no detectable difference in cytokine expression for GM-CSF, IFN-γ, IL-1β, IL-2, IL-8, IL-17, IP-10, MCP-1, MCP-3, MDC, TNF-α, and Fractalkine. However, significantly higher expression was noted for IL-6, IL-10, MIP-1α, MIP-1β, RANTES, IL-1α, and GRO in Serpin B1 treated cells compared to PBS cell controls. RANTES and IL-1α were also elevated following treatment with BSA. Therefore, the increase observed in Serpin B1 treated cultures for these two cytokines/chemokines does not appear to be unique to this antiprotease but rather to stimulation with any protein. Nevertheless, a 6.61-fold increase was observed in IL-6 concentration (pg/mL) in Serpin B1 treated PBMCs compared to PBS treated PBMCs ($p = 0.0501$). Similarly, a 3.32-fold ($p = 0.0503$), 13.27-fold ($p = 0.0196$), 3.50-fold ($p = 0.0169$), and 11.27-fold ($p = 0.00002$) increase were observed in IL-10, MIP-1α, MIP-1β and GRO, respectively (Figure 45A).

To determine if Serpin B1 exhibits the same effect on the expression of these cytokines/chemokines when HIV is present, PBMCs were again incubated with PBS, BSA or Serpin B1, however, this time, they were first infected with HIV_{1956}. Following analysis of the milliplex data it was determined that the expression of IFN-γ, IL-1β, IL-2, IL-8, IL-17, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-1β, RANTES, IL-1α, Fractalkine, and GRO were not

affected by Serpin B1 treatment. However, GM-CSF (3.06-fold increase, p = 0.0155), IL-6 (15.07-fold increase, p = 0.0004), IL-10 (4.34-fold increase, p = 0.0087) and TNF-α (6.21-fold increase, p = 0.0015) were significantly up-regulated following treatment of Serpin B1 compared to PBS cell controls, none of which were altered following treatment with BSA (Figure 45B).

To assess how quickly these cytokines/chemokines are up-regulated following Serpin B1 exposure, results from all six time points were plotted and analyzed (Figure 46). These results demonstrate that IL-6, IL-10, MIP-1α, MIP-1β, GM-CSF, TNF-α and GRO increased expression very early (3-12 hours post-infection), and systematically increased over time.

Since the goal of this thesis work is to determine whether Serpin B1 would be an appropriate microbicide candidate. The ability of Serpin B1 to induce cytokines and chemokines was also assessed in three FGT epithelial cell lines. These adherent cell lines were either treated, or not, with HIV_{1956}, and treated with PBS, BSA (50μg/mL) or Serpin B1 (50μg/mL) for three days with supernatants collected following 3 hours, 12 hours, 18 hours, 24hours and 3 day incubations, and analyzed using Milliplex. Many of the cytokines/chemokines analyzed were not in high enough abundance to be detected with the milliplex platform resulting in only eight analytes (GM-CSF, IL-6, IL-8, IL-1α, MIP-3α, RANTES, GRO, Fractalkine) detectable for each condition. Results were nearly identical between HIV treated and untreated cultures, and thus, results are only presented for uninfected cultures (Figure 47). Results indicated that for all three epithelial cell lines, GM-CSF had the largest fold change induction in response to Serpin B1, compared to PBS controls. Serpin B1 treated vaginal epithelial cells (VK2) demonstrated increased expression of GM-CSF by 13.13-fold (p = 0.0375) (Figure 47A). Similarly, endocervical (End1), and ectocervial (Ect1) epithelial cells showed a 44.08-fold (p = 0.0068), and 28.11-fold (p = 0.0011) increase in GM-CSF, respectively. IL-6 (9.72-fold, p = 0.0064), IL-8 (8.12-fold, p = 0.0402), IL-1α (8.97-fold, p = 0.0188) and GRO (3.34-fold, p = 0.0034) were

also significantly induced by Serpin B1 treatment within endocervical epithelial cell line cultures compared to PBS controls (Figure 47B). Ectocervical epithelial cell lines demonstrated increased expression of IL-6 (3.44-fold, $p = 0.0188$), and IL-1α (2.90-fold, $p = 0.0129$) when treated with Serpin B1 compared to PBS (Figure 47C).

Cumulatively these results suggest that Serpin B1 consistently induces the expression of GM-CSF, IL-6, and IL-1α at early time points following exposure to HIV in both PBMCs and FGT cell lines. Additionally, Serpin B1 induces the expression of IL-10, GRO, TNF-α, MIP-1α and MIP-1β within PBMC cultures.

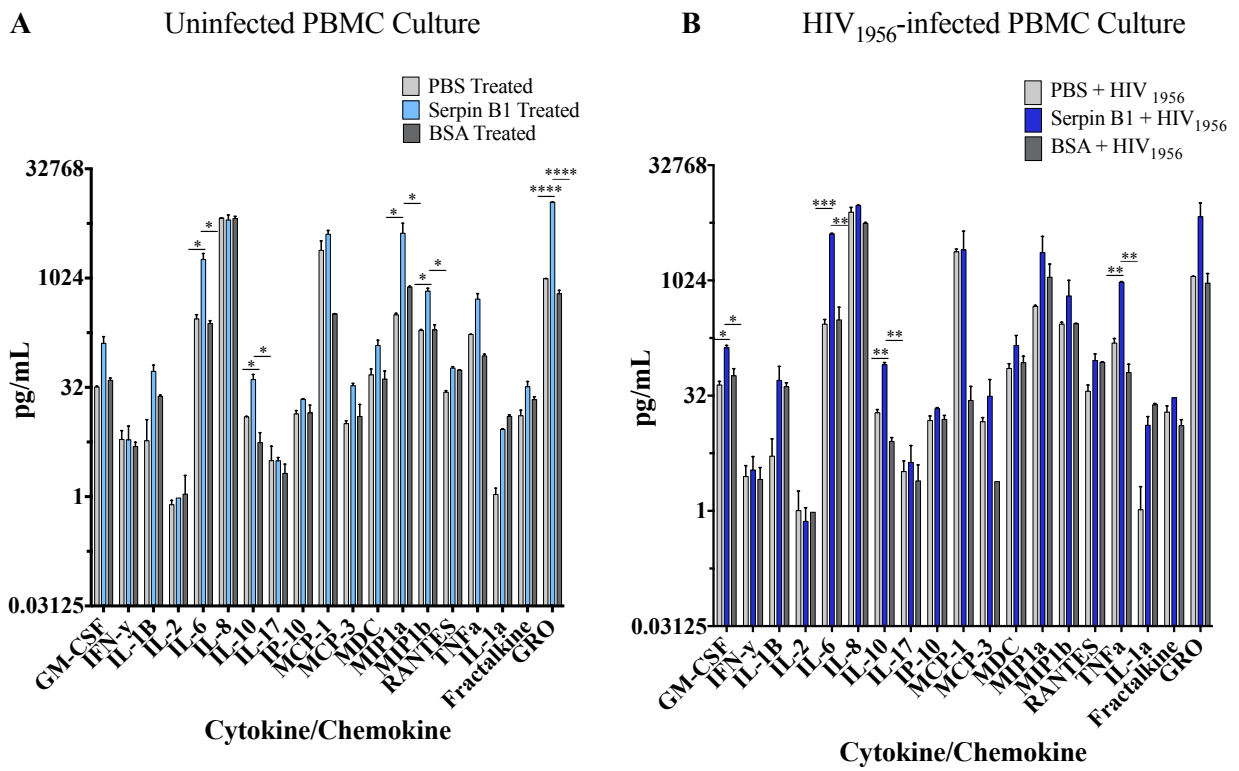


Figure 45: Effect of Serpin B1 treatment on cytokine and chemokine production by PBMCs

PHA Stimulated PBMCs were treated with PBS, Serpin B1, or BSA for five days. Cytokine and chemokine levels in culture supernatants were detected by Milliplex assays. Expression of each cytokine at time point 0 hour, was used to normalize data. Each bar represents the mean $\pm$ SEM for two biological replicates, each comprised of two technical replicates. Omnibus K2 Normality test was performed followed by unpaired Mann Whitney U-tests with an alpha of 0.05 between cell controls and experimental cultures.

A: PHA-stimulated, uninfected PBMCs treated with Serpin B1, BSA or PBS. B: PHA-stimulated, HIV_{1956}-infected PBMCs treated with Serpin B1, BSA, or PBS.

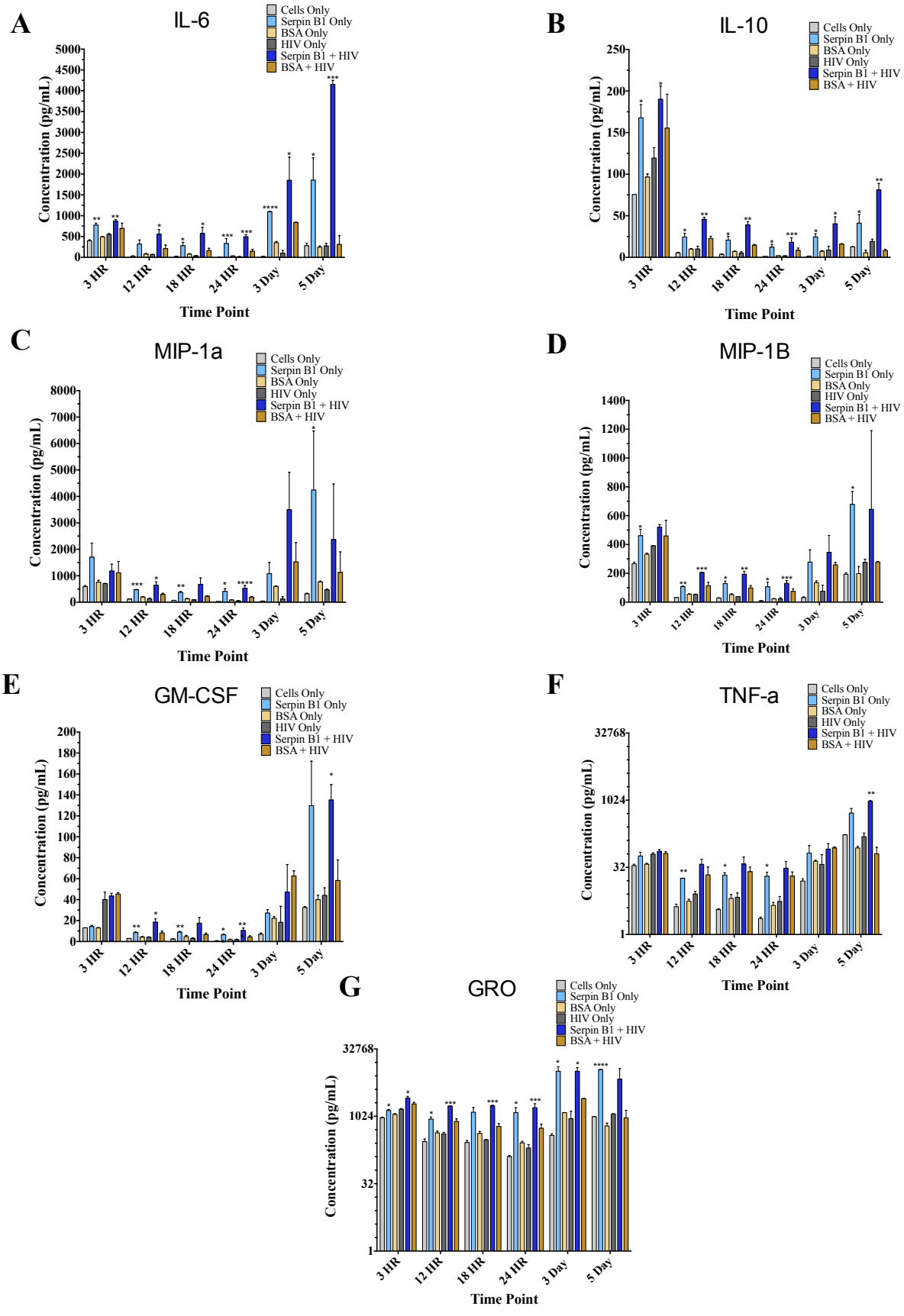

A
IL-6
B
IL-10
C
MIP-1a
D
MIP-1B
E
GM-CSF
F
TNF-a
G
GRO
Cells Only
Serpin B1 Only
BSA Only
HIV Only
Serpin B1 + HIV
BSA + HIV
Concentration (pg/mL)
Time Point
3 HR
12 HR
18 HR
24 HR
3 Day
5 Day

Figure 46: Cytokine/Chemokine expression following Serpin B1 treatment of PBMCs at multiple time points

PBMCs were stimulated with PHA for three days and either infected, or not, with HIV_{1956} followed by treatment with PBS, Serpin B1 or BSA for 3 hours, 12 hours, 18 hours, 24 hours, 3 days, and 5 days. Cytokine and chemokine levels in culture supernatants were detected by Milliplex assays. Expression of each cytokine at time point 0 hour was used to normalize data. Each bar represents the mean $\pm$ SEM for two biological replicates consisting of two technical replicates. Omnibus K2 Normality test was performed followed by unpaired Mann Whitney U-tests with an alpha of 0.05 between cell controls and experimental cultures.

A: Expression of IL-6. B: Expression of IL-10. C: Expression of MIP-1α. D: Expression of MIP-1β. E: Expression of GM-CSF. F: Expression of TNF-α. G: Expression of GRO.

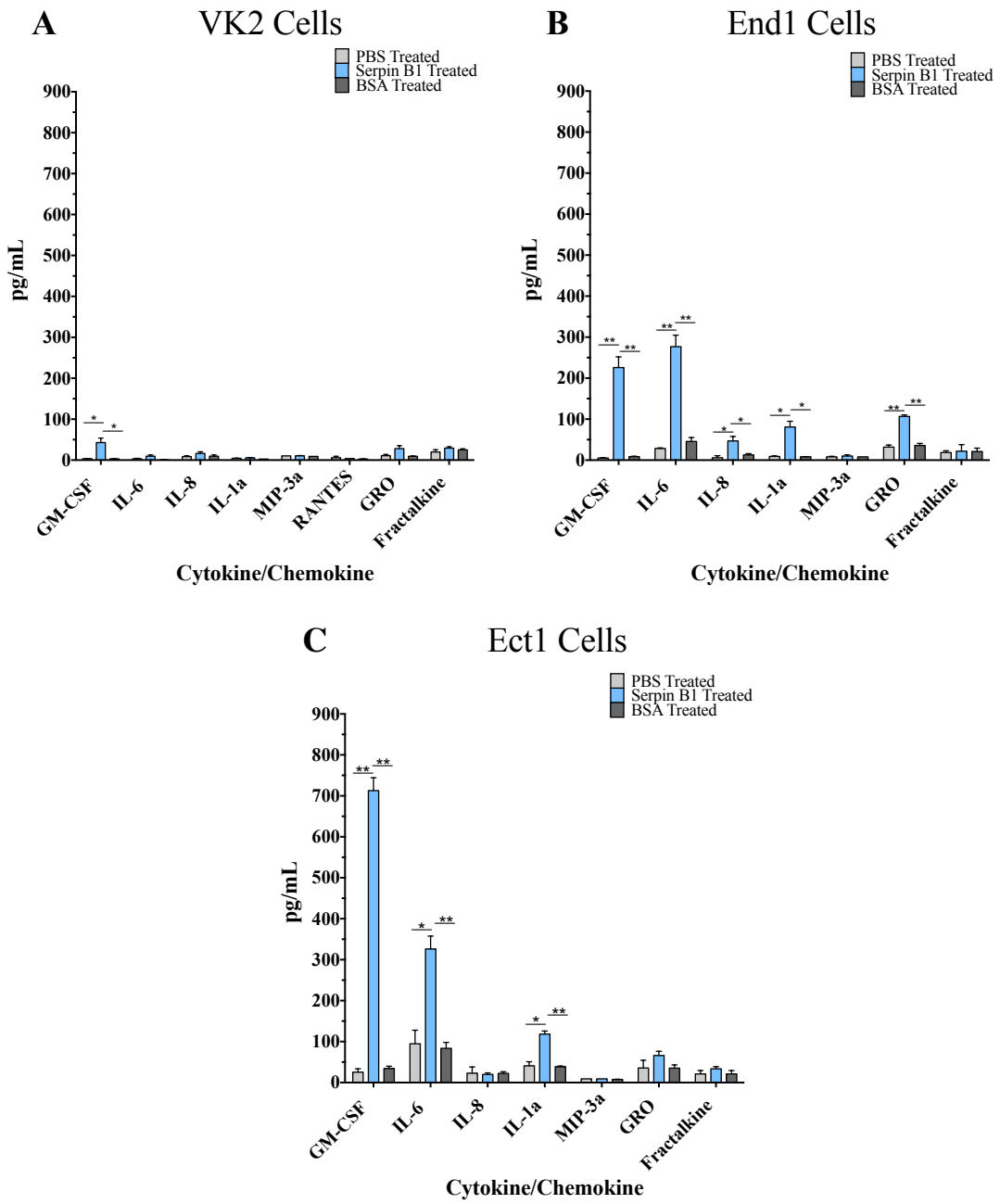


Figure 47: Effect of Serpin B1 on the production of specific cytokines and chemokines by female genital tract epithelial cell lines

FGT epithelial cell lines were treated with PBS, Serpin B1 or BSA for three days. Cytokine and chemokine levels in culture supernatants were detected by Milliplex assays. Expression of each cytokine at time point 0 hour was used to normalize data. Each bar represents the mean $\pm$ SEM for two biological replicates each comprised of two technical replicates. Omnibus K2 Normality test was performed followed by unpaired Mann Whitney U-tests with an alpha of 0.05 between cell controls and experimental cultures.

A: VK2 (vaginal) cell line, treated with PBS, Serpin B1 or BSA for three days. B: Endocervical cell line, treated with PBS, Serpin B1 or BSA for three days. C: Ectocervical cell line, treated with PBS, Serpin B1 or BSA for three days.

7.5 Summary

Serpin B1 appears to affect cellular proliferation and apoptosis, which may be influencing HIV replication. Specifically, it was determined that Serpin B1 inhibits cellular proliferation in PBMCs, C8166 T, ACH2, and A3.01 cells. This reduction in cellular proliferation may be due, in part, to the observed induction of an early apoptotic state.

Serpin B1 was also shown to increase the expression of various cytokines and chemokines within PBMCs and specific FGT epithelial cell lines. Specifically, GM-CSF, IL-6, IL-10, MIP-1α, MIP-1β, TNF-α, and GRO were up-regulated within either or both of the infected and uninfected PBMC cultures treated with Serpin B1. GM-CSF was also shown to be up-regulated among all the epithelial cell cultures treated with Serpin B1, along with IL-6 and IL-1α in the endocervical and ectocervical epithelial cells.

One cellular function that was not affected by Serpin B1 was that of activation. Following treatment with Serpin B1, there was no significant increase in early activation markers, namely CD69, CD38, HLA-DR, compared to levels present within PBS treated cells. Taken together these results began to suggest possible mechanisms by which Serpin B1 inhibited HIV-1 replication, however, a better understanding of the pathways responsible for decreased proliferation and increase in early apoptotic markers is necessary.

CHAPTER 8: EFFECT OF SERPIN B1 ON CELLULAR PROTEIN EXPRESSION

8.1 Rationale

Serpin B1 has been shown to inhibit multiple HIV-1 strains in multiple cell types. This inhibitory activity appears to act thru general cellular mechanisms (Chapter 7) and possibly thru direct inhibition of the HIV-1 virus, likely at post-transcriptional stages (Chapter 6). Serpin B1 altered cellular proliferation, up-regulated early apoptotic markers, and induced specific cytokines and chemokines (GM-CSF, IL-6, MIP-1α, MIP-1β, IL-1α, GRO). Furthermore, the data from Chapter 6 suggests that there may be a direct effect exhibited by Serpin B1 on HIV protein translation or protein assembly at the host cell membrane. Determining which proteins and which pathways are most affected by Serpin B1 will aid in deciphering the exact mechanism of action by which this antiprotease neutralizes HIV-1. Furthermore, since Serpin B1 alone only partially inhibits HIV-1 replication, determining which other antiproteases correlate with its expression may identifty candidates that act in a synergistic manner with Serpin B1.

8.2 Hypothesis

Serpin B1 interferes with signaling pathways involved in cellular proliferation and up-regulates the expression and activity of molecules involved in apoptotic pathways.

8.3 Objectives

- Perform label-free mass spectrometry on PHA-stimulated and unstimulated PBMC cell culture supernatants, and cell lysates following Serpin B1 treatment to determine which proteins, and hence which cellular pathways, are most significantly affected by Serpin B1.
- Determine which antiproteases within CVL are induced, and correlate with, the expression of Serpin B1.

8.4 Results

8.4.1 Mass spectrometry analysis of secreted proteins from Serpin B1 treated PBMCs

8.4.1.1 Protein differential expression analysis

Determining which proteins are secreted (present in supernatants) or induced intracellularly (within cell lysates) by Serpin B1 provides insight into which pathways it may be affecting. PBMCs from three local Winnipeg donors were stimulated, or not, with PHA for three days followed by infection with HIV_{1956} for three hours, and treatment with either PBS (cell control), BSA (50μg/mL, protein control), or Serpin B1 (50μg/mL). After five days, supernatants were collected and analyzed via label-free mass spectrometry. Supernatants from the infected cultures were also analyzed by p24 ELISA to confirm the efficiency of infection. Mass spectrometry data was analyzed by comparing the $_{LOG}2$ relative expression of each protein within Serpin B1 treated supernatants to that of PBS treated controls, or by comparing BSA treated protein controls to PBS cell controls. Conducting these analyzes on the BSA treated protein control allowed for the identification of proteins that were overexpressed in the presence of any protein stimulus. Comparing these results to those obtained following analysis of Serpin B1 treated supernatants allowed for the elimination of those proteins not induced specifically by Serpin B1.

8.4.1.1.1 Protein differential expression analysis of Serpin B1 treated, unstimulated, uninfected supernatants

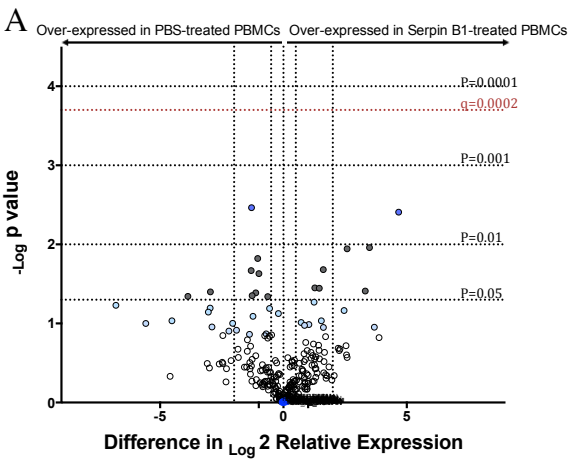
Determining the effect that Serpin B1 has on unstimulated cells provided information on the impact that it may have, if employed in a microbicide, on bystander cells within the FGT. Supernatants from unstimulated, uninfected PBMC cell cultures, treated with Serpin B1 were assessed through label-free mass spectrometry. Two-hundred, and fifty-three proteins were identified as having percent covariance below 25. Relative expressions were calculated by comparing the abundance of each protein within a specific condition to that of the average

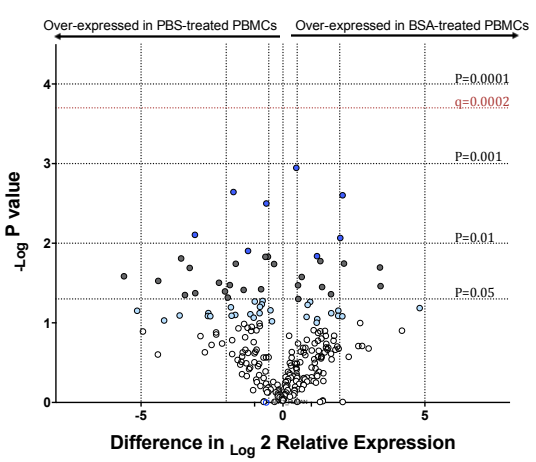
abundance across all samples. The expression of each of these proteins was compared between Serpin B1, or BSA treated cell cultures and PBS treated controls. Following Benjamani-Hochberg multiple comparisons analysis none of the differentially expressed proteins stood up to the stringent q value of 0.0002 and, thus, p-value frequency histograms were constructed to determine if results were due to actual effects. As can be observed in Figure 48C, there was a trend towards a true effect, with an increase in p-value frequency seen in the lower values compared to the p-values approaching 1.0. For this reason, the statistical significance criterion was reduced to consist of a less stringent alpha of 0.05. Further analysis was performed on the 16 specific differentially expressed proteins in the Serpin B1 treated cultures that met the new criterion for significance. However, this was done with caution since the p-value distribution did not definitively confirm results based on an actual effect. Of these 16 differentially expressed proteins within the Serpin B1 treated cultures (Figure 48A, Table 6), seven were up-regulated and nine down-regulated, compared to PBS treated PBMC supernatants. However, two of the up-regulated poteins (Extended Synaptotagmin-1, and AMP Deaminase 2) were also up-regulated within BSA treated cultures (Table 7). Hence, both of these proteins likely become induced during stimulation of PBMCs with any foreign protein stimulus and are, therefore, not the result of Serpin B1-specific effects exerted on the cells.

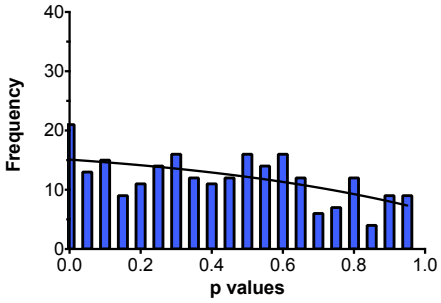
Following IPA and Uniprot analysis of the individual biological functions for each of the remaining 14 proteins it was determined that amoung the up-regulated proteins there were strong associations with ATP binding (T-complex protein 1 subunit delta $p = 0.0389$, ATP synthase F(0) complex subunit B1 mitochondrial $p = 0.0114$), hydrolase activity (Xaa-Pro aminopeptidase 1 $p = 0.011$), aminopeptidase activity (Xaa-Pro aminopeptidase 1 $p = 0.011$), increased apoptosis (Programmed cell death 6-interacting protein $p = 0.0355$), and decreased transcription (Histone H1.5 $p = 0.0358$) (Table 6).

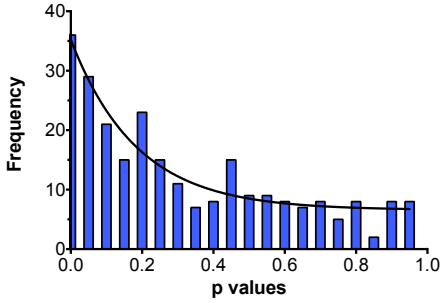
Alternatively, the nine down-regulated proteins were found to be associated with cell death/survival functions of the cell (Importin subunit beta-, p = 0.0151), cell migration (CD82 antigen, p = 0.0454), transcription (Staphylococcal nuclease domain-containing protein 1 p = 0.0215, Chromobox protein homolog 3 p = 0.0034, Core histone macro-H2A.1 p = 0.0446), EIF2 signaling in translation (60S ribosomal protein L35a, p = 0.0399), serine peptidase inhibitory activity (Antithrombin III, p = 0.0458), as well as cell growth and proliferation (Core histone macro-H2A.1 p = 0.0446 and FACT complex subunit SPT16, p = 0.0409) (Table 6).

It, thus, appears as though Serpin B1 may exhibit a response within unstimulated PBMCs that results in the regulated expression of proteins involved in the production of cellular energy, as well as proteins associated with increased apoptosis and decreased transcription.


A
Over-expressed in PBS-treated PBMCs
Over-expressed in Serpin B1-treated PBMCs
P=0.0001
q=0.0002
P=0.001
P=0.01
P=0.05
-Log p value
Difference in Log 2 Relative Expression


B
Over-expressed in PBS-treated PBMCs
Over-expressed in BSA-treated PBMCs
P=0.0001
q=0.0002
P=0.001
P=0.01
P=0.05
-Log P value
Difference in Log 2 Relative Expression


C
Frequency
p values


D
Frequency
p values

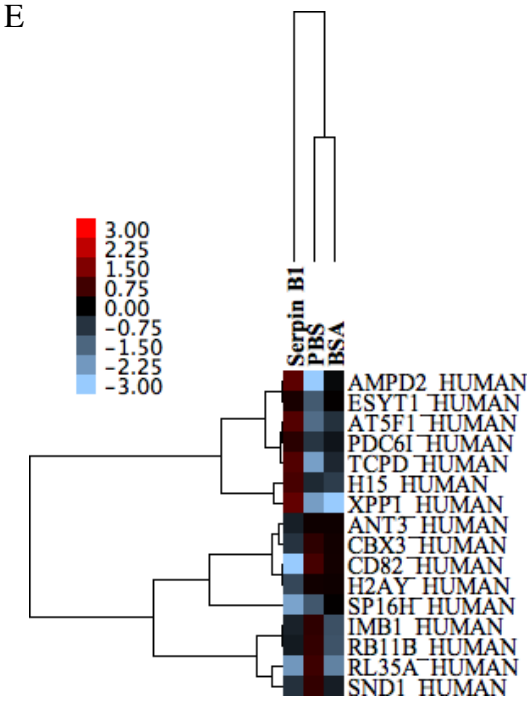

E
3.00
2.25
1.50
0.75
0.00
-0.75
-1.50
-2.25
-3.00
Serpin B1
PBS
BSA
AMPD2_HUMAN
ESYT1_HUMAN
AT5F1_HUMAN
PDC6I_HUMAN
TCPD_HUMAN
H15_HUMAN
XPP1_HUMAN
ANT3_HUMAN
CBX3_HUMAN
CD82_HUMAN
H2AY_HUMAN
SP16H_HUMAN
IMB1_HUMAN
RB11B_HUMAN
RL35A_HUMAN
SND1_HUMAN

Figure 48: Protein expression in supernatants from unstimulated, uninfected Serpin B1, and BSA treated cultures compared to PBS treated controls

A: Volcano plot depicting differential protein expression from the 253 proteins identified via label-free mass spectrometry, in Serpin B1 treated unstimulated PBMC supernatants compared to PBS treated supernatants, B: Volcano plot representing differential protein expression in BSA treated, unstimulated PBMC supernatants compared to PBS treated controls. C: P-value frequency distribution for relative protein expression between Serpin B1 and PBS treated PBMCs for the 253 proteins. D: P value frequency distribution for relative expression comparison between BSA and PBS treated PBMCs for the 253 proteins. E: Dendogram depicting the clustering patterns of the 16 differentially expressed proteins in the Serpin B1 treated supernatants compared to the PBS and BSA treated controls.

%CV was set to 0.25. $_{LOG}2$ relative expressions were determined and compared between culture conditions using multiple student t-tests, without assuming consistent SD. Alpha= 0.05. P values for each t-test were log transformed and plotted against the difference between $_{LOG}2$ relative expression values to create the volcano plot.

Table 6: Differentially expressed proteins in unstimulated PBMC culture supernatants treated with Serpin B1 compared to cultures treated with PBS

Accession Number	Protein Name	PBS $_{Log}$ 2 Rel. Exp.	SB1 $_{Log}$2 Rel. Exp.	Diff. in $_{Log}$2 Rel. Exp.	P value	^{Log} Trans. P value	q value	# unique peptide	Biological Function
UP-REGULATED IN SERPIN B1 TREATED CULTURES									
PDC6I_HUMAN	Programmed cell death 6-interacting protein	-0.7829	0.4879	1.2708	0.0355	1.4499	0.0018	17	Apoptosis, viral reproduction
H15_HUMAN	Histone H1.5	-0.6101	0.845	1.4551	0.0358	1.4456	0.0020	29	Chromatin organization, negative regulation of RNA polymerase II promoter transcription
ESYT1_HUMAN	Extended synaptotagmin-1	-1.3292	0.2892	1.6184	0.0209	1.6801	0.0012	42	Lipid binding/transport
AT5F1_HUMAN	ATP synthase F(0) complex subunit B1, mitochondrial	-1.5945	0.989	2.5835	0.0114	1.9442	0.0008	23	ATPase activty, respiratory electron transport chain
TCPD_HUMAN	T-complex protein 1 subunit delta	-2.3595	0.959	3.3185	0.0389	1.4098	0.0022	51	ATP binding, de novo post-translational protein folding
XPP1_HUMAN	Xaa-Pro aminopeptidase 1	-2.3237	1.1682	3.4919	0.011	1.9588	0.0006	8	Aminopeptidase activity, hydrolase activity, peptidase activity
AMPD2_HUMAN	AMP deaminase 2	-3.5765	1.0955	4.672	0.0039	2.4076	0.0004	5	AMP deaminase, hydrolase activity
DOWN-REGULATED IN SERPIN B1 TREATED PBMCs									
CD82_HUMAN	CD82 antigen	0.8173	-3.0559	-3.8732	0.0454	1.3425	0.0030	11	Cell migration, transcription
RL35A_HUMAN	60S ribosomal protein L35a	0.7219	-2.2386	-2.9605	0.0399	1.3991	0.0024	10	EIF2 signaling, translation
SND1_HUMAN	Staphylococcal nuclease domain-containing protein 1	0.5911	-0.7149	-1.306	0.0215	1.6679	0.0014	14	DNA-dependent transcription, viral reproduction
CBX3_HUMAN	Chromobox protein homolog 3	0.5321	-0.7609	-1.293	0.0034	2.4642	0.0002	15	Negative regulator of DNA-dependent transcription
H2AY_HUMAN	Core histone macro-H2A.1	0.2085	-1.0609	-1.2694	0.0446	1.3511	0.0028	23	Negative regulator of transcription from RNA polymerase II promoter, regulator of cell growth
SP16H_HUMAN	FACT complex subunit SPT16	-1.2975	-2.4172	-1.1197	0.0409	1.3878	0.0026	10	poly A RNA binding, DNA repair, DNA replication
IMB1_HUMAN	Importin subunit beta-1	0.5494	-0.4932	-1.0426	0.0151	1.8211	0.0010	56	Nuclear protein import, apoptotic process, intracellular transport of virus, viral life cycle
RB11B_HUMAN	Ras-related protein Rab-11B	0.6044	-0.389	-0.9934	0.0234	1.6303	0.0016	16	Endocytosis, GTPase activity,
ANT3_HUMAN	Antithrombin-III (Serpin C1)	0.1717	-0.4658	-0.6375	0.0458	1.339	0.0032	4	Serine peptidase inhibitor, blood coagulation

All biological functions were determined using IPA software and uniprot.org

Table 7: Differentially expressed proteins in BSA treated PBMC culture supernatants compared to PBS treated controls

Accession Number	Protein Name	PBS $_{Log}$ 2 Rel Exp.	BSA $_{Log}$ 2 Rel. Exp.	Difference in $_{Log}$ 2 Rel. Exp.	P value	$_{Log}$ Trans. P value	BSA Sample Protein was Identified In
UP-REGULATED IN BSA TREATED PBMC CULTURES							
EHD4_HUMAN	EH domain-containing protein 4	-3.1746	1.6073	4.7819	0.0235	1.6292	PHA-Stimulated Sups
AMPD2_HUMAN	AMP deaminase 2	-3.5766	-0.1389	3.4376	0.0345	1.4617	Unstimulated Sups
ZAP70_HUMAN	Tyrosine-protein kinase ZAP-70	-2.9066	0.5125	3.4192	0.0201	1.6962	Unstimulated Sups
LCK_HUMAN	Tyrosine-protein kinase Lck	-1.5884	0.8505	2.4389	0.0004	3.4050	PHA-Stimulated, ML1956-Infected Sups
FA49B_HUMAN	Protein FAM98B	-1.4535	0.6992	2.1526	0.0180	1.7455	Unstimulated Sups
TES_HUMAN	Testin	-1.3593	0.7430	2.1023	0.0025	2.6029	Unstimulated Sups
TES_HUMAN	Testin	-1.4421	0.3403	1.7824	0.0417	1.3795	PHA-Stimulated, ML1956-Infected Sups
IF4H_HUMAN	Eukaryotic translation initiation factor 4H	-2.7725	-0.7526	2.0199	0.0086	2.0664	Unstimulated Sups
CUL4A_HUMAN	Cullin-4A	-1.2531	0.4391	1.6923	0.0437	1.3590	Unstimulated Sups
EFTU_HUMAN	Elongation factor Tu, mitochondrial	-1.3333	0.3554	1.6887	0.0162	1.7905	PHA-Stimulated Sups
ESYT1_HUMAN	Extended synaptotagmin-1	-1.3292	0.0526	1.3817	0.0355	1.4494	Unstimulated Sups
SNAA_HUMAN	Alpha-soluble NSF attachment protein	-0.6542	0.6652	1.3194	0.0168	1.7756	Unstimulated Sups
TBB5_HUMAN	Tubulin beta chain	-0.5917	0.6107	1.2024	0.0145	1.8381	Unstimulated Sups
ENPL_HUMAN	Endoplasmin	-0.3093	0.7693	1.0786	0.0023	2.6411	PHA-Stimulated Sups
HCLS1_HUMAN	Hematopoietic lineage cell-specific protein	-0.5150	0.4600	0.9749	0.0092	2.0349	PHA-Stimulated, ML1956-Infected Sups
ERP29_HUMAN	Endoplasmic reticulum resident protein 29	-1.0539	-0.0840	0.9700	0.0352	1.4536	PHA-Stimulated, ML1956-Infected Sups
FEN1_HUMAN	Flap endonuclease 1	-0.3235	0.3386	0.6621	0.0265	1.5765	Unstimulated Sups
KPYM_HUMAN	Pyruvate kinase PKM	-0.3042	0.2363	0.5406	0.0501	1.3002	Unstimulated Sups
GBB1_HUMAN	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	-0.2036	0.3294	0.5330	0.0337	1.4719	Unstimulated Sups

ALBU_HUMAN	Serum albumin	-0.1169	0.3529	0.4697	0.0011	2.9475	Unstimulated Sups
DOWN-REGULATED IN BSA TREATED PBMC CULTURES							
CBX3_HUMAN	Chromobox protein homolog 3	0.5321	0.2205	-0.3115	0.0182	1.7398	Unstimulated Sups
FBLN1_HUMAN	Fibulin 1	0.4891	-0.0399	-0.5290	0.0148	1.8304	Unstimulated Sups
APOM_HUMAN	Apolipoprotein M	0.2962	-0.2881	-0.5843	0.0032	2.4992	Unstimulated Sups
PRS8_HUMAN	Protein RCC2	0.0623	-0.5545	-0.6168	0.0148	1.8291	Unstimulated Sups
XPP1_HUMAN	Xaa-Pro aminopeptidase 1	-1.2321	-1.9963	-0.7642	0.0146	1.8349	PHA-Stimulated, ML1956-Infected Sups
ROA2_HUMAN	Heterogeneous nuclear ribonucleoproteins A2/B1	0.3539	-0.4157	-0.7696	0.0379	1.4219	Unstimulated Sups
PRDX5_HUMAN	Peroxiredoxin-5, mitochondrial	0.3299	-0.7983	-1.1282	0.0409	1.3888	PHA-Stimulated, ML1956-Infected Sups
FINC_HUMAN	Fibronectin	0.1724	-0.9651	-1.1375	0.0281	1.5509	PHA-Stimulated Sups
PABP1_HUMAN	Polyadenylate-binding protein 1	0.2638	-0.9637	-1.2275	0.0125	1.9031	Unstimulated Sups
CYBP_HUMAN	Calcyclin-binding protein	0.5780	-0.7987	-1.3767	0.0448	1.3487	PHA-Stimulated, ML1956-Infected Sups
ZCCHV_HUMAN	Zinc finger CCCH-type antiviral protein 1	0.7829	-0.6029	-1.3858	0.0386	1.4139	Unstimulated Sups
HBD_HUMAN	Hemoglobin subunit delta	0.3206	-1.2585	-1.5791	0.0029	2.5389	PHA-Stimulated Sups
SYWC_HUMAN	Tryptophan--tRNA ligase, cytoplasmic	0.5854	-1.0771	-1.6624	0.0181	1.7426	Unstimulated Sups
DCTP1_HUMAN	dCTP pyrophosphatase 1	-0.0124	-1.7499	-1.7375	0.0496	1.3046	PHA-Stimulated, ML1956-Infected Sups
IMB1_HUMAN	Importin subunit beta-1	0.5494	-1.1918	-1.7412	0.0023	2.6432	Unstimulated Sups
SF3A1_HUMAN	Splicing factor 3A subunit 1	0.5049	-1.3647	-1.8697	0.0335	1.4752	Unstimulated Sups
GMFG_HUMAN	Glia maturation factor gamma	0.3625	-1.5801	-1.9426	0.0481	1.3177	Unstimulated Sups
UBA1_HUMAN	Ubiquitin-like modifier-activating enzyme 1	-0.0037	-2.0459	-2.0421	0.0403	1.3943	Unstimulated Sups
RS5_HUMAN	40S ribosomal protein S5	0.6298	-1.6231	-2.2529	0.0313	1.5040	Unstimulated Sups
ITB1_HUMAN	Integrin beta-1	0.4665	-2.6236	-3.0901	0.0424	1.3728	Unstimulated Sups
RFA2_HUMAN	Replication protein A 32 kDa subunit	0.6544	-2.4463	-3.1007	0.0078	2.1058	Unstimulated Sups

PSB3_HUMAN	Proteasome subunit beta type-3	0.2513	-3.0347	-3.2860	0.0204	1.6896	Unstimulated Sups
ATG3_HUMAN	Ubiquitin-like-conjugating enzyme ATG3	-0.6293	-4.0799	-3.4506	0.0448	1.3489	Unstimulated Sups
PSB1_HUMAN	Proteasome subunit beta type-1	0.7781	-2.8078	-3.5859	0.0155	1.8085	Unstimulated Sups
PIN1_HUMAN	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1	-0.1006	-4.2870	-4.1864	0.0353	1.4527	PHA-Stimulated Sups
MDHM_HUMAN	Malate dehydrogenase, mitochondrial	0.3050	-4.0901	-4.3950	0.0298	1.5265	Unstimulated Sups
LIS1_HUMAN	Platelet-activating factor acetylhydrolase IB subunit alpha	-0.4640	-6.0641	-5.6001	0.0260	1.5852	Unstimulated Sups
ITB1_HUMAN	Integrin beta-1	0.4748	-7.8377	-8.3126	0.0262	1.5813	PHA-Stimulated, ML1956-Infected Sups

Blue text indicates proteins that were also up-regulated in Serpin B1 treated PBMC culture for the matching sample.
Red text indicates proteins that were also down-regulated in Serpin B1 treated PBMC cultures for the matching sample.

8.4.1.1.2 Protein differential expression analysis for Serpin B1 treated, PHA-stimulated, uninfected supernatants

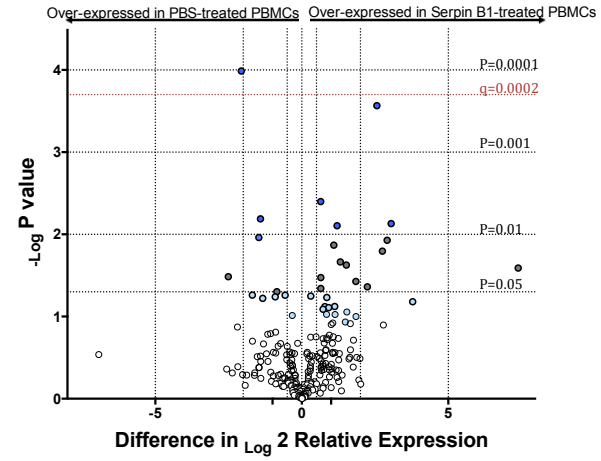
PBMCs from three local Winnipeg donors were stimulated for three days with PHA before treatment with Serpin B1 (50μg/mL), BSA (50μg/mL), or PBS (cell control) for five days. Two-hundred, and fifty-three proteins were confidently identified and met the 25% covariance cut-off. Relative expressions were calculated by comparing the abundance of each protein in a particular condition to that of the average abundance of all samples. These relative expressions were $_{LOG}2$ transformed, and multiple t-tests were performed between the expression of each protein in Serpin B1 treated cultures and the corresponding expression in PBS treated cultures. Benjamini-Hochberg multiple comparison analysis was performed with an FDR of 5% yielding only two proteins (Rho GDP-dissociation inhibitor 2, and Guanine nucleotide-binding protein subunit beta-1) that met the necessary p-values to be considered significant. Therefore, a p-value frequency histogram was constructed to ensure that the data was not due to random chance (Figure 49C). Since the histogram was not evenly distributed it was concluded that the proteins identified were likely due to true effects and all subsequent analysis was performed with an alpha of 0.05 as the statistically significant criterion. With this adjusted alpha, there were 19 proteins (7.5% of the total 253 proteins) identified as significantly differentially expressed, all of which were displayed in volcano plots (Figure 49A). These volcano plots were used to compare the differential expression of proteins following Serpin B1 or BSA (Figure 49B) treatment compared to PBS treated PBMC controls in PHA-stimulated cell cultures. These analyzes provided the identification of proteins, and thus potential pathways, that are affected by Serpin B1 in cells that are already in an activated/stimulated state.

It was determined that none of the proteins that were identified to be differentially expressed within PHA-stimulated, Serpin B1 treated culture supernatants were also induced in the presence of BSA. As such, the regulation of these proteins' expression is likely due to direct effects that Serpin B1 is having on the PBMCs rather than a non-specific effect caused by the addition of any protein, such as BSA, to the culture (Figure 49, Table 7 and 8). BSA treated

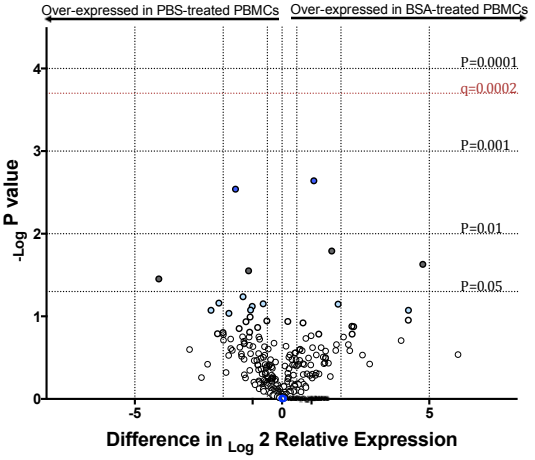
supernatants did overexpress three proteins and under-express three proteins compared to the PBS treated control cells (Table 7). However, the p-value frequency histogram (Figure 49D) did not convincingly reveal that these results were due to actual effects caused by BSA and as such the proteins that were differentially regulated in BSA cultures may have been due to random chance alone.

Of the 19 differentially expressed proteins identified in Serpin B1 treated culture supernatants, 14 were found to be up-regulated while the remaining five were down-regulated. Furthermore, According to individual analysis, using IPA software and Uniprot, of biological functions for each differentially regulated protein (Table 8), the proteins found to be over-expressed within Serpin B1 treated cultures were most highly associated with ATP binding (ATP-dependent RNA helicase DDX1 p = 0.016, Structural maintenance of chromosomes protein 3 p = 0.0118, Ubiquitin-like-modifier-activating enzyme 1 p = 0.0375), hydrolase activity (dCTP pyrophosphatase 1 p = 0.0236), increased apoptosis (Glyceraldehyde-3-phosphate-dehydrogenase p = 0.004, Reticulon-4 p = 0.0079), decreased transcription (Histone H1.5 p = 0.0135, structural maintenance of chromosomes protein 3 p = 0.0118), redox response (Rho GDP-dissociation inhibitor 2 p = 0.0003, Cytochrome b-c1 complex subunit 1 mitochondrial p = 0.0074), and reduced proliferation (Histone H1.5 p = 0.0135, Reticulon-4 p = 0.0079). In addition one protein was associated with glycolysis/gluconeogenesis (Glyceraldehyde-3-phosphate-dehydrogenase p = 0.004), endopeptidase inhibitory activity (Calpastatin p = 0.0335), and cell migration (Reticulon-4 p = 0.0079) (Table 8). All of which suggests a role for Serpin B1 in the regulation of cellular energy metabolism as well as in regulation of cellular transcription, proliferation, and the oxidative stress response.

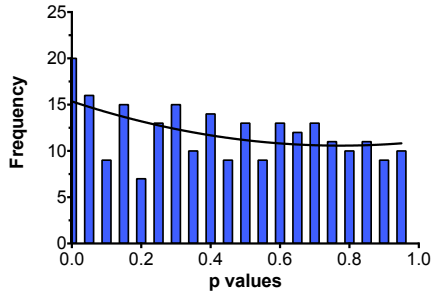
A


Over-expressed in PBS-treated PBMCs
Over-expressed in Serpin B1-treated PBMCs
P=0.0001
q=0.0002
P=0.001
P=0.01
P=0.05
-Log P value
Difference in Log 2 Relative Expression

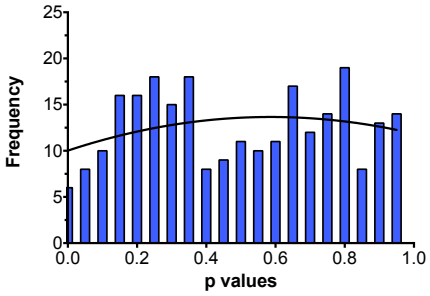
B


Over-expressed in PBS-treated PBMCs
Over-expressed in BSA-treated PBMCs
P=0.0001
q=0.0002
P=0.001
P=0.01
P=0.05
-Log P value
Difference in Log 2 Relative Expression

C


Frequency
p values

D


Frequency
p values

E

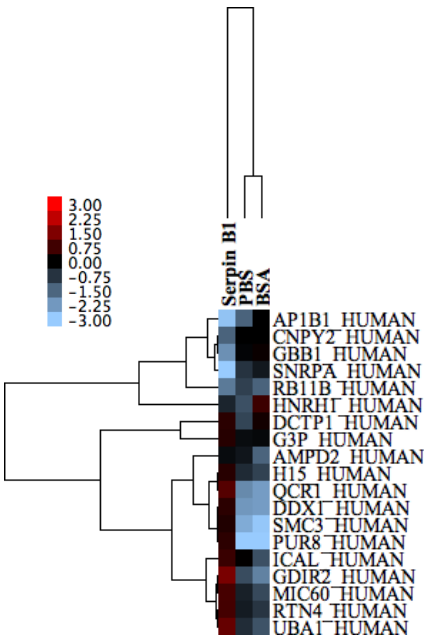

3.00
2.25
1.50
0.75
0.00
-0.75
-1.50
-2.25
-3.00
Serpin B1
PBS
BSA
AP1B1_HUMAN
CNPY2_HUMAN
GBB1_HUMAN
SNRPA_HUMAN
RB11B_HUMAN
HNRH1_HUMAN
DCTP1_HUMAN
G3P_HUMAN
AMPD2_HUMAN
H15_HUMAN
QCR1_HUMAN
DDX1_HUMAN
SMC3_HUMAN
PUR8_HUMAN
ICAL_HUMAN
GDIR2_HUMAN
MIC60_HUMAN
RTN4_HUMAN
UBA1_HUMAN

Figure 49: Protein expression in supernatants from PHA-stimulated, uninfected Serpin B1, and BSA treated PBMC cultures compared to PBS treated controls

A: Volcano plot depicting differential protein expression from the 253 proteins identified via label-free mass spectrometry, in Serpin B1 treated, PHA-stimulated PBMC supernatants compared to PBS treated supernatants, B: Volcano plot illustrating differential protein expression in BSA treated, PHA-stimulated PBMC supernatants compared to PBS treated control. C: P value frequency distribution for relative protein expression between Serpin B1 and PBS treated PHA-stimulated PBMCs for the 253 proteins. D: P-value frequency distribution for relative expression comparison between BSA and PBS treated PHA-stimulated PBMCs for the 253 proteins. E: Dendrogram depicting the clustering patterns of the 19 differentially expressed proteins in the Serpin B1 treated supernatants compared to the PBS and BSA treated controls.

%CV was set to 0.25. $_{LOG}2$ relative expressions were determined for 253 common proteins and compared between culture conditions using multiple student t-tests, without assuming consistent SD, alpha= 0.05. P values for each t-test were log transformed and plotted against the difference between $_{LOG}2$ relative expression values to create the volcano plot.

Table 8: Differentially expressed proteins in the supernatants of uninfected, PHA-stimulated, Serpin B1 treated PBMC cultures compared to PBS treated controls

Accession Number	Protein Name	PBS $_{Log}$ 2 Rel Exp.	SB1 $_{Log}$ 2 Rel. Exp.	Diff. in $_{Log}$ 2 Rel. Exp.	P value	$_{Log}$ Trans. P value	q value	# unique peptide	Biological Function
UP-REGULATED IN SERPIN B1 TREATED CULTURES									
PUR8_HUMAN	Adenylosuccinate lyase	-6.8634	0.5220	7.3854	0.0258	1.5892	0.0026	14	Catalytic activity, lyase activity
QCR1_HUMAN	Cytochrome b-c1 complex subunit 1, mitochondria l	-2.0386	1.0110	3.0496	0.0074	1.4843	0.0010	37	Aerobic respiration, oxidation-reduction process
SMC3_HUMAN	Structural maintenance of chromosome s protein 3	-2.5352	0.3793	2.9145	0.0118	1.3619	0.0016	18	ATP binding, cell cycle, cell division, post-translational protein modification, regulation of DNA replication
DDX1_HUMAN	ATP-dependent RNA helicase DDX1	-2.2384	0.5117	2.7501	0.0160	2.1884	0.0020	16	ATP binding, ATP-dependent RNA helicase activity
GDIR2_HUMAN	Rho GDP-dissociation inhibitor 2	-1.1275	1.4343	2.5618	0.0003	1.9609	0.0004	104	Cellular response to redox response, immune response, actin cytoskeleton organization
UBA1_HUMAN	Ubiquitin-like modifier-activating enzyme 1	-0.6530	1.1945	1.8475	0.0375	1.3010	0.0032	98	ATP binding, ligase activity, protein ubiquitination
DCTP1_HUMAN	dCTP pyrophosphatase 1	-1.0221	0.4982	1.5203	0.0236	2.3993	0.0024	9	Hydrolase activity
MIC60_HUMAN	MICOS complex subunit MIC60	-0.4819	0.8338	1.3157	0.0216	1.6269	0.0022	29	Protein binding
RTN4_HUMAN	Reticulon-4	-0.4018	0.8036	1.2054	0.0079	1.4263	0.0012	9	Apoptotic process, negative regulation of cell growth, regulation of cell migration
H15_HUMAN	Histone H1.5	-0.6264	0.4653	1.0917	0.0135	1.9279	0.0018	29	Chromatin organization, negative regulation of transcription from RNA polymerase II promoter, negative regulation of cell growth
ICAL_HUMAN	Calpastatin	0.0331	0.6836	0.6505	0.0335	1.7952	0.0030	28	Calpain inhibitor, calcium-dependent cysteine-type endopeptidase inhibitor
HNRH1_HUMAN	Heterogeneous nuclear ribonucleoprotein H	-1.1874	-0.5379	0.6495	0.0455	1.8689	0.0002	82	Gene expression, mRNA processing, nuclear mRNA splicing
G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	-0.1862	0.4586	0.6448	0.0040	2.1307	0.0006	129	Gluconeogenesis, glycolysis, apoptosis, cellular response to IFN-y
AMPD2_HUMAN	AMP deaminase 2	-0.3215	-0.1701	0.1514	0.0435	3.9879	0.0034	5	AMP deaminase, hydrolase activity
DOWN-REGULATED IN SERPIN B1 TREATED CULTURES									
RB11B_HUMAN	Ras-related protein Rab-11B	-0.9548	-1.8060	-0.8512	0.0500	1.4744	0.0038	16	GDP/GTP binding, GTPase activity, viral entry/replication , clathrin-mediated endocytosis
AP1B1_HUMAN	AP-1 complex subunit beta-1	-1.4732	-2.8815	-1.4083	0.0065	3.5653	0.0008	12	Clathrin binding, protein kinase binding, antigen processing and presentation via MHC class II

CNPY2_HUMAN	Protein canopy homolog 2	-0.0028	-1.4698	-1.4670	0.0109	2.7698	0.0014	7	Negative regulation of gene expression, protein binding, cell death
GBB1_HUMAN	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	-0.0725	-2.1357	-2.0632	0.0001	2.1050	0.0002	44	G-protein coupled receptor binding, GTPase activity,
SNRPA_HUMAN	U1 small nuclear ribonucleoprotein A	-0.7605	-3.2665	-2.5060	0.0328	1.3420	0.0028	8	Gene expression, spliceosome induced nuclear mRNA splicing

All biological functions were determined using IPA software and uniprot.org

8.4.1.1.3 Protein differential expression analysis for Serpin B1 treated, PHA-stimulated, HIV_{1956}-infected supernatants

Following 3-day stimulation with PHA, PBMCs from three local Winnipeg donors were infected with HIV_{1956} and treated with Serpin B1 (50µg/mL), BSA (50µg/mL), or PBS (cell control) for five days. These conditions most closely mimic those in the initial PBMC neutralization assays and as such will provide information on potential pathways affected in those assays where HIV neutralizing activity was observed.

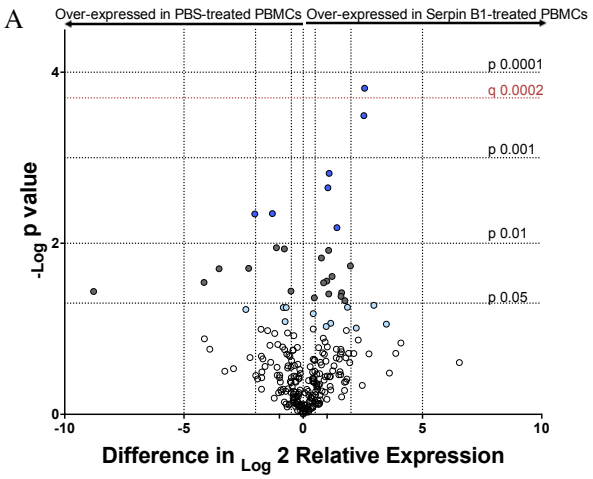
Two-hundred, and fifty-three proteins were confidently identified and met the 25% covariance cut-off. Relative expressions were calculated by comparing the abundance of each protein in a particular condition to that of the average abundance of all samples. These relative expressions were $_{LOG}2$ transformed, and multiple t-tests were performed between the expression of each protein in Serpin B1 treated cultures and the corresponding expression in PBS treated cultures. Benjamini-Hochberg multiple comparison analysis was performed with an FDR of 5% which yielded only two proteins (Tyrosine-protein kinase Lck, and Xaa-Pro aminopeptidase 1) that met the necessary p values to be considered significant. Therefore, p value frequency histograms were constructed to ensure data was not due to random chance (Figure 50C). All further analysis was conducted with an adjusted alpha of 0.05. Using this adjusted criterion for statistical significance, 25 proteins were identified as differentially expressed within Serpin B1 treated supernatants compared to PBS treated cultures (Figure 50A). Of these 25 proteins, 17 were identified as up-regulated within Serpin B1 treated cultures, two of which (Endoplasmic reticulum resident protein 29, and Tyrosine-protein kinase Lck) were also identified as over-expressed within BSA treated cultures compared to PBS treated cell controls (Figure 50B, Table 7 and 9). However since the p-value distribution for the BSA comparison indicates results that are likely due to random chance rather than a true effect these two proteins were not removed from further analysis of the differentially expressed proteins in Serpin B1 treated cultures.

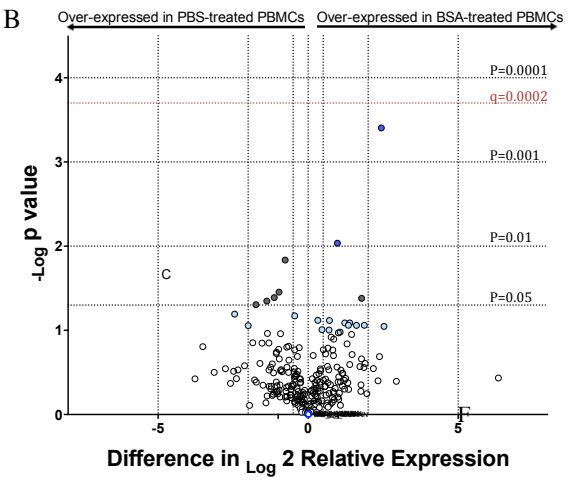
According to IPA and Uniprot analysis of the individual biological functions for each of the 17 over-expressed proteins, these proteins were most strongly associated with functions involved in redox reactions (Peroxiredoxin-6 p = 0.0066, Isocitrate dehydrogenase NADP p = 0.0121, Cytochrome b-c1 complex subunit 1 mitochondria p = 0.0184, Annexin A1 p = 0.029, Pyruvate dehydrogenase E1 component p = 0.0391), and decreased proliferation (Tyrosine-protein kinase-Lck p = 0.0002, Tubulin beta chain p = 0.0277, Annexin A1 p = 0.029, Testin p = 0.0468), which together accounted for 8 of the total 24 biological functions identified for these proteins (Table 9). Additionally, proteins involved in ATP binding (T-complex protein 1 subunit beta mitochondrial p = 0.0378), hydrolase activity (Xaa-Pro aminopeptidase p = 0.0003), increased apoptosis (Tyrosine-protein kinase-Lck p = 0.0002, Hematopoietic lineage cell-specific protein p = 0.0015, Annexin A1 p = 0.029), decreased transcription (Endoplasmic reticulum resident protein 29 p = 0.0022, Hematopoietic lineage cell-specific protein p = 0.0015, Endoplasmic reticulum resident protein 29 p = 0.0022), cell migration (Tyrosine-protein kinase-Lck p = 0.0002, Annexin A1 p = 0.029), endopeptidase inhibitory activity (Alpha-2-HS-glycoprotein p = 0.0435), and gluconeogenesis (Isocitrate dehydrogenase, NADP mitochondrial p = 0.0184, Triophosphate isomerase p = 0.0149) were identified (Table 9). Furthermore, two proteins associated with the NK cell response (Tubulin beta-4B chain p = 0.0244, Tubulin beta chain p = 0.0277) were also identified as up-regulated within the supernatants of Serpin B1 treated, HIV-infected PBMCs compared to PBS treated controls (Table 9). IPA pathway analysis also identified 14-3-3-mediated signaling (Tubulin beta-4B chain, Tubulin beta chain, p = 4.10×10^{-3}), Acetyl-CoA biosynthesis (Pyruvate dehydrogenase E1 complex subunit, mitochondrial, p = 5.76×10^{-3}), phagosome maturation (Peroxiredoxin-6, Tubulin beta-4B chain, Tubulin beta chain, p = 1.23×10^{-4}), and remodeling (Tubulin beta-4B chain, Tubulin beta chain, p = 1.41×10^{-3})/signaling (Tubulin beta-4B chain, Tubulin beta chain, p = 6.31×10^{-3}) of

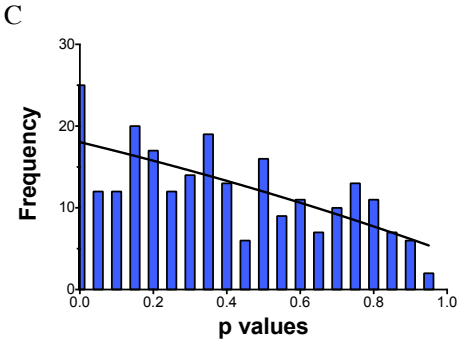
epithelial adherens junctions as the top canonical pathways associated with these up-regulated proteins (Figure 50F).

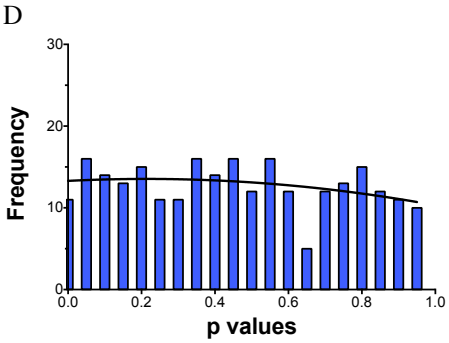
The remaining eight down-regulated proteins (Figure 50A, Table 9) were most strongly associated with mammalian target of rapamycin (mTOR; Eukaryotic initiation factor 4A-I, Eukaryotic initiation factor 4A-II,2.21 x 10^{-3}), EIF-(eukaryotic initiation factor)2 (Eukaryotic initiation factor 4A-I, Eukaryotic initiation factor 4A-II, 2.14 x 10^{-3}) and EIF4/p70S6K (Eukaryotic initiation factor 4A-I, Eukaryotic initiation factor 4A-II,p = 1.35 x 10^{-3}) signaling pathways (Figure 50G). The individual cellular functions of these proteins were also found to be associated with cellular proliferation (Insulin-like growth factor II, p = 0.0362), apoptosis (Lymphocyte-specific protein I, p = 0.0117), cell migration (Lymphocyte-specific protein I, p = 0.0117), translation (Eukaryotic initiation factor 4A-II, p = 0.0045 and Eukaryotic initiation factor 4A-I, p = 0.027), and cell cycle (Alpha endosulfine, p = 0.0198) (Table 9).

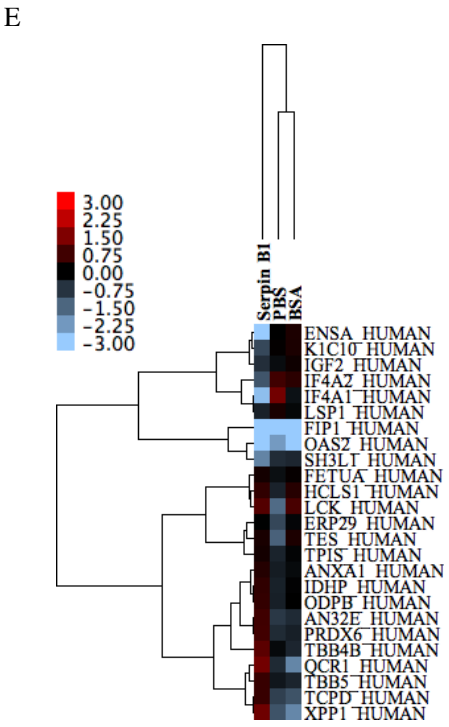
These results support the data from Chapter 6 and 7, which suggest that Serpin B1 interfered with protein translation, potentially through interference with the mTOR and EIF pathways as well as with cellular replication. However, these findings would need to be validated.

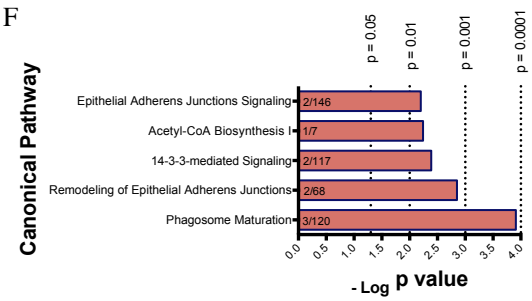

A
Over-expressed in PBS-treated PBMCs
Over-expressed in Serpin B1-treated PBMCs
p 0.0001
q 0.0002
p 0.001
p 0.01
p 0.05
-Log p value
Difference in Log 2 Relative Expression


B
Over-expressed in PBS-treated PBMCs
Over-expressed in BSA-treated PBMCs
P=0.0001
q=0.0002
P=0.001
P=0.01
P=0.05
-Log p value
Difference in Log 2 Relative Expression


C
Frequency
p values


D
Frequency
p values


E
3.00
2.25
1.50
0.75
0.00
-0.75
-1.50
-2.25
-3.00
Serpin B1
PBS
BSA
ENSA_HUMAN
K1C10_HUMAN
IGF2_HUMAN
IF4A2_HUMAN
IF4A1_HUMAN
LSP1_HUMAN
FIP1_HUMAN
OAS2_HUMAN
SH3L1_HUMAN
FETUA_HUMAN
HCLS1_HUMAN
LCK_HUMAN
ERP29_HUMAN
TES_HUMAN
TPIS_HUMAN
ANXA1_HUMAN
IDHP_HUMAN
ODPB_HUMAN
AN32E_HUMAN
PRDX6_HUMAN
TBB4B_HUMAN
QCR1_HUMAN
TBB5_HUMAN
TCPD_HUMAN
XPP1_HUMAN


F
p = 0.05
p = 0.01
p = 0.001
p = 0.0001
Canonical Pathway
Epithelial Adherens Junctions Signaling
Acetyl-CoA Biosynthesis I
14-3-3-mediated Signaling
Remodeling of Epithelial Adherens Junctions
Phagosome Maturation
2/146
1/7
2/117
2/68
3/120
- Log p value

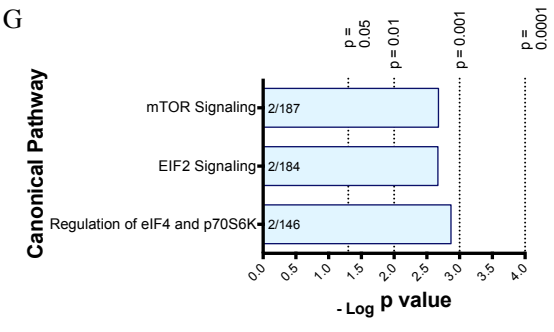

G
p = 0.05
p = 0.01
p = 0.001
p = 0.0001
Canonical Pathway
mTOR Signaling
EIF2 Signaling
Regulation of eIF4 and p70S6K
2/187
2/184
2/146
- Log p value

Figure 50: Protein expression in supernatants from PHA-stimulated, HIV_{1956}-infected PBMC cultures treated with Serpin B1, and BSA compared to PBS treated controls

A: Volcano plot depicting differential protein expression from the 253 proteins identified via label-free mass spectrometry, in Serpin B1 treated, PHA-stimulated, HIV_{1956}-infected, PBMC supernatants compared to PBS treated supernatants, B: Volcano plot illustrating differential protein expression in BSA treated, PHA-stimulated, HIV_{1956}-infected, PBMC supernatants compared to PBS treated control. C: P value frequency distribution for relative protein expression between Serpin B1, and PBS treated PHA-stimulated, HIV_{1956}-infected PBMCs for the 253 proteins. D: P value frequency distribution for relative expression comparison between BSA and PBS treated PHA-stimulated, HIV_{1956}-infected PBMCs for the 253 proteins. E: Dendrogram depicting the clustering patterns of the 25 differentially expressed proteins in the Serpin B1 treated supernatants compared to the PBS and BSA treated controls. F: Depiction of the top canonical pathways, according to IPA, associated with the 17 up-regulated proteins within the Serpin B1 treated culture supernatants. G: The top canonical pathways, according to IPA, associated with the eight down-regulated proteins within the Serpin B1 treated culture supernatants.

%CV was set to 0.25. $_{LOG}2$ relative expressions were determined for 253 common proteins and compared between culture conditions using multiple student t-tests, without assuming consistent SD, alpha= 0.05. P values for each t-test were log transformed and plotted against the difference between $_{LOG}2$ relative expression values to create the volcano plot.

Table 9: Differentially expressed proteins in the supernatants of Serpin B1 treated, PHA-stimulated, HIV_{1956}-infected PBMCs compared to PBS treated controls

Accession Number	Protein Name	PBS $_{Log}$ 2 Rel Exp.	SB1 $_{Log}$ 2 Rel. Exp.	Diff. in $_{Log}$ 2 Rel. Exp.	P value	$_{Log}$ Trans. P value	q value	# unique peptide	Biological Function
UP-REGULATED IN SERPIN B1 TREATED CULTURES									
FIP1_HUMAN	Pre-mRNA 3'-end-processing factor FIP1	-16.609	-8.6837	7.9253	0.0366	1.4364	0.0040	2	Poly (A) RNA binding, mRNA processing
LCK_HUMAN	Tyrosine-protein kinase Lck	-1.5884	0.9876	2.576	0.0002	3.8124	0.0002	33	Activation of cysteine-type endopeptidase activity involved in apoptotic process, aging, blood coagulation, innate immune response, leukocyte migration, positive regulation of gamma-delta T cell differentiation, regulation of cell proliferation, regulation of defense to virus
XPP1_HUMAN	Xaa-Pro aminopeptidase 1	-1.2321	1.3085	2.5406	0.0003	3.4921	0.0004	8	Aminopeptidase activity, hydrolase activity, metallopeptidase activity
QCR1_HUMAN	Cytochrome b-c1 complex subunit 1, mitochondrial	-0.6237	1.356	1.9797	0.0184	1.7359	0.0024	37	Aerobic respiration, oxidation-reduction process
TES_HUMAN	Testin	-1.442	0.3057	1.7477	0.0468	1.3296	0.0049	43	Negative regulation of cell proliferation
TCPD_HUMAN	T-complex protein 1 subunit delta	-0.9562	0.6542	1.6104	0.0378	1.4224	0.0042	51	ATP binding, poly (A) RNA binding, de novo posttranslational protein folding
AN32E_HUMAN	Acidic leucine-rich nuclear phosphoprotein 32 family member E	-0.8287	0.7549	1.5836	0.0417	1.3798	0.0045	27	Histone binding, phosphatase inhibitor activity
PRDX6_HUMAN	Peroxiredoxin-6	-0.6377	0.7765	1.4142	0.0066	2.1821	0.0014	42	Hydrogen peroxide catabolic process, oxidation-reduction process, response to reactive oxygen species
TBB4B_HUMAN	Tubulin beta-4B chain	-0.1342	1.0777	1.2119	0.0244	1.6126	0.0030	24	Double-stranded RNA binding, de novo posttranslational protein folding, NK cell mediated cytotoxicity, sturcutral molecule
HCLS1_HUMAN	Hematopoietic lineage cell-specific protein	-0.515	0.5712	1.0862	0.0015	2.8176	0.0006	92	Negative regulation of leukocyte apoptotic process and transcription from RNA polymerase II promoter, positive regulation of cell proliferation and macrophage differentiation
ODPB_HUMAN	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	-0.4649	0.6076	1.0725	0.0392	1.4071	0.0043	19	Catalytic, oxidoreductase, pyruvate dehydrogenase activity
IDHP_HUMAN	Isocitrate dehydrogenase [NADP], mitochondrial	-0.4875	0.5786	1.0661	0.0121	1.9172	0.0020	63	Carbohydrate metabolic process, glyoxylate cycle, oxidation-reduction process
ERP29_HUMAN	Endoplasmic reticulum resident protein 29	-1.0539	-0.0177	1.0362	0.0022	2.6488	0.0008	31	Chaperone binding, activation of MAPK activity, negative regulation of gene expression, positive regulation of protein phosphorylation

TBB5_HUMAN	Tubulin beta chain	-0.3344	0.656	0.9904	0.0277	1.5578	0.0032	179	Cell division, NK cell mediated cytotoxicity, protein polymerization, structural molecule
ANXA1_HUMAN	Annexin A1	-0.4234	0.4324	0.8558	0.029	1.5382	0.0036	49	Negative regulation of apoptosis and IL-8 production, response to IL-1, proliferation, alpha-beta T cell differentiation, double-stranded DNA-dependent ATPase activity, helicase activity, cellular response to hydropgen peroxide,
TPIS_HUMAN	Triosephosphat e isomerase	-0.5253	0.2407	0.766	0.0149	1.8275	0.0022	160	Catalytic activity, isomerase activity, gluconeogenesis
FETUA_HUMAN	Alpha-2-HS-glycoprotein	-0.2222	0.2419	0.4641	0.0435	1.3617	0.0047	11	Cysteine-type endopeptidase inhibitor, kinase inhibitory, acute-phase response
DOWN-REGULATED IN SERPIN B1 TREATED CULTURES									
IGF2_HUMAN	Insulin-like growth factor II	-0.1795	-0.6913	-0.5118	0.0362	1.4409	0.0038	3	Blood coagulation, cell proliferation, negative regulation of NK cell mediated cytotoxicity, positive regulation of activated T cell proliferation, positive regulation of MAPK cascade, positive regulation of transcription from RNA polymerase II promoter and DNA-dependent
LSP1_HUMAN	Lymphocyte-specific protein 1	0.2959	-0.4935	-0.7894	0.0117	1.9335	0.0018	96	Apoptotic process, chemotaxis, defense response
K1C10_HUMAN	Keratin, type I cytoskeletal 10	0.0553	-1.0658	-1.1211	0.0113	1.9479	0.0016	86	Cellular response to calcium, keratinocyte differentiation
SH3L1_HUMAN	SH3 domain-binding glutamic acid-rich-like protein	-0.661	-1.9511	-1.2901	0.0045	2.3477	0.0010	23	Positive regulation of signal transduction
IF4A2_HUMAN	Eukaryotic initiation factor 4A-II	0.7601	-1.2682	-2.0283	0.0045	2.3425	0.0012	5	Poly (A) RNA binding, translation initiation, virus process, ATP binding
OAS2_HUMAN	2'-5'-oligoadenylate synthase 2	-2.2876	-4.5727	-2.2851	0.0197	1.7061	0.0026	16	Cytokine-mediated signaling pathway, defense response to virus, immune response, IFN-y mediated signaling pathway, response to virus
ENSA_HUMAN	Alpha-endosulfine	-0.0084	-3.5334	-3.525	0.0198	1.7023	0.0028	5	Negative regulation of catalytic activity, cell cycle, cell division, phosphatase inhibitor
IF4A1_HUMAN	Eukaryotic initiation factor 4A-I	1.3548	-2.8051	-4.1599	0.0287	1.5428	0.0034	77	double-stranded RNA binding, translation initiation, gene expression, virus process, ATP binding

All biological functions were determined using IPA software and uniprot.org

8.4.2 Mass spectrometry analysis of proteins within cell lysates of Serpin B1 treated PBMC cultures

8.4.2.1 Protein differential expression analysis

Analysis of the secreted proteins following Serpin B1 treatment provided information on the extracellular proteins, and the possible cellular pathways associated with them, that may be playing a role in Serpin B1's HIV inhibitory mechanism. However, analysis of cell lysates had the potential to provide an even more complete picture by providing the intracellular proteins, and thus pathways, being affected by Serpin B1. Following five-day incubations with either Serpin B1 (50μg/mL), BSA (50μg/mL, protein control), or PBS (cell control), PBMCs were lysed and analyzed via label-free mass spectrometry. Relative expression was determined for each of the 253 identified proteins that met the 25% CV designated criterion and multiple student t-tests were performed for each protein between the Serpin B1 and PBS treated cultures. Benjamini-Hochberg multiple comparison analysis rendered all of the studies underpowered and thus, following the construction of p-value distribution histograms, only those experiments that appeared to have results due to actual effects, and not random chance were subsequently analyzed using an alpha of 0.05 and assessed with IPA pathway analysis. Once again, an analysis was performed on the BSA treated cultures as well as the Serpin B1 treated cell lysates for determination of the proteins that were differentially expressed explicitly in the Serpin B1 cultures as compared to those that were regulated following treatment with any non-specific protein.

Following analysis of the individual p-value distribution histograms it became apparent that the cell lysates obtained from unstimulated (Figure 51) and PHA-stimulated (Figure 52), uninfected PBMC cultures did not contain enough differentially abundant proteins, compared to

the PBS treated control, for results to be considered statistically significant. Since the results from these two conditions could not be guaranteed to be due to true effects of Serpin B1 subsequent analysis was only performed on the data obtained from the PHA-stimulated, HIV-infected cell lysates (Figure 53).

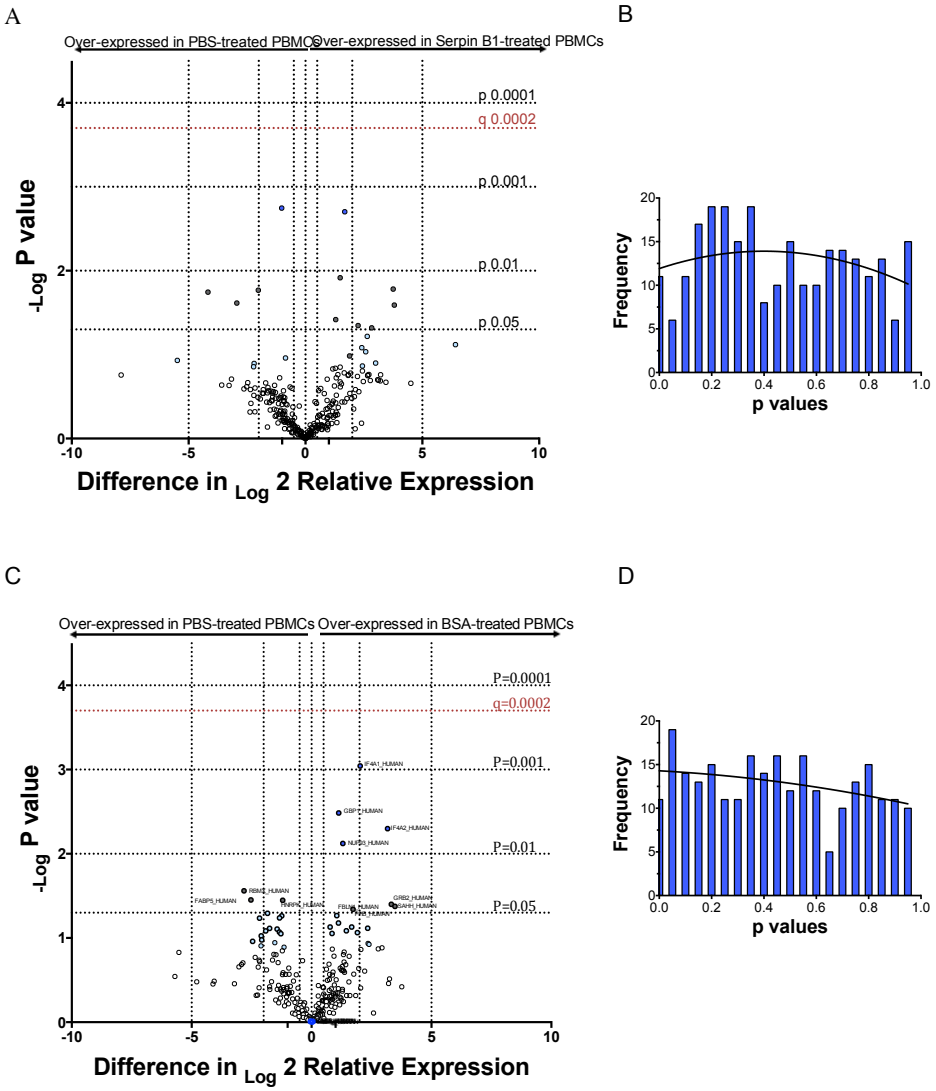


Figure 51: Protein expression in cell lysates from unstimulated, uninfected Serpin B1, and BSA treated cultures compared to PBS treated controls

A: Proteins identified through label-free mass spectrometry in Serpin B1 treated, unstimulated PBMC cell lysates compared to PBS treated cell control cell lysates. B: P value frequency distribution for relative expression comparisons between Serpin B1 and PBS treated PBMCs for the 253 identified proteins. C: BSA treated, unstimulated PBMC culture cell lysates compared to PBS treated control. D: P value frequency distribution for relative expression comparisons between BSA and PBS treated PBMCs.

%CV was set to 0.25. $_{LOG}2$ relative expressions were determined for 253 common proteins and compared between culture conditions using multiple student t-tests, without assuming consistent SD, alpha= 0.05. P values for each t-test were log transformed and plotted against the difference between $_{LOG}2$ relative expression values to create the volcano plot.

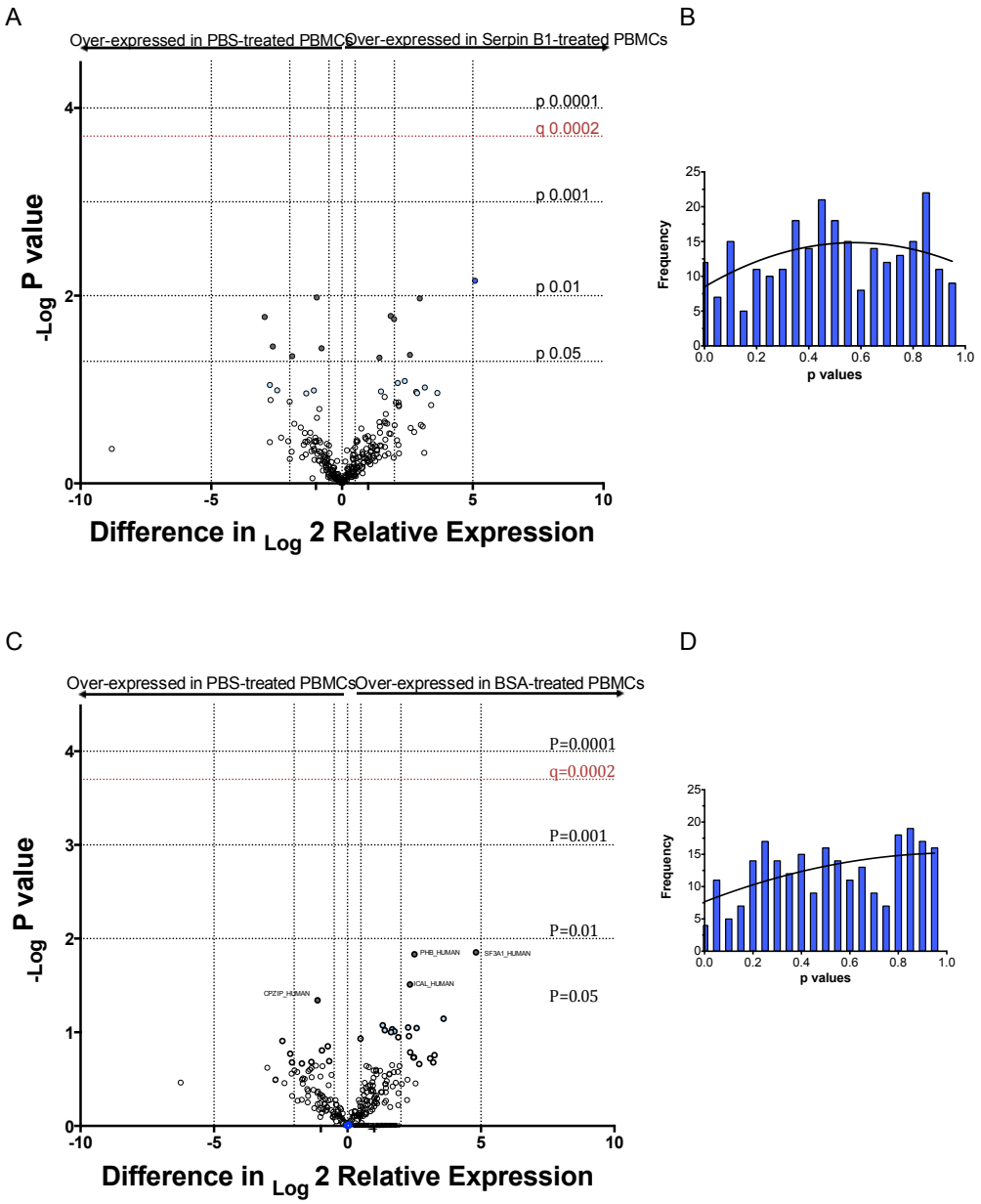


Figure 52: Protein expression in cell lysates from PHA-stimulated, uninfected Serpin B1, and BSA treated cultures compared to PBS treated controls

A: Proteins identified through label-free mass spectrometry in Serpin B1 treated, PHA-stimulated PBMC cell lysates compared to PBS treated cell control cell lysates. B: P value frequency distribution for relative expression comparisons between Serpin B1 and PBS treated PBMCs for the 254 identified proteins. C: BSA treated, PHA-stimulated PBMC culture cell lysates compared to PBS treated control. D: P value frequency distribution for relative expression comparisons between BSA and PBS treated PBMCs.

%CV was set to 0.25. $_{LOG}2$ relative expressions were determined for 253 common proteins and compared between culture conditions using multiple student t-tests, without assuming consistent SD, alpha= 0.05. P values for each t-test were log transformed and plotted against the difference between $_{LOG}2$ relative expression values to create the volcano plot.

8.4.2.1.1 Protein differential expression analysis of Serpin B1 treated, PHA-stimulated, HIV_{1956}-infected PBMC cell lysates

Cell lysates from PHA-stimulated, HIV_{1956}-infected cultures were collected and analyzed by label-free mass spectrometry as well as through differential protein analysis. These results provided information on the cellular processes that Serpin B1 affects within cells that have been treated with HIV as would be the case in cells within the FGT that have been exposed and potentially infected with HIV before the addition of Serpin B1.

From the 253 proteins that were identified via mass spectrometry, 21 were identified as differentially expressed in Serpin B1 treated cells compared to PBS controls (Figure 53A, Table 10). Of these 21 proteins, 20 were over-expressed within Serpin B1 cultures with only one (SH3 domain-binding glutamic acid-rich-like protein 3) also identified as over-expressed within BSAtreated controls (Figure 53B, Table 11). However, again, due to the relatively even spread of p-value distribution (Figure 53D) in the BSA treated culture, it is not clear as to whether these results are due to true effects or rather due to random effects. For this reason, SH3-domain binding glutamic acid-rich-like protein 3 was not removed from subsequent analysis. As such, the specific biological functions of each of the 20 over-expressed proteins (Table 10) was determined using IPA software and uniprot.org. Cumulatively, these proteins were found to be associated with a total of 35 different functional roles within various specific biological pathways. Specifically, seven of these proteins were associated with increased apoptosis (SH3 domain-binding glutamic acid-rich-like protein 3 $p = 0.0075$, Lymphocyte-specific protein 1 $p = 0.0082$, Coronin-1A $p = 0.0114$, Protein S100-A4 $p = 0.0151$, Nucleoside diphosphate kinase A $p = 0.0284$, Proliferation-associated protein 2G4 $p = 0.0313$, Programmed cell death 6-interacting protein $p = 0.0372$), six with decreased proliferation (Coronin-1A, ADP-ribosylation factor 1 $p = 0.0191$, Septin-6 $p = 0.0194$, Nucleoside diphosphate kinase A, Proliferation-associated protein 2G4, Programmed cell death 6-interacting protein), six with decreased mRNA processing/transcription/translation (Cytochrome c oxidase subunit 4 isoform 1 $p = 0.0001$, 40S

ribosomal protein S14 p = 0.0039, U2 small nuclear ribonucleoprotein A′ p = 0.0098, Proliferation-associated protein 2G4, 60S ribosomal protein L10 p = 0.0345, Histone H1.5 p = 0.0448), five with cell migration (Lymphocyte-specific protein 1, Coronin-1A, Protein S100-A4, SH3 domain binding protein 1 p = 0.0075, Integrin beta-1 p = 0.0459) one with cell redox homeostasis (SH3 domain-binding glutamic acid-rich-like protein 3 p = 0.0075), and one with ATP binding (Nucleoside diphosphate kinase A). There were also two proteins (Coronin-1A, SH3 domain-binding protein 1) associated with NK cell function that were identified within these infected cell lysates treated with Serpin B1. There were also five proteins (40S ribosomal protein S14, ADP-ribosylation factor 1, 60S ribosomal protein L10, Programmed cell death 6-interacting protein, Integrin beta-1) associated with different biological functions involved in viral reproduction, which had not been previously identified in any other examined conditions (Table 10). These 20 up-regulated proteins were also analyzed using IPA pathway analysis, which revealed associations with specific canonical pathways, namely, Rho family GTPase signaling (Actin-related protein 2/3 complex subunit 1B, Integrin beta-1, Septin-6, $p = 1.42 \times 10^{-3}$), clathrin-mediated endocytosis signaling (Clathrin-light chain B, Actin-related protein 2/3 complex subunit 1B, Integrin beta-1, $p = 7.23 \times 10^{-4}$), integrin signaling (ADP-ribosylation factor 1, Actin-related protein 2/3 complex subunit 1B, Integrin beta-1, $p = 1.00 \times 10^{-3}$) Actin nucleation by ARP-WASP complex (Actin-related protein 2/3 complex subunit 1B, Integrin beta-1, $p = 1.18 \times 10^{-3}$), and Granzyme A signaling (Nucleoside diphosphate kinase A, Histone H1.5, $p = 1.68 \times 10^{-4}$) (Figure 53E).

These results suggest that the intracellular proteins that are being regulated by Serpin B1 are associated with similar pathways to those proteins that were identified extracellularly within the supernatants of treated PBMC cultures. The specific cellular pathways that may be affected by Serpin B1 include Granzyme A signaling, mTOR signaling, EIF signaling, 14-3-3-mediated signaling as well as endocytic/phagocytic, and general cellular maintenance pathways.

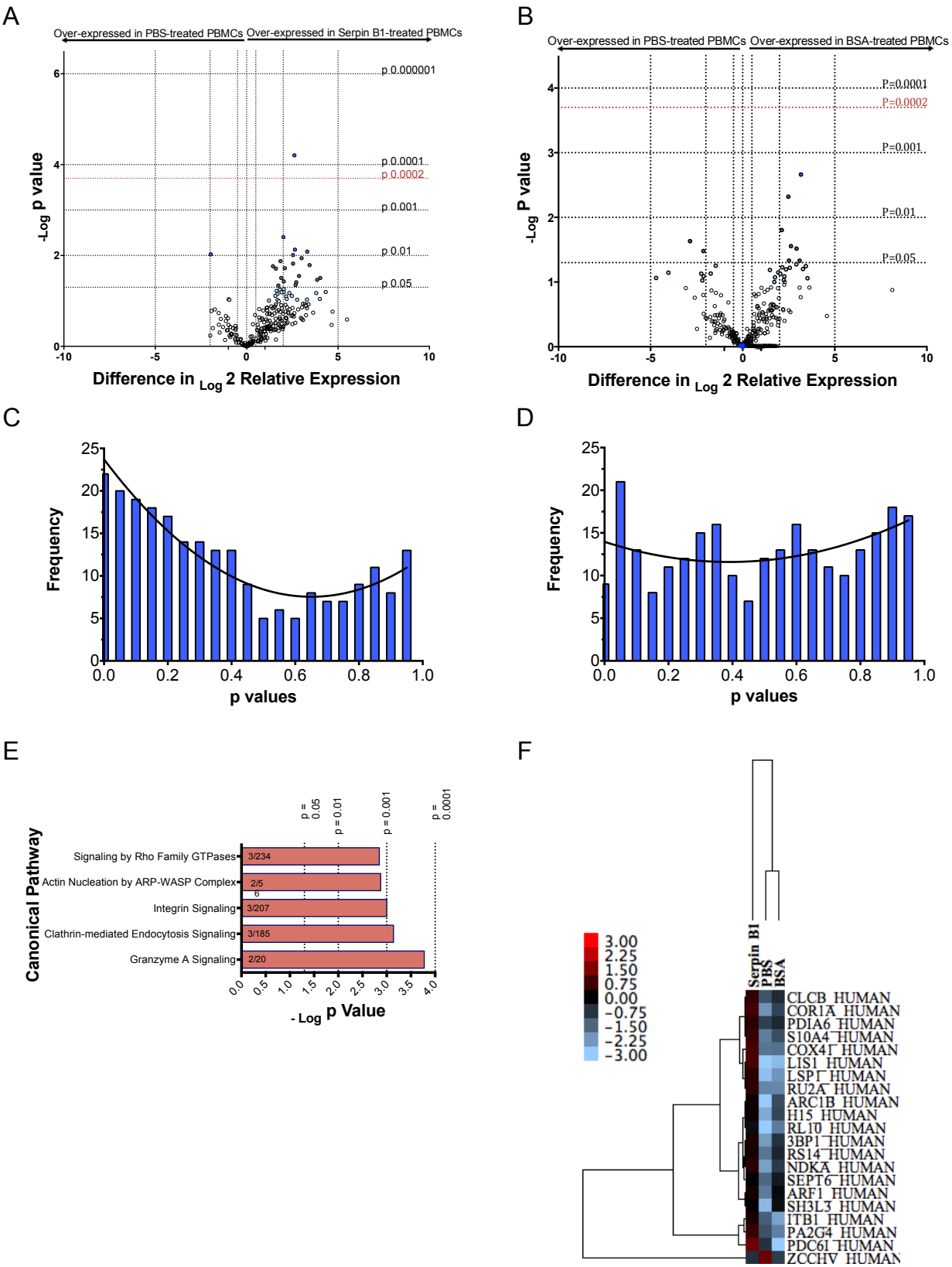

A
Over-expressed in PBS-treated PBMCs
Over-expressed in Serpin B1-treated PBMCs
p 0.000001
p 0.0001
p 0.0002
p 0.001
p 0.01
p 0.05
-Log p value
Difference in Log 2 Relative Expression
B
Over-expressed in PBS-treated PBMCs
Over-expressed in BSA-treated PBMCs
P=0.0001
P=0.0002
P=0.001
P=0.01
P=0.05
-Log P value
Difference in Log 2 Relative Expression
C
Frequency
p values
D
Frequency
p values
E
p = 0.05
p = 0.01
p = 0.001
p = 0.0001
Canonical Pathway
Signaling by Rho Family GTPases
Actin Nucleation by ARP-WASP Complex
Integrin Signaling
Clathrin-mediated Endocytosis Signaling
Granzyme A Signaling
3/234
2/5
3/207
3/185
2/20
- Log p Value
F
3.00
2.25
1.50
0.75
0.00
-0.75
-1.50
-2.25
-3.00
Serpin B1
PBS
BSA
CLCB_HUMAN
COR1A_HUMAN
PDIA6_HUMAN
S10A4_HUMAN
COX4I_HUMAN
LIS1_HUMAN
LSP1_HUMAN
RU2A_HUMAN
ARC1B_HUMAN
H15_HUMAN
RL10_HUMAN
3BP1_HUMAN
RS14_HUMAN
NDKA_HUMAN
SEPT6_HUMAN
ARF1_HUMAN
SH3L3_HUMAN
ITB1_HUMAN
PA2G4_HUMAN
PDC6I_HUMAN
ZCCHV_HUMAN

Figure 53: Protein expression in cell lysates from PHA-stimulated, HIV_{1956}-infected Serpin B1, and BSA treated cultures compared to PBS treated controls

A: Volcano plot depicting differential protein expression from the 253 proteins identified via label-free mass spectrometry, in Serpin B1 treated PHA-stimulated, HIV_{1956}-infected, PBMC cell lysates compared to PBS treated supernatants, B: Volcano plot illustrating the differential protein expression in BSA treated, PHA-stimulated, HIV_{1956}-infected, PBMC cell lysates compared to PBS treated control. C: P value frequency distribution for relative protein expression between Serpin B1 and PBS treated PHA-stimulated, HIV_{1956}-infected PBMCs for the 253 proteins. D: P value frequency distribution for relative expression comparison between BSA and PBS treated PHA-stimulated, HIV_{1956}-infected PBMCs for the 253 proteins. E: Depiction of the top canonical pathways, according to IPA, associated with the 20 up-regulated proteins in the Serpin B1 treated culture cell lysates. F: Dendrogram depicting the clustering patterns of the 21 differentially expressed proteins within the Serpin B1 treated cell lysates compared to the PBS and BSA treated controls

%CV was set to 0.25. $_{LOG}2$ relative expressions were determined for 253 common proteins and compared between culture conditions using multiple student t-tests, without assuming consistent SD, alpha= 0.05. P values for each t-test were log transformed and plotted against the difference between $_{LOG}2$ relative expression values to create the volcano plot.

Table 10: Differentially expressed proteins in the cell lysates of Serpin B1 treated, PHA-stimulated, HIV_{1956}-infected PBMCs compared to PBS treated controls

Accession Number	Protein Name	PBS $_{Log}$ 2 Rel Exp.	SB1 $_{Log}$ 2 Rel. Exp.	Diff. in $_{Log}$ 2 Rel. Exp.	P value	^{Log} TranS. P value	q value	# unique peptide	Biological Function
UP-REGULATED IN SERPIN B1 TREATED CULTURES									
LIS1_HUMAN	Platelet-activating factor acetylhydrolase IB subunit alpha	-3.2039	0.8243	4.0282	0.0313	1.5051	0.0030	12	Actin cytoskeleton organization, mitotic cell cycle, regulation of GTPase activity, negative regulation of JNK cascade
RL10_HUMAN	60S ribosomal protein L10	-4.0511	-0.1911	3.86	0.0345	1.4624	0.0034	9	Structural component of ribosomes, translation, viral reproduction, viral transcription
ARC1B_HUMAN	Actin-related protein 2/3 complex subunit 1B	-3.2813	0.1613	3.4426	0.0163	1.788	0.0020	61	Actin binding, structural constituent of cytoskeleton
LSP1_HUMAN	Lymphocyte-specific protein 1	-2.8343	0.4818	3.3161	0.0082	2.0848	0.0008	94	Actin binding, apoptosis, cellular defense response, chemotaxis
COR1A_HUMAN	Coronin-1A	-2.1989	0.8103	3.0092	0.0114	1.9414	0.0014	125	Negative regulation of apoptosis, leukocyte chemotaxis, NK cell degranulation, proliferation, actin binding,
NDKA_HUMAN	Nucleoside diphosphate kinase A	-2.3707	0.4995	2.8702	0.0284	1.547	0.0028	12	ATP binding, single-stranded DNA binding, cell differentiation, endocytosis, negative regulation of cellular proliferation, regulation of apoptotic process,
SH3L3_HUMAN	SH3 domain-binding glutamic acid-rich-like protein 3	-2.7378	-0.0226	2.7152	0.0389	1.4101	0.0038	28	Cytolysis, apoptosis, cell redox homeostasis, positive regulation of GTPase activity, electron carrier activity
H15_HUMAN	Histone H1.5	-2.5012	0.1757	2.6769	0.0448	1.349	0.0040	27	Chromatin organization, negative regulation of transcription from RNA polymerase II promoter
3BP1_HUMAN	SH3 domain-binding protein 1	-2.3119	0.3323	2.6442	0.0075	2.1276	0.0006	4	CCR5 chemokine receptor binding, chemoattractant activity, kinase activity, cellular response to IL-1, negative regulation by host of viral transcription, neutrophil and NK cell chemotaxis
COX41_HUMAN	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	-1.7451	0.8655	2.6106	0.0001	4.2045	0.0002	30	Cytochrome-c oxidase, gene expression, respiratory electron transport chain, transcription initiation from RNA polymerase II promoter
RU2A_HUMAN	U2 small nuclear ribonucleoprotein A'	-1.9661	0.5755	2.5416	0.0098	2.0096	0.0012	10	Poly (A) RNA binding, mRNA splicing via spliceosome
S10A4_HUMAN	Protein S100-A4	-1.8679	0.6723	2.5402	0.0151	1.8214	0.0018	26	Positive regulation of I-kappaB kinase/NK-kB cascade, RAGE receptor binding, apoptosis, chemotaxis
ARF1_HUMAN	ADP-ribosylation factor 1	-1.8227	0.2649	2.0876	0.0191	1.7186	0.0024	14	Actin filament organization, antigen processing and presentation via MHC class II, viral reproduction, proliferation
PDC6I_HUMAN	Programmed cell death 6-interacting protein	-0.7421	1.2926	2.0347	0.0372	1.429	0.0036	15	Apoptotic process, cell division, viral infectious cycle, viral reproduction, SH3 domain binding

RS14_HUMAN	40S ribosomal protein S14	-1.7459	0.2652	2.0111	0.0039	2.4035	0.0004	25	Structural component of ribosome, negative regulation of transcription from RNA polymerase II promoter, regulation of translation, viral infectious cycle
PA2G4_HUMAN	Proliferation-associated protein 2G4	-1.205	0.6868	1.8918	0.0313	1.5048	0.0032	50	Cell cycle arrest, cell proliferation, negative regulation of apoptotic process, negative regulation of DNA-dependent transcription, regulation of translation
CLCB_HUMAN	Clathrin light chain B	-1.2621	0.5904	1.8525	0.0134	1.8737	0.0016	3	Clathrin-mediated endocytosis, intracellular protein transport
ITB1_HUMAN	Integrin beta-1	-1.4063	0.3271	1.7334	0.0459	1.3384	0.0042	7	Actin binding, cell adhesion, blood coagulation, leukocyte migration, negative regulation of Rho protein signal transduction, positive regulation of MAPK cascade, viral entry into host cell
SEPT6_HUMAN	Septin-6	-1.5443	0.0557	1.6	0.0194	1.7119	0.0026	51	Cell cycle, cell division, viral reproduction, GTP binding
PDIA6_HUMAN	Protein disulfide-isomerase A6	-0.9454	0.4956	1.441	0.0173	1.7619	0.0022	27	Apoptotic cell clearance, platelet activation and aggregation, isomerase activity
DOWN-REGULATED IN SERPIN B1 TREATED CULTURES									
ZCCHV_HUMAN	Zinc finger CCCH-type antiviral protein 1	1.269	-0.6975	-1.9665	0.0094	2.0249	0.0010	19	DEAD/H-box RNA helicase binding, cellular response to exogenous dsRNA, defense response to virus, negative regulation of viral genome replication, positive regulation of I-kappa kinase/NK-kappaB cascade, positive regulation of IFN-alpha and IFN-beta production, positive regulation of RIG-I signaling pathway

All biological functions were determined using IPA software and uniprot.org

Table 11: Differentially expressed proteins in BSA treated, PHA-stimulated, HIV_{1956}-infected PBMC culture cell lysates compared to PBS treated controls

Accession Number	Protein Name	PBS $_{Log}$ 2 Rel Exp.	BSA $_{Log}$ 2 Rel. Exp.	Difference in $_{Log}$ 2 Rel. Exp.	P value	$_{Log}$ Trans. P value
UP-REGULATED IN BSA TREATED CULTURES						
SRP09_HUMAN	Signal recognition particle 9 kDa protein	-2.4306	0.7313	3.1619	0.0022	2.6629
HNRH1_HUMAN	Heterogeneous nuclear ribonucleoprotein H	-2.8244	0.2684	3.0928	0.0467	1.3305
GDIR2_HUMAN	Rho GDP-dissociation inhibitor 2	-3.7000	-0.7768	2.9232	0.0304	1.5176
MIF_HUMAN	Macrophage migration inhibitory factor	-2.3282	0.2956	2.6238	0.0278	1.5554
SH3L3_HUMAN	SH3 domain-binding glutamic acid-rich-like protein 3	-2.7378	-0.2238	2.5140	0.0467	1.3308
TBB5_HUMAN	Tubulin beta chain	-2.6689	-0.1883	2.4806	0.0048	2.3194
AMPB_HUMAN	Aminopeptidase B	-1.4627	0.6589	2.1217	0.0157	1.8034
DOWN-REGULATED IN BSA TREATED CULTURES						
C1TC_HUMAN	C-1-tetrahydrofolate synthase, cytoplasmic	-0.6258	-2.7573	-2.1315	0.0331	1.4800
PUR8_HUMAN	Adenylosuccinate lyase	0.5044	-2.3523	-2.8568	0.0234	1.6316

Blue font indicates proteins that were up-regulated in HIV-infected, PHA-stimulated, Serpin B1 treated PBMC culture cell lysates.

8.4.3 Antiproteases correlated with Serpin B1 expression

Due to the overlapping function for many antiproteases, namely that of serine and cysteine protease inhibition, specific antiproteases may exhibit synergistic effects when present in *in vitro* or *in vivo* cultures simultaneously. To determine which antiproteases are up-regulated at the same time as Serpin B1, a Pearson correlation analysis was performed comparing Serpin B1 expression to that of all other identified antiproteases within CVL samples identified via label-free mass spectrometry.

Within the CVL samples of women from the Winnipeg and Pumwani cohorts, several antiproteases positively correlated with Serpin B1 expression in at least one of the cohorts. Specifically, Serpin B2, Cystatin A, and SLPI, exhibited significant correlation in both cohorts, while Serpin B3, B4, B12, and B13, as well as Elafin, were positively correlated in CVL samples from Pumwani women only (Figure 54 and Table 12). Alternatively, A2ML1, Serpin A1, Serpin F1, and angiotensinogen exhibited inversely correlated concentrations compared to Serpin B1, in both cohorts, while Serpin B10, C1, D1, and G1 were all significantly inversely correlated to the concentration of Serpin B1 in only one of the two cohorts

It is, therefore, clear that the expression of several antiproteases are positively correlated with that of Serpin B1. As such, it may be possible to design a microbicide that would incorporate these antiproteases along with Serpin B1, thus representing proteins that are naturally expressed simultaneously within the FGT, thereby reducing the overall immune response to the exogenously added proteins. Furthermore, since the functions of these antiproteases are so diverse (Table 16, Appendix), they may act synergistically against HIV and may also balance the potential adverse effects exhibited by Serpin B1 alone (cell migration, apoptosis).

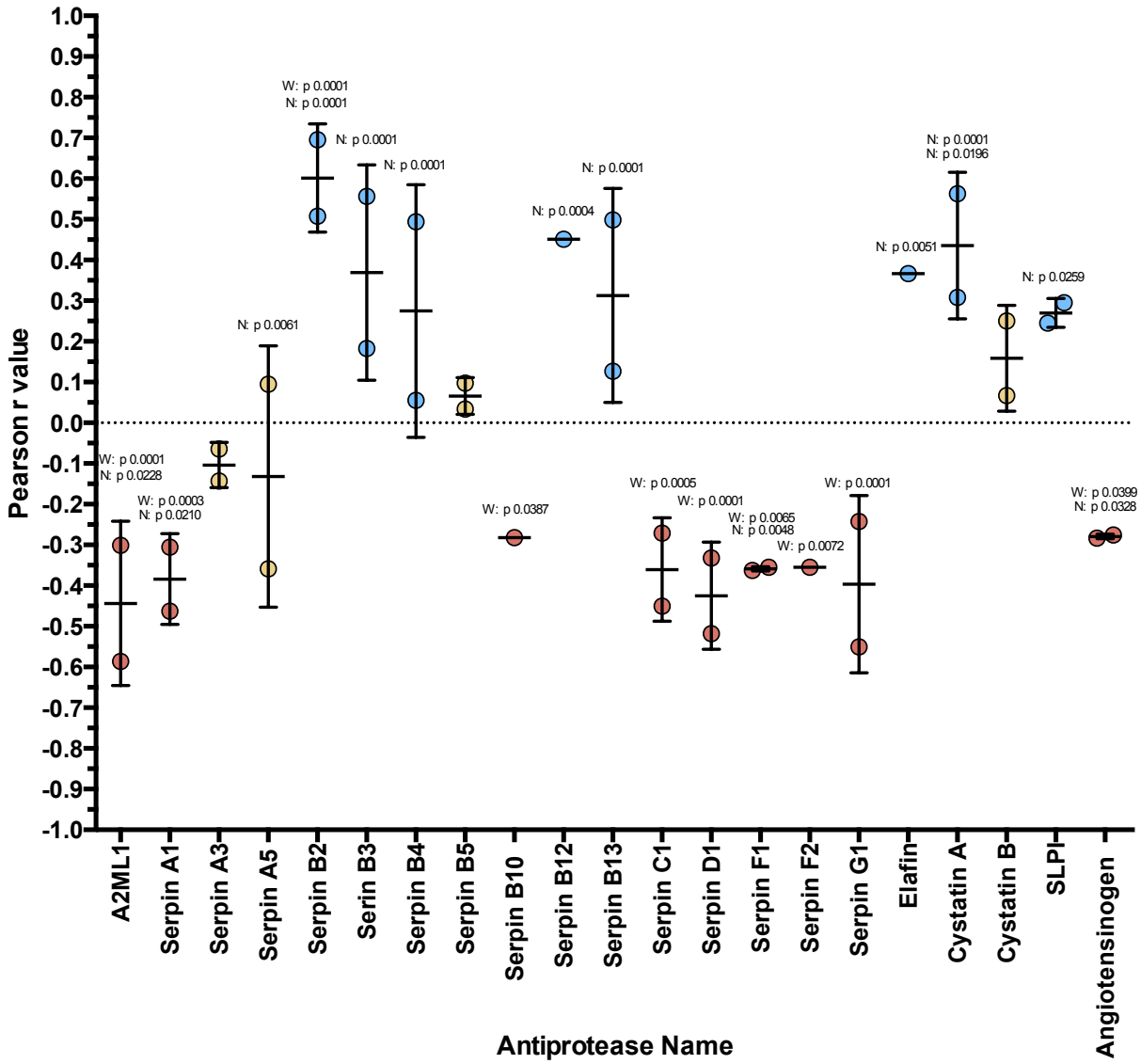


Figure 54: Antiproteases correlated with Serpin B1 in the CVL of women from the Winnipeg and Pumwani cohorts

All antiprotease data points in red were identified as having concentrations from at least one cohort, significantly inversely correlated to Serpin B1 concentration. All data points in blue represent antiproteases that in at least one cohort exhibited significant positive correlation. All data points in yellow did not exhibit statistically significant r values in either cohort or had extreme varying results between the two cohorts. Each point represents the r value for that antiprotease within one of the cohorts. Those data sets with only one point were identified within only that specific cohort.

All statistically significant p values are presented above the data points, with Winnipeg cohort values prefaced by W:, and Pumwani, Nairobi women by N:

Parametric Pearson correlation r values were calculated for Serpin B1 vs. each antiprotease. Correlations were two-tailed, with a 95% confidence interval, and an alpha of 0.05

Table 12: Correlation of antiprotease concentration in CVL with Serpin B1 concentration

Antiprotease Name	Cohort	r value	p value	Correlation description	Significance
A2ML1	Pumwani	-0.301146	0.0228	Inverse	*
	Winnipeg	-0.5863	< 0.0001	Inverse	****
Serpin A1	Pumwani	-0.305204	0.021	Inverse	*
	Winnipeg	-0.4628	0.0003	Inverse	***
Serpin A3	Pumwani	-0.235821	0.0774	--	--
	Winnipeg	-0.1432	0.2925	--	--
Serpin A5	Pumwani	-0.358951	0.0061	Inverse	**
	Winnipeg	0.09518	0.4853	--	--
Serpin B2	Pumwani	0.695447	< 0.0001	Positive	****
	Winnipeg	0.5074	< 0.0001	Positive	****
Serpin B3	Pumwani	0.55637	< 0.0001	Positive	****
	Winnipeg	0.1824	0.1786	--	--
Serpin B4	Pumwani	0.516861	< 0.0001	Positive	****
	Winnipeg	0.05536	0.6853	--	--
Serpin B5	Pumwani	0.097746	0.4695	--	--
	Winnipeg	-0.03402	0.8034	--	--
Serpin B10	Pumwani	--	--	--	--
	Winnipeg	-0.2821	0.0387	Inverse	*
Serpin B12	Pumwani	0.450898	0.0004	Positive	***
	Winnipeg	--	--	--	--
Serpin B13	Pumwani	0.556371	< 0.0001	Positive	****
	Winnipeg	0.1268	0.3519	--	--
Serpin C1	Pumwani	-0.070767	0.6009	--	--
	Winnipeg	-0.4503	0.0005	Inverse	***
Serpin D1	Pumwani	-0.237269	0.0755	--	--
	Winnipeg	-0.5178	< 0.0001	Inverse	****
Serpin F1	Pumwani	--	--	--	--
	Winnipeg	-0.3624	0.0065	Inverse	**
Serpin F2	Pumwani	--	--	--	--
	Winnipeg	-0.3551	0.0072	Inverse	**
Serpin G1	Pumwani	-0.197987	0.1399	--	--
	Winnipeg	-0.5505	< 0.0001	Inverse	****
Elafin	Pumwani	0.366301	0.0051	Positive	**
	Winnipeg	--	--	--	--
Cystatin A	Pumwani	0.308401	0.0196	Positive	*
	Winnipeg	0.5629	< 0.0001	Positive	****
Cystatin B	Pumwani	0.250373	0.0603	--	--
	Winnipeg	0.06689	0.6243	--	--
SLPI	Pumwani	0.294911	0.0259	Positive	*
	Winnipeg	0.2451	0.0687	--	--
Angiotensinogen	Pumwani	-0.283173	0.0328	Inverse	*
	Winnipeg	-0.2755	0.0399	Inverse	*

8.5 Summary

Several common functions were identified as being associated with the differentially expressed proteins in all Serpin B1 treated PBMC supernatants and cell lysates. These functions were related to increased apoptosis, decreased transcription, ATP binding, cellular response to redox reactions, and decreased proliferation, all of which suggest a potential mechanism for the observed decrease in proliferation, and induction of early apoptotic cell phenotypes observed following Serpin B1 treatment (Chapter 7). However, there were also unique functions identified for proteins that were differentially expressed in cultures infected with HIV following treatment with Serpin B1. Specifically, these functions included: phagosome maturation, post-transcriptional modification, NK cell response to stimulus, and protein translation. These additional pathways suggest that Serpin B1 affects cellular transcription, and translation, even though it was shown to not interfere directly with viral transcription (Chapter 7).

The key canonical pathways and networks affected by Serpin B1 treatment included those associated with cell signaling, cell death/survival, cell proliferation and translation of proteins. Specifically, proteins associated with pathways that focused on regulation of the EIF signaling, mTOR, Rho and Granzyme A signaling were affected by Serpin B1. It appears as though proteins that were down-regulated following Serpin B1 treatment of HIV-infected PBMCs were most associated with pathways involved in protein translation, namely EIF and mTOR.

Analysis was also performed to determine which antiproteases' correlate directly with Serpin B1 levels within CVL samples obtained from women in the Winnipeg and Pumwani cohorts. Numerous antiproteases were found to be positively correlated withthe presence of Serpin B1, specifically Serpin B family members, Elafin, Cystatin A and SLPI. The expression of other antiproteases were found to be inversely correlated with that of Serpin B1. These

antiproteases consisted of Serpins belonging to all other clades aside from the B clade (save for Serpin B10), as well as angiotensinogen.

Taken together these results suggest that Serpin B1 treatment is associated with changes in cellular apoptosis and proliferation, which suggests that Serpin B1 may be eliciting a portion of its inhibitory effect on HIV-1 through interference with proper cellular protein translation through the EIF and mTOR pathways, as well as through other protein-protein interactions. However all of these results would need to be validated.

“The aim of argument, or of discussion, should not be victory, but progress.”
~Joseph Joubert

CHAPTER 9: DISCUSSION

Understanding natural protective mucosal mechanisms within HESN populations may provide critical information for the design and development of novel microbicides for HIV. This thesis describes the effect of select antiproteases in FGT secretions on HIV-1 replication, with the ultimate goal of identifying potential candidates for the development of future microbicides. The role, if any, that complete CVL has in limiting HIV replication was also a primary focus. The main findings were that Serpin B1 was identified as an antiprotease that exhibited the strongest, consistent neutralization of HIV-1, and the determination that the primary mechanism for this inhibitory capacity, associates with the regulation of cellular proliferation and apoptotic pathways.

The central hypothesis of this thesis was that: Antiproteases identified as up-regulated within the CVL of HESN women, exhibit both direct anti-HI,V and indirect cellular mechanisms to regulate HIV replication. This hypothesis was partially proven in that Serpin B1, an antiprotease found to be up-regulated in the CVL of HESN women, exhibits definitive indirect cellular mechanisms of inhibition against HIV-1 replication.

9.1 Effect of complete CVL on HIV infection

The objective of this section was to compare the inhibitory capacity of CVL samples from HESN women in the Pumwani, cohort to samples from HIV-susceptible and HIV-infected women as well as to samples from women in an HIV low-exposure cohort in Winnipeg, Canada. The overarching hypothesis of this section was that: ***CVL fluids from HESN women inhibits HIV-1 infection more effectively than CVL from susceptible high-exposure commercial sex workers from Nairobi, Kenya or low-exposure women from Winnipeg, Canada.*** This hypothesis was partially proven in that CVL from all women in the high-exposure HIV cohort exhibited stronger anti-HIV effects compared to CVL from the low-exposure cohort. However,

no significant differences were observed between the samples from HESN and HIV-susceptible women from the Pumwani cohort.

9.1.1 HESN CVL does not exhibit enhanced HIV-1 neutralization capacity compared to CVL from other highly-exposed women

HESN CVL did not exhibit significantly higher levels of HIV-1 inhibition *in vitro*. The absence of significant HIV-1 inhibition by HESN CVL compared to the other study groups may be due to the large dispersion of individual effects within each group. Both significant inhibition and enhancement of infection were observed within CVL from individual HESN, HIV-susceptible, and HIV-infected women. This large variability in neutralization may be due to numerous confounding factors such as age[371], contraceptive method[83,372], menstrual phase[84,373], the make-up of the natural microbiome within the FGT[374], or the presence of secondary infections[194,375]. However, there may also be a more simple explanation, in that HESN women seem to be protected from HIV infection by multiple mechanisms[201,202,218,235,361,376]. These correlates include not only secreted factors within mucosal compartments but also those that affect systemic immune cells, endothelial cells, platelets, as well as those within tissues that would not be represented by CVL samples[201,202,218,235,361,376]. Furthermore, the concentration of specific factors in CVL may not have been in high enough abundance, either due to dilution during sampling or due to individual fluctuations between women, to exhibit a strong enough effect on HIV-1 alone to be detected by the assay. Hence, the factors that exhibit the strongest HIV-neutralizing capacity within HESN women may not have functioned at their optimal efficiency in an *in vitro* system. This would likely have been true for the HESN samples that exhibited significant enhancement of HIV-1 infection. The proviral factors that were present in these samples may not be capable of exhibiting such strong effects *in vivo* due to the presence of other protective mechanisms such as increased epithelial integrity, decreased immune cell targets

at the site of infection, or a reduced immune state within target cells both in the mucosal compartment and the blood.

The variability observed in neutralization capacity between HIV-1 Bal and IIIB infections, for CVL from all three Pumwani study groups, may be due to the mechanism of action of select correlates of protection such as defensins[230,234,371], MIP-3a[83,151], and SLPI[228] exhibiting protective capabilities in a tropic-dependent manner. These correlates may demonstrate stronger effects in cells infected with Bal R5-tropic viruses compared to IIIB X4-tropic HIV[373].

9.1.2 CVL from HIV highly-exposed women exhibit more potent neutralizing effects compared to HIV low-exposure women

The comparison of HIV-1 neutralization between low-exposure, Winnipeg women and high-exposure, commercial sex workers from Nairobi confirmed our hypothesis that CVL fluids from high-exposure women exhibit significantly stronger neutralization of HIV compared to low-exposure women. There was, however, no significant difference in neutralization between the postpartum and non-postpartum low-exposure groups. This was in opposition to the expected results, which, due to previous reports, predicted that high levels of pro-inflammatory cytokines (IL-6, IL-1, IFN-γ)[64,374], and elevated levels of immune activation within serum lymphocyte populations in postpartum women[251,377], would cause the CVL from these women to enhance the levels of HIV infection compared to non-postpartum women. Alternatively, lower levels of estrogen and progesterone were observed in lactating postpartum women compared to control non-postpartum women and non-lactating postpartum women[377,378,372], which has been associated with lower levels of HIV acquisition in other studies[83,372]. Taken together this data provides possible explanations for the individual variability in HIV-specific neutralizing effects exhibited by the CVL of postpartum women.

The significant differences in HIV-1 neutralization observed in CVL samples from HIV-high-exposure (Pumwani cohort) women, and low-exposure (Winnipeg) women were as expected by the proposed hypothesis. This hypothesis proposed despite the evidence that healthy women residing within HIV high endemic regions exhibit higher cervicovaginal concentrations of pro-inflammatory cytokines such as IL-1β, IL-6, and IL-8 compared to women from the United States of America, Europe, or Canada[379,380]. It is also well-documented that women within high-risk areas exhibit greater numbers and proportions of $CD4^+$ T cells expressing the CD69 activation marker and CCR5, as well as higher proportions of activated $CD8^+$ T cells[379,381]. Alternatively, specific reports have described the levels of specific anti-inflammatory, anti-viral proteins, such as SLPI, at higher abundance within women from high-endemic areas[220,226,228]. Despite this information I predicted that high-exposure women would exhibit stronger anti-HIV effects due to the presence of specific anti-HIV factors within the FGT of women who are regularly exposed to the virus[201].

9.1.3 The HIV-1 neutralizing capacity of CVL remains relatively stable over time

Determining the consistent ability of an individual woman's CVL to affect HIV-1 replication may provide insight into a woman's overall susceptibility to HIV infection. If the CVL of HESN or HIV-susceptible women contains inhibitory anti-viral factors, it would be expected that the expression of these factors would be relatively stable. The opposite is also true, for those women whose CVL consistently enhances HIV-1 infection, it can be argued that they may be at higher risk of acquiring HIV-1 infection over time, and this data may provide information on risk factors for HIV-1 acquisition.

The results of this section revealed limited variability in neutralizing activity and proteomic content of CVL from HESN and HIV-susceptible women in sample points collected over three to five years. This was the expected result for women who did not undergo significant changes in health, contraceptive method, and pregnancy or for those that did not acquire

secondary infections. However, a much higher proportion of samples from HIV-infected women demonstrated changes in HIV-1 neutralization capacity over time. This may be due to fluctuations in viral load and thus, in the overall immune response to the virus that occurs in HIV-infected individuals that are not on ARVs. Alternatively, these women may have been experiencing immune dysfunction due to prolonged HIV infection. When the viral load is high, a strong immune response will be mounted resulting in an increase in specific HIV-1 responses within the blood as well as potentially at the mucosal surfaces, such as the FGT, however whether these responses are adequate, or effective in limiting HIV *in vitro* is unknown [382-385]. The presence of secondary infections, which HIV-infected women are more susceptible to when compared to HIV-uninfected individuals[9,15,386], also likely results in variability in inflammatory mediators present within the FGT. Many infections, including *Neisseria gonorrhoea*[375] or *Chlamydia trachomatis*[387], could potentially cause activation and proliferation of immune cells that will begin to produce anti-viral or anti-bacterial factors. These factors may alter neutralization or enhancement profiles.

9.1.4 Summary of complete CVL's effect on HIV-1 infection

In this study, we disproved our hypothesis that CVL samples from HESN women would inhibit HIV-1 more strongly than CVL from HIV-susceptible women from both high and low endemic areas. Instead, CVL from both HESN and HIV-susceptible women in high-endemic areas both exhibited stronger anti-HIV activity compared to CVL obtained from HIV-susceptible women in a low-endemic setting.

9.1.5 Limitations and Future Directions

There were several limitations to this study, many of which were due to the limited availability of patient samples and epidemiological data. TZM-bl cells are an immortalized cell line that has been proven useful in preliminary neutralization assays. However, TZM-bl cells are not primary cells and thus, may lack relevance to what is occurring *in vivo*.

Additional HIV-1 strains would have been useful in demonstrating the effect that individual CVL samples have on different clades. Unfortunately, the lack of sample availability was a limiting factor in these situations.

The choice of cohorts was also a limitation of this study. Ideally, a cohort in Winnipeg consisting of female sex workers as well as a cohort in Nairobi that was made up of healthy women from the general public would have been beneficial. These additional cohorts would have provided a group of women in Nairobi that are engaged in low-risk behavior but still live in a high-endemic area, as well as a group of women involved in high-risk behavior in a low-endemic area in Winnipeg. These cohorts would have also allowed for controlling of sexual activity as a confounding factor in both countries. However, while the work in this study was performed with a well-established cohort with a strong history in research studies in Nairobi, and a strong, newly developed cohort in Winnipeg, there were no additional cohorts in existence at this time that would have provided the samples necessary for these desired comparisons

9.2 Proteomic analysis of CVL from HIV high-exposure and low-exposure women

The specific hypothesis for this section was that: ***CVL from women in HIV low-endemic areas (Winnipeg cohort) contain distinct protein profiles compared to women in high-endemic areas (Pumwani cohort). Low-exposure women will express a lower abundance of known antiviral proteins, including antiproteases.*** This hypothesis was partially proven in that women from both cohorts did express unique proteins compared to the other, which following cluster analysis clustered primarily based on the cohort. However, the level of antiproteases varied between cohorts with some elevated in Winnipeg women and others in the CVL of women from the Pumwani cohort.

9.2.1 Proteomic profiles differ significantly between CVL from highly-exposed Pumwani women, and Winnipeg women with low exposure to HIV-1.

Label-free mass spectrometry analysis of the proteomic content present in CVL samples from Winnipeg and Nairobi women revealed numerous proteins differentially expressed between the two cohorts. (Figure 17 and Table 2). The most striking differences in protein content between the two cohorts involved proteins related to innate immune responses. Women from Nairobi expressed higher levels of proteins associated with apoptosis, complement activation, and overall immune activation, as well as higher levels of proteins associated with nucleobase salvaging and the Atherosclerosis signaling pathway. This data is in line with what is specified in literature, with women from Kenya exhibiting higher cervicovaginal concentrations of pro-inflammatory cytokines, including IL-1, IL-6, and SLPI as well as higher levels of activated cells, expressing the CD69 activation marker, compared to North American women[381,388]. The up-regulation of three proteins associated with the Atherosclerosis pathway may simply suggest that proteins are present in the CVL of women from the Pumwani cohort that are involved in chronic immune activation since this is one of the defining characteristics of Atherosclerosis[389,390]. The extended presence of immune activation may lead to the observed increase in pro-apoptotic proteins and perhaps subsequent damage to cells. Increased apoptotic pathways/damage to cells would result in the an increased attempt to repair damaged cellular DNA, which involves the salvaging of nucleobases (both for pyrimidines and purines), which are then used in the biosynthesis of nucleotides of DNA[391]. Nevertheless, the increased level of pro-apoptotic proteins within the CVL of women from the Pumwani cohort, may account, in part, for the increased neutralizing effects against HIV-1 compared to women in the Winnipeg cohort.

The CVL samples from Winnipeg women were found to express higher levels of proteins associated with increased viral replication and neutrophil function (including IL-8) as well as regulation of peptidase activity (Table 2) when compared with CVL samples from women in the

Pumwani cohort. Winnipeg women's CVL also contained elevated levels of proteins associated with the acute phase response as well as the LXR/RXR and FXR/RXR activation pathways. Again a portion of this data is in line with what is observed in literature; specifically, IL-8, among other soluble mucosal immune mediators such as IP-10, are up-regulated in the CVL of women from the USA compared to women from Uganda and South Africa[392]. The presence of proteins associated with neutrophil function and pro-viral functions in Winnipeg women may be due to differing immune responses to the normal microbiota within the FGT between women of European ancestry compared to those of African ancestry. The former has been identified as containing *Lactobacillus*-dominated microbiomes while the latter exhibit a much more diverse microbiome consisting of *Gardnerella* species as well as *mycoplasma and Sneathia*[393,394]. This highly variable microbiome may result in the observed widespread basal immune activation within women from Nairobi and, due to the higher prevalence of HIV-1 and HSV among African women, these women may also have a more robust anti-viral response in place compared to Winnipeg women. Conversely, women from Winnipeg may not have adapted a strong anti-viral response due to more limited exposure to such pathogens. However, the neutrophil-dominated immune response may be the result of a higher proportion of bacterial infections in these women compared to viruses. The remaining over-expressed proteins within these women, (liver C receptor/LXR and Farnesoid bile X receptor/FXR) are associated with cholesterol/lipid metabolism and are known to regulate the innate immune response within mucosal tissues specifically within the mucosa of the small intestine[395,396]. The up-regulation of proteins associated with these pathways may simply suggest a difference in diet and, thus, the need for an increase in proteins involved in lipid or cholesterol metabolism within Winnipeg women. Alternatively, it may suggest an increase in regulation of the immune state within the FGT of these women, mediated by both the LXR/FXR pathways and acute phase proteins[397,398].

The expression of particular antiproteases was also noted to differ between the two study cohorts. SLPI, Serpin B12, Elafin, and Serpin C1 were over-expressed within the CVL of women from the Pumwani cohort while Serpin B5, Serpin B10, Serpin F2, and angiotensinogen were up-regulated within Winnipeg women's CVL fluids (Figure 18). These differences may be due to differential expression of mucosal proteins that exhibit similar functions, within different ethnic groups which has been noted for SLPI[64]. The observed differential expression between Serpin C1 and Serpin F2 in these two cohorts may be due to preferential expression of proteins that overall have similar functions between different populations, in this case, each is involved in regulation of the blood coagulation pathway. However, Serpin F2 also has more broad-spectrum functions as an inhibitor of trypsin and chymotrypsin, which may account for the decreased expression of some of the other antiproteases with similar functions to this, such as SLPI and Serpin B12 in the Winnipeg cohort. Also, over-expression of Serpin B5 within the CVL of Winnipeg women may associate with higher levels of female cancers in Canada (277.4/100,000 women) compared to Kenya (196.6/100,000 women)[399]. These elevated rates of cancer in women in Canada may result in increased expression of tumor-suppressor proteins such as Serpin B5[400]. Elafin and SLPI may have been over-expressed within the CVL of women from the Pumwani cohort as a result of the strong ability of these antiproteases to mediate immune responses which would be necessary for women who exhibit higher overall levels of immune activation compared to women within HIV-low endemic areas such as Winnipeg.

Cluster analysis of the proteomic data revealed three distinct clusters that clearly separated approximately two-thirds of the samples based primarily on the cohort that the samples were collected from (Figure 19, Table 3). Two distinct clusters consisted mostly of samples from the Pumwani cohort. Following analysis of the potential epidemiological factors that may have caused this divergence in cohort sample clustering, it was apparent that age, contraceptive method and menstrual phase may all be contributing factors. These three factors are all

interconnected with women in Cluster five, who have a higher median age, higher proportion of women having gone through menopause (50%), and higher proportion of women not using hormonal contraception compared to women comprising Cluster two (Table 3). Generally, women who have undergone menopause will not feel the need to use contraceptive methods to prevent pregnancy, however, when still active in sex work they may choose to use condoms as a means to protect themselves against sexually transmitted infections. This may explain the overwhelming proportion (80%) of women, whose samples comprised Cluster five, who were not using any form of hormonal contraception compared to women in Cluster two (31.3%). Additionally, the samples in Cluster five may not have been grouped together with those in Cluster two due to the dramatic changes in their mucosal proteome that may be affected by estrogen and progesterone that occur during menopause, and the subsequent changes in cellular metabolism and rates of glycolysis/gluconeogenesis[401,402], for example.

The other two clusters that segregated independently were Cluster three and four. However, again, differing proportions of individuals in different menstrual phases appeared to play a role in the differential clustering of these samples. Specifically, a large percentage of the women (30%) within Cluster three were in the follicular phase of their menstrual cycle while a similar proportion of women within Cluster four (37.5%) were in the luteal phase. Different stages of the menstrual cycle are directly associated with distinct cellular processes, which would alter the specific proteins within the FGT, and thus the proteins that would appear within the CVL. For example, the proliferative/follicular phase is characterized by regeneration of the endometrial stratum functionalis layer and a gradual increase in estrogen levels which causes a dramatic increase in the volume of cervical mucus, and a change in the overall texture/composition[403]. Thus, many cellular processes involved in cervical mucus production (mucins, albumin, etc.), cellular integrity, maintenance, regeneration, and mitosis, would be up-regulated during this phase compared to the later secretory/luteal phase. Alternatively, the

hallmarks of the luteal phase consist of a gradual decline in estrogen levels, which causes a progressive thickening and decreased volume in cervical mucus. There is also a continuous increase in progesterone, produced by the corpus luteum (follicle that released egg during ovulation) during this phase. In addition, the epithelial cells of the uterus and cervix will begin to store glycogen, and mitosis has essentially been halted[404,405]. Thus, this stage of the menstrual cycle may contain proteins associated with cellular pathways involved in carbohydrate metabolism (glycogen production and storage) or reduced cellular integrity/breakdown of cell-to-cell junctions in the late stages of the luteal phase. It is, therefore, plausible that the differences in the protein compositions within the samples that made up Clusters three and four were due in part to the differences in menstrual phases. However, it is also likely that alternative factors, including genetic polymorphisms, exposure to pathogenic microbes, composition of the vaginal microbiome, etc. are playing important roles in creating unique proteomic profiles in the women whose samples comprised these two clusters.

9.2.2 Proteins within CVL correlated with HIV replication

Previous studies have identified numerous candidates for protein correlates of protection within the CVL of HESN women [222,236,365,406,407]. However the actual concentrations of these factors may not correlate with the rate of infection *in vitro*. The most striking finding obtained from this study is the correlation of various proteins known to affect HIV replication within the CVL of women from the Winnipeg cohort while only a select number of proteins were found to be correlated within the Pumwani cohort. This may be due to the requirement of multiple factors to act in concert to offer protection against HIV, which may not have been identified through mass spectrometry. Alternatively, there may have been more unique responses to HIV-1 within the Pumwani cohort due to the selective pressure experienced by the mucosal immune system within each individual's FGT following repeated exposures to HIV as well as other pathogens. With a fluctuating repertoire of proteins expressed differentially among the individuals within

the Pumwani cohort, correlations would be difficult to identify. Conversely, the women in Winnipeg who would rarely be exposed to HIV-1 may express factors that are common among the participants with little fluctuation due to a relatively stable microbiome, reduced immune activation compared to women in Kenya and limited HIV-specific responses[394]. The observed variability in CVL composition between women in the Pumwani cohort suggests unique challenges to determining consistent correlates of protection within the CVL of women from HIV high-exposure areas compared to women in low-exposure settings.

Despite these challenges some very specific correlates were identified, primarily in the Winnipeg women. Not surprisingly, many of the proteins are involved in acute phase responses, and in mediating the innate immune response through endopeptidase inhibitory functions, which has been previously associated with protection against HIV infection[408]. Many antiproteases were correlated with increased HIV-1 inhibitory activity (Table 4), including multiple members of the Serpin B family (B1, B2, B3, B4, B5, B13), as well as Serpin A5, A2ML1, and Cystatin B. Serpin B2 exhibited the strongest correlation, save for Elongation factor Tu which belongs to *Lactobacillus acidophilus* species. The question arises whether these antiproteases are being down-regulated by the increased exposure to HIV-1 or whether the infection rates of HIV-1 are being decreased by increased levels of the antiproteases. It can be postulated that the latter is, at least in part, accurate based on the known functions of these proteins. Three of the six Serpin B family members (B3, B4, B5) exhibit known roles in control of tumor cell suppression and control, while both Serpin B2 and B13 exhibit functions in control of cellular apoptosis and proliferation. Together with the functions demonstrated by Serpin B1 in this thesis, it can be hypothesized that the control of cellular proliferation that these antiproteases exhibit may be important in the control of HIV-1 replication. This is likely through limiting the number of potential target cells for HIV-1 to infect or alternatively, by affecting the efficiency by which the infected cells replicate. Serpin A5, A2ML1, and Cystatin B all exhibit more traditional

endopeptidase inhibitory activity, specifically mediating immune responses that could cause hyperactivation of immune cells and subsequent damage to the surrounding tissues allowing for easier transport of HIV-1 through the mucosal tissue or increased proportions of activated cells which are prime targets for HIV-1 to replicate within. Hence, these three antiproteases within CVL may play an important role in protection against HIV-1 infection *in vivo* through proper control of the immune response within the local FGT mucosal compartments, and *in vitro* by reducing the overall activation state of target cells. Furthermore, the top protein that correlated with an increase in HIV inhibition is the Elongation factor Tu of *Lactobacillus acidophilus*. This correlation lends to the known protective role for the commonly found *Lactobacillus* bacteria species within the FGT microbiome[70,197,393]. As previously mentioned, these species predominate within the microbiome of women with European ancestry compared to women of African ancestry[394], which may result in subsequently increased protection against infection with HIV-1 *in vivo* and a less favorable environment for HIV survival *in vitro*.

The 28 proteins (Table 5) that were correlated with enhanced HIV-1 infection were highly associated with the acute phase immune response. These proteins are usually produced by hepatocytes, are highly variable, and include factors expressed in response to trauma such as infection, stress, or inflammation[397,408]. Many of these proteins are directly involved in positive regulation of apoptosis, up-regulation of the inflammatory response, induction of the complement system, endopeptidase activity, recruitment of pro-inflammatory cytokines and/or chemokines, and stimulation/activation of immune cells, all of which would assist in creating an optimal environment for HIV-1 replication and rapid dissemination to new target cells.

9.2.3 Summary of proteomic analysis of CVL from HIV low-exposure and high-exposure women

Determining the differences in the proteomic profiles of CVL from women at high and low-exposure to HIV, allows for characterization of factors associated with an individual's risk of acquiring HIV.

This study indicates that women from HIV high-endemic areas, express proteins associated with higher levels of inflammation and immune activation compared to women in low-exposure cohorts. However, both cohorts demonstrated high levels of specific antiproteases, some of which had overlapping functions. Thus, it appears that while women from different areas of the world may express varying levels of proteins, specifically antiproteases, they may do so to limit the immune activation in the FGT.

Cluster analysis revealed the majority of clustering was based on cohorts alone. However, specific clusters included women from both cohorts who had epidemiological commonalities including those who have experienced menopause or those that were using similar contraceptive methods. This suggests that significant variability exists in protein expression at an individual level, which depends on numerous epidemiological factors.

Together this portion of the study provides evidence for the highly variable nature of the proteome within the FGT, including differing levels of proteins associated with immune activation, inflammatory responses and potentially variable microbiomes between groups. Variation in differing levels of exposure to HIV-1 and other infectious genital/mucosal pathogens, and women experiencing dramatic changes in their menstrual cycle, i.e. menopause or postpartum are all factors that likely alter the FGT proteome, and thus the ability to affect HIV replication. These differences in proteomes are important to consider when designing an HIV microbicide in that one formula may not work for all women at all times.

9.2.4 Limitations and Future Directions

This study would have been strengthened by having higher volumes of CVL samples to be able to perform more in-depth assays for determination of inhibitory capacity against HIV-1. Additionally, an increase in the number of samples available would have been beneficial to test larger numbers of CVL capable of enhancing HIV-1 infection, both within the Winnipeg and Pumwani cohorts. An increase in enhancing CVL numbers would have provided better comparison between inhibitory and non-inhibitory samples.

It would have also been useful to collect matched blood samples for each CVL sample. This would have provided the opportunity to be able to correlate proteins that were determined to be up-regulated or down-regulated within the CVL in an effort to make more definitive conclusions about the causative determinants for many of the differences observed in protein expression between cohorts, and ultimately the effect this would have on mucosal fluids' ability to alter HIV replication.

9.3 Screening antiproteases for HIV-neutralization capacity

The overarching hypothesis of this section was that: ***Antiproteases, Serpin A1, A3, B1, B13, C1, Cystatin A, and B, and A2ML1, that have been shown to be over-expressed within the CVL of HESN women are capable of neutralizing HIV-1 infection of TZM-bl cells and PBMCs,*** which was proven for specific antiproteases (Serpin B1, B13, C1, Cystatin A, Cystatin B and A2ML1) while others (Serpin A1, A3) did not exhibit any significant inhibitory activity in this *in vivo* system.

9.3.1 Six antiproteases significantly inhibit HIV-1 infection in a TZM-bl cell assay while only one significantly inhibited HIV-1 within an assay using PBMC targets

Eight individual antiproteases were screened for HIV inhibitory activity using a TZM-bl assay infected with either HIV-1 Bal or IIIB. Six antiproteases exhibited significant inhibitory

activity; A2ML1, Cystatin A, Cystatin B, Serpin C1, Serpin B1 and Serpin B13 (Figure 21, 22, 23). However, when neutralization capacity was measured in a PBMC assay, Serpin B1 was the only antiprotease that showed significant inhibitory activity against HIV-1 in this system (Figure 25 & 26). A2ML1, Cystatin B, and Serpin B13 exhibited trends in inhibitory activity in PBMCs, however, this was not reproducible. The inhibitory activity of Serpin B13 (Figure 22) was approximately equal to the level of cytotoxicity observed (Figure 20) in TZM-bl cells (24%). Hence, this inhibitory activity is not likely specific for HIV but rather due to Serpin B13 induced cell death. The overall lack of inhibitory activity observed within the PBMC cultures was likely due to the low concentrations of antiproteases used. Within these assays, 10μg/mL was the highest concentration employed so as to determine which antiproteases exhibit inhibitory activity at a low enough concentration that would be feasible for use in further in-depth analyzes. However, for all antiproteases, save for Serpin B1, concentrations of 30-40μg/mL in TZM-bl was required before inhibitory activity was observed.

The differences observed in inhibitory activity between HIV-1 Bal and HIV-1 IIIB in TZM-bl cultures, was likely due to the differential expression levels of the HIV co-receptors CCR5 and CXCR4 on these cells. TZM-bl cells over-express CCR5 and while they also express CXCR4 they do so at a much lower level. Due to this reduced expression of CXCR4, a higher viral MOI was used when infecting with IIIB so as to achieve detectable levels of infection. At an MOI of 1.0, compared to the MOI of 0.2 for Bal, IIIB continued to exhibit β-galactosidase levels 10-fold lower than what was observed in TZM-bl cells infected with Bal. However, the use of an increased MOI may have required that higher concentrations of antiprotease be used to achieve significant inhibition with Cystatin A and Serpin C1 (Figure 21).

Surprisingly, Serpin A1 was not one of the antiproteases identified as inhibitory in these studies (Figure 23). This antiprotease has previously been associated with protection against

HIV-1 within *in vitro* studies utilizing PBMCs[315,409], and *in vivo*, through inhibition of neutrophil elastase within a murine model, which has been associated with decreased HIV-infectivity[222,315]. Serpin A1 has also been shown to compete with gp120 binding and reduces fusion of HIV-1 with the host cell membrane[315,409]. However, the C-terminal peptide fragment is responsible for the bulk of the anti-HIV activity exhibited by this protein[315]. The lack of inhibitory activity observed in the current study may be due to the difference in source of Serpin A1, and concentrations employed between this study and that performed by Shapiro *et al.*[409]. Within the latter study, Serpin A1 was isolated from the blood of patients rather than synthesized commercially. Also, the concentrations used in Shapiro's study were in the range of 1-5 mg/mL, concentrations that are relevant within the blood however 33-166 fold higher than that observed in the mucosal fluids of the female genital tract[235,236]. It is also possible that the commercially produced Serpin A1 utilized in our study does not efficiently produce the C36 fragment at levels high enough to exhibit an effect within either TZM-bl or PBMC cell cultures. TZM-bl cells are derived from HeLa cells, which are purified, immortalized cervical carcinoma cells, and do not contain elastase producing cells which are the primary targets of Serpin A1's extracellular anti-HIV activity, explaining the lack of inhibitory activity.

Any antiprotease that abrogates HIV-1 infection primarily through immunoregulatory activity or through interference with late stages in replication (post-translational) would not demonstrate significant inhibitory activity within the TZM-bl cell assay. These cells do not need to be activated for HIV to infect them. The β-galactosidase gene within these cells is under the control of HIV Tat protein, and thus, the measured relative luminescence units (RLUs) are directly related to the number of virus particles infecting the cells, irrespective of the degree of cellular activation. Furthermore, the β-galactosidase gene is transcribed and translated simultaneously with HIV-1 genes and detected following cell lysis hence there is no effective way to utilize this assay for the detection of inhibitory activity against HIV-1 assembly and

budding from the cells. It may have been due to these reasons that inhibitory activity was not observed in this assay by Serpin A3, which exhibits the bulk of its antiprotease activity through inhibition of cathepsin G and mast cell chymase which would inhibit HIV *in vivo* however would be less efficient in a TZM-bl *in vitro* model.

The previously described HIV-specific inhibitory activity exhibited by Serpin C1 (antithrombin III)[410,411] was supported by this study. Serpin C1 exhibits HIV inhibitory activity through increasing $CD8^+$ T-cells, $CD4^+$ T-cells and NK cell migration as well as through induction of a G-protein-coupled signal transduction process and inhibition of TNF-α-induced NF-κB activation[410]. The latter two may have been associated with the observed inhibitory activity within the current study, in that they would slow the overall transcription of viral mRNA. However, migration of $CD8^+$ T cells, $CD4^+$ T cells and NK cells to the site of infection could not have been the cause of the effects observed within a TZM-bl cell culture.

While there is little concrete evidence in the literature to suggest that either Cystatin A or Cystatin B exhibits direct anti-HIV activity, there is documented evidence that Cystatin A is associated with decreased p24 antigen expression within tonsillar lymphoid follicles[356]. Furthermore, this antiprotease demonstrates significant anti-inflammatory capabilities through the inhibition of cathepsins B, L, and S, all of which are released by macrophages and participate in the inflammatory response[340,343]. Cystatin A has also been identified as anti-apoptotic[356], which would limit the number of virions being released by infected cells via apoptosis. Similarly, Cystatin B exhibits inhibitory activity against Cathepsin B produced by macrophages; however there is contradictory data as to how this affects HIV-1 replication. One study has demonstrated that Cystatin B correlates with increased HIV replication within monocyte-derived macrophages[412] and has been shown to inhibit the IFN-induced antiviral response through suppression of tyrosine phosphorylated STAT-1, suggesting a role in the persistence of HIV within reservoir cells[413]. Alternatively, Cystatin B has been associated with restricted HIV

replication within placental macrophages[414]. This conflicting evidence suggests that neither Cystatin A nor Cystatin B demonstrated direct anti-HIV mechanisms, but rather exhibit broad ranges of regulatory effects on cellular inflammatory processes which in specific situations are protective in nature and, in others, potentially exacerbate the infection. Further studies would have to be performed within mucosal physiologically relevant cell and tissue models to truly understand the role that these antiproteases play in HIV acquisition within the FGT.

While Serpin A3, B1, B13, and A2ML1 are up-regulated within the CVL of HESN women[236], few studies describe the role that these antiproteases has in HIV-1 infection. In fact, aside from the known anti-inflammatory functions of all these proteins, this is the first study that has examined the direct role that they have on HIV-1 *in vitro.*

9.3.2 Summary of antiprotease neutralization screening assays

This study identified HIV-1 inhibitory activity in six of the eight antiproteases, shown to be up-regulated within the CVL of HESN women[235]. While Serpin A1 and A3 did not exhibit significant anti-HIV activity within either TZM-bl or PBMC cell cultures Serpin C1, Cystatin A, Cystatin B, A2ML1 and Serpin B1 demonstrated significant effects. This data complements what is found in the literature. It also provides evidence for a role in protection against HIV-infection for Cystatin A and B as well as for A2ML1. Finally, the finding that Serpin B1 exhibited the most consistent inhibition requires further analysis to decipher the mechanism employed.

9.3.3 Limitations and Future Directions

This study would have provided more complete data for effects exhibited within PBMC cultures, had higher concentrations, matching those used within the TZM-bl assays, been used. However, the availability of protein was a limiting factor. Also, the use of additional cell lines/cell types that are more physiologically relevant to the FGT would build upon the data generated from this study and would thus provide a more comprehensive understanding of the

role these antiproteases play. The most physiologically relevant cell type would be cervical mononuclear cells (CMCs). However, isolation of an adequate number of CMCs from frozen samples followed by HIV-1 infection *in vitro* is incredibly challenging and often unsuccessful.

9.4 HIV-specific mechanisms of inhibition exhibited by Serpin B1

The hypothesis for this section was: ***Serpin B1 inhibits HIV-1 through interference with virus binding to host cells or through inhibiting HIV-1 enzymes required for virus replication and assembly***. This hypothesis was partially proven as none of the examined stages of the HIV replication cycle were directly inhibited by Serpin B1. However, the studies performed demonstrated Serpin B1 may be acting on post-transcriptional stages in HIV replication.

9.4.1 Commercially produced Serpin B1 inhibits Neutrophil Elastase and localizes to cytoplasm and nucleus of PBMCs

Serpin B1 is best characterized as an inhibitor of the serine protease, leukocyte/neutrophil elastase. This antiprotease inhibits elastase through binding of the ser-195 within the active site of the protease. A covalent bond is formed between the ser-195 and a carbonyl group on the backbone of Serpin B1, followed by irreversible conformational changes in both the protease and the Serpin, rendering them both inactive[282,286]. Determining that commercially produced Serpin B1, used throughout this thesis, exhibited anti-elastase activity at the concentrations employed throughout the experiments (0.78μg/mL-50μg/mL, Figure 27) suggests that this protein is biologically active and thus its HIV inhibitory activity would also likely be preserved.

The confocal imaging of fluorescently-labeled Serpin B1 indicates that Serpin B1 is taken up by PBMCs where it localizes to both the cytoplasm as well as the nucleus (Figure 28 and 29), suggesting that exogenous Serpin B1 is present within the cellular compartments that the natural protein would be found[328]. This study also demonstrates that PBMCs express detectable levels of endogenous Serpin B1 within both the nucleus and cytoplasm which agrees with literature[328] and provides evidence for its role as an intracellular protease inhibitor. Most relevant to the

current study, however, is the increase in intracellular Serpin B1 observed within the first three hours, which would be the timeframe HIV-1 requires for entry into target cells (1 hour after initiation of infection) and initiation of reverse transcription (approximately 5 hours after initiation of infection)[415], suggesting its ability to limit HIV replication.

9.4.2 Serpin B1 inhibits multiple strains of HIV-1 in multiple cell cultures

The HIV-1 inhibitory activity exhibited by Serpin B1 was apparent in PBMC and TZM-bl cultures infected with both HIV-1 IIIB and HIV-1 Bal. However, it was important to also determine its inhibitory capacity within the C8166 T cell line, which was used in subsequent mechanistic studies as well as within a physiologically relevant, ectocervical tissue explant model. Additionally, Serpin B1 was shown to inhibit a Kenyan primary HIV isolate (HIV_{1956}). Inhibition within these assays, ranged from 30-60% compared to untreated control cultures infected with HIV-1 (Figure 30) and regardless of the cell type or HIV strain employed.

9.4.3 Serpin B1 does not affect early stages of the HIV-1 replication cycle

Since Serpin B1 demonstrated consistent HIV inhibitory activity in multiple cell cultures, it was important to begin by determining whether Serpin B1 directly interferes with HIV binding.

Neutralization assays with Serpin B1 treated C8166 T cells infected with a VSV-G-pseudotyped single-cycle-replicating pNL-Bru-ΔBgl/Luc^{+}/R^{-} virus was used to determine the effect that Serpin B1 had on virus binding/fusion and cellular uptake into the cells. However, this assay was not able to detect effects on post-translational stages of HIV replication since the readout is based on luciferase activity activated by Tat expression (Figure 31).

The absence of inhibition in this system by Serpin B1 suggests that the mechanism either involves blocking binding to host cell receptors or in stages post-translation including viral assembly and budding. However, subsequent experiments using qRT-PCR quantitation of viral DNA levels following Serpin B1 treatment, indicated that early, pre-uptake stages are not being

affected by Serpin B1 treatment but rather it was affecting later stages of viral infection (Figure 32). This assay was designed to measure the relative abundance of early viral DNA (single stranded negative sense DNA strand) and late viral DNA (double stranded DNA) within the cell cytoplasm as well as 2-LTR circular DNA within the nucleus. It was evident through comparison of the relative abundance in all three of these types of viral DNA that Serpin B1 did not interfere with binding/fusion, reverse transcription, nuclear import or integration. Had there been inhibition at the stage of viral binding or fusion, reduced levels of all HIV DNA would have occurred due to lower abundance of virus present within the cells, as was observed in cultures treated with ENF. Similarly, had reverse transcription been affected, lower levels of early and late viral cDNA would have been apparent as was seen in cultures treated with AZT.

Quantitation of the relative abundance of 2-LTR provided evidence that Serpin B1 did not interfere with either nuclear import nor integration of HIV cDNA into the host genome. 2-LTR unintegrated circular viral DNA is produced within the nucleus through non-homologous end joining[416,417]. Therefore, should decreased abundance of 2-LTR circular HIV DNA be detected in treated cells, that did not exhibit lower levels of late viral cDNA, it could be concluded that nuclear import was being impaired. Conversely, should higher levels of 2-LTR circular DNA be detected within treated cells, it would be concluded that integration is being impaired. Should the double-stranded HIV cDNA be prevented from integrating into the host genome, more of it would become circularized the longer it remains unintegrated within the nucleus. It was evident however that Serpin B1 treatment did not affect levels of HIV-1 DNA. It can, therefore, be concluded that Serpin B1 does not interfere with viral binding/fusion, reverse transcription, nuclear import or integration but rather appears to affect HIV in the stages of its lifecycle following provirus integration.

9.4.4 Serpin B1 may inhibit late stages of HIV-1 replication

The late stages of HIV replication begin at transcription and continue through to viral assembly and budding at the host cell membrane. To determine if Serpin B1 interfered with any of these stages an ACH2 cell line containing an integrated provirus was employed.

The provirus in ACH2 cells is induced to produce infectious HIV-1 viral particles following treatment with phorbol myristate acetate (PMA) or TNF-α. This study indicated that following Serpin B1 treatment, there was significantly reduced levels of extracellular p24 at 48 and 72 hours (Figure 33). These results suggest that Serpin B1 may interfere with late stages of the viral lifecycle. Alternatively, since ACH2 cells have a 24-hour replication cycle, these results may indicate that Serpin B1 is interfering with the proliferative capacity of cells since significant effects were not observed until 48 hours, which would account for at least two cell cycles. Thus, a reduction in the number of cells producing virus may also be a plausible explanation for the observed results. It may also be possible that both of these explanations are valid, and that Serpin B1 interferes with late stages in the HIV lifecycle, accounting for the minimal decrease in p24 production following 24 hours incubation, while also interfering with cellular proliferation required for continued propagation of viral particles and would account for the increased inhibitory effect observed following 48 and 72-hour incubation (Figure 33).

To define the effects of Serpin B1 on transcription HIV mRNA splice variants were quantified in both ACH2 and PBMC cell lysates (Figure 34). For HIV-1 to replicate it relies on a myriad of host factors for RNA processing which generates over 40 different mRNAs[38,418,419], all of which are required for the synthesis of the 15 viral proteins[38-40]. Once mRNA undergoes splicing, three classes of mRNAs result, unspliced, single-spliced and multi-spliced mRNA[38,40]. Alterations in the normal RNA splice patterns would identify if undersplicing occurs which would reduce the amount of multi-spliced and single-spliced RNA. Alternatively, over-splicing

would lessen the amount of unspliced RNA and subsequent loss of structural and accessory proteins[38]. Following analysis of the results, it was apparent that no significant differences were observed in the relative abundance of any splice variant within the ACH2 or PBMC cell lysates. Thus, Serpin B1 does not interfere with transcription or splicing of the HIV-1 pre-mRNA.

The final direct HIV-1 mechanistic study performed with Serpin B1 involved quantification of the total intracellular p24 levels after HIV-1 infection via flow cytometry. Through comparison of intracellular to extracellular p24 levels, it could be inferred as to whether Serpin B1 inhibits p24 production, resulting in equally reduced intracellular and extracellular p24 levels, or if it interferes with assembly or budding resulting in reduced extracellular p24 levels. The results indicated that intracellular p24 levels were significantly reduced, with approximately 20% of lymphocytes expressing intracellular p24 in HIV-infected, untreated cells compared to 8.68% in Serpin B1 treated cells (Figure 36). This results in a 56.6% reduction in infection which was comparable to the previously observed reduction in extracellular p24 levels (57.7%, Figure 30C). Thus, this data suggests that Serpin B1 interferes with protein production and not likely viral assembly or budding.

9.4.5 Summary of HIV-specific mechanisms exhibited by Serpin B1

This study aimed to identify HIV-specific inhibitory mechanisms of Serpin B1. Through multiple assays, it was shown that Serpin B1 does not interfere directly with HIV binding or uptake by the host cell, reverse transcription, nuclear import, integration, transcription or viral assembly and budding from the host cell. Rather, Serpin B1 likely interferes with translation/post-translational modification, which was inferred by the indentification of reduced intracellular p24 levels, however, this was the only stage not directly examined in this section. Thus, it is fair to conclude that translation is being affected, an effect which may be due to direct interference with cellular pathways required for protein translation or that cellular replication is being reduced resulting in lower output of virus.

9.4.6 Limitations and Future Directions

The use of a clade C virus throughout all the experiments would have been beneficial. This is the clade that accounts for approximately 50% of diagnosed infections worldwide and predominates in southern Africa, Ethiopia, and India. Together with the viruses employed within this study, the inclusion of a clade C HIV would have allowed for coverage of the most prominent clades causing infection (approximately 70-75% of global infections are caused by clade A, B, and C)[23,24]. While it is not expected that Serpin B1 would effect clades differently, the inclusion of a clade C virus would have provided substantial evidence for this.

Lastly, this study would have benefited from a direct analysis of the effect that Serpin B1 has on protein translation. However due to the expertise of our laboratory with mass spectrometry-based proteomic techniques, we chose this methodology to examine the possible effects of Serpin B1 on translation and its related cellular pathways, which is presented in Chapter eight.

9.5 Cellular response to Serpin B1

The specific hypotheses of this section were that: ***Serpin B1 will regulate the expression of factors, including proteases and anti-viral cytokines/chemokines, that have previously been shown to result in reduced HIV replication; Serpin B1 reduces the level of immune activation and proliferative capacity of cells. Thereby, reducing the amount of HIV virions produced by infected cells.*** The latter hypothesis was partially proven in that Serpin B1 reduces the level of proliferation in cells, however, there was no detectable effect on cellular immune activation. Alternatively, the first hypothesis yielded results that were inconclusive.

9.5.1 Serpin B1 does not affect cellular activation

HIV-1 preferentially targets and replicates in activated $CD4^+$ T cells and thus immune activation is required for HIV infection[241,376]. Immune activation also impacts an individual's

risk of HIV acquisition[241,420]. Activated T cells undergo proliferation and express activation markers and effector molecules that are involved in energy metabolism[241]. Specifically, increased expression of HLA-DR, CD25, CD69 and CCR5[421] has been associated with increased immune activation and HIV replication[376].

The HIV inhibitory activity of Serpin B1 may be due to a reduction in the activation state of susceptible $CD4^+$ T cells thereby reducing the efficiency by which HIV-1 infects and replicates. To address this question PBMC immune activation assays were conducted using a flow cytometric technique with CD3/CD28 beads and PMA/Ionomycin employed as positive controls[422,423].

Results from this portion of the study revealed that Serpin B1 does not significantly affect the expression of immune activation markers (CD69, HLA-DR, CCR5) and thus does not affect cellular activation (Figure 37). Since PBMCs treated with Serpin B1 did not exhibit altered activation this is not likely the mechanism by which this antiprotease exhibits its anti-HIV-1 activity.

9.5.2 Serpin B1 interferes with cellular proliferation

Reducing the number of actively replicating HIV-1 target cells would result in decreased levels of HIV replication. Hence, the decreased level of infection observed in Serpin B1 treated cells may be due to an overall reduction of actively proliferating cells (Figures 38, 40, 41). Within PBMC cultures treated with Serpin B1, no significant differences were observed in lymphocyte viability compared to PBS treated cells. However, the proportion of proliferating lymphocytes was significantly reduced.

Similarly, within PMA-stimulated cells, Serpin B1 treatment of A3.01 and ACH2 cells, markedly reduced proliferation compared to PMA only stimulated cells (Figure 41). There was, however, a larger effect observed in ACH2 cells, which contain an HIV provirus, suggesting a direct interaction in cells already infected with HIV-1.

9.5.3 Serpin B1 increases expression of cellular early apoptotic markers

HIV infection has been associated with both inducing and inhibiting apoptosis depending on the stage of infection. In fact, many of the HIV proteins have demonstrated either anti- or pro-apoptotic capabilities. Specifically, gp120 binding results in apoptosis of both infected $CD4^+$ T cells and uninfected bystander cells[424]. Furthermore, binding to CXCR4 by gp120 is a more potent inducer of apoptosis than binding with CCR5[424,425]. Also, T cells that express Nef up-regulate Fas and FasL and PD-1, while expressing lower levels of Bcl-2 and Bcl-XL[426]. Alternatively, Nef has also been identified as an inhibitor of the pro-apoptotic serine/threonine kinase ASK-1 and p53[427] while also inhibiting phosphorylation of Bcl-2 associated death promoter (BAD, pro-apoptotic protein). A current hypothesis suggests that induced apoptosis of early, acute HIV-1 infected cells would not only prevent the spread of the virus but would also limit the exacerbated killing of important bystander immune cells. To this end, specific compounds are currently being examined for their potential to overcome retroviral-induced resistance to apoptosis in the acute phase of infection. Specifically, Ciclopirox (CPX) and Deferiprone (DEF) are drugs that have been shown to increase apoptosis differentially in HIV-1 infected cells through the activation of the intrinsic mitochondrial pathway, without causing significant damage to bystander cells or surrounding tissue/epithelial integrity[428]. Thus factors capable of preferentially inducing apoptosis within acute HIV-1 infected cells may contribute to the control of the spread and establishment of efficient HIV-1 infection.

The results of this study suggest that Serpin B1 likely induces an early apoptotic state within both PBMCs (Figure 43) and ACH2/A3.01 cells (Figure 44). In Serpin B1 treated PBMCs, the early apoptotic marker Annexin V was up-regulated in HIV-infected and uninfected cells. There were also increased proportions of markers for actively apoptotic cells within Serpin B1 treated cells HIV-infected cells. However, this increase in active apoptosis may be due to one donor data point skewing the results enough to create differences between the Serpin B1 and

PBS treated conditions (Figure 43), but early apoptotic markers were clearly upregulated in Serpin B1 treated cells. Similarly, in Serpin B1 treated ACH2/A3.01 cultures induction of early apoptosis (Annexin V +, Live/Dead -) within both cell types was clearly observed, however, Serpin B1 treatment did not induce active apoptosis in either of the cell types.

The consistent induction of early apoptotic markers within all cell subsets suggests that Serpin B1 may be inducing a pre-apoptotic state within these cells, which may result in cell "silencing" and a subsequent autophagic state. Autophagy causes reduced transcription and translation as well as a slowing of cell division, accounting for the decrease in cellular proliferation[429,430]. It is not clear if long-term Serpin B1 treatment would eventually trigger full apoptosis of these cells. If more prolonged treatment with Serpin B1 does induce complete apoptosis in cells, this could be explained by the previously observed endonuclease activity of this protein upon cleavage[329]. If this antiprotease were to be integrated into a potential microbicide, careful consideration would have to be given to the timing that it would be applied. Elevated levels of Serpin B1 during early acute infection may act to interfere with the survival of infected cells thereby preventing dissemination of the virus to the circulatory/lymph systems. Alternatively, prolonged exposure to elevated levels of this antiprotease may result in apoptosis or "silencing" of critical immune cells or even structural epithelial cells of the FGT. Thus, further studies must be performed to determine on which cell types Serpin B1 imparts its pro-apoptotic effectss.

9.5.4 Serpin B1 alters expression of pro-inflammatory cytokines in PBMCs and FGT epithelial cell lines

Cytokines and chemokines have pivotal roles in both the control and exacerbation of HIV-1 infection[431] (Appendix, Table 17). It has been established that the FGT mucosa of HESN women exhibits a unique cytokine profile compared to susceptible women. Specifically, HESN

women express significantly lower levels of MIG, IP-10 and IL-1α in their CVL compared to HIV-susceptible controls[370].

Pro-inflammatory cytokines activate HIV-1 replication and maintain expression through the induction of NF-κB binding to the HIV-1 LTR[86]. A cell's susceptibility to HIV infection is dependent on the level of $CD4^+$ T cell activation and the stage of monocyte/macrophage differentiation[36,376]. Therefore, it is reasonable to conclude that an increase in pro-inflammatory cytokines present at the site of infection, would result in an increase in the number of activrated/proliferating cells recruited for productive infection by HIV-1. Additionally, it has been reported that TNF-α, IL-1 and IL-6 directly stimulate HIV-1 replication in T lymphocytes and monocyte-derived macrophages (MDMs). Alternatively, IL-10 inhibits HIV-1 replication in MDMs (Appendix, Table 17).

The over-expression of pro-inflammatory cytokines/chemokines can up-regulate the antiviral responses. However, it may also act to increase the pool of target cells for HIV at the site of infection[432]. Conversely, anti-inflammatory cytokines may dampen the anti-viral response while also reducing the number of potential target cells available[432]. Furthermore, the cytokine/chemokine profile during acute HIV infection has been highly associated with the viral set-point (stabilized viral load following acute infection)[177]. Specifically, elevated levels of IL-12, IL-10 and IFN-γ during acute infection are associated with lower viral set-points and are thus beneficial to the individual[433-436]. It is thus supported throughout the literature that specific cytokine/chemokines present during acute infection offer protection against HIV replication, which may be different from the cytokine/chemokine profile exhibited during chronic infection. Providing an explanation for the observed pleiotropic and often contrasting effects administered by many cytokines during HIV infection (Appendix, Table 17).

The results of this study suggest that Serpin B1 induces both pro-inflammatory (TNF-α, IL-1, IL-8 and IL-6) and anti-inflammatory cytokines (IL-10) as well as specific chemokines (MIP-1α, MIP-1β, GM-CSF, GRO) which is not a function usually ascribed to antiproteases. Antiproteases primarily mediate the function of proteases, which themselves induce pro-inflammatory immune responses and thus antiproteases indirectly reduce pro-inflammatory responses rather than inducing them. However, upon closer examination of the data presented here, while Serpin B1 has been shown to induce expression of IL-6 and TNF-α within PBMC cultures, at all time points examined (3 hours to 5 days, Figure 45 and 46), there were also significantly elevated levels of IL-10 in these same cultures. IL-10 may act to counter the pro-inflammatory effects exhibited by IL-6 and TNF-α. Furthermore, in the absence of elevated IFN-γ, the pro-HIV effects of TNF-α may be minimal[437]. MIP-1α and MIP-1β, both of which exhibit anti-HIV effects, were found to be up-regulated within PBMC cultures treated with Serpin B1. GM-CSF and GRO were also elevated in PBMC cultures treated with Serpin B1 however GM-CSF was not significantly increased until day five while GRO was induced by day 3. Hence, the up-regulation of GM-CSF may have been in response to the increased expression of GRO. *In vivo* GRO would function to limit the amount of migrating neutrophils after three days of infection, thereby reducing the overall level of inflammation in the tissues, which is in keeping with the known function of Serpin B1 as a regulator of neutrophil activity.

Within FGT epithelial cell lines, Serpin B1 did not have as strong of an effect on cytokine/chemokine expression as was observed in the PBMC cultures (Figures 47). Within all three cell lines (vaginal, endocervical and ectocervical), one of the most strongly up-regulated chemokines was GM-CSF which functions to limit neutrophil recruitment to the site of infection while also ensuring efficient survival and proliferation of MDMs as well as protecting MDMs against HIV infection[438] (Appendix, Table 17). Therefore, up-regulation of this chemokine in

the epithelial cells of the FGT would serve to reduce the level of inflammation as well as protect the targets of HIV, namely macrophages, from infection. However, pro-inflammatory cytokines were up-regulated, namely IL-6, IL-8, IL-1α and GRO, primarily at 3 and five days post-infection. These were all induced in the absence of up-regulated anti-inflammatory cytokines, such as IL-10. This could imply that should elevated levels of Serpin B1 be present for longer than three days prior to exposure to HIV-1, induction of a pro-inflammatory state would result and theoretically increase the risk of HIV-1 infection. These studies would have to be repeated in a tissue or animal model to better understand the interplay between Serpin B1 and the induction of cytokines from various cell types *in vivo*. Within the FGT, immune cells are present that may be capable of expressing immune-suppressive cytokines, such as IL-10, which would counteract the pro-inflammatory cytokines produced by the epithelial cells. Additionally, *in vivo* studies would provide the opportunity to examine temporal effects of Serpin B1 on cytokine/chemokine expression along the course of HIV infection.

9.5.5 Summary of cellular response to Serpin B1

The HIV inhibitory effects exhibited by Serpin B1 were determined to not be related to the activation state of PBMCs as Serpin B1 treatment did not affect the expression of activation markers. However, Serpin B1 does significantly reduce the number of actively proliferating cells in either PBMC or ACH2/A3.01 cells, with a stronger effect observed in ACH2 cells. The decrease in proliferation is associated with an increase in early apoptotic (Annexin V) markers. Again this effect was observed in both PBMC lymphocyte populations as well as with ACH2 and A3.01 cells cultures, with slightly stronger effects seen in HIV treated cultures. This suggests Serpin B1 may be inducing an early apoptotic state, resulting in a slowing of cellular processes, including proliferation, and thereby reducing the number of active target cells for HIV to infect as well as slowing the rate at which HIV-1 is being generated within infected cells.

Serpin B1 appears to have a dynamic effect on cytokine and chemokine expression in PBMCs as well as in FGT cell lines, inducing both pro-inflammatory and anti-inflammatory cytokines as well as proviral and antiviral cytokines. A temporal and spatial balance in the expression of these factors may result in an overall protective effect against HIV-1 infection. Taken together these results suggest that if Serpin B1 is to be employed as a candidate within a microbicide it would have to be done with great care and temporal precision. Inducing apoptosis/reduced cellular proliferation is beneficial at very early stages of infection, before the propagation of the founder virus to more than the initial infected cells, however beyond this point, induction of these process may result in the death/dysfunction of immune cells. As for the effects on cytokines and chemokines, a pro-inflammatory response exhibited during early acute infection would bring effector cells to the site of infection for control of the virus. Additionally, if anti-HIV cytokines (MIP-1α, MIP-1β, GM-CSF) are present prior to initial infection or early acute infection, inhibition of HIV-1 uptake may be possible. While a clearer picture is beginning to emerge as to how Serpin B1 implements its anti-HIV activity, the exact cellular pathways that it engages with to do so are not yet known, which is the focus of the last chapter of this thesis - determining through proteomics, the cellular targets of Serpin B1.

9.5.6 Limitations and Future Directions

In the proliferation studies, it would have been beneficial to measure various exhaustion markers (PD-1, TIM-3) to determine whether the cells that exhibited reduced proliferation were also demonstrating reduced functional responses. Within the apoptotic studies, additional markers (caspases, Fas/FasL) could have been stained for to give a clearer picture of which apoptotis pathways were being affected. Additionally, within these studies, it would have been beneficial to have stained for both Annexin V as well as p24. This duo staining would have determined if the increased apoptosis in infected cultures was occurring in HIV-infected cells or bystander cells. This information would prove to be an important piece for determining whether

Serpin B1 is a suitable candidate for a microbicide. However, this staining was not possible due to reagent limitations; as both 7-AAD and p24 were on the same fluorochrome (FITC). Lastly, all of these studies would have been stronger had a Serpin B1-specific antibody also been used in complimentary assays to block the antiprotease function so as to ensure the effects observed were truly caused by Serpin B1 and not a contaminate present in the 80-85% pure protein solution.

9.6 Effect of Serpin B1 on cellular protein expression

The specific hypothesis for this section was: **Serpin B1 interferes with signalling pathways involved in cellular signaling involved in proliferation, and up-regulates the expression and activity of molecules involved in apoptotic pathways.** This was partially proven in that Serpin B1 appears to affect multiple proteins involved in apoptosis and proliferation. One pathway that may be affected by Serpin B1 is the mTOR pathway, which is a central regulator of protein translation and cell proliferation.

9.6.1 Serpin B1 induces expression of proteins involved in oxidative stress, which may induce an autophagic state through inhibition of the mTOR signalling pathway

Many proteins with overlapping functions were associated with Serpin B1 treatment and PHA-stimulation in HIV-infected and uninfected PBMCs. Determining the commonalities between these functions allows for the generation of a hypothesis for a potential mechanism for the observed increase in apoptotic markers and decreased proliferation, which associates with Serpin B1's ability to inhibit HIV.

Many of the proteins that were identified as up-regulated by Serpin B1 were linked directly to general antiprotease activity. Specifically, in all cell lysates analyzed, specific proteins were identified that associated with ubiquitin C and general protein ubiquitination. Serpin B1, along with all other serpins that exhibit antiprotease activity, upon binding to a

protease will distort the structure of both the protease as well as itself, thereby acting as a suicide inhibitor and rendering both substrate and enzyme inactive[285,286,296,298]. Furthermore, when proteins become misfolded they are tagged by ubiquitin for degradation by the cell[44]. Therefore, the natural function of Serpin B1 binding to and distorting the structure of its target protease would result in increased induction of ubiquitination.

Within the Serpin B1 treated PHA-stimulated cell lysates, several up-regulated proteins were also associated with NK cell function within HIV-infected cultures. Suggesting that Serpin B1 is inducing activation of NK cells. Furthermore, increased IL-10 production results in NK cell proliferation, cytotoxicity and IFN-γ production when combined with IL-18[439]. Therefore, the increased IL-10 production observed in the cytokine analysis of this study may explain the increased expression of proteins associated with NK cell response in the PHA-stimulated, HIV-infected supernatants and cell lysates. Induction of NK cell function has been associated with protection against HIV acquisition within HESN populations of women[182]. Hence, the increased function of NK cells induced by Serpin B1 may offer protection against *in vivo* HIV infection and may contribute to the role of Serpin B1 within the FGT of HESN women. However, this function would have contributed minimally to the observed neutralizing effects in *in vitro* studies since NK cells were not present in pure cultures consisting of T cell and epithelial cell lines.

Another common function observed in both cell lysates and supernatants of Serpin B1 treated PBMCs was the presence of proteins associated with oxidative stress/redox response. There were also multiple proteins up-regulated within both supernatants and cell lysates that are related to cell migration[337] as well as the production of NO and ROS in macrophages. Thus, Serpin B1 may be inducing activation of macrophages resulting in the observed increase in proteins associated with oxidative stress present within Serpin B1 treated PBMC cultures. This induction of oxidative stress may account for the observed up-regulation of proteins associated with mitochondrial dysfunction within Serpin B1 treated cell lysates. Increased levels of

oxidative stress have been related to mitochondrial dysfunction in numerous studies, causing a disruption in membrane potential thereby significantly reducing, if not obliterating, the production of ATP[146]. A significant reduction in ATP production would result in reduced cellular energy and thus reduced non-essential cellular activity including genetic transcription, translation and division. These processes were identified as being associated with the up-regulated proteins of Serpin B1 treated cell lysates and supernatants.

An alternative explanation for the increase in ROS within Serpin B1 treated PBMC cultures may be attributed to the up-regulation of proteins associated with Granzyme A signaling, which was apparent in unstimulated and stimulated, HIV- infected cultures alike. Granzyme A is known to be an inducer of caspase-independent apoptosis[440]. Thus, activation of Granzyme A signaling may be the mechanism by which Serpin B1 induces, the previously described, caspase-independent apoptosis[441-443].

If the mitochondria are no longer functioning the cell will attempt to compensate by scavenging ATP from outside the cell as well as through the production of energy through glycolysis and the citric acid cycle, accounting for the increased expression of ATP-binding proteins identified following Serpin B1 treatment. It also may account for the increased expression of proteins associated with acetyl-coA biosynthesis, which were found to be up-regulated in Serpin B1 treated cultures. Acetyl-CoA enters into the citric acid cycle within the cytoplasm of cells to produce 3 NADH molecules, 1 $FADH_2$ and 1 GTP molecule (1 ATP)[444]. Additionally, the observed increase in proteins associated with carbohydrate metabolism, following Serpin B1 treatment of PBMCs, would also lend support to the concept that Serpin B1 forces the cells to scavenge for energy once an apoptotic-like state is induced. Breaking down carbohydrates to yield glucose will further fuel the glycolysis cycle, resulting in additional ATP production[444].

With an altered energy metabolism, cells treated with Serpin B1 may not succumb immediately to apoptosis but rather may be able to accumulate enough energy to maintain basic, necessary, cellular functions, thereby inducing an autophagic state rather than a one characterized by complete apoptosis[445]. Autophagy is induced by numerous cellular molecules including Annexin V[429,446] and thus, may explain the increased early apoptotic Annexin V markers on cells treated with Serpin B1. The presence of AMP-activated protein kinase (AMPK) also promotes Autophagy. AMPK is stimulated by the overabundance of AMP and IMP within the cytosol of a cell[430,447]. The presence of AMP deaminase 2 in the supernatants of Serpin B1 treated PBMCs would convert AMP to IMP[430]. AMP, coupled together with ROS, acts as an upstream regulator of AMPK. Increased levels of AMPK result in inhibition of the mTOR pathway and down-regulation of the anti-apoptotic proteins Bcl-2[430], which was previously determined to associate directly with Serpin B1/L-DNaseII resulting in a reduction of the pro-apoptotic nature of this endonuclease [329]. Therefore, down-regulation of Bcl-2 would limit the protection of the cell from apoptosis. Inhibition of the mTOR pathway also results in down-regulation of the EIF and p60S6K signalling pathways[144,430]. This was represented within Serpin B1 treated PBMC cultures, where EIF proteins were significantly down-regulated. Both EIF and p60S6K are highly associated with efficient protein translation[448-450] and thus, the reduced levels of proteins directly involved in these pathways may partially explain the observed decrease in cellular proliferation and reduced virus output *in vitro*. Hence, by down-regulating the mTOR pathway, there would be a direct reduction in the level of protein translation, which would cause reduced cellular replication/proliferation, as was observed following Serpin B1 treatment of cells in this study. Furthermore, mTOR inhibitors, including Sirolimus are currently in Phase I and II clinical trials to determine its safety in HIV-infected individuals on ARVs. Researchers expect that Sirolimus will decrease the level of inflammation and immune activation in the body, which will subsequently reduce the level of HIV in the blood [630,451,452]. Serpin B1

may be a potential candidate to add to the repertoire of agents capable of inducing such effects. The additional benefit that Serpin B1 would exhibit compared to agents such as Sirolimus is that Serpin B1 is a natural factor and as such would limit any increased immune activation caused by administering a foreign molecule/agent into the FGT.

9.6.2 Serpin B1 expression is correlated with expression of other Serpin B family members, which may contribute to its antiviral effects

Identification of additional antiproteases whose expression is correlated with Serpin B1 expression allowed for a more complete understanding of the physiological environment that is present when Serpin B1 is up-regulated *in vivo*. This study identified other members of the Serpin B family (Serpin B2, B3, B4, B12, B13) that strongly positively correlate with Serpin B1 within human cervicovaginal lavage from women within the Pumwani and Winnipeg cohorts. Elafin, SLPI and Cystatin A were also significantly positively correlated (Figure 54 and Table 12). Conversely, a high proportion of the non-Serpin B family members, as well as A2ML1 were inversely correlated with Serpin B1 both within the CVL of women as well as within Serpin B1 treated PBMC cultures (Figure 54, Tables 12).

To determine how the regulation of these factors may be controlled the chromosomal loci was examined. It was clear that four distinct loci are responsible for encoding all of the antiproteases up-regulated with Serpin B1. Specifically, chromosome loci 3q21.1, 6p25.2, 18q21.33 and 20q13.12 encode all of the positively correlated antiproteases, with Serpin B6 at the same locus as Serpin B1 (6p25.2) (Appendix, Table 16). All other Serpin B family members are encoded on chromosome 18q21.33 while both Elafin and SLPI are encoded on chromosome 20q13.12. Suggesting that these loci are regulated by similar transcriptional factors, which would explain the correlated expression of proteins encoded on all four of these loci. While limited studies have been performed on regulators of the activation of Serpin B family members, there has been extensive work done on Elafin and SLPI, which may be used to infer what is

causing these other genes to be up-regulated. There is a vast array of stimulators that regulate Elafin and SLPI, including progesterone (progesterone response element in SLPI promoter), IL-1β, TNF-α and neutrophil elastase[406,453]. Many of the Serpin B family members inhibit Elastase, which would suggest that its overabundance would trigger the expression of its regulators so as to protect surrounding tissues/cells. Furthermore, the membrane-associated progesterone receptor was identified as up-regulated within Serpin B1 treated cell lysates which may indicate that progesterone also, has a role in the stimulation of Serpin B1 expression as well as that of other antiproteases with correlated expression.

Specific antiproteases that have a correlated expression with Serpin B1 may contribute to the observed HIV inhibitory activity *in vitro*. The anti-apoptotic function of Serpin B2, B3 and B13 may counterbalance the apoptotic activity of Serpin B1, once cleaved into L-DNase II, thereby protecting bystander cells from damage and increased immune activation at the site of infection, thus providing further protection against HIV-1. The function of Serpin B4 in protection against NK cell cytotoxicity may also protect bystander cells from excessive NK cell stimulation by Serpin B1. While NK cell activation has been shown to be protective against the acquisition of HIV-1 during early acute infection, prolonged, uncontrolled activation would result in damage to tissue and bystander immune cells, which could theoretically enhance HIV infection. It, therefore, appears as though the antiproteases that are up-regulated along with Serpin B1 may offer improved protective functions against HIV-1 infection through control of immune activation caused by Serpin B1's induction of NK cell function and through prevention of complete apoptosis following induction of an autophagic state by Serpin B1.

Many of the antiproteases that were inversely correlated with Serpin B1 exhibit diverse functions and were located on a broad range of chromosomes, likely with unique promoters and stimuli. For instance, a human serpin gene cluster at 14q32.1 consists of 11 serpin genes and encodes for six distinct serpin antiproteases, namely Serpin A1, A2, A3, A5, A6 and A10[454].

Upstream of the Serpin A1 gene is a locus control region that is required for gene activation within this cluster[454,455]. It is therefore not surprising that all six of these antiproteases were found to be coordinately expressed. Also, many of the other antiproteases inversely correlated with Serpin B1, including Serpin C1, A8, D1, F1, F2 and G1, are all highly associated with regulation of the blood coagulation pathway and are likely regulated by anti-clotting factors such as heparin[282]. Since these antiproteases appear to have a much different function compared to many of the Serpin B family members that are up-regulated with Serpin B1, it may be that they are up-regulated at differing times in the infection - perhaps during tissue and wound repair following infection rather than actively during clearance of the infection.

9.6.3 Summary of Serpin B1's effect on cellular protein expression

Serpin B1 has demonstrated significant anti-HIV activity, the mechanism for which may be due to its ability to induce a pre-apoptotic state within target cells and subsequently reduce cellular proliferation. Evidence from this proteomic/pathway analysis provided evidence that Serpin B1 treatment was associated with the induction of proteins involved in ATP-binding, apoptosis, mitochondrial dysfunction, reduced transcription, reduced proliferation and ROS production. The oxidative stress caused by this ROS production may dysregulate the mitochondrial membrane resulting in reduction in ATP production and possibly induction of an autophagic state with reduced non-essential cellular processes. Many of the proteins that were up-regulated by Serpin B1, such as AMPD, EIF 4A-1, EIF 4A-II and H1.5, associate with the mTOR pathway. This pathway regulates multiple cellular pathways involved in cellular replication, specifically protein translation. Reduced translation and cellular replication could account for the decreased cellular proliferation observed in Serpin B1 treated cell cultures. Considering that an mTOR inhibitor (Sirolimus) is currently in phase II trials for effectiveness as a therapeutic agent against HIV, it is plausible to postulate that should Serpin B1 inhibit the

activity of the mTOR pathway, it may provide a successful means of inhibiting HIV within the context of the FGT.

9.6.4 Limitations and Future Directions

All of the discussion in this section is based on theoretical postulations formed through piecing together the individual functions of up-regulated proteins within Serpin B1 treated cell cultures. However, no further molecular studies were performed to determine: the extent to which mitochondria is affected; whether macrophages and NK cells are activated; whether the mTOR pathway is inhibited; or to determine the level of oxidative stress present in cells following Serpin B1 treatment. All of these studies would be imperative next steps to prove the current hypothesis for the mechanism employed by Serpin B1.

9.7 Major Findings & Contributions to the field of HIV mucosal immunology

The work in this thesis resulted in many novel findings. Firstly, I have demonstrated that the ability of CVL alone to inhibit HIV is not enough to define the protective HESN phenotype since CVL from HESN women, as a group, did not significantly inhibit HIV to a greater degree than that of HIV-susceptible or HIV-infected women. Furthermore, I demonstrated that the effects that an individual's CVL has on HIV is relatively stable over time. It was also demonstrated that the CVL from women at high exposure to HIV exhibit stronger HIV inhibitory activity compared to women in a low-exposure settings. Also, that majority of the women from these two different settings contain distinct proteomic profiles, more related to other women in their current geographic location than women in the other settings. These findings are important to understand the variability of host factors that affect HIV replication.

Serpin B1 was determined to be the antiprotease with the strongest capacity for inhibiting HIV among those that were up-regulated within the CVL of HESN women. This is the first study that has examined Serpin B1 in the context of HIV. While it does not appear as though

Serpin B1 directly affects any of the stages in the HIV replication cycle, it does seem to exhibit HIV inhibitory activity by reducing cellular proliferation and induction of early apoptotic markers. Serpin B1 was also shown to increase the expression of MIP-1α and MIP-1β, which have both been shown to down-regulate CCR5, thereby preventing efficient up-take of R5-tropic HIV by activated macrophages[456], potentially offering an additional mechanism of inhibition.

While the pro-apoptotic nature of Serpin B1 has been previously identified[329], determining the potential mechanism is novel. Studies in this thesis demonstrated individually that Serpin B1 decreases the replicative capacity of cells while simultaneously increasing the expression of early apoptotic markers (Annexin V). Furthermore, it appears as though Serpin B1 induces the expression of proteins that associate with the mTOR signaling pathway. Down-regulation of the mTOR pathway would result in the effects observed by Serpin B1 treatment including, reduced protein transcription, translation and cellular replication, thereby reducing the efficiency of HIV replication (Figure 58).

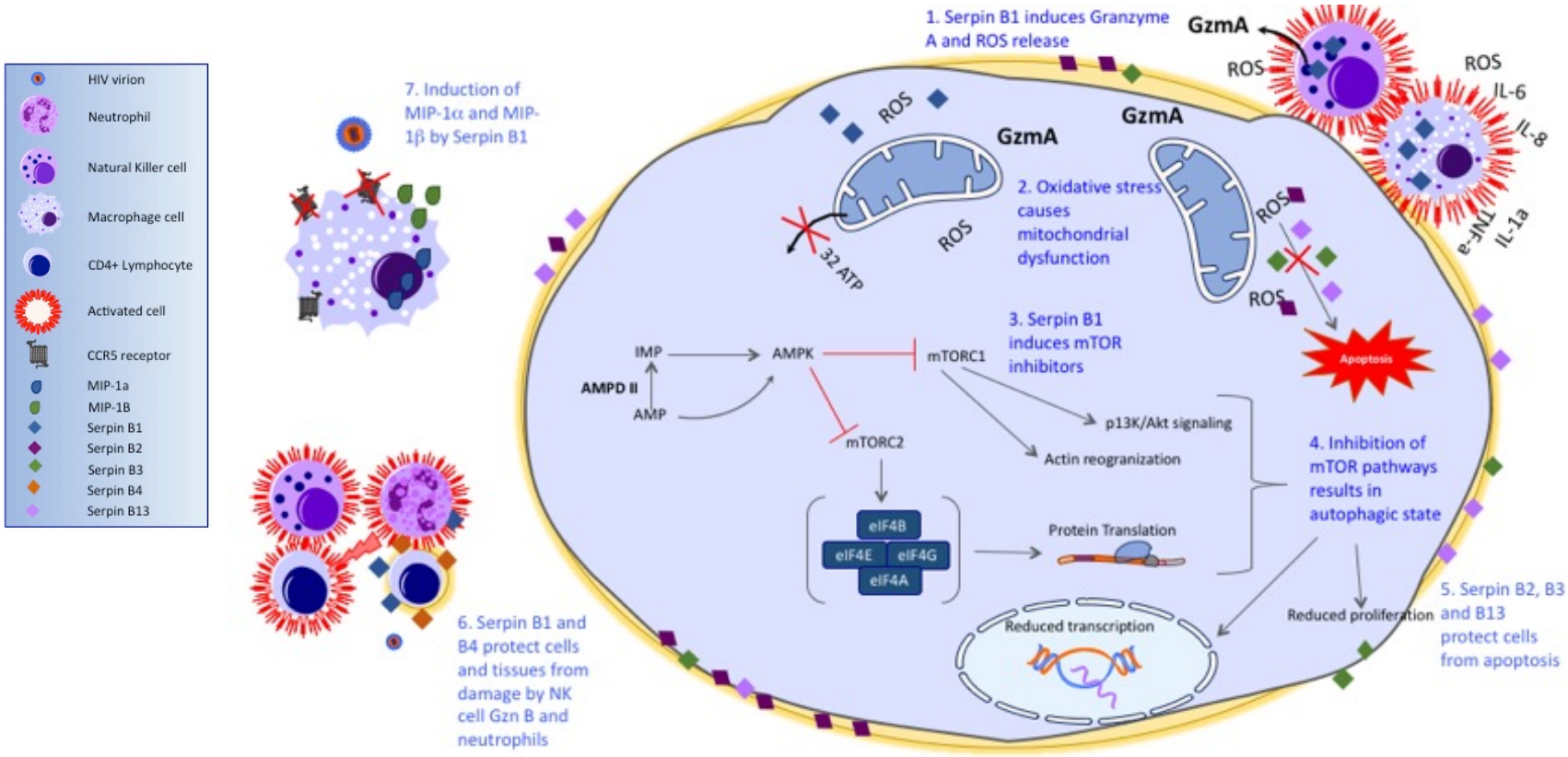


Figure 55: Potential mechanism employed by Serpin B1 in neutralizing HIV activity

1) Serpin B1 may stimulate macrophages to produce ROS and pro-inflamatory cytokines and stimulate NK cells to release Granzyme A resulting in additional ROS production. 2) Oxidative stress causes mitochondrial dysfunction. 3) Energy stress experienced by the cell following mitochondrial dysfunction causes an increase in cytoplasmic AMP, which is converted to IMP by AMPD. AMP and IMP activate AMPK, effectively inhibiting mTOR complex I/ II. 4) mTOR complex I regulates p13K/Akt signaling and actin reorganization, and mTOR complex II regulates the EIF signalling pathway which regulates protein translation. Inhibition of these complexes induces an autophagic state and subsequently reduced transcription and cellular proliferation thereby slowing the replication of of HIV-1. 5) Serpin B2, B3 and B13, may function to protect autophagic cells from complete apoptosis and 6) over production of cytotoxic granules (Granzyme B, Granzyme H) by NK cells and neutrophils. 7) Serpin B1 induces the production of MIP-1α and MIP-1β, which down-regulate CCR5, preventing efficient up-take of R5-tropic HIV by activated macrophage

9.8 Future Directions and Concluding Remarks

The work in this thesis has the potential to contribute to the design of future microbicides and other therapeutics for HIV. However, further studies must first be performed in order to confirm the observed results. All cellular mechanistic studies carried out in this thesis should be duplicated in the presence of an anti-Serpin B1 antibody to confirm that all observed effects are in fact caused by Serpin B1 and not an unknown factor present in the protein solution. In addition, the effect that Serpin B1 has on the translation of HIV proteins was not directly examined in this thesis but is an important component of the inhibitory mechanism that needs to be confirmed. This can be achieved through the detection and quantification of viral polyproteins through western blot analysis.

The experiments performed in this thesis should also be repeated in the presence of a combination of antiproteases, the expression of which positively correlated with that of Serpin B1. This will allow for determination of which antiproteases will work synergistically with Serpin B1 in inhibiting HIV.

Additionally, molecular studies involving micro-gene array, western-blotting and gene silencing techniques should be performed to confirm the effect that Serpin B1 has on the mTOR pathway. Specifically, the activity of mTOR could be detected by measuring direct levels of phosphorylation by P70S6 via Western Blotting and cellular knockdown of P70S6[457]. An alternative place in the mTOR pathway that may be affected, as was supported by the data in this thesis, is the up-regulation of AMPK activity. This kinase phosphorylates Ulk1, which promotes the development of autophagy[430], while mTOR inhibits the activation of Ulk1, thereby inhibiting the development of autophagy[430]. Therefore, detecting the level of phosphorylation of Ulk1, through Western Blotting, following treatment with Serpin B1, would provide insight into the activity levels of both AMPK and mTOR as well as the potential for an autophagic state within the treated cells. Direct analysis of autophagy can also be achieved by performing specific assays that detect levels of processed LC3B-II, which is a cellular readout for autophagy[458].

It is also important to identify the degree of apoptosis induced by Serpin B1. A simple method to determine if the majority of cells exposed to Serpin B1 succumb to complete apoptosis is to perform a longer experiment (10-14 days) identical to the six-day assay that was conducted in this thesis. Staining, again, with Annexin V and 7-AAD or live/dead stain would allow for the determination of whether the cells that only express the early apoptotic marker, Annexin V, after six days will undergo complete apoptosis in the days that follow, compared to PBS treated controls.

Deciphering the exact mechanism by which Serpin B1 induces apoptotic markers is also important. It is possible, due to the results in this thesis, that the induction of Granzyme A activity is a major factor in the observed increase in apoptotic markers. However, determining whether this is the case is challenging since Granzyme A does not induce caspase expression or cause large enough breaks in the DNA, which would be detectable via agarose gel. Literature also suggests that the DNA fragments, created by Granzyme A activity, cannot be labeled with deoxynucleotidyl transferase and are too large to be released from dying cells[440]. However, the nicked DNA, caused by Granzyme A, can be observed using alkaline agarose gels, which are formulated to denature DNA into its constituent strands followed by labeling with Klenow fragment of DNA polymerase I[440]. Thus, this would be the most optimal method for detecting whether any damage has been done to the DNA and if so whether that damage was due to increased Granzyme A activity.

Although previous papers, as well as the work in this thesis, suggest that the pro-apoptotic nature of Serpin B1 is caspase-independent, it would also be beneficial to test this definitively. The most efficient way to examine this would be through flow cytometry staining of caspase-3 and -7 as well as PARP, which is a cleavage product of these two caspases and is considered a classical characteristic of active apoptosis[459,460].

Furthermore, the effect of Serpin B1 on HIV must be examined within an animal model in order to determine the true effect within an *in vivo* system. This will allow for confirmation

that the observed inhibitory effects *in vitro* are not over-shadowed by other cellular processes when in an animal model. This will also demonstrate the impact that exogenous Serpin B1 has on the tissue of the FGT when present for various amounts of time. Thus, providing information on the optimal timing of delivery of Serpin B1 in order to be effective against HIV, while limiting the amount of tissue and bystander cell death/damage, which will determine if this antiprotease would truly make an effective candidate for a microbicide.

Continuing to identify mucosally derived protective factors within the FGT will provide novel candidates for use in microbicides, which is currently one of the most promising options for HIV prevention in women. It is imperative that we continue to identify natural-based measures that will limit the amount of immune activation within the FGT while also providing the necessary protection against HIV. It is naïve to think that one factor alone will provide adequate inhibitory activity against HIV so as to protect every woman while simultaneously protecting against secondary infections and pregnancy. However, the only way for a preventative measure to be accepted by the women, that it is designed to protect, is to ensure that all of these criteria are met. Thus, finding a factor capable of inhibiting HIV is only the first step, extensive studies must also be performed to determine which additional factors will be employed within the Multipurpose Preventative Technology (MPT) to ensure all reproductive health needs are being met. With women accounting for over 60% of the new infections every year[5], I believe that continued research into safe and effective vaginal MPTs that protect against HIV as well as other STIs should be a top priority for the HIV mucosal immunology research field. I am proud and honoured to have been granted the opportunity to play a small role towards this end.

CHAPTER 10: REFERENCES:

1. Montagnier, L. *Virus*. (2008).
2. Fauci, A. S. HIV and AIDS: 20 years of science. *Nat. Med.* **9,** 839–843 (2003).
3. Sharp, P. M. & Hahn, B. H. Origins of HIV and the AIDS pandemic. *Cold Spring Harb Perspect Med* **1,** a006841 (2011).
4. Hirsch, V. M., Olmsted, R. A., Murphey-Corb, M., Purcell, R. H. & Johnson, P. R. An African primate lentivirus (SIVsm) closely related to HIV-2. *Nature* **339,** 389–392 (1989).
5. UNAIDS, U. N. P. O. H. A. *The gap report*. (Geneva: UNAIDS, 2014).
6. WHO, U.Unicef. *Global report: UNAIDS report on the global AIDS epidemic 2013*. (Geneva: UNAIDS, 2013).
7. Sidibé, M. GLOBAL REPORT (UNAIDS REPORT ON THE GLOBAL AIDS EPIDEMIC| 2010). (2013).
8. Morocco, K. Tackling HIV/AIDS Among Key Populations: Essential to Achieving an AIDS-Free Generation. (2013).
9. Musyoki, H. *et al.* Prevalence of HIV, sexually transmitted infections, and risk behaviours among female sex workers in Nairobi, Kenya: results of a respondent driven sampling study. *AIDS Behav* **19 Suppl 1,** 58 (2015).
10. Tun, W. *et al.* HIV and STI prevalence and injection behaviors among people who inject drugs in Nairobi: results from a 2011 bio-behavioral study using respondent-driven sampling. *AIDS Behav* **19 Suppl 1,** S24–35 (2015).
11. Ramjee, G. & Daniels, B. Women and HIV in Sub-Saharan Africa. *AIDS Research and Therapy 2013 10:1* **10,** 1 (2013).
12. Schwandt, M., Morris, C., Ferguson, A., Ngugi, E. & Moses, S. Anal and dry sex in commercial sex work, and relation to risk for sexually transmitted infections and HIV in Meru, Kenya. *Sex Transm Infect* **82,** 392–396 (2006).
13. Behets, F. M. T. F. *et al.* Evidence-based treatment guidelines for sexually transmitted infections developed with and for female sex workers. *Tropical Medicine & International Health* **8,** 251–258 (2003).
14. Hall, H. I. *et al.* Epidemiology of HIV in the United States and Canada: Current Status and Ongoing Challenges. *JAIDS Journal of Acquired Immune Deficiency Syndromes* **51,** S13–S20 (2009).
15. Hladik, F. & Hope, T. J. HIV infection of the genital mucosa in women. *Curr HIV/AIDS Rep* **6,** 20–28 (2009).
16. WHO, U. *UNAIDS. Global update on HIV treatment 2013: results, impact and opportunities*. (Geneva, 2013).
17. De Leys, R. *et al.* Isolation and partial characterization of an unusual human immunodeficiency retrovirus from two persons of west-central African origin. *J. Virol.* **64,** 1207–1216 (1990).
18. Mauclère, P. *et al.* Serological and virological characterization of HIV-1 group O infection in Cameroon. *AIDS* **11,** 445 (1997).
19. Vallari, A. *et al.* Confirmation of putative HIV-1 group P in Cameroon. *J. Virol.* **85,** 1407 (2011).
20. Baeten, J. M. *et al.* HIV-1 subtype D infection is associated with faster disease progression than subtype A in spite of similar plasma HIV-1 loads. *J. Infect. Dis.*

195, 1180 (2007).
21. Renjifo, B. *et al.* Preferential in-utero transmission of HIV-1 subtype C as compared to HIV-1 subtype A or D. *AIDS* **18,** 1629–1636 (2004).
22. John-Stewart, G. C. *et al.* Subtype C Is associated with increased vaginal shedding of HIV-1. *J. Infect. Dis.* **192,** 496 (2005).
23. Abecasis, A. B. *et al.* HIV-1 subtype distribution and its demographic determinants in newly diagnosed patients in Europe suggest highly compartmentalized epidemics. *Retrovirology* **10,** 7 (2013).
24. Hemelaar, J., Gouws, E., Ghys, P. D. & Osmanov, S. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. *AIDS* **20,** W13–23 (2006).
25. van der Loeff, M. F. S. *et al.* Sixteen years of HIV surveillance in a West African research clinic reveals divergent epidemic trends of HIV-1 and HIV-2. *Int J Epidemiol* **35,** 1322–1328 (2006).
26. Rowland-Jones, S. L. & Whittle, H. C. Out of Africa: what can we learn from HIV-2 about protective immunity to HIV-1? *Nat. Immunol.* **8,** 329 (2007).
27. Turner, B. G. & Summers, M. F. Structural biology of HIV. *J. Mol. Biol.* **285,** 32 (1999).
28. Sainski, A. M., Cummins, N. W. & Badley, A. D. HIV Life Cycle. *antimicrobe.org*
29. Didigu, C. A. & Doms, R. W. Novel Approaches to Inhibit HIV Entry. *Viruses 2012, Vol. 4, Pages 309-324* **4,** 324 (2012).
30. Loftin, L. M. Regulation of CCR5 use on primary CD4+ lymphocytes by R5X4 HIV-1. (2010).
31. Berlier, W. *et al.* Selective sequestration of X4 isolates by human genital epithelial cells: Implication for virus tropism selection process during sexual transmission of HIV. *J. Med. Virol.* **77,** 465 (2005).
32. Siliciano, R. F. & Greene, W. C. HIV latency. *Cold Spring Harb Perspect Med* **1,** a007096 (2011).
33. Karn, J. The molecular biology of HIV latency: breaking and restoring the Tat-dependent transcriptional circuit. *Curr Opin HIV AIDS* **6,** 4–11 (2011).
34. Karn, J. & Stoltzfus, C. M. Transcriptional and posttranscriptional regulation of HIV-1 gene expression. *Cold Spring Harb Perspect Med* **2,** a006916 (2012).
35. Berkhout, B., Silverman, R. H. & Jeang, K. T. Tat trans-activates the human immunodeficiency virus through a nascent RNA target. *Cell* **59,** 273–282 (1989).
36. Berkhout, B. & Jeang, K. T. trans activation of human immunodeficiency virus type 1 is sequence specific for both the single-stranded bulge and loop of the trans-acting-responsive hairpin: a quantitative analysis. *J. Virol.* **63,** 5501–5504 (1989).
37. Mancebo, H. S. *et al.* P-TEFb kinase is required for HIV Tat transcriptional activation in vivo and in vitro. *Genes Dev.* **11,** 2633–2644 (1997).
38. Wong, R. W., Balachandran, A., Ostrowski, M. A. & Cochrane, A. Digoxin suppresses HIV-1 replication by altering viral RNA processing. *PLoS Pathog.* **9,** e1003241 (2013).
39. McLaren, M., Marsh, K. & Cochrane, A. Modulating HIV-1 RNA processing and utilization. *Front. Biosci.* **13,** 5693–5707 (2008).
40. Stoltzfus, C. M. Chapter 1. Regulation of HIV-1 alternative RNA splicing and its

role in virus replication. *Adv. Virus Res.* **74,** 1–40 (2009).
41. Cullen, B. R. Nuclear mRNA export: insights from virology. *Trends Biochem. Sci.* **28,** 419 (2003).
42. de Breyne, S., Soto-Rifo, R., López-Lastra, M. & Ohlmann, T. Translation initiation is driven by different mechanisms on the HIV-1 and HIV-2 genomic RNAs. *Virus Res.* **171,** 366–381 (2013).
43. VerPlank, L. *et al.* Tsg101, a homologue of ubiquitin-conjugating (E2) enzymes, binds the L domain in HIV type 1 Pr55(Gag). *Proc. Natl. Acad. Sci. U.S.A.* **98,** 7724–7729 (2001).
44. Sette, P., Jadwin, J. A., Dussupt, V., Bello, N. F. & Bouamr, F. The ESCRT-associated protein Alix recruits the ubiquitin ligase Nedd4-1 to facilitate HIV-1 release through the LYPXnL L domain motif. *J. Virol.* **84,** 8181–8192 (2010).
45. Briggs, J. A. G. *et al.* The mechanism of HIV-1 core assembly: insights from three-dimensional reconstructions of authentic virions. *Structure* **14,** 15–20 (2006).
46. Royce, R. A., Seña, A., Cates, W. & Cohen, M. S. Sexual transmission of HIV. *N. Engl. J. Med.* **336,** 1072–1078 (1997).
47. Tebit, D. M., Ndembi, N., Weinberg, A. & Quiñones-Mateu, M. E. Mucosal transmission of human immunodeficiency virus. *Curr. HIV Res.* **10,** 3–8 (2012).
48. Ariën, K. K., Jespers, V. & Vanham, G. HIV sexual transmission and microbicides. *Rev. Med. Virol.* **21,** 110–133 (2011).
49. Boily, M.-C. *et al.* Heterosexual risk of HIV-1 infection per sexual act: systematic review and meta-analysis of observational studies. *Lancet Infect Dis* **9,** 129 (2009).
50. Boily, M.-C., Baggaley, R. F. & Masse, B. The role of heterosexual anal intercourse for HIV transmission in developing countries: are we ready to draw conclusions? *Sex Transm Infect* **85,** 408–410 (2009).
51. Keele, B. F. *et al.* Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 7557 (2008).
52. Tindall, B. & Cooper, D. A. Primary HIV infection: host responses and intervention strategies. *AIDS* **5,** 1 (1991).
53. Pantaleo, G., Graziosi, C. & Fauci, A. S. New concepts in the immunopathogenesis of human immunodeficiency virus infection. *N. Engl. J. Med.* **328,** 327 (1993).
54. Levy, J. A. HIV pathogenesis: 25 years of progress and persistent challenges. *AIDS* **23,** 147–160 (2009).
55. Brenchley, J. M. *et al.* Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat. Med.* **12,** 1371 (2006).
56. McCune, J. M. The dynamics of CD4+ T-cell depletion in HIV disease. *Nature* **410,** 974–979 (2001).
57. Grossman, Z., Meier-Schellersheim, M., Paul, W. E. & Picker, L. J. Pathogenesis of HIV infection: what the virus spares is as important as what it destroys. *Nat. Med.* **12,** 289–295 (2006).
58. Nguyen, P. V., Kafka, J. K., Ferreira, V. H., Roth, K. & Kaushic, C. Innate and adaptive immune responses in male and female reproductive tracts in homeostasis and following HIV infection. *Cell. Mol. Immunol.* **11,** 410–427 (2014).
59. Rafferty, H., Sibeko, S. & Rowland-Jones, S. How can we design better vaccines to prevent HIV infection in women? *Front Microbiol* **5,** 572 (2014).

60. Marieb, E. N. & Hoehn, K. *Human Anatomy & Physiology Plus Masteringa&p with Etext Package and 80 Readings for Composition*. (Pearson College Division, 2013).
61. Blaskewicz, C. D., Pudney, J. & Anderson, D. J. Structure and function of intercellular junctions in human cervical and vaginal mucosal epithelia. *Biol. Reprod.* **85,** 104 (2011).
62. Anderson, D. J., Marathe, J. & Pudney, J. The structure of the human vaginal stratum corneum and its role in immune defense. *American Journal of Reproductive Immunology* **71,** 618–623 (2014).
63. Southern, P. J., Horbul, J. E., Miller, B. R. L. & Maher, D. M. Coming of age: reconstruction of heterosexual HIV-1 transmission in human ex vivo organ culture systems. *Mucosal Immunol* **4,** 383–396 (2011).
64. Cohen, M. S. Genitourinary mucosal defenses. *Sexually transmitted diseases* (1999).
65. Huggins, G. R. & Preti, G. Vaginal odors and secretions. *Clin Obstet Gynecol* **24,** 355–377 (1981).
66. Cole, A. M. & Cole, A. L. Antimicrobial polypeptides are key anti-HIV-1 effector molecules of cervicovaginal host defense. **59,** 34 (2008).
67. Cole, A. M. in *Antimicrobial Peptides and Human Disease* **306,** 199–230 (Springer Berlin Heidelberg, 2006).
68. Wira, C. R., Fahey, J. V., Rodriguez-Garcia, M., Shen, Z. & Patel, M. V. Regulation of Mucosal Immunity in the Female Reproductive Tract: The Role of Sex Hormones in Immune Protection Against Sexually Transmitted Pathogens. *American Journal of Reproductive Immunology* **72,** 236–258 (2014).
69. Petrova, M. I., Lievens, E., Malik, S., Imholz, N. & Lebeer, S. Lactobacillus species as biomarkers and agents that can promote various aspects of vaginal health. *Front Physiol* **6,** 81 (2015).
70. Martin, H. L. *et al.* Vaginal lactobacilli, microbial flora, and risk of human immunodeficiency virus type 1 and sexually transmitted disease acquisition. *J. Infect. Dis.* **180,** 1863–1868 (1999).
71. Jones, R. E. & Lopez, K. H. *Human Reproductive Biology*. (Academic Press, 2013).
72. Telzak, E. E. *et al.* HIV-1 seroconversion in patients with and without genital ulcer disease. A prospective study. *Ann. Intern. Med.* **119,** 1181–1186 (1993).
73. Shen, R., Richter, H. E. & Smith, P. D. Interactions between HIV-1 and mucosal cells in the female reproductive tract. *American Journal of Reproductive Immunology* **71,** 608–617 (2014).
74. M.D, P. D. S., MacDonald, T. T., Immunology, S. F. M. & Blumberg, R. S. *Principles of Mucosal Immunology*. (Garland Pub, 2012).
75. Stoddard, E. *et al.* gp340 promotes transcytosis of human immunodeficiency virus type 1 in genital tract-derived cell lines and primary endocervical tissue. *J. Virol.* **83,** 8596–8603 (2009).
76. Gorodeski, G. I. *Endocrinology* (2007). doi:10.1210/en.2006-1120
77. Gorodeski, G. I. Estrogen modulation of epithelial permeability in cervical-vaginal cells of premenopausal and postmenopausal women. *Menopause* **14,** 1012–1019 (2007).
78. Wira, C. R., Rodriguez-Garcia, M. & Patel, M. V. The role of sex hormones in immune protection of the female reproductive tract. *Nat. Rev. Immunol.* **15,** 217–230 (2015).

79. Charles R Wira, J. V. F. A new strategy to understand how HIV infects women: identification of a window of vulnerability during the menstrual cycle. *AIDS* **22,** 1909–1917 (2008).
80. Morris, M. R. *et al.* Relationship of menstrual cycle and vaginal infection in female rhesus macaques challenged with repeated, low doses of SIVmac251. *J. Med. Primatol.* n/a–n/a (2015). doi:10.1111/jmp.12177
81. Kersh, E. N. *et al.* SHIV susceptibility changes during the menstrual cycle of pigtail macaques. *J. Med. Primatol.* **43,** 310–316 (2014).
82. Sodora, D. L., Gettie, A., Miller, C. J. & Marx, P. A. Vaginal transmission of SIV: assessing infectivity and hormonal influences in macaques inoculated with cell-free and cell-associated viral stocks. *AIDS Res. Hum. Retroviruses* **14 Suppl 1,** S119–23 (1998).
83. Ferreira, V. H. *et al.* Medroxyprogesterone Acetate Regulates HIV-1 Uptake and Transcytosis but Not Replication in Primary Genital Epithelial Cells, Resulting in Enhanced T-Cell Infection. *J. Infect. Dis.* jiu832 (2014). doi:10.1093/infdis/jiu832
84. Wira, C. R., Fahey, J. V., Rodriguez-Garcia, M., Shen, Z. & Patel, M. V. Regulation of mucosal immunity in the female reproductive tract: the role of sex hormones in immune protection against sexually transmitted pathogens. *American Journal of Reproductive Immunology* **72,** 236–258 (2014).
85. Wira, C. R., Rodriguez-Garcia, M., Shen, Z., Patel, M. & Fahey, J. V. The role of sex hormones and the tissue environment in immune protection against HIV in the female reproductive tract. *American Journal of Reproductive Immunology* **72,** 171–181 (2014).
86. Patel, M. V., Fahey, J. V., Rossoll, R. M. & Wira, C. R. Innate immunity in the vagina (part I): estradiol inhibits HBD2 and elafin secretion by human vaginal epithelial cells. *American Journal of Reproductive Immunology* **69,** 463–474 (2013).
87. Han, J. H. *et al.* Modulation of human beta-defensin-2 expression by 17beta-estradiol and progesterone in vaginal epithelial cells. *Cytokine* **49,** 209–214 (2010).
88. Yeaman, G. R. *et al.* Unique CD8+ T cell-rich lymphoid aggregates in human uterine endometrium. *J. Leukoc. Biol.* **61,** 427–435 (1997).
89. Mackay, I. R., Rosen, F. S., Medzhitov, R. & Janeway, C., Jr. Innate Immunity. *N. Engl. J. Med.* **343,** 338–344 (2000).
90. Abbas, A. K. & Lichtman, A. H. H. *Basic Immunology Updated Edition*. (2010).
91. Pauza, C. D., Poonia, B., Li, H., Cairo, C. & Chaudhry, S. γδ T Cells in HIV Disease: Past, Present, and Future. *Front Immunol* **5,** (2014).
92. Schwarzenberger, P. *et al.* Requirement of endogenous stem cell factor and granulocyte-colony-stimulating factor for IL-17-mediated granulopoiesis. *J. Immunol.* **164,** 4783–4789 (2000).
93. Borregaard, N. Neutrophils, from marrow to microbes. *Immunity* **33,** 657–670 (2010).
94. Theilgaard-Mönch, K. *et al.* Haptoglobin is synthesized during granulocyte differentiation, stored in specific granules, and released by neutrophils in response to activation. *Blood* **108,** 353 (2006).
95. Theilgaard-Mönch, K. *et al.* Highly glycosylated alpha1-acid glycoprotein is synthesized in myelocytes, stored in secondary granules, and released by activated neutrophils. *J. Leukoc. Biol.* **78,** 462–470 (2005).

96. Johansson, B. *et al.* Alpha-1-antitrypsin is present in the specific granules of human eosinophilic granulocytes. *Clin. Exp. Allergy* **31,** 379–386 (2001).
97. Borregaard, N. & Cowland, J. B. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* **89,** 3503–3521 (1997).
98. Theilgaard-Mönch, K., Knudsen, S., Follin, P. & Borregaard, N. The transcriptional activation program of human neutrophils in skin lesions supports their important role in wound healing. *J. Immunol.* **172,** 7684–7693 (2004).
99. Smale, S. T. Transcriptional regulation in the innate immune system. *Curr. Opin. Immunol.* **24,** 51–57 (2012).
100. Theilgaard-Mönch, K. *et al.* The transcriptional program of terminal granulocytic differentiation. *Blood* **105,** 1785–1796 (2005).
101. Auffray, C. *et al.* Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science* **317,** 666 (2007).
102. Bain, C. C. & Mowat, A. M. Macrophages in intestinal homeostasis and inflammation. *Immunol. Rev.* **260,** 102 (2014).
103. Erwig, L.-P. & Henson, P. M. Clearance of apoptotic cells by phagocytes. *Cell Death Differ.* **15,** 243–250 (2008).
104. Erwig, L.-P. & Henson, P. M. Immunological consequences of apoptotic cell phagocytosis. *Am. J. Pathol.* **171,** 2–8 (2007).
105. Kono, H. & Rock, K. L. How dying cells alert the immune system to danger. *Nat. Rev. Immunol.* **8,** 289 (2008).
106. Mosser, D. M. & Edwards, J. P. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* **8,** 969 (2008).
107. Grainger, J. R. *et al.* Inflammatory monocytes regulate pathologic responses to commensals during acute gastrointestinal infection. *Nat. Med.* **19,** 713–721 (2013).
108. Mackaness, G. B. Cellular immunity and the parasite. *Adv. Exp. Med. Biol.* **93,** 65–73 (1977).
109. O'Shea, J. J. & Murray, P. J. Cytokine signaling modules in inflammatory responses. *Immunity* **28,** 487 (2008).
110. Cavaillon, J. M. Cytokines and macrophages. *Biomed. Pharmacother.* **48,** 445–453 (1994).
111. Mosser, D. M. The many faces of macrophage activation. *J. Leukoc. Biol.* **73,** 209–212 (2003).
112. Mantovani, A. *et al.* The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* **25,** 677–686 (2004).
113. Gordon, S. Alternative activation of macrophages. *Nat. Rev. Immunol.* **3,** 23–35 (2003).
114. Kadowaki, N. *et al.* Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J. Exp. Med.* **194,** 863–869 (2001).
115. Akira, S. Mammalian Toll-like receptors. *Curr. Opin. Immunol.* **15,** 5–11 (2003).
116. Reis e Sousa, C. Activation of dendritic cells: translating innate into adaptive immunity. *Curr. Opin. Immunol.* **16,** 21 (2004).
117. Reis e Sousa, C. Toll-like receptors and dendritic cells: for whom the bug tolls. *Seminars in Immunology* **16,** 27 (2004).
118. Alvarez, D., Vollmann, E. H. & Andrian, von, U. H. Mechanisms and consequences

of dendritic cell migration. *Immunity* **29,** 342 (2008).
119. Barreira da Silva, R. & Münz, C. Natural killer cell activation by dendritic cells: balancing inhibitory and activating signals. *Cell. Mol. Life Sci.* **68,** 3505–3518 (2011).
120. Krummen, M. *et al.* Release of IL-12 by dendritic cells activated by TLR ligation is dependent on MyD88 signaling, whereas TRIF signaling is indispensable for TLR synergy. *J. Leukoc. Biol.* **88,** 189–199 (2010).
121. Warger, T. *et al.* Synergistic activation of dendritic cells by combined Toll-like receptor ligation induces superior CTL responses in vivo. *Blood* **108,** 544–550 (2006).
122. Takeuchi, O. & Akira, S. Pattern Recognition Receptors and Inflammation. *Cell* **140,** 805–820 (2010).
123. Interferon-alpha and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets. **195,** 1507–1512 (2002).
124. Muzio, M. *et al.* Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J. Immunol.* **164,** 5998–6004 (2000).
125. Visintin, A. *et al.* Regulation of Toll-like receptors in human monocytes and dendritic cells. *J. Immunol.* **166,** 249–255 (2001).
126. Luo, G., Niesel, D. W., Shaban, R. A., Grimm, E. A. & Klimpel, G. R. Tumor necrosis factor alpha binding to bacteria: evidence for a high-affinity receptor and alteration of bacterial virulence properties. *Infect. Immun.* **61,** 830–835 (1993).
127. Gerosa, F. *et al.* The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions. *J. Immunol.* **174,** 727–734 (2005).
128. Nguyen, K. B. *et al.* Coordinated and distinct roles for IFN-alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. *J. Immunol.* **169,** 4279–4287 (2002).
129. Flacher, V. *et al.* Human Langerhans cells express a specific TLR profile and differentially respond to viruses and Gram-positive bacteria. *J. Immunol.* **177,** 7959–7967 (2006).
130. Serbina, N. V. & Pamer, E. G. Immunology. Giving credit where credit is due. *Science* **301,** 1857 (2003).
131. Di Santo, J. P. Natural killer cell developmental pathways: a question of balance. *Annu. Rev. Immunol.* **24,** 257–286 (2006).
132. Lanier, L. L., Le, A. M., Civin, C. I., Loken, M. R. & Phillips, J. H. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J. Immunol.* **136,** 4480–4486 (1986).
133. Campbell, J. J. *et al.* Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. *J. Immunol.* **166,** 6477–6482 (2001).
134. Jacobs, R. *et al.* CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. *Eur. J. Immunol.* **31,** 3121–3127 (2001).
135. Moretta, A. *et al.* Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu. Rev. Immunol.* **19,** 223 (2001).
136. Moretta, L., Bottino, C., Cantoni, C., Mingari, M. C. & Moretta, A. Human natural

killer cell function and receptors. *Curr Opin Pharmacol* **1,** 387–391 (2001).
137. Lanier, L. L. NK cell recognition. *Annu. Rev. Immunol.* **23,** 274 (2005).
138. Mandelboim, O. *et al.* Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* **409,** 1055–1060 (2001).
139. Arnon, T. I. *et al.* Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur. J. Immunol.* **31,** 2680 (2001).
140. Long, E. O., Kim, H. S., Liu, D., Peterson, M. E. & Rajagopalan, S. Controlling NK Cell Responses: Integration of Signals for Activation and Inhibition. *Annu. Rev. Immunol.* **31,** 227–258 (2013).
141. Wira, C. R., Fahey, J. V., Sentman, C. L., Pioli, P. A. & Shen, L. Innate and adaptive immunity in female genital tract: cellular responses and interactions. *Immunol. Rev.* **206,** 306–335 (2005).
142. Abbas, A. K., Lichtman, A. H. H. & Pillai, S. *Cellular and Molecular Immunology*. (Elsevier Health Sciences, 2014).
143. Duprez, L., Wirawan, E., Berghe, T. V. & Vandenabeele, P. Major cell death pathways at a glance. *Microbes and Infection* **11,** 1050–1062 (2009).
144. Sangiuliano, B. *et al.* Cell Death-Associated Molecular-Pattern Molecules: Inflammatory Signaling and Control. *Mediators of Inflammation* **2014,** 1–14 (2014).
145. Kaczmarek, A., Vandenabeele, P. & Krysko, D. V. Necroptosis: The Release of Damage-Associated Molecular Patterns and Its Physiological Relevance. *Immunity* **38,** 209–223 (2013).
146. Sesso, A. *et al.* Mitochondrial Swelling and Incipient Outer Membrane Rupture in Preapoptotic and Apoptotic Cells. *The Anatomical Record* **295,** 1647–1659 (2012).
147. Youle, R. J. & Strasser, A. The BCL-2 protein family: opposing activities that mediate cell death. *Nature Reviews Molecular Cell Biology* **9,** 47–59 (2008).
148. Krysko, D. V. Cell death: initiation, propagation and clearance. *... of Anatomy* (2006).
149. Krysko, D. V., D'Herde, K. & Vandenabeele, P. Clearance of apoptotic and necrotic cells and its immunological consequences. **11,** 1709–1726 (2006).
150. King, A. E., Horne, A. W., Hombach-Klonisch, S., Mason, J. I. & Critchley, H. O. D. Differential expression and regulation of nuclear oligomerization domain proteins NOD1 and NOD2 in human endometrium: a potential role in innate immune protection and menstruation. *Mol. Hum. Reprod.* **15,** 311–319 (2009).
151. Schaefer, T. M., Fahey, J. V., Wright, J. A. & Wira, C. R. Innate immunity in the human female reproductive tract: antiviral response of uterine epithelial cells to the TLR3 agonist poly(I:C). *J. Immunol.* **174,** 992–1002 (2005).
152. Schaefer, T. M., Desouza, K., Fahey, J. V., Beagley, K. W. & Wira, C. R. Toll-like receptor (TLR) expression and TLR-mediated cytokine/chemokine production by human uterine epithelial cells. *Immunology* **112,** 428–436 (2004).
153. Pioli, P. A. *et al.* Differential expression of Toll-like receptors 2 and 4 in tissues of the human female reproductive tract. *Infect. Immun.* **72,** 5799–5806 (2004).
154. Nazli, A. *et al.* Differential induction of innate anti-viral responses by TLR ligands against Herpes simplex virus, type 2, infection in primary genital epithelium of women. *Antiviral Res.* **81,** 103 (2009).
155. Ferreira, V. H. *et al.* Endometrial epithelial cell responses to coinfecting viral and bacterial pathogens in the genital tract can activate the HIV-1 LTR in an

NF{kappa}B-and AP-1-dependent manner. *J. Infect. Dis.* **204,** 308 (2011).
156. Harwani, S. C., Lurain, N. S., Zariffard, M. R. & Spear, G. T. Differential inhibition of human cytomegalovirus (HCMV) by toll-like receptor ligands mediated by interferon-beta in human foreskin fibroblasts and cervical …. *Virol J* (2007).
157. Pudney, J., Quayle, A. J. & Anderson, D. J. Immunological microenvironments in the human vagina and cervix: mediators of cellular immunity are concentrated in the cervical transformation zone. *Biol. Reprod.* **73,** 1253–1263 (2005).
158. Trifonova, R. T., Lieberman, J. & Baarle, D. Distribution of Immune Cells in the Human Cervix and Implications for HIV Transmission. *American Journal of Reproductive Immunology* **71,** 252–264 (2014).
159. Givan, A. L. *et al.* Flow Cytometric Analysis of Leukocytes in the Human Female Reproductive Tract: Comparison of Fallopian Tube, Uterus, Cervix, and Vagina. *American Journal of Reproductive Immunology* **38,** 350–359 (1997).
160. P M Henson, R. B. J. J. Tissue injury in inflammation. Oxidants, proteinases, and cationic proteins. *Journal of Clinical Investigation* **79,** 669–674 (1987).
161. The dual roles of neutrophils and macrophages in inflammation: a critical balance between tissue damage and repair. (2006).
162. Anne Croy, B., Van Den Heuvel, M. J., Borzychowski, A. M. & Tayade, C. Uterine natural killer cells: a specialized differentiation regulated by ovarian hormones. *Immunol. Rev.* **214,** 161–185 (2006).
163. Koopman, L. A. *et al.* Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J. Exp. Med.* **198,** 1201–1212 (2003).
164. Shanmugasundaram, U. *et al.* Phenotype and Functionality of CD4+ and CD8+ T Cells in the Upper Reproductive Tract of Healthy Premenopausal Women. *American Journal of Reproductive Immunology* **71,** 95–108 (2014).
165. Russell, M. W. & Mestecky, J. Humoral immune responses to microbial infections in the genital tract. *Microbes and Infection* **4,** 667–677 (2002).
166. Kutteh, W. H., Moldoveanu, Z. & Mestecky, J. Mucosal immunity in the female reproductive tract: correlation of immunoglobulins, cytokines, and reproductive hormones in human cervical mucus around the time of ovulation. *AIDS Res. Hum. Retroviruses* **14 Suppl 1,** S51–5 (1998).
167. Firoz Mian, M. & Ashkar, A. A. Induction of Innate Immune Responses in the Female Genital Tract: Friend or Foe of HIV-1 Infection? *American Journal of Reproductive Immunology* **65,** 344 (2011).
168. Russo, C. L. *et al.* Mucin gene expression in human male urogenital tract epithelia. *Hum. Reprod.* **21,** 2793 (2006).
169. Wira, C. R., Grant Tschudy, K. S. & Crane Godreau, M. A. Epithelial Cells in the Female Reproductive Tract: a Central Role as Sentinels of Immune Protection. *American Journal of Reproductive Immunology* **53,** 65–76 (2005).
170. Cha, L. *et al.* Interferon-alpha, immune activation and immune dysfunction in treated HIV infection. *Clinical & Translational Immunology* **3,** e10 (2014).
171. Poli, G., Kinter, A. L., Vicenzi, E. & Fauci, A. S. Cytokine regulation of acute and chronic HIV infection in vitro: from cell lines to primary mononuclear cells. *Res. Immunol.* **145,** 578–582 (1994).
172. Poli, G., Biswas, P. & Fauci, A. S. Interferons in the pathogenesis and treatment of human immunodeficiency virus infection. *Antiviral Res.* **24,** 221–233 (1994).

173. Hardy, G. A. D. *et al.* Interferon-α Is the Primary Plasma Type-I IFN in HIV-1 Infection and Correlates with Immune Activation and Disease Markers. *PLoS ONE* **8,** e56527 (2013).
174. Fraietta, J. A. *et al.* Type I Interferon Upregulates Bak and Contributes to T Cell Loss during Human Immunodeficiency Virus (HIV) Infection. *PLoS Pathog.* **9,** e1003658 (2013).
175. Sivaraman, V., Zhang, L. & Su, L. Type I interferon contributes to CD4+ T cell depletion induced by infection with HIV-1 in the human thymus. *J. Virol.* **85,** 9243–9246 (2011).
176. McMichael, A. J., Borrow, P., Tomaras, G. D., Goonetilleke, N. & Haynes, B. F. The immune response during acute HIV-1 infection: clues for vaccine development. *Nat. Rev. Immunol.* **10,** 11–23 (2010).
177. Stacey, A. R. *et al.* Induction of a striking systemic cytokine cascade prior to peak viremia in acute human immunodeficiency virus type 1 infection, in contrast to more modest and delayed responses in acute hepatitis B and C virus infections. *J. Virol.* **83,** 3733 (2009).
178. Beignon, A.-S. *et al.* Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor‚Äì
viral RNA interactions. *Journal of Clinical Investigation* **115,** 3265–3275 (2005).
179. Mandl, J. N. *et al.* Divergent TLR7 and TLR9 signaling and type I interferon production distinguish pathogenic and nonpathogenic AIDS virus infections. *Nat. Med.* **14,** 1087 (2008).
180. Granelli-Piperno, A., Golebiowska, A., Trumpfheller, C., Siegal, F. P. & Steinman, R. M. HIV-1-infected monocyte-derived dendritic cells do not undergo maturation but can elicit IL-10 production and T cell regulation. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 7674 (2004).
181. Hu, J., Gardner, M. B. & Miller, C. J. *J. Virol.* **74,** (2000).
182. Shacklett, B. L. Cell-mediated immunity to HIV in the female reproductive tract. *J. Reprod. Immunol.* **83,** 190–195 (2009).
183. Borrow, P. & Bhardwaj, N. Innate immune responses in primary HIV-1 infection. *Curr Opin HIV AIDS* **3,** 36–44 (2008).
184. Montoya, C. J., Velilla, P. A., Chougnet, C., Landay, A. L. & Rugeles, M. T. Increased IFN-γ production by NK and CD3+/CD56+ cells in sexually HIV-1-exposed but uninfected individuals. *Clinical Immunology* **120,** 138–146 (2006).
185. Paiardini, M. & Müller-Trutwin, M. HIV-associated chronic immune activation. *Immunol. Rev.* **254,** 78–101 (2013).
186. Giorgi, J. V. *et al.* Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *J. Infect. Dis.* **179,** 859–870 (1999).
187. Hunt, P. W. *et al.* Relationship between T cell activation and CD4+ T cell count in HIV-seropositive individuals with undetectable plasma HIV RNA levels in the absence of therapy. *J. Infect. Dis.* **197,** 133 (2008).
188. Zhang, Z. *et al.* Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. *Science* **286,** 1353 (1999).
189. Lederman, M. M. *et al.* Immunologic failure despite suppressive antiretroviral

therapy is related to activation and turnover of memory CD4 cells. *J. Infect. Dis.* **204,** 1226 (2011).
190. Corbett, E. L., Steketee, R. W., Kuile, ter, F. O. & Latif, A. S. HIV-1/AIDS and the control of other infectious diseases in Africa. *The Lancet* (2002).
191. Iqbal, S. M. & Kaul, R. REVIEW ARTICLE: Mucosal Innate Immunity as a Determinant of HIV Susceptibility. *American Journal of Reproductive Immunology* **59,** 44–54 (2008).
192. Plummer, F. A. *Heterosexual transmission of HIV-1: Interactions of conventional sexually transmitted diseases, hormone contraception and HIV-1*. (AIDS Res Hum Retroviruses, 1998).
193. Plummer, F. A. Heterosexual transmission of human immunodeficiency virus type 1 (HIV): interactions of conventional sexually transmitted diseases, hormonal contraception and HIV-1. *AIDS Res. Hum. Retroviruses* **14 Suppl 1,** S5–10 (1998).
194. Herpes simplex virus 2 infection increases HIV acquisition in men and women: systematic review and meta-analysis of longitudinal studies. **20,** 73 (2006).
195. Mole, L., Ripich, S., Margolis, D. & Holodniy, M. The Impact of Active Herpes Simplex Virus Infection on Human Immunodeficiency Virus Load. *J. Infect. Dis.* **176,** 766 (1997).
196. Taha, T. E. *et al.* Bacterial vaginosis and disturbances of vaginal flora: association with increased acquisition of HIV. *AIDS* **12,** 1699–1706 (1998).
197. Mitchell, C. *et al.* Interaction between lactobacilli, bacterial vaginosis-associated bacteria, and HIV Type 1 RNA and DNA Genital shedding in U.S. and Kenyan women. *AIDS Res. Hum. Retroviruses* **29,** 13–19 (2013).
198. Sha, B. E. *et al.* Female genital-tract HIV load correlates inversely with Lactobacillus species but positively with bacterial vaginosis and Mycoplasma hominis. *J. Infect. Dis.* **191,** 25–32 (2005).
199. Ferreira, V. H., Kafka, J. K. & Kaushic, C. Influence of Common Mucosal Co-Factors on HIV Infection in the Female Genital Tract. *American Journal of Reproductive Immunology* **71,** 543–554 (2014).
200. Chun, T. W., Engel, D., Mizell, S. B., Ehler, L. A. & Fauci, A. S. Induction of HIV-1 replication in latently infected CD4+ T cells using a combination of cytokines. *J. Exp. Med.* **188,** 83–91 (1998).
201. Tomescu, C., Abdulhaqq, S. & Montaner, L. J. Evidence for the innate immune response as a correlate of protection in human immunodeficiency virus (HIV)-1 highly exposed seronegative subjects (HESN). *Clin. Exp. Immunol.* **164,** 158–169 (2011).
202. Horton, R. E., McLaren, P. J., Fowke, K., Kimani, J. & Ball, T. B. Cohorts for the study of HIV-1-exposed but uninfected individuals: benefits and limitations. *J. Infect. Dis.* **202 Suppl 3,** 81 (2010).
203. Fowke, K. R. *et al.* Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya. *The Lancet* **348,** 1347–1351 (1996).
204. Desimmie, B. A. *et al.* Multiple APOBEC3 restriction factors for HIV-1 and one Vif to rule them all. *J. Mol. Biol.* **426,** 1220–1245 (2014).
205. Lecossier, D., Bouchonnet, F., Clavel, F. & Hance, A. J. Hypermutation of HIV-1 DNA in the absence of the Vif protein. *Science* **300,** 1112–1112 (2003).
206. Conticello, S. G., Harris, R. S. & Neuberger, M. S. The Vif protein of HIV triggers

degradation of the human antiretroviral DNA deaminase APOBEC3G. *Curr. Biol.* **13,** 2009–2013 (2003).
207. Altfeld, M. *et al.* HLA Alleles Associated with Delayed Progression to AIDS Contribute Strongly to the Initial CD8(+) T Cell Response against HIV-1. *PLoS Med.* **3,** e403 (2006).
208. Martin, M. P. *et al.* Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. *Nature Genetics* **39,** 733–740 (2007).
209. Ravet, S. *et al.* Distinctive NK-cell receptor repertoires sustain high-level constitutive NK-cell activation in HIV-exposed uninfected individuals. *Blood* **109,** 4296–4305 (2007).
210. Liu, R. *et al.* Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86,** 367–377 (1996).
211. Dean, M. *et al.* Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science* **273,** 1856–1862 (1996).
212. Tang, J. *et al.* Distribution of chemokine receptor CCR2 and CCR5 genotypes and their relative contribution to human immunodeficiency virus type 1 (HIV-1) seroconversion, early HIV-1 RNA concentration in plasma, and later disease progression. *J. Virol.* **76,** 662–672 (2002).
213. Race-specific HIV-1 disease-modifying effects associated with CCR5 haplotypes. **96,** 12004–12009 (1999).
214. Kaslow, R. A., Dorak, T. & Tang, J. J. Influence of host genetic variation on susceptibility to HIV type 1 infection. **191 Suppl 1,** S68–77 (2005).
215. McNeil, A. J. *et al.* Association of HLA types A1-B8-DR3 and B27 with rapid and slow progression of HIV disease. *QJM* **89,** 177–185 (1996).
216. Migueles, S. A. *et al.* HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 2709–2714 (2000).
217. Sivro, A. *et al.* Interferon regulatory factor 1 polymorphisms previously associated with reduced HIV susceptibility have no effect on HIV disease progression. *PLoS ONE* **8,** e66253 (2013).
218. Shen, R. & Smith, P. D. Mucosal correlates of protection in HIV-1-exposed sero-negative persons. *American Journal of Reproductive Immunology* **72,** 219–227 (2014).
219. Iqbal, S. M. *et al.* Elevated elafin/trappin-2 in the female genital tract is associated with protection against HIV acquisition. *AIDS* **23,** 1669–1677 (2009).
220. Drannik, A. G., Henrick, B. M. & Rosenthal, K. L. War and peace between WAP and HIV: role of SLPI, trappin-2, elafin and ps20 in susceptibility to HIV infection. *Biochem. Soc. Trans.* **39,** 1427–1432 (2011).
221. Burgener, A., Sainsbury, J., Plummer, F. A. & Ball, T. B. Systems biology-based approaches to understand HIV-exposed uninfected women. *Curr HIV/AIDS Rep* **7,** 53–59 (2010).
222. Drannik, A. G. *et al.* Anti-HIV-1 activity of elafin is more potent than its precursor's, trappin-2, in genital epithelial cells. *J. Virol.* **86,** 4599–4610 (2012).
223. Furci, L. *et al.* Antigen-driven C-C chemokine-mediated HIV-1 suppression by

CD4(+) T cells from exposed uninfected individuals expressing the wild-type CCR-5 allele. *J. Exp. Med.* **186,** 455–460 (1997).
224. Hasselrot, K. *et al.* HIV-1 exposed uninfected men who have sex with men have increased levels of salivary CC-chemokines associated with sexual behavior. *AIDS* **24,** 1569–1575 (2010).
225. Suresh, P., Wanchu, A., Bhatnagar, A., Sachdeva, R. K. & Sharma, M. Spontaneous and antigen-induced chemokine production in exposed but uninfected partners of HIV type 1-infected individuals in North India. *AIDS Res. Hum. Retroviruses* **23,** 261–268 (2007).
226. Novak, R. M. *et al.* Cervicovaginal levels of lactoferrin, secretory leukocyte protease inhibitor, and RANTES and the effects of coexisting vaginoses in human immunodeficiency virus (HIV)-seronegative women with a high risk of heterosexual acquisition of HIV infection. *Clin. Vaccine Immunol.* **14,** 1107 (2007).
227. Romas, L. M. *et al.* A comparative proteomic analysis of the soluble immune factor environment of rectal and oral mucosa. *PLoS ONE* **9,** e100820 (2014).
228. McNeely, T. B. *et al.* Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. *J. Clin. Invest.* **96,** 456–464 (1995).
229. Groot, F. *et al.* Lactoferrin prevents dendritic cell-mediated human immunodeficiency virus type 1 transmission by blocking the DC-SIGN--gp120 interaction. *J. Virol.* **79,** 3009–3015 (2005).
230. Kuhn, L. *et al.* Alpha-defensins in the prevention of HIV transmission among breastfed infants. *J. Acquir. Immune Defic. Syndr.* **39,** 138 (2005).
231. Wang, W. *et al.* Activity of alpha- and theta-defensins against primary isolates of HIV-1. *J. Immunol.* **173,** 515–520 (2004).
232. Venkataraman, N. *et al.* Reawakening Retrocyclins: Ancestral Human Defensins Active Against HIV-1. *PLoS Biol.* **7,** e1000095 (2009).
233. Münk, C. *et al.* The θ-Defensin, Retrocyclin, Inhibits HIV-1 Entry. *AIDS Res. Hum. Retroviruses* **19,** 875–881 (2004).
234. Levinson, P. *et al.* Levels of innate immune factors in genital fluids: association of alpha defensins and LL-37 with genital infections and increased HIV acquisition. *AIDS* **23,** 309–317 (2009).
235. Burgener, A. *et al.* Comprehensive proteomic study identifies serpin and cystatin antiproteases as novel correlates of HIV-1 resistance in the cervicovaginal mucosa of female sex workers. *J. Proteome Res.* **10,** 5139 (2011).
236. Burgener, A. *et al.* Identification of differentially expressed proteins in the cervical mucosa of HIV-1-resistant sex workers. *J. Proteome Res.* **7,** 4446–4454 (2008).
237. Program, W. B. G. H. A.UNAIDS. *The Global Economic Crisis and HIV Prevention and Treatment Programmes: Vulnerabilities and Impact*. (2009).
238. HIV-CAUSAL Collaboration *et al.* The effect of combined antiretroviral therapy on the overall mortality of HIV-infected individuals. *AIDS* **24,** 123–137 (2010).
239. Nakagawa, F. *et al.* Projected life expectancy of people with HIV according to timing of diagnosis. *AIDS* **26,** 335–343 (2012).
240. Carpenter, C. C. *et al.* Antiretroviral therapy in adults: updated recommendations of the International AIDS Society-USA Panel. in **283,** 381–390 (2000).
241. Ndesendo, V. M. K. *et al.* A review of current intravaginal drug delivery approaches

employed for the prophylaxis of HIV/AIDS and prevention of sexually transmitted infections. *AAPS PharmSciTech* **9,** 505–520 (2008).
242. Lei, J. H. *et al.* Circumcision Status and Risk of HIV Acquisition during Heterosexual Intercourse for Both Males and Females: A Meta-Analysis. *PLoS ONE* **10,** e0125436 (2015).
243. Abdool Karim, Q. *et al.* Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science* **329,** 1168–1174 (2010).
244. Beyrer, C., Bekker, L.-G., Pozniak, A. & Barré-Sinoussi, F. Pre-exposure prophylaxis works--it's time to deliver. *Lancet* **385,** 1482 (2015).
245. Gay, C. L. & Cohen, M. S. Antiretrovirals to prevent HIV infection: pre- and postexposure prophylaxis. *Curr Infect Dis Rep* **10,** 323–331 (2008).
246. Hankins, C. A. & Dybul, M. R. The promise of pre-exposure prophylaxis with antiretroviral drugs to prevent HIV transmission: a review. *Curr Opin HIV AIDS* **8,** 50–58 (2013).
247. Robb, M. L. *et al.* Risk behaviour and time as covariates for efficacy of the HIV vaccine regimen ALVAC-HIV (vCP1521) and AIDSVAX B/E: a post-hoc analysis of the Thai phase 3 efficacy trial RV 144. *Lancet Infect Dis* **12,** 531–537 (2012).
248. Gilbert, P. B. *et al.* Statistical interpretation of the RV144 HIV vaccine efficacy trial in Thailand: a case study for statistical issues in efficacy trials. *J. Infect. Dis.* **203,** 969–975 (2011).
249. Obiero, J., Mwethera, P. G., Hussey, G. D. & Wiysonge, C. S. Vaginal microbicides for reducing the risk of sexual acquisition of HIV infection in women: systematic review and meta-analysis. *BMC Infect. Dis.* **12,** 289 (2012).
250. Watts, C. & Zimmerman, C. Violence against women: global scope and magnitude. *Lancet* **359,** 1232–1237 (2002).
251. Hladik, F. *et al.* Initial events in establishing vaginal entry and infection by human immunodeficiency virus type-1. *Immunity* **26,** 270 (2007).
252. Liang, X. J. *Nanopharmaceutics: the potential application of nanomaterials*. (2013).
253. Thurman, A. R., Clark, M. R. & Doncel, G. F. Multipurpose prevention technologies: biomedical tools to prevent HIV-1, HSV-2, and unintended pregnancies. *Infect Dis Obstet Gynecol* **2011,** 1–10 (2011).
254. Thurman, A. R. & Doncel, G. F. Innate immunity and inflammatory response to Trichomonas vaginalis and bacterial vaginosis: relationship to HIV acquisition. *American Journal of Reproductive Immunology* **65,** 98 (2011).
255. Porter, D. P., Kulkarni, R., Fralich, T., Miller, M. D. & White, K. L. Characterization of HIV-1 drug resistance development through week 48 in antiretroviral naive subjects on rilpivirine/emtricitabine/tenofovir DF or efavirenz/emtricitabine/tenofovir DF in the STaR study (GS-US-264-0110). *J. Acquir. Immune Defic. Syndr.* **65,** 318–326 (2014).
256. Cutler, B. & Justman, J. Vaginal microbicides and the prevention of HIV transmission. *Lancet Infect Dis* **8,** 697 (2008).
257. Moulard, M. *et al.* Selective interactions of polyanions with basic surfaces on human immunodeficiency virus type 1 gp120. *J. Virol.* **74,** 1948–1960 (2000).
258. Mondor, I., Ugolini, S. & Sattentau, Q. J. Human immunodeficiency virus type 1 attachment to HeLa CD4 cells is CD4 independent and gp120 dependent and

requires cell surface heparans. *J. Virol.* **72,** 3623–3634 (1998).
259. Callahan, L. N., Phelan, M., Mallinson, M. & Norcross, M. A. Dextran sulfate blocks antibody binding to the principal neutralizing domain of human immunodeficiency virus type 1 without interfering with gp120-CD4 interactions. *J. Virol.* **65,** 1543–1550 (1991).
260. Ramjee, G. Microbicide research: current and future directions. *Curr Opin HIV AIDS* **5,** 316–321 (2010).
261. Cervico-vaginal tissue ex vivo as a model to study early events in HIV-1 infection. **65,** 268–278 (2011).
262. Abdool Karim, S. S. & Baxter, C. Overview of microbicides for the prevention of human immunodeficiency virus. *Best Pract Res Clin Obstet Gynaecol* **26,** 427–439 (2012).
263. Pirrone, V., Wigdahl, B. & Krebs, F. C. The rise and fall of polyanionic inhibitors of the human immunodeficiency virus type 1. *Antiviral Res.* **90,** 168–182 (2011).
264. Antimisiaris, S. G. & Mourtas, S. Recent advances on anti-HIV vaginal delivery systems development. *Adv. Drug Deliv. Rev.* (2015). doi:10.1016/j.addr.2015.03.015
265. De Clercq, E. The role of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in the therapy of HIV-1 infection1Presented at the Eleventh International Conference on Antiviral Research, San Diego, CA, 5–10 April 1998.1. *Antiviral Res.* **38,** 153–179 (1998).
266. Moss, D. M. *et al.* Divalent metals and pH alter raltegravir disposition in vitro. *Antimicrob. Agents Chemother.* **56,** 3020–3026 (2012).
267. Lv, Z., Chu, Y. & Wang, Y. HIV protease inhibitors: a review of molecular selectivity and toxicity. *HIV/AIDS (Auckland, N.Z.)* **7,** 95–104 (2015).
268. Network, M. T. *MTN statement on decision to discontinue use of oral tenofovir tablets in VOICE, a major HIV prevention study in women. 2011*. (2013).
269. Amico, K. R., Mansoor, L. E., Corneli, A., Torjesen, K. & van der Straten, A. Adherence support approaches in biomedical HIV prevention trials: experiences, insights and future directions from four multisite prevention trials. *AIDS Behav* **17,** 2155 (2013).
270. DR, M., Jr, M, T., AF, P. & DL, M. Contraception by means of a Silastic vaginal ring impregnated with medroxyprogesterone acetate. *American Journal of Obstetrics and Gynecology* **107,** (1970).
271. Montgomery, E. T. *et al.* Vaginal Ring Adherence in Sub-Saharan Africa: Expulsion, Removal, and Perfect Use. *AIDS Behav* **16,** 1787–1798 (2012).
272. Malcolm, R. K., Edwards, K.-L., Kiser, P., Romano, J. & Smith, T. J. Advances in microbicide vaginal rings. *Antiviral Res.* **88,** S30–S39 (2010).
273. Roumen, F. J., Apter, D., Mulders, T. M. & Dieben, T. O. Efficacy, tolerability and acceptability of a novel contraceptive vaginal ring releasing etonogestrel and ethinyl oestradiol. *Hum. Reprod.* **16,** 469–475 (2001).
274. Novak, A., la Loge, De, C., Abetz, L. & Van der Meulen, E. A. The combined contraceptive vaginal ring, NuvaRing®: an international study of user acceptability. *Contraception* **67,** 187–194 (2003).
275. Network, M. T. *ASPIRE—a study to prevent infection with a ring for extended use*. (2013).
276. van der Straten, A., Musara, P. & Etima, J. Disclosure of Pharmacokinetic (PK)

Drug Results Promotes Open Discourse on Non-adherence among Women in VOICE. *AIDS research and …* (2014).
277. van der Straten, A. *et al.* Perspectives on use of oral and vaginal antiretrovirals for HIV prevention: the VOICE-C qualitative study in Johannesburg, South Africa. *Journal of the International AIDS Society* **17,** 19146 (2014).
278. Smith, D. K., Toledo, L., Smith, D. J., Adams, M. A. & Rothenberg, R. Attitudes and program preferences of African-American urban young adults about pre-exposure prophylaxis (PrEP). *AIDS Educ Prev* **24,** 408–421 (2012).
279. Gunawardana, M. *et al.* Microbial biofilms on the surface of intravaginal rings worn in non-human primates. *Journal of Medical Microbiology* **60,** 828–837 (2011).
280. Gettins, P. G. W. Serpin structure, mechanism, and function. *Chem. Rev.* **102,** 4751–4804 (2002).
281. Bock, S. C. *et al.* Human C1 inhibitor: primary structure, cDNA cloning, and chromosomal localization. *Biochemistry* **25,** 4292–4301 (1986).
282. Law, R. H. P. *et al.* An overview of the serpin superfamily. *Genome Biol.* **7,** (2006).
283. Cabrita, L. D. & Bottomley, S. P. How do proteins avoid becoming too stable? Biophysical studies into metastable proteins. *Eur. Biophys. J.* **33,** 83–88 (2004).
284. Ye, S. *et al.* The structure of a Michaelis serpin-protease complex. *Nat. Struct. Biol.* **8,** 979–983 (2001).
285. Gettins, P., Patston, P. A. & Olson, S. T. *Serpins: Structure, Function and Biology, RG Landes, Austin, TX*. (View in Article, 1996).
286. Silverman, G. A. *et al.* The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. *J. Biol. Chem.* **276,** 33293–33296 (2001).
287. Stein, P. & Chothia, C. Serpin tertiary structure transformation. *J. Mol. Biol.* **221,** 615–621 (1991).
288. Huntington, J. A., Read, R. J. & Carrell, R. W. Structure of a serpin-protease complex shows inhibition by deformation. *Nature* **407,** 923–926 (2000).
289. Bode, W. & Huber, R. Natural protein proteinase inhibitors and their interaction with proteinases. *Eur. J. Biochem.* **204,** 433–451 (1992).
290. Kaslik, G. *et al.* Effects of serpin binding on the target proteinase: global stabilization, localized increased structural flexibility, and conserved hydrogen bonding at the active site. *Biochemistry* **36,** 5455 (1997).
291. Kaslik, G., Patthy, A., Bálint, M. & Gráf, L. Trypsin complexed with alpha 1-proteinase inhibitor has an increased structural flexibility. *FEBS Lett.* **370,** 179–183 (1995).
292. Kounnas, M. Z., Church, F. C., Argraves, W. S. & Strickland, D. K. Cellular internalization and degradation of antithrombin III-thrombin, heparin cofactor II-thrombin, and alpha 1-antitrypsin-trypsin complexes is mediated by the low density lipoprotein receptor-related protein. *J. Biol. Chem.* **271,** 6523–6529 (1996).
293. Lomas, D. A. & Carrell, R. W. Serpinopathies and the conformational dementias. *Nature Reviews Genetics* **3,** 759–768 (2002).
294. Wright, H. T. Ovalbumin is an elastase substrate. *J. Biol. Chem.* **259,** 14335–14336 (1984).
295. Hunt, L. T. & Dayhoff, M. O. A surprising new protein superfamily containing ovalbumin, antithrombin-III, and alpha 1-proteinase inhibitor. *Biochem. Biophys.*

Res. Commun. **95,** 864–871 (1980).
296. Whisstock, J. C. *et al.* Serpins flex their muscle: II. Structural insights into target peptidase recognition, polymerization, and transport functions. *J. Biol. Chem.* **285,** 24307–24312 (2010).
297. Whitney, J. B., Asmal, M. & Geiben-Lynn, R. Serpin induced antiviral activity of prostaglandin synthetase-2 against HIV-1 replication. *PLoS ONE* **6,** e18589 (2011).
298. Silverman, G. A. *et al.* Serpins flex their muscle: I. Putting the clamps on proteolysis in diverse biological systems. *J. Biol. Chem.* **285,** 24299–24305 (2010).
299. Ashton Rickardt, P. G. Serine protease inhibitors and cytotoxic T lymphocytes. *Immunol. Rev.* **235,** 147–158 (2010).
300. Heutinck, K. M., Berge, ten, I. J. M., Hack, C. E., Hamann, J. & Rowshani, A. T. Serine proteases of the human immune system in health and disease. *Molecular Immunology* **47,** 1943 (2010).
301. Askew, D. J. & Silverman, G. A. Intracellular and extracellular serpins modulate lung disease. *Journal of Perinatology* **28,** S127–S135 (2008).
302. Sun, J. *et al.* A cytosolic granzyme B inhibitor related to the viral apoptotic regulator cytokine response modifier A is present in cytotoxic lymphocytes. *J. Biol. Chem.* **271,** 27802–27809 (1996).
303. Beltran, A. *et al.* Re-activation of a dormant tumor suppressor gene maspin by designed transcription factors. *Oncogene* **26,** 2791–2798 (2007).
304. Jeunemaitre, X. *et al.* Molecular basis of human hypertension: Role of angiotensinogen. *Cell* **71,** 169–180 (1992).
305. Perogamvros, I. *et al.* Novel Corticosteroid-Binding Globulin Variant That Lacks Steroid Binding Activity. *The Journal of Clinical Endocrinology & Metabolism* **95,** E142–E150 (2013).
306. Domingues, R., Font, P., Sobrinho, L. & Bugalho, M. J. A novel variant in Serpina7 gene in a family with thyroxine-binding globulin deficiency. *Endocr* **36,** 83–86 (2009).
307. Belin, D. Biology and facultative secretion of plasminogen activator inhibitor-2. *Thrombosis and haemostasis* **70,** 144–147 (1993).
308. Dickinson, J. L., Bates, E. J., Ferrante, A. & Antalis, T. M. Plasminogen activator inhibitor type 2 inhibits tumor necrosis factor alpha-induced apoptosis. Evidence for an alternate biological function. *J. Biol. Chem.* **270,** 27894 (1995).
309. Rees, D. D. *et al.* Recombinant Human Monocyte/Neutrophil Elastase Inhibitor Protects Rat Lungs against Injury from Cystic Fibrosis Airway Secretions. *American Journal of Respiratory Cell and Molecular Biology* **20,** 69–78 (2012).
310. Dahlen, J. R., Foster, D. C. & Kisiel, W. Human Proteinase Inhibitor 9 (PI9) Is a Potent Inhibitor of Subtilisin A. *Biochem. Biophys. Res. Commun.* **238,** 329–333 (1997).
311. Dahlen, J. R., Jean, F., Thomas, G., Foster, D. C. & Kisiel, W. Inhibition of Soluble Recombinant Furin by Human Proteinase Inhibitor 8. *J. Biol. Chem.* **273,** 1851–1854 (1998).
312. Chertov, O. *et al.* Identification of human neutrophil-derived cathepsin G and azurocidin/CAP37 as chemoattractants for mononuclear cells and neutrophils. *J. Exp. Med.* **186,** 739–747 (1997).
313. Sambrano, G. R. *et al.* Cathepsin G activates protease-activated receptor-4 in human

platelets. *J. Biol. Chem.* **275,** 6819–6823 (2000).
314. Münch, J. *et al.* Discovery and optimization of a natural HIV-1 entry inhibitor targeting the gp41 fusion peptide. *Cell* **129,** 275 (2007).
315. Congote, L. F. Serpin A1 and CD91 as host instruments against HIV-1 infection: are extracellular antiviral peptides acting as intracellular messengers? *Virus Res.* **125,** 134 (2007).
316. Janciauskiene, S. *et al.* Inhibition of lipopolysaccharide-mediated human monocyte activation, in vitro, by alpha1-antitrypsin. *Biochem. Biophys. Res. Commun.* **321,** 592–600 (2004).
317. Kaseba-Sata, C. *et al.* Increased Risk of Intrauterine Transmission of HIV-1 Associated With Granulocyte Elastase in Endocervical Mucus. *J. Acquir. Immune Defic. Syndr.* **41,** 249–251 (2006).
318. Al-Omari, M. *et al.* Acute-phase protein α1-antitrypsin inhibits neutrophil calpain I and induces random migration. *Mol. Med.* **17,** 865–874 (2011).
319. Congote, L. F. The C-terminal 26-residue peptide of serpin A1 is an inhibitor of HIV-1. *Biochem. Biophys. Res. Commun.* **343,** 617–622 (2006).
320. Kaiserman, D. *et al.* Comparison of human chromosome 6p25 with mouse chromosome 13 reveals a greatly expanded ov-serpin gene repertoire in the mouse. *Genomics* **79,** 349–362 (2002).
321. Wang, L. *et al.* Identification of SERPINB1 as a physiological inhibitor of human granzyme H. *J. Immunol.* **190,** 1330 (2013).
322. Remold-O'Donnell, E., Nixon, J. C. & Rose, R. M. Elastase inhibitor. Characterization of the human elastase inhibitor molecule associated with monocytes, macrophages, and neutrophils. *J. Exp. Med.* **169,** 1071–1086 (1989).
323. Mangan, M. S. J., Kaiserman, D. & Bird, P. I. The role of serpins in vertebrate immunity. *Tissue Antigens* **72,** 10 (2008).
324. Cooley, J., Takayama, T. K., Shapiro, S. D., Schechter, N. M. & Remold-O'Donnell, E. The serpin MNEI inhibits elastase-like and chymotrypsin-like serine proteases through efficient reactions at two active sites. *Biochemistry* **40,** 15762–15770 (2001).
325. Chua, F. & Laurent, G. J. Neutrophil elastase: mediator of extracellular matrix destruction and accumulation. *Proc Am Thorac Soc* **3,** 427 (2006).
326. Benarafa, C., Cooley, J., Zeng, W., Bird, P. I. & Remold-O'Donnell, E. Characterization of four murine homologs of the human ov-serpin monocyte neutrophil elastase inhibitor MNEI (SERPINB1). *J. Biol. Chem.* **277,** 42028–42033 (2002).
327. Farley, K., Stolley, J. M., Zhao, P., Cooley, J. & Remold-O'Donnell, E. A serpinB1 regulatory mechanism is essential for restricting neutrophil extracellular trap generation. *J. Immunol.* **189,** 4574–4581 (2012).
328. Padron-Barthe, L., Leprêtre, C., Martin, E., Counis, M.-F. & Torriglia, A. Conformational modification of serpins transforms leukocyte elastase inhibitor into an endonuclease involved in apoptosis. *Mol. Cell. Biol.* **27,** 4036 (2007).
329. Jaadane, I., Nagbou, A., Behar-Cohen, F. & Torriglia, A. Interaction of Leukocyte Elastase Inhibitor/L-DNase II with BCL-2 and BAX. *Biochim. Biophys. Acta* **1843,** 2807–2815 (2014).
330. Loison, F. *et al.* Proteinase 3–dependent caspase-3 cleavage modulates neutrophil

death and inflammation. *J. Clin. Invest.* **124,** 4445–4458 (2014).
331. Gong, D. *et al.* Critical role of serpinB1 in regulating inflammatory responses in pulmonary influenza infection. *J. Infect. Dis.* **204,** 592–600 (2011).
332. Cooley, J., Sontag, M. K., Accurso, F. J. & Remold-O'Donnell, E. SerpinB1 in cystic fibrosis airway fluids: quantity, molecular form and mechanism of elastase inhibition. *Eur Respir J* **37,** 1083–1090 (2011).
333. Pastor, M. D. *et al.* Identification of proteomic signatures associated with lung cancer and COPD. *Journal of Proteomics* **89,** 227–237 (2013).
334. Cui, X. *et al.* Decreased expression of SERPINB1 correlates with tumor invasion and poor prognosis in hepatocellular carcinoma. *J Mol Hist* **45,** 59–68 (2014).
335. Chou, R.-H. *et al.* Suppression of the invasion and migration of cancer cells by SERPINB family genes and their derived peptides. *Oncol. Rep.* **27,** 238–245 (2012).
336. Huasong, G. *et al.* Serine protease inhibitor (SERPIN) B1 suppresses cell migration and invasion in glioma cells. *Brain Res.* (2014). doi:10.1016/j.brainres.2014.06.017
337. Tseng, M.-Y. *et al.* Serine protease inhibitor (SERPIN) B1 promotes oral cancer cell motility and is over-expressed in invasive oral squamous cell carcinoma. *Oral Oncol.* **45,** 771–776 (2009).
338. Hui, L., Yongxia, Z., Zhili, G. & Jun, G. Calcitriol inhibits keratinocyte proliferation by upregulating leukocyte elastase inhibitor (serpin B1). *J. Dermatol.* **41,** 393–398 (2014).
339. Lowes, M. A., Bowcock, A. M. & Krueger, J. G. Pathogenesis and therapy of psoriasis. *Nature* **445,** 866–873 (2007).
340. Turk, V. & Bode, W. The cystatins: Protein inhibitors of cysteine proteinases. *FEBS Lett.* **285,** 213–219 (1991).
341. Groce, N. E. & Trani, J.-F. Millennium Development Goals and people with disabilities. *Lancet* **374,** 1800–1801 (2009).
342. Cornwall, G. A. & Hsia, N. A new subgroup of the family 2 cystatins. *Molecular and Cellular Endocrinology* **200,** 1–8 (2003).
343. Josiah Ochieng, G. C. Cystatin Superfamily. *Journal of health care for the poor and underserved* **21,** 51–70 (2010).
344. Vorster, B. *et al.* The Cysteine Protease–Cysteine Protease Inhibitor System Explored in Soybean Nodule Development. *Agronomy 2013, Vol. 3, Pages 550-570* **3,** 550–570 (2013).
345. Creasy, B. M. & McCoy, K. L. Cytokines regulate cysteine cathepsins during TLR responses. *Cell. Immunol.* **267,** 56–66 (2011).
346. Suzuki, T. *et al.* Comprehensive gene expression profile of LPS-stimulated human monocytes by SAGE. *Blood* **96,** 2584–2591 (2000).
347. Tamura, T., Thotakura, P., Tanaka, T. S., Ko, M. S. H. & Ozato, K. Identification of target genes and a unique cis element regulated by IRF-8 in developing macrophages. *Blood* **106,** 1938 (2005).
348. Verdot, L. *et al.* Cystatins Up-regulate Nitric Oxide Release from Interferon-γ-activated Mouse Peritoneal Macrophages. *J. Biol. Chem.* **271,** 28077–28081 (1996).
349. Xu, Y. *et al.* IL-10 controls cystatin C synthesis and blood concentration in response to inflammation through regulation of IFN regulatory factor 8 expression. *J. Immunol.* **186,** 3673 (2011).
350. Marletta, M. A. *Nitric oxide synthase structure and mechanism*. (1993).

351. Das, L., Datta, N., Bandyopadhyay, S. & Das, P. K. Successful therapy of lethal murine visceral leishmaniasis with cystatin involves up-regulation of nitric oxide and a favorable T cell response. *J. Immunol.* **166,** 4020–4028 (2001).
352. Chapman, H. A., Reilly, J. J., Yee, R. & Grubb, A. Identification of cystatin C, a cysteine proteinase inhibitor, as a major secretory product of human alveolar macrophages in vitro. *Am. Rev. Respir. Dis.* **141,** 698 (1990).
353. Pierre, P. & Mellman, I. Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. *Cell* **93,** 1135 (1998).
354. Riese, R. J. *et al.* Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading. *Immunity* **4,** 357–366 (1996).
355. Venkataraman, N., Cole, A. L., Svoboda, P., Pohl, J. & Cole, A. M. Cationic polypeptides are required for anti-HIV-1 activity of human vaginal fluid. *J. Immunol.* **175,** 7560–7567 (2005).
356. Voltersvik, P. *et al.* Cystatin A and HIV-1 p24 antigen expression in tonsillar lymphoid follicles during HIV-1 infection and during highly active antiretroviral therapy. *J. Acquir. Immune Defic. Syndr.* **41,** 277 (2006).
357. Ao, Z., Fowke, K. R., Cohen, É. A. & Yao, X. Contribution of the C-terminal tri-lysine regions of human immunodeficiency virus type 1 integrase for efficient reverse transcription and viral DNA nuclear import. *Retrovirology* **2,** 62 (2005).
358. Reed, L. J. & Muench, H. *A simple method of estimating fifty per cent endpoints*. (American journal of epidemiology, 1938).
359. Yamamoto, N. *et al.* Analysis of human immunodeficiency virus type 1 integration by using a specific, sensitive and quantitative assay based on real-time polymerase chain reaction. *Virus Genes* **32,** 105–113 (2006).
360. Li, X.-Y., Guo, F., Zhang, L., Kleiman, L. & Cen, S. APOBEC3G inhibits DNA strand transfer during HIV-1 reverse transcription. *J. Biol. Chem.* **282,** 32065–32074 (2007).
361. Drannik, A. G. *et al.* Anti-HIV-1 activity of elafin depends on its nuclear localization and altered innate immune activation in female genital epithelial cells. *PLoS ONE* **7,** (2012).
362. Lu, X.-S., Bélec, L. & Pillot, J. Anti-gp160 IgG and IgA Antibodies Associated with a Large Increase in Total IgG in Cervicovaginal Secretions from Human Immunodeficiency Virus Type 1-Infected Women. *J. Infect. Dis.* **167,** 1189–1192 (1993).
363. Musey, L. *et al.* HIV-1 induces cytotoxic T lymphocytes in the cervix of infected women. *J. Exp. Med.* **185,** 293–303 (1997).
364. Sampathkumar, R. *et al.* Influence of HLA Class I Haplotypes on HIV-1 Seroconversion and Disease Progression in Pumwani Sex Worker Cohort. *PLoS ONE* **9,** e101475 (2014).
365. Rahman, S. *et al.* Mucosal serpin A1 and A3 levels in HIV highly exposed sero-negative women are affected by the menstrual cycle and hormonal contraceptives but are independent of epidemiological confounders. **69,** 64–72 (2013).
366. Uchiyama, K. *et al.* Serpin B1 protects colonic epithelial cell via blockage of neutrophil elastase activity and its expression is enhanced in patients with ulcerative colitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* **302,** G1163–70 (2012).
367. Salazar-Gonzalez, J. F. *et al.* Genetic identity, biological phenotype, and

evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. *J. Exp. Med.* **206,** 1289 (2009).
368. Yasumatsu, R. *et al.* SERPINB1 upregulation is associated with in vivo complex formation with neutrophil elastase and cathepsin G in a baboon model of bronchopulmonary dysplasia. *Am. J. Physiol. Lung Cell Mol. Physiol.* **291,** L619–27 (2006).
369. Kaiserman, D. & Bird, P. I. Control of granzymes by serpins. *Cell Death Differ.* **17,** 586–595 (2010).
370. Lajoie, J. *et al.* A distinct cytokine and chemokine profile at the genital mucosa is associated with HIV-1 protection among HIV-exposed seronegative commercial sex workers. *Mucosal Immunol* **5,** 277–287 (2012).
371. Wira, C. R. *et al.* Sex hormone regulation of innate immunity in the female reproductive tract: the role of epithelial cells in balancing reproductive potential with protection against sexually transmitted pathogens. *American Journal of Reproductive Immunology* **63,** 544–565 (2010).
372. Africander, D., Louw, R., Verhoog, N., Noeth, D. & Hapgood, J. P. Differential regulation of endogenous pro-inflammatory cytokine genes by medroxyprogesterone acetate and norethisterone acetate in cell lines of the female genital tract. *Contraception* **84,** 423–435 (2011).
373. Wira, C. R. & Veronese, F. Hormone regulation of the mucosal environment in the reproductive tract and the prevention of HIV infection. *American Journal of Reproductive Immunology* **71,** 487–489 (2014).
374. MacIntyre, D. A. *et al.* The vaginal microbiome during pregnancy and the postpartum period in a European population. *Sci Rep* **5,** 8988 (2015).
375. Fichorova, R. N., Desai, P. J., Gibson, F. C. & Genco, C. A. Distinct proinflammatory host responses to Neisseria gonorrhoeae infection in immortalized human cervical and vaginal epithelial cells. *Infect. Immun.* **69,** 5840–5848 (2001).
376. Card, C. M., Ball, T. B. & Fowke, K. R. Immune Quiescence: a model of protection against HIV infection. *Retrovirology* (2013).
377. Oosthuyse, T. & Bosch, A. N. The effect of the menstrual cycle on exercise metabolism: implications for exercise performance in eumenorrhoeic women. *Sports Med* **40,** 207–227 (2010).
378. Groer, M. W. *et al.* Immunity, inflammation and infection in post-partum breast and formula feeders. **54,** 222–231 (2005).
379. Murphy, K. *et al.* Levels of Genital Tract Defensins and Cytokines Differ between HIV-Uninfected US and African Women. *American Journal of Reproductive Immunology* n/a–n/a (2015). doi:10.1111/aji.12411
380. Mitchell, C. *et al.* Associations between genital tract infections, genital tract inflammation, and cervical cytobrush HIV-1 DNA in US versus Kenyan women. *J. Acquir. Immune Defic. Syndr.* **62,** 143–148 (2013).
381. Cohen, C. R. *et al.* Increased levels of immune activation in the genital tract of healthy young women from sub-Saharan Africa. *AIDS* **24,** 2069–2074 (2010).
382. Ogg, G. S. *et al.* Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* **279,** 2103–2106 (1998).
383. Kiepiela, P. *et al.* CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat. Med.* **13,** 46–53 (2007).

384. Betts, M. R. *et al.* Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection. *J. Virol.* **75,** 11983–11991 (2001).
385. Rosenberg, E. S. *et al.* Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* **278,** 1447 (1997).
386. van de Wijgert, J. H. H. M. *et al.* Disentangling contributions of reproductive tract infections to HIV acquisition in African Women. *Sexually transmitted diseases* **36,** 357–364 (2009).
387. Bas, S. *et al.* The proinflammatory cytokine response to Chlamydia trachomatis elementary bodies in human macrophages is partly mediated by a lipoprotein, the macrophage infectivity potentiator, through TLR2/TLR1/TLR6 and CD14. *J. Immunol.* **180,** 1158–1168 (2008).
388. Iqbal, S. M. *et al.* Elevated T cell counts and RANTES expression in the genital mucosa of HIV-1-resistant Kenyan commercial sex workers. *J. Infect. Dis.* **192,** 738 (2005).
389. Libby, P., Ridker, P. M. & Maseri, A. Inflammation and atherosclerosis. *Circulation* **105,** 1135–1143 (2002).
390. Moore, K. J. & Tabas, I. Macrophages in the pathogenesis of atherosclerosis. *Cell* **145,** 341–355 (2011).
391. Nyhan, W. L. *Nucleotide Synthesis via Salvage Pathway*. (John Wiley & Sons, Ltd, 2005). doi:10.1002/9780470015902.a0001399.pub3
392. Masson, L. *et al.* Genital Inflammation and the Risk of HIV Acquisition in Women. *Clin. Infect. Dis.* **61,** 260–269 (2015).
393. Teixeira, G. S. *et al.* Characteristics of Lactobacillus and Gardnerella vaginalis from women with or without bacterial vaginosis and their relationships in gnotobiotic mice. *Journal of Medical Microbiology* **61,** 1074–1081 (2012).
394. Fettweis, J. M. *et al.* Differences in vaginal microbiome in African American women versus women of European ancestry. *Microbiology* **160,** 2272–2282 (2014).
395. Im, S.-S. & Osborne, T. F. Liver x receptors in atherosclerosis and inflammation. *Circ. Res.* **108,** 996–1001 (2011).
396. Renga, B. *et al.* The bile acid sensor FXR is required for immune-regulatory activities of TLR-9 in intestinal inflammation. *PLoS ONE* **8,** e54472 (2013).
397. Cray, C., Zaias, J. & Altman, N. H. Acute phase response in animals: a review. *Comp. Med.* **59,** 517–526 (2009).
398. Kramer, H. B. *et al.* Elevation of Intact and Proteolytic Fragments of Acute Phase Proteins Constitutes the Earliest Systemic Antiviral Response in HIV-1 Infection. *PLoS Pathog.* **6,** e1000893 (2010).
399. Ferlay, J., Soerjomataram, I., Ervi, M., Dikshit, R. & Eser, S. *Cancer incidence and mortality worldwide: IARC CancerBase No. 11 [Internet]. GLOBOCAN 2012 v1. 0 Lyon, France: International Agency for Research ….* (2012).
400. Patston, P. A. & Gettins, P. G. W. Significance of secondary structure predictions on the reactive center loop region of serpins: a model for the folding of serpins into a metastable state. *FEBS Lett.* **2,** 239 (1996).
401. Chappell, C. A. *et al.* The effect of menopause on the innate antiviral activity of cervicovaginal lavage. *American Journal of Obstetrics and Gynecology* **213,** 204.e1–204.e6 (2015).

402. Moorthy, K., Sharma, D., Basir, S. F. & Baquer, N. Z. Administration of estradiol and progesterone modulate the activities of antioxidant enzyme and aminotransferases in naturally menopausal rats. *Exp. Gerontol.* **40,** 295–302 (2005).
403. Jones, R. E. & Lopez, K. H. *Human reproductive biology*. (2013).
404. Rubulis, A., Jacobs, R. D. & Hughes, E. C. Glycogen synthetase in mammalian uterus. *Biochimica et Biophysica Acta (BBA) - Enzymology and Biological Oxidation* **99,** 584–586 (1965).
405. ZONDEK, B. & STEIN, L. PGLYCOGEN CONTENT OF THE HUMAN UTERINE MUCOSA GLYCOPENIA UTERI. *Endocrinology* **27,** 395–399 (2013).
406. Drannik, A. G. *et al.* Trappin-2/elafin modulate innate immune responses of human endometrial epithelial cells to PolyI:C. *PLoS ONE* **7,** (2012).
407. Drannik, A. G., Nag, K., Sallenave, J.-M. & Rosenthal, K. L. Antiviral activity of trappin-2 and elafin in vitro and in vivo against genital herpes. *J. Virol.* **87,** 7526–7538 (2013).
408. Dezube, B. J. *et al.* The effect of tenidap on cytokines, acute-phase proteins, and virus load in human immunodeficiency virus (HIV)-infected patients: correlation between plasma HIV-1 RNA and proinflammatory cytokine levels. *J. Infect. Dis.* **176,** 807–810 (1997).
409. Shapiro, L., Pott, G. B. & Ralston, A. H. Alpha-1-antitrypsin inhibits human immunodeficiency virus type 1. *FASEB J.* **15,** 115–122 (2001).
410. Elmaleh, D. R., Brown, N. V. & Geiben-Lynn, R. Anti-viral activity of human antithrombin III. *Int. J. Mol. Med.* **16,** 191–200 (2005).
411. Geiben-Lynn, R., Brown, N., Walker, B. D. & Luster, A. D. Purification of a modified form of bovine antithrombin III as an HIV-1 CD8+ T-cell antiviral factor. *J. Biol. Chem.* **277,** 42352–42357 (2002).
412. Rivera, L. E., Colón, K., Cantres-Rosario, Y. M., Zenon, F. M. & Melendez, L. M. Macrophage derived cystatin B/cathepsin B in HIV replication and neuropathogenesis. *Curr. HIV Res.* **12,** 111–120 (2014).
413. Rivera-Rivera, L., Perez-Laspiur, J., Colón, K. & Meléndez, L. M. Inhibition of interferon response by cystatin B: implication in HIV replication of macrophage reservoirs. *J. Neurovirol.* **18,** 20–29 (2012).
414. Luciano-Montalvo, C., Ciborowski, P., Duan, F., Gendelman, H. E. & Meléndez, L. M. Proteomic analyses associate cystatin B with restricted HIV-1 replication in placental macrophages. *Placenta* **29,** 1016–1023 (2008).
415. Murray, J. M., Kelleher, A. D. & Cooper, D. A. Timing of the components of the HIV life cycle in productively infected CD4+ T cells in a population of HIV-infected individuals. *J. Virol.* **85,** 10798–10805 (2011).
416. Farnet, C. M. & Haseltine, W. A. Circularization of human immunodeficiency virus type 1 DNA in vitro. *J. Virol.* **65,** 6942–6952 (1991).
417. Romerio, F., Gabriel, M. N. & Margolis, D. M. Repression of human immunodeficiency virus type 1 through the novel cooperation of human factors YY1 and LSF. *J. Virol.* **71,** 9375 (1997).
418. Schwartz, S., Felber, B. K., Benko, D. M., Fenyö, E. M. & Pavlakis, G. N. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. *J. Virol.* **64,** 2519–2529 (1990).
419. Martin Stoltzfus, C. in **74,** 1–40 (Elsevier, 2009).

420. Miedema, F. *et al.* Immune Activation and Collateral Damage in AIDS Pathogenesis. *Front Immunol* **4,** 298 (2013).
421. Portales, P. *et al.* The intensity of immune activation is linked to the level of CCR5 expression in human immunodeficiency virus type 1-infected persons. *Immunology* **137,** 89–97 (2012).
422. Belouski, S. S. *et al.* Utility of lyophilized PMA and ionomycin to stimulate lymphocytes in whole blood for immunological assays. *Cytometry* **9999B,** NA–NA (2009).
423. Lock, P. A. *et al.* The Mitogen-Induced Increase in T Cell Size Involves PKC and NFAT Activation of Rel-NF-kB-Dependent c-myc Expression. (2004). at <http://arrow.latrobe.edu.au:8080/vital/access/manager/Repository/latrobe:31299>
424. Cummins, N. W. & Badley, A. D. Mechanisms of HIV-associated lymphocyte apoptosis: 2010. *Cell Death & Disease* **1,** e99 (2010).
425. Corasaniti, M. T. *et al.* Evidence that the HIV-1 coat protein gp120 causes neuronal apoptosis in the neocortex of rat via a mechanism involving CXCR4 chemokine receptor. *Neuroscience Letters* **312,** 67–70 (2001).
426. Muthumani, K. *et al.* Human immunodeficiency virus type 1 Nef induces programmed death 1 expression through a p38 mitogen-activated protein kinase-dependent mechanism. *J. Virol.* **82,** 11536–11544 (2008).
427. Greenway, A. L. *et al.* Human immunodeficiency virus type 1 Nef binds to tumor suppressor p53 and protects cells against p53-mediated apoptosis. *J. Virol.* **76,** 2692–2702 (2002).
428. Hanauske-Abel, H. M. *et al.* Drug-induced reactivation of apoptosis abrogates HIV-1 infection. *PLoS ONE* **8,** e74414 (2013).
429. Ghislat, G., Aguado, C. & Knecht, E. Annexin A5 stimulates autophagy and inhibits endocytosis. *J Cell Sci* **125,** 92–107 (2012).
430. Kim, J., Kundu, M., Viollet, B. & Guan, K.-L. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nature Cell Biology* **13,** 132–141 (2011).
431. Kedzierska, K. & Crowe, S. M. Cytokines and HIV-1: interactions and clinical implications. *Antivir Chem Chemother* **12,** 133–150 (2001).
432. Kornfeld, C. *et al.* Antiinflammatory profiles during primary SIV infection in African green monkeys are associated with protection against AIDS. *J. Clin. Invest.* **115,** 1082–1091 (2005).
433. Roberts, L. *et al.* Plasma cytokine levels during acute HIV-1 infection predict HIV disease progression. *AIDS* **24,** 819–831 (2010).
434. Mueller, Y. M. *et al.* IL-15 treatment during acute simian immunodeficiency virus (SIV) infection increases viral set point and accelerates disease progression despite the induction of stronger SIV-specific CD8+ T cell responses. *J. Immunol.* **180,** 350–360 (2008).
435. Deeks, S. G. *et al.* Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load. *Blood* **104,** 942–947 (2004).
436. Katsikis, P. D., Mueller, Y. M. & Villinger, F. The cytokine network of acute HIV infection: a promising target for vaccines and therapy to reduce viral set-point? *PLoS Pathog.* **7,** e1002055 (2011).

437. Sol, V. V.-D., Punzón, C. & Fresno, M. IFN-γ-induced TNF-α expression is regulated by interferon regulatory factors 1 and 8 in mouse macrophages. *The Journal of Immunology* (2008).
438. Kedzierska, K. *et al.* Granulocyte-macrophage colony-stimulating factor inhibits HIV-1 replication in monocyte-derived macrophages. *AIDS* **14,** 1739–1748 (2000).
439. Cai, G., Kastelein, R. A. & Hunter, C. A. IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-γ when combined with IL-18. *Eur. J. Immunol.* **29,** 2658–2665 (1999).
440. Lieberman, J. & Fan, Z. Nuclear war: the granzyme A-bomb. *Curr. Opin. Immunol.* **15,** 553–559 (2003).
441. Martinvalet, D., Zhu, P. & Lieberman, J. Granzyme A Induces Caspase-Independent Mitochondrial Damage, a Required First Step for Apoptosis. *Immunity* **22,** 355–370 (2005).
442. Martinvalet, D. & Thiery, J. A novel caspase-independent apoptotic pathway triggered by Granzyme A. *Med Sci (Paris)* **24,** 901–903 (2008).
443. Martinvalet, D., Dykxhoorn, D. M., Ferrini, R. & Lieberman, J. Granzyme A Cleaves a Mitochondrial Complex I Protein to Initiate Caspase-Independent Cell Death. *Cell* **133,** 681–692 (2008).
444. Voet, D., Voet, J. & Pratt, C. *Fundamentals of Biochemistry; Life at the Molecular Level 2nd*. (Hoboken: Wiley, 2006).
445. Badadani, M. Autophagy mechanism, regulation, functions, and disorders. *ISRN Cell Biology* (2012).
446. Moreau, K. *et al.* Transcriptional regulation of Annexin A2 promotes starvation-induced autophagy. *Nature Communications* **6,** 8045 (2015).
447. Towler, M. C. & Hardie, D. G. AMP-activated protein kinase in metabolic control and insulin signaling. *Circ. Res.* **100,** 328–341 (2007).
448. Anne-Claude Gingras, Brian Raught, A. & Sonenberg, N. eIF4 Initiation Factors: Effectors of mRNA Recruitment to Ribosomes and Regulators of Translation. *http://dx.doi.org/10.1146/annurev.biochem.68.1.913* **68,** 913–963 (2003).
449. Pyronnet, S. *et al.* Human eukaryotic translation initiation factor 4G (eIF4G) recruits Mnk1 to phosphorylate eIF4E. *The EMBO Journal* **18,** 270–279 (1999).
450. Khaleghpour, K., Pyronnet, S., Gingras, A. C. & Sonenberg, N. Translational homeostasis: eukaryotic translation initiation factor 4E control of 4E-binding protein 1 and p70 S6 kinase activities. *Mol. Cell. Biol.* **19,** 4302–4310 (1999).
451. Boratyńska, M. & Smolska, D. Inhibition of mTOR by sirolimus induces remission of post-transplant lymphoproliferative disorders. *Transplant International* **21,** 605–608 (2008).
452. Lebbe, C. *et al.* Sirolimus Conversion for Patients with Posttransplant Kaposi's Sarcoma. *American Journal of Transplantation* **6,** 2164–2168 (2006).
453. King, A. E., Morgan, K., Sallenave, J.-M. & Kelly, R. W. Differential regulation of secretory leukocyte protease inhibitor and elafin by progesterone. *Biochem. Biophys. Res. Commun.* **310,** 594–599 (2003).
454. Marsden, M. D. & Fournier, R. E. K. Chromosomal elements regulate gene activity and chromatin structure of the human serpin gene cluster at 14q32.1. *Mol. Cell. Biol.* **23,** 3516–3526 (2003).
455. Baxter, E. W., Cummings, W. J. & Fournier, R. E. K. Formation of a large, complex

domain of histone hyperacetylation at human 14q32.1 requires the serpin locus control region. *Nucl. Acids Res.* **33,** 3313–3322 (2005).
456. Blanpain, C. *et al.* CCR5 Binds Multiple CC-Chemokines: MCP-3 Acts as a Natural Antagonist. *Blood* **94,** 1899–1905 (1999).
457. Chiang, G. G. & Abraham, R. T. Phosphorylation of mammalian target of rapamycin (mTOR) at Ser-2448 is mediated by p70S6 kinase. *J. Biol. Chem.* **280,** 25485–25490 (2005).
458. Barth, S., Glick, D. & Macleod, K. F. Autophagy: assays and artifacts. *The Journal of Pathology* **221,** 117–124 (2010).
459. Soldani, C. *et al.* Poly(ADP-ribose) Polymerase Cleavage during Apoptosis: When and Where? *Experimental Cell Research* **269,** 193–201 (2001).
460. Yang, Y., Zhao, S. & Song, J. Caspase-dependent apoptosis and -independent poly(ADP-ribose) polymerase cleavage induced by transforming growth factor β1. *The International Journal of Biochemistry & Cell Biology* **36,** 223–234 (2004).
461. Briz, V., Poveda, E. & Soriano, V. HIV entry inhibitors: mechanisms of action and resistance pathways. *J. Antimicrob. Chemother.* **57,** 619–627 (2006).
462. Poveda, E., Briz, V. & Soriano, V. Enfuvirtide, the first fusion inhibitor to treat HIV infection. *AIDS Rev* **7,** 139–147 (2005).
463. Poveda, E., Barreiro, P., Rodés, B. & Soriano, V. Enfuvirtide is active against HIV type 1 group O. *AIDS Res. Hum. Retroviruses* **21,** 583–585 (2005).
464. Allaway, G. P. *et al.* Expression and characterization of CD4-IgG2, a novel heterotetramer that neutralizes primary HIV type 1 isolates. *AIDS Res. Hum. Retroviruses* **11,** 533–539 (1995).
465. Moore, J. P., Sattentau, Q. J., Klasse, P. J. & Burkly, L. C. A monoclonal antibody to CD4 domain 2 blocks soluble CD4-induced conformational changes in the envelope glycoproteins of human immunodeficiency virus type 1 (HIV-1) and HIV-1 infection of CD4+ cells. *J. Virol.* **66,** 4784–4793 (1992).
466. Vermeire, K. *et al.* CADA inhibits human immunodeficiency virus and human herpesvirus 7 replication by down-modulation of the cellular CD4 receptor. *Virology* **302,** 342–353 (2002).
467. Vermeire, K. *et al.* The Anti-HIV potency of cyclotriazadisulfonamide analogs is directly correlated with their ability to down-modulate the CD4 receptor. *Mol. Pharmacol.* **63,** 203–210 (2003).
468. Lin, P.-F. *et al.* A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding. *PNAS* **100,** 11013–11018 (2003).
469. Guo, Q. *et al.* Biochemical and genetic characterizations of a novel human immunodeficiency virus type 1 inhibitor that blocks gp120-CD4 interactions. *J. Virol.* **77,** 10528–10536 (2003).
470. Pancera, M., Druz, A., Zhou, T. & O'Dell, S. Structure of BMS-806, a small-molecule HIV-1 entry inhibitor, bound to BG505 SOSIP. 664 HIV-1 Env trimer. ... *human retroviruses* (2014).
471. Madani, N. *et al.* Localized changes in the gp120 envelope glycoprotein confer resistance to human immunodeficiency virus entry inhibitors BMS-806 and #155. *J. Virol.* **78,** 3742–3752 (2004).
472. Dragic, T. *et al.* A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. *PNAS* **97,** 5639–5644 (2000).

473. Lai, W.-H., Huang, L. & Chen, C.-H. HIV entry inhibitors: progress in development and application. *Yao Xue Xue Bao* **45,** 131–140 (2010).
474. Trkola, A. *et al.* Potent, broad-spectrum inhibition of human immunodeficiency virus type 1 by the CCR5 monoclonal antibody PRO 140. *J. Virol.* **75,** 579–588 (2001).
475. Tsamis, F. *et al.* Analysis of the mechanism by which the small-molecule CCR5 antagonists SCH-351125 and SCH-350581 inhibit human immunodeficiency virus type 1 entry. *J. Virol.* **77,** 5201–5208 (2003).
476. Castonguay, L. A. *et al.* Binding of 2-aryl-4-(piperidin-1-yl)butanamines and 1,3,4-trisubstituted pyrrolidines to human CCR5: a molecular modeling-guided mutagenesis study of the binding pocket. *Biochemistry* **42,** 1544–1550 (2003).
477. Sax, P. E. FDA approval: maraviroc. *AIDS clinical care* **19,** 75 (2007).
478. Sax, P. E. Maraviroc for treatment-naive patients with HIV-1 infection: is the glass half empty or half full? *J. Infect. Dis.* **201,** 797–799 (2010).
479. Stone, N. D. *et al.* Multiple-dose escalation study of the safety, pharmacokinetics, and biologic activity of oral AMD070, a selective CXCR4 receptor inhibitor, in human subjects. *Antimicrob. Agents Chemother.* **51,** 2351–2358 (2007).
480. Ichiyama, K. *et al.* A duodenally absorbable CXC chemokine receptor 4 antagonist, KRH-1636, exhibits a potent and selective anti-HIV-1 activity. *PNAS* **100,** 4185–4190 (2003).
481. Johnson, V. A., Brun-Vézinet, F., Clotet, B. & Gunthard, H. F. Update of the drug resistance mutations in HIV-1: December 2009. *Top HIV Med* (2009).
482. Singh, K. *et al.* Biochemical mechanism of HIV-1 resistance to rilpivirine. *J. Biol. Chem.* **287,** 38110–38123 (2012).
483. Feng, M. *et al.* In vitro resistance selection with doravirine (MK-1439), a novel nonnucleoside reverse transcriptase inhibitor with distinct mutation development pathways. *Antimicrob. Agents Chemother.* **59,** 590–598 (2015).
484. Sarafianos, S. G. *et al.* Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with beta-branched amino acids. *PNAS* **96,** 10027–10032 (1999).
485. Gao, H.-Q., Boyer, P. L., Sarafianos, S. G., Arnold, E. & Hughes, S. H. The role of steric hindrance in 3TC resistance of human immunodeficiency virus type-1 reverse transcriptase. *J. Mol. Biol.* **300,** 403–418 (2000).
486. Matsumi, S. *et al.* Pathways for the emergence of multi-dideoxynucleoside-resistant HIV-1 variants. *AIDS* **17,** 1127 (2003).
487. Shafer, R. W. *et al.* Combination Therapy with Zidovudine and Didanosine Selects for Drug-Resistant Human Immunodeficiency Virus Type 1 Strains with Unique Patterns of pol Gene Mutations. *J. Infect. Dis.* **169,** 722–729 (1994).
488. Maeda, Y., Venzon, D. J. & Mitsuya, H. Altered Drug Sensitivity, Fitness, and Evolution of Human Immunodeficiency Virus Type 1 with pol Gene Mutations Conferring Multi-Dideoxynucleoside Resistance. *J. Infect. Dis.* **177,** 1207–1213 (1998).
489. Meyer, P. R., Matsuura, S. E., Mian, A. M., So, A. G. & Scott, W. A. A Mechanism of AZT Resistance. *Molecular Cell* **4,** 35–43 (1999).
490. Brenner, B. G. *et al.* HIV-1 subtype C viruses rapidly develop K65R resistance to tenofovir in cell culture. *AIDS* **20,** F9–F13 (2006).

491. Evering, T. H. & Markowitz, M. *HIV-1 Integrase Inhibitors*. (The PRN Notebook, 2008).
492. Evering, T. H. & Markowitz, M. Raltegravir: an integrase inhibitor for HIV-1. *Expert Opinion on Investigational Drugs* **17,** 413–422 (2008).
493. Matsuzaki, Y., Watanabe, W., Yamataka, K. & Sato, M. *JTK-303/GS 9137, a novel small-molecule inhibitor of HIV-1 integrase: anti-HIV activity profile and pharmacokinetics in animals*. (13th Conference on …, 2006).
494. Fantauzzi, A. & Mezzaroma, I. Dolutegravir: clinical efficacy and role in HIV therapy. *Therapeutic Advances in Chronic Disease* **5,** 2040622314530461–177 (2014).
495. Brower, E. T., Bacha, U. M., Kawasaki, Y. & Freire, E. Inhibition of HIV-2 Protease by HIV-1 Protease Inhibitors in Clinical Use. *Chemical Biology & Drug Design* **71,** 298–305 (2008).
496. McCoy, C. Darunavir: A Nonpeptidic Antiretroviral Protease Inhibitor. *Clinical Therapeutics* **29,** 1559–1576 (2007).
497. Schmit, J.-C. *et al.* Resistance-related mutations in the HIV-1 protease gene of patients treated for 1 year with the protease inhibitor ritonavir (ABT-538). *AIDS* **10,** 995 (1996).
498. Rhee, S.-Y. *et al.* HIV-1 protease mutations and protease inhibitor cross-resistance. *Antimicrob. Agents Chemother.* **54,** 4253–4261 (2010).
499. Ohtaka, H., Velázquez Campoy, A., Xie, D. & Freire, E. Overcoming drug resistance in HIV-1 chemotherapy: The binding thermodynamics of Amprenavir and TMC-126 to wild-type and drug-resistant mutants of the HIV-1 protease. *Protein Science* **11,** 1908–1916 (2002).
500. Larder, B. A. *et al.* Tipranavir inhibits broadly protease inhibitor-resistant HIV-1 clinical samples. *AIDS* **14,** 1943 (2000).
501. Lefebvre, E. & Schiffer, C. A. Resilience to resistance of HIV-1 protease inhibitors: profile of darunavir. *AIDS Rev* **10,** 131–142 (2008).
502. Poli, G., Kinter, A. L. & Fauci, A. S. Interleukin 1 induces expression of the human immunodeficiency virus alone and in synergy with interleukin 6 in chronically infected U1 cells: inhibition of inductive effects by the interleukin 1 receptor antagonist. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 108–112 (1994).
503. Smith, K. A. Interleukin-2: inception, impact, and implications. *Science* **240,** 1169–1176 (1988).
504. Fan, J., Li, P., Kok, T. W. & Burrell, C. J. AZT blocks down-regulation of IL-2 and IFN-gamma gene expression in HIV acutely infected cells. *Arch. Virol.* **142,** 1035–1043 (1997).
505. Hu, R., Oyaizu, N., Kalyanaraman, V. S. & Pahwa, S. HIV-1 gp160 as a modifier of Th1 and Th2 cytokine response: gp160 suppresses interferon-gamma and interleukin-2 production concomitantly with enhanced interleukin-4 production in vitro. *Clin. Immunol. Immunopathol.* **73,** 245–251 (1994).
506. Kinter, A. & Fauci, A. S. Interleukin-2 and human immunodeficiency virus infection: pathogenic mechanisms and potential for immunologic enhancement. *Immunol. Res.* **15,** 1–15 (1996).
507. Hong, M. A. *et al.* IL-2 and IFN-gamma, but not IL-4 secretion by peripheral blood mononuclear cells (PBMC) are related to CD4+ T cells and clinical status in

Brazilian HIV-1-infected subjects. *Rev. Inst. Med. Trop. Sao Paulo* **40,** 351–354 (1998).
508. Al-Harthi, L., Roebuck, K. A. & Landay, A. Induction of HIV-1 replication by type 1-like cytokines, interleukin (IL)-12 and IL-15: effect on viral transcriptional activation, cellular proliferation, and endogenous cytokine production. *J. Clin. Immunol.* **18,** 124–131 (1998).
509. Tuosto, L. *et al.* Ligation of either CD2 or CD28 rescues CD4+ T cells from HIV-gp120-induced apoptosis. *Eur. J. Immunol.* **25,** 2917–2922 (1995).
510. Emery, S. *et al.* Pooled analysis of 3 randomized, controlled trials of interleukin-2 therapy in adult human immunodeficiency virus type 1 disease. *J. Infect. Dis.* **182,** 428–434 (2000).
511. Barouch, D. H. *et al.* Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* **290,** 486–492 (2000).
512. Paul, W. E. Interleukin-4: a prototypic immunoregulatory lymphokine. *Blood* **77,** 1859–1870 (1991).
513. Imami, N., Antonopoulos, C., Hardy, G. A., Gazzard, B. & Gotch, F. M. Assessment of type 1 and type 2 cytokines in HIV type 1-infected individuals: impact of highly active antiretroviral therapy. *AIDS Res. Hum. Retroviruses* **15,** 1499–1508 (1999).
514. Novak, R. M., Holzer, T. J., Kennedy, M. M., Heynen, C. A. & Dawson, G. The effect of interleukin 4 (BSF-1) on infection of peripheral blood monocyte-derived macrophages with HIV-1. *AIDS Res. Hum. Retroviruses* **6,** 973–976 (1990).
515. Rincón, M., Anguita, J., Nakamura, T., Fikrig, E. & Flavell, R. A. Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4+ T cells. *J. Exp. Med.* **185,** 461–469 (1997).
516. Birx, D. L. *et al.* Induction of interleukin-6 during human immunodeficiency virus infection. *Blood* **76,** 2303–2310 (1990).
517. Baqui, A. A. *et al.* Enhanced interleukin-1beta, interleukin-6 and tumor necrosis factor-alpha production by LPS stimulated human monocytes isolated from HIV+ patients. *Immunopharmacol Immunotoxicol* **22,** 401–421 (2000).
518. Namen, A. E. *et al.* B cell precursor growth-promoting activity. Purification and characterization of a growth factor active on lymphocyte precursors. *J. Exp. Med.* **167,** 988–1002 (1988).
519. Conlon, P. J. *et al.* Murine thymocytes proliferate in direct response to interleukin-7. *Blood* **74,** 1368–1373 (1989).
520. He, Y. W. & Malek, T. R. Interleukin-7 receptor alpha is essential for the development of gamma delta + T cells, but not natural killer cells. *J. Exp. Med.* **184,** 289–293 (1996).
521. Fry, T. J. & Mackall, C. L. Interleukin-7: from bench to clinic. *Blood* **99,** 3892–3904 (2002).
522. Moran, P. A., Diegel, M. L., Sias, J. C., Ledbetter, J. A. & Zarling, J. M. Regulation of HIV production by blood mononuclear cells from HIV-infected donors: I. Lack of correlation between HIV-1 production and T cell activation. *AIDS Res. Hum. Retroviruses* **9,** 455–464 (1993).
523. Smithgall, M. D., Wong, J. G., Critchett, K. E. & Haffar, O. K. IL-7 up-regulates HIV-1 replication in naturally infected peripheral blood mononuclear cells. *J. Immunol.* **156,** 2324–2330 (1996).

524. Ferrari, G. *et al.* IL-7 enhancement of antigen-driven activation/expansion of HIV-1-specific cytotoxic T lymphocyte precursors (CTLp). *Clin. Exp. Immunol.* **101,** 239–248 (1995).
525. Olivier, A. J. *et al.* Distinct cytokine patterns in semen influence local HIV shedding and HIV target cell activation. *J. Infect. Dis.* **209,** 1174–1184 (2014).
526. Harada, A. *et al.* Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J. Leukoc. Biol.* **56,** 559–564 (1994).
527. Matsumoto, T. *et al.* Elevated serum levels of IL-8 in patients with HIV infection. *Clin. Exp. Immunol.* **93,** 149–151 (1993).
528. Esser, R., Glienke, W., Briesen, von, H., Rübsamen-Waigmann, H. & Andreesen, R. Differential regulation of proinflammatory and hematopoietic cytokines in human macrophages after infection with human immunodeficiency virus. *Blood* **88,** 3474–3481 (1996).
529. Esser, R. *et al.* Secretory repertoire of HIV-infected human monocytes/macrophages. *Pathobiology* **59,** 219–222 (1991).
530. Denis, M. & Ghadirian, E. Dysregulation of interleukin 8, interleukin 10, and interleukin 12 release by alveolar macrophages from HIV type 1-infected subjects. *AIDS Res. Hum. Retroviruses* **10,** 1619–1627 (1994).
531. Weissman, D., Poli, G. & Fauci, A. S. Interleukin 10 blocks HIV replication in macrophages by inhibiting the autocrine loop of tumor necrosis factor alpha and interleukin 6 induction of virus. *AIDS Res. Hum. Retroviruses* **10,** 1199–1206 (1994).
532. Trinchieri, G. & Scott, P. Interleukin-12: basic principles and clinical applications. *Curr. Top. Microbiol. Immunol.* **238,** 57–78 (1999).
533. Marshall, J. D. *et al.* The interleukin-12-mediated pathway of immune events is dysfunctional in human immunodeficiency virus-infected individuals. *Blood* **94,** 1003–1011 (1999).
534. Akridge, R. E. & Reed, S. G. Interleukin-12 decreases human immunodeficiency virus type 1 replication in human macrophage cultures reconstituted with autologous peripheral blood mononuclear cells. *J. Infect. Dis.* **173,** 559–564 (1996).
535. de Vries, J. E. The role of IL-13 and its receptor in allergy and inflammatory responses. *J. Allergy Clin. Immunol.* **102,** 165–169 (1998).
536. Keegan, A. D. IL-4 and IL-13: From 'supe' to nuts. *Cytokine* **75,** 1–2 (2015).
537. Wang, T. & Secombes, C. J. The evolution of IL-4 and IL-13 and their receptor subunits. *Cytokine* **75,** 8–13 (2015).
538. Bailer, R. T. *et al.* IL-13 and IFN-gamma secretion by activated T cells in HIV-1 infection associated with viral suppression and a lack of disease progression. *J. Immunol.* **162,** 7534–7542 (1999).
539. Bailer, R. T., Lee, B. & Montaner, L. J. IL-13 and TNF-alpha inhibit dual-tropic HIV-1 in primary macrophages by reduction of surface expression of CD4, chemokine receptors CCR5, CXCR4 and post-entry viral gene expression. *Eur. J. Immunol.* **30,** 1340–1349 (2000).
540. Lodolce, J. P., Burkett, P. R., Koka, R. M., Boone, D. L. & Ma, A. Regulation of lymphoid homeostasis by interleukin-15. *Cytokine & Growth Factor Reviews* **13,** 429–439 (2002).
541. and, T. A. W. & Tagaya, Y. THE MULTIFACETED REGULATION OF

INTERLEUKIN-15 EXPRESSION AND THE ROLE OF THIS CYTOKINE IN NK CELL DIFFERENTIATION AND HOST RESPONSE TO INTRACELLULAR PATHOGENS1. *http://dx.doi.org/10.1146/annurev.immunol.17.1.19* **17,** 19–49 (2003).
542. Mueller, Y. M. *et al.* IL-15 enhances survival and function of HIV-specific CD8+ T cells. *Blood* **101,** 1024–1029 (2003).
543. Mueller, Y. M. *et al.* Interleukin-15 increases effector memory CD8+ t cells and NK Cells in simian immunodeficiency virus-infected macaques. *J. Virol.* **79,** 4877–4885 (2005).
544. Eberly, M. D. *et al.* Increased IL-15 production is associated with higher susceptibility of memory CD4 T cells to simian immunodeficiency virus during acute infection. *J. Immunol.* **182,** 1439–1448 (2009).
545. Maciaszek, J. W. *et al.* IL-16 represses HIV-1 promoter activity. *J. Immunol.* **158,** 5–8 (1997).
546. Amiel, C. *et al.* Interleukin-16 (IL-16) inhibits human immunodeficiency virus replication in cells from infected subjects, and serum IL-16 levels drop with disease progression. *J. Infect. Dis.* **179,** 83–91 (1999).
547. Kolls, J. K. & Lindén, A. Interleukin-17 Family Members and Inflammation. *Immunity* **21,** 467–476 (2004).
548. Chung, D. R. *et al.* CD4+ T cells mediate abscess formation in intra-abdominal sepsis by an IL-17-dependent mechanism. *J. Immunol.* **170,** 1958–1963 (2003).
549. Huang, W., Na, L., Fidel, P. L. & Schwarzenberger, P. Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. *J. Infect. Dis.* **190,** 624–631 (2004).
550. Maek-A-Nantawat, W., Buranapraditkun, S., Klaewsongkram, J. & Ruxrungthum, K. Increased Interleukin-17 Production Both in Helper T Cell Subset Th17 And CD4-Negative T Cells in Human Immunodeficiency Virus Infection. *http://dx.doi.org/10.1089/vim.2006.0063* **20,** 66–75 (2007).
551. Johnson, T. P. *et al.* Induction of IL-17 and nonclassical T-cell activation by HIV-Tat protein. *Proc. Natl. Acad. Sci. U.S.A.* **110,** 13588–13593 (2013).
552. Kim, C. J. *et al.* Mucosal Th17 cell function is altered during HIV infection and is an independent predictor of systemic immune activation. *J. Immunol.* **191,** 2164–2173 (2013).
553. Dufour, J. H. *et al.* IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *J. Immunol.* **168,** 3195–3204 (2002).
554. Simmons, R. P. *et al.* HIV-1 infection induces strong production of IP-10 through TLR7/9-dependent pathways. *AIDS* **27,** 2505–2517 (2013).
555. Ramirez, L. A. *et al.* High IP-10 levels decrease T cell function in HIV-1-infected individuals on ART. *J. Leukoc. Biol.* **96,** 1055–1063 (2014).
556. Lane, B. R. *et al.* The C-X-C chemokine IP-10 stimulates HIV-1 replication. *Virology* **307,** 122–134 (2003).
557. Rho, M. B. *et al.* A potential role for interferon-alpha in the pathogenesis of HIV-associated dementia. *Brain Behav. Immun.* **9,** 366–377 (1995).
558. Gessani, S. *et al.* Induction of cytokines by HIV-1 and its gp120 protein in human peripheral blood monocyte/macrophages and modulation of cytokine response

during differentiation. *J. Leukoc. Biol.* **62,** 49–53 (1997).
559. Gessani, S. *et al.* Role of endogenous interferon-beta in the restriction of HIV replication in human monocyte/macrophages. *J. Leukoc. Biol.* **56,** 358–361 (1994).
560. Gessani, S. *et al.* Role of interferons in the restriction of HIV replication in human monocytes/macrophages. *Res. Immunol.* **145,** 659–62– discussion 662–3 (1994).
561. Fan, S. X., Turpin, J. A., Aronovitz, J. R. & Meltzer, M. S. Interferon-gamma protects primary monocytes against infection with human immunodeficiency virus type 1. *J. Leukoc. Biol.* **56,** 362–368 (1994).
562. Han, X., Becker, K., Degen, H. J., Jablonowski, H. & Strohmeyer, G. Synergistic stimulatory effects of tumour necrosis factor alpha and interferon gamma on replication of human immunodeficiency virus type 1 and on apoptosis of HIV-1-infected host cells. *Eur. J. Clin. Invest.* **26,** 286–292 (1996).
563. Armitage, J. O. Emerging applications of recombinant human granulocyte-macrophage colony-stimulating factor. *Blood* **92,** 4491–4508 (1998).
564. Esser, R. *et al.* Individual cell analysis of the cytokine repertoire in human immunodeficiency virus-1-infected monocytes/macrophages by a combination of immunocytochemistry and in situ hybridization. *Blood* **91,** 4752–4760 (1998).
565. Kutza, J. *et al.* Macrophage colony-stimulating factor antagonists inhibit replication of HIV-1 in human macrophages. *J. Immunol.* **164,** 4955–4960 (2000).
566. Wang, J., Roderiquez, G., Oravecz, T. & Norcross, M. A. Cytokine regulation of human immunodeficiency virus type 1 entry and replication in human monocytes/macrophages through modulation of CCR5 expression. *J. Virol.* **72,** 7642–7647 (1998).
567. Vyakarnam, A., McKeating, J., Meager, A. & Beverley, P. C. Tumour necrosis factors (alpha, beta) induced by HIV-1 in peripheral blood mononuclear cells potentiate virus replication. *AIDS* **4,** 21–27 (1990).
568. Muñoz-Fernández, M. A. *et al.* Immunological and virological markers of disease progression in HIV-infected children. *Acta Paediatr Suppl* **421,** 46–51 (1997).
569. Folks, T. M., Justement, J., Kinter, A., Dinarello, C. A. & Fauci, A. S. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. *Science* **238,** 800–802 (1987).
570. Kumar, A., Abbas, W. & Herbein, G. TNF and TNF receptor superfamily members in HIV infection: new cellular targets for therapy? *Mediators of Inflammation* **2013,** 484378–13 (2013).
571. Herbein, G., Montaner, L. J. & Gordon, S. Tumor necrosis factor alpha inhibits entry of human immunodeficiency virus type 1 into primary human macrophages: a selective role for the 75-kilodalton receptor. *J. Virol.* **70,** 7388–7397 (1996).
572. Rollins, B. J. Chemokines. *Blood* **90,** 909–928 (1997).
573. Greco, G., Fujimura, S. H., Mourich, D. V. & Levy, J. A. Differential effects of human immunodeficiency virus isolates on beta-chemokine and gamma interferon production and on cell proliferation. *J. Virol.* **73,** 1528–1534 (1999).
574. Schmidtmayerova, H., Sherry, B. & Bukrinsky, M. Chemokines and HIV replication. *Nature* **382,** 767–767 (1996).
575. Cocchi, F. *et al.* Higher macrophage inflammatory protein (MIP)-1alpha and MIP-1beta levels from CD8+ T cells are associated with asymptomatic HIV-1 infection. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 13812–13817 (2000).

576. Coffey, M. J., Woffendin, C., Phare, S. M., Strieter, R. M. & Markovitz, D. M. RANTES inhibits HIV-1 replication in human peripheral blood monocytes and alveolar macrophages. *Am. J. Physiol.* **272,** L1025–9 (1997).
577. Cocchi, F. *et al.* Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science* **270,** 1811–1815 (1995).
578. Capobianchi, M. R. *et al.* Inhibition of HIV type 1 BaL replication by MIP-1alpha, MIP-1beta, and RANTES in macrophages. *AIDS Res. Hum. Retroviruses* **14,** 233–240 (1998).
579. Jiang, Y. & Jolly, P. E. Effect of beta-chemokines on human immunodeficiency virus type 1 replication, binding, uncoating, and CCR5 receptor expression in human monocyte-derived macrophages. *J. Hum. Virol.* **2,** 123–132 (1999).
580. Hieshima, K. *et al.* Molecular Cloning of a Novel Human CC Chemokine Liver and Activation-regulated Chemokine (LARC) Expressed in Liver CHEMOTACTIC ACTIVITY FOR LYMPHOCYTES AND GENE LOCALIZATION ON CHROMOSOME 2. *J. Biol. Chem.* **272,** 5846–5853 (1997).
581. Ghosh, M. *et al.* ORIGINAL ARTICLE: CCL20/MIP3α is a Novel Anti-HIV-1 Molecule of the Human Female Reproductive Tract. *American Journal of Reproductive Immunology* **62,** 60–71 (2009).
582. Deshmane, S. L., Kremlev, S., Amini, S. & Sawaya, B. E. Monocyte Chemoattractant Protein-1 (MCP-1): An Overview. *http://dx.doi.org/10.1089/jir.2008.0027* **29,** 313–326 (2010).
583. Conant, K. *et al.* Induction of monocyte chemoattractant protein-1 in HIV-1 Tat-stimulated astrocytes and elevation in AIDS dementia. *PNAS* **95,** 3117–3121 (1998).
584. Chang, L., Ernst, T., Hillaire, C. S. & Conant, K. Antiretroviral treatment alters relationship between MCP-1 and neurometabolites in HIV patients. *Antivir. Ther. (Lond.)* (2004).
585. JW, H. *et al.* Inhibition of morphine-potentiated HIV-1 replication in peripheral blood mononuclear cells with the nuclease-resistant 2-5A agonist analog, 2-5A(N6B). *JAIDS Journal of Acquired Immune Deficiency Syndromes* **30,** 9–20 (2002).
586. Schols, D., Proost, P., Van Damme, J. & De Clercq, E. RANTES and MCP-3 inhibit the replication of T-cell-tropic human immunodeficiency virus type 1 strains (SF-2, MN, and HE). *J. Virol.* **71,** 7300–7304 (1997).
587. Lambert, J. S. *et al.* Production of the HIV-Suppressive Chemokines CCL3/MIP-1α and CCL22/MDC Is Associated with More Effective Antiretroviral Therapy in HIV-Infected Children. *The Pediatric Infectious Disease Journal* **26,** 935–944 (2007).
588. Yoneda, O. *et al.* Membrane-bound form of fractalkine induces IFN-γ production by NK cells. *Eur. J. Immunol.* **33,** 53–58 (2003).
589. Suzuki, M. *et al.* Fractalkine/CX3CL1 protects striatal neurons from synergistic morphine and HIV-1 Tat-induced dendritic losses and death. *Mol Neurodegeneration* **6,** 1–18 (2011).
590. Lane, B. R., Strieter, R. M., Coffey, M. J. & Markovitz, D. M. Human immunodeficiency virus type 1 (HIV-1)-induced GRO-alpha production stimulates HIV-1 replication in macrophages and T lymphocytes. *J. Virol.* **75,** 5812–5822 (2001).

591. Poli, G. *et al.* Transforming growth factor beta suppresses human immunodeficiency virus expression and replication in infected cells of the monocyte/macrophage lineage. *J. Exp. Med.* **173,** 589–597 (1991).
592. Reinhold, D., Wrenger, S. & Kähne, T. *HIV-1 Tat: immunosuppression via TGF-β1 induction*. (Immunology …, 1999).
593. Garba, M. L., Pilcher, C. D., Bingham, A. L., Eron, J. & Frelinger, J. A. HIV antigens can induce TGF-beta(1)-producing immunoregulatory CD8+ T cells. *J. Immunol.* **168,** 2247–2254 (2002).

CHAPTER 11: APPENDIX

11.1 Abbreviations

A2ML1	Alpha-2-Macroglobulin-Like protein I
Ab	Antibody
ADCC	Antibody-Dependent Cell Cytotoxicity
AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen Presenting Cell
ARVs	Antiretrovirals
cFLIP	FADD-Like IL-1β-converting enzyme-inhibitory Protein
CTLs	Cytotoxic T Lymphocytes
CR1	type 1 Complement Receptor
CVL	Cervico-Vaginal Lavage
DCs	Dendritic Cells
DMPA	Depot Medroxyprogesterone Acetate
EIF	Eukaryotic Initiation Factor
FADD	FAS-Associated Death Domain protein
FGT	Female Genital Tract
FSH	Follicle-Stimulating Hormone
FSW	Female Sex Worker
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HESN	HIV Exposed Sero-Negative
HIV	Human Immunodeficiency Virus
Ig	Immunoglobulin
IL-	Interleukin
IN	Integrase
IFN	Interferon
IRF1	Interferon Regulatory Factor I
iTreg	induced T regulatory cell
KIRs	Killer cell Immunoglobulin-like Receptors
LCs	Langerhans Cells
LH	Luteinizing Hormone
LEI	Leukocyte Elastase Inhibitor
LTR	Long Terminal Repeat
MAC	Membrane Attack Complex
MALT	Mucosal-Associated Lymphoid Tissue
MCP	Monocyte Chemoattractant Protein
MDG	Millennium Development Goals
M-GSF	Macrophage Colony-Stimulating Factor
MIP	Macrophage Inflammatory Protein
MNEI	Monocyte/Neutrophil Elastase Inhibitor
MPA	Medroxyprogesterone Acetate
MPT	Multipupose Prevention Technology
MSM	Men who have Sex with Men
mTOR	Mammalian Target of Rapamycin
MyD88	Myeloid differentiation primary-response gene 88

NCR	Natural Cytotoxicity Receptor
Nef	Negative Factor
NET	Nuetrophil Extracellular Trap
NF-Kβ	Nuclear Factor Kβ
NK cells	Natural Killer Cells
NLRs	Nod-Like Receptors
NNRTIs	Non-Nucleoside Reverse Transcriptase Inhibitors
NO	Nitric Oxide
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
NSP	Neutrophil Serine Proteases
PAMPs	Pattern Recognition Receptors
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PHAC	Public Health Agency of Canada
PIs	Protease Inhibitors
PMNs	Polymorphonuclear cells (Neutrophils)
PR	Protease (HIV)
PRRs	Pattern Recognition Receptors
PrEP	Pre-Exposure Prophylaxis
RA	Retinoic Acid
RANTES	Regulated upon Activation Normal T cell Expressed and Secreted
RCL	Reactive Centre Loop
Rev	Regulator of Expression of Virion proteins
ROS	Reactive Oxygen Species
RRE	Rev-Responsive Element
RT	Reverse Transcriptase
SB1	Serpin B1
SERPIN	Serine Protease Inhibitor
SLPI	Secretory Leukocyte Protease Inhibitor
SNPs	Single Nucleotide Polymorphisms
STAT	Signal Transducer and Activator of Transcription
STIs	Sexually Transmitted Infections
Tat	Trans-Activator of Transcription
TAR	Transactivation-Response Region
Tfh	Follicular Helper T cell
TGF-β	Transforming Growth Factor Beta
T_H	T Helper cell
TLRs	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor alpha
TNFR	Tumor Necrosis Factor Receptor
TLRs	Toll-Like Receptors
TRADD	TNFR1-Associated Death Domain protein
uNK	Uterine Natural Killer cell
Vif	Virion Infectivity Factor
Vpr	Viral Protein r
Vpu	Viral Protein u
WHO	World Health Organization

11.2 Description of current HIV inhibitors

Table 13: Classification and description of anti-HIV mechanisms for current ARV agents

Name of HIV Inhibitor	Classification of Inhibitor	Mechanism of Action	Stage of Development	Efficiency of anti-HIV activity / Presence of Resistance
Enfuvirtide	Fusion Inhibitor[461]	Prevents fusion of HIV and cellular membranes by mimicking an HR2 fragment of gp41, thereby blocking formation of the critical six-helix bundle structure required for fusion[462]	Approved for clinical use[462]	Yes, has been shown to efficiently inhibit M and O HIV viruses[463].
PRO-542 (CD4-IgG2)	gp120-CD4 binding inhibitor[464]	Mimics the CD4 receptor thereby interfering with binding of gp120 to real CD4[464]	Has undergone Phase I and II clinical trials and has been examined in combination with Enfuvirtide in phase I and II trials.	Most efficient in combination with Enfuvirtide
TNX-355 (Ibalizumab)	gp120-CD4 binding inhibitor[465]	Monoclonal Ab specific for CD4. Competes with gp120 for CD4 binding[465]	Has completed Phase I and II in HIV infected and uninfected individuals. Is now in Phase III trials (clinicaltrials.gov last verified June 2015, obtained Oct.5, 2015).	Yes, however requires administration through infusion which limits its use in a clinical setting
CADA (Cyclotriazadi-sulfonamide)	gp120-CD4 binding inhibitor[466]	Down-regulates post-translational expression of CD4[467]	Has not been tested in clinical trials to date.	Exhibited efficiency *in vitro* only.
BMS-806	gp120-CD4 binding inhibitor[468]	Binds HIV-1 gp120 and prevents the necessary conformational changes in this protein for CD4 binding to occur[468,469]	In pre-clinical trials[470]	No, resistance has been identified[471]
TAK-779	CCR5 antagonist[461]	Binds to CCR5 and blocks subsequent HIV co-receptor binding [472]	Phase II discontinued[473]	Caused local reactions at the site of injection so was discontinued however led to the identification of TAK-220 which shares a similar mechanism with TAK-779
PRO-140	CCR5 antagonist[461]	Monoclonal Ab specific for CCR5 thereby inhibiting gp120 binding[474]. Inhibits HIV-1 subtypes A, C, B, E and F[474]	Currently in phase II clinical trials (aidsinfo.nih.gov last updated June 2015, obtained October 5, 2015)	Efficient at inhibiting R5 HIV viruses
Vicriviroc	CCR5 antagonist[461]	Bind directly to CCR5 transmembrane portion, thereby inhibiting gp120 binding[475]	As of July 2010 Merck haulted all development on vicriviroc due to unimpressive phase I and II trial results. Trials are ongoing in combination with other anti-HIV agents in an effort to increase efficacy (aidsinfo.nih.gov last updated June 2015, obtained October 5,	Alone does not significantly suppress HIV replication in clinical trials.

			2015)	
Maravirok	CCR5 antagonist[461]	Bind directly to CCR5 transmembrane portion, thereby inhibiting gp120 binding[476].	Available for clinical use. FDA approved and produced by Pfizer[477]	Yes, however only against R5-viruses. It is the first and only CCR5-antagonist available for clinical use[478]
AMD070	CXCR4 antagonist[461]	Binds directly to CXCR4, preventing binding of X-tropic viruses to host cells[461]	Has undergone phase I and II clinical trials. Caused increased white blood cell count and tachycardia in participants of one study[479]	Efficiently inhibits X4 HIV viruses.
KRH-1636	CXCR4 antagonist[461]	Binds directly to CXCR4, preventing binding of X-tropic viruses to host cells[480]	Not in clinical trials	Has only demonstrated efficiency *in vitro*[461].
KRH-2731	CXCR4 antagonist[461]	Binds directly to CXCR4, preventing binding of X-tropic viruses to host cells[461]	Not in clinical trials	Has only demonstrated efficiency *in vitro* and in rat studies[461].
Efavirenz	NNRTI	Bind to RT and prevent proper reverse transcription of HIV RNA to DNA[265]	Available for clinical use	Resistance detected in HIV RT, preventing proper binding of NNRTI to RT[481].
Rilpivirine	NNRTI	Bind to RT and prevent proper reverse transcription of HIV RNA to DNA[265]	Available for clinical use	Resistance detected in HIV RT, preventing proper binding of NNRTI to RT[482].
Etravirine	NNRTI	Bind to RT and prevent proper reverse transcription of HIV RNA to DNA[265]	Available for clinical use	Resistance detected in HIV RT, preventing proper binding of NNRTI to RT[481]
Delavirdine	NNRTI	Bind to RT and prevent proper reverse transcription of HIV RNA to DNA[265]	Available for clinical use	Resistance has not yet been detected/recorded.
Nevirapine	NNRTI	Bind to RT and prevent proper reverse transcription of HIV RNA to DNA[265]	Available for clinical use	Resistance detected in HIV RT, preventing proper binding of NNRTI to RT [481]
Doravirine	NNRTI	Bind to RT and prevent proper reverse transcription of HIV RNA to DNA[265].	Currently in Phase IIb clinical trials[483].	Resistance has not yet been detected/recorded.
Lamivudine	NRTI	Incorporates into the growing HIV DNA chain however lacks a 3'-hydroxyl group thus inhibiting elongation of DNA strand[265]. Cytidine analog.	Available for clinical use	Resistance detected in HIV RT[484] Resistance detected in HIV pol gene[485,486]
Zidovudine (AZT)	NRTI	Incorporates into the growing HIV DNA chain however lacks a 3'-hydroxyl group thus inhibiting elongation of DNA strand [265]. Thymidine analog.	Available for clinical use	Resistance detected in HIV pol gene[486-488]. Excision enhancement mutations have been detected which prevents AZT from blocking DNA elongation[489]
Didanosine	NRTI	Incorporates into the growing HIV DNA chain however lacks a 3'-hydroxyl group thus inhibiting elongation of DNA strand[265]. Adenosine analog.	Available for clinical use	Resistance detected in HIV pol gene[486-488]
Stavudine	NRTI	Incorporates into the growing HIV DNA chain however lacks a 3'-hydroxyl group thus inhibiting elongation of DNA strand [265]. Thymidine analog.	Available for clinical use	Resistance detected in HIV pol gene[486-488].
Emtricitabine	NRTI	Incorporates into the growing HIV DNA chain however lacks a 3'-	Available for clinical use	Resistance detected in HIV pol gene[485,486]

		hydroxyl group thus inhibiting elongation of DNA strand[265]. Cytidine analog.		
Abacavir	NtRTI	Incorporates into the growing HIV DNA chain however lacks a 3'-hydroxyl group thus inhibiting elongation of DNA strand[265]. Guanosine analog.	Available for clinical use	Resistance detected in HIV pol gene[486-488]
Tenofovir	NtRTI	Incorporates into the growing HIV DNA chain however lacks a 3'-hydroxyl group thus inhibiting elongation of DNA strand[265]. Adenosine analog.	Available for clinical use	Resistance has been detected in RT of HIV subtype C viruses reducing inhibitory activity of Tenofovir[490]
Raltegravir	Integrase Inhibitor	Inhibits integration of HIV DNA into cellular DNA.	Available for clinical use	Inhibits both HIV-1 clades and HIV-2[491,492]
Elvitegravir	Integrase Inhibitor	Inhibits integration of HIV DNA into cellular DNA.	Available for clinical use	Exhibits inhibitory activity against HIV strains that contain NRTI, NNRTI or Protease inhibitor resistance[493]
Dolutegravir	Integrase Inhibitor	Inhibits integration of HIV DNA into cellular DNA.	Available for clinical use	Is effective against HIV strains that contain raltegravir and elvitegravir resistance mutations[494]
Saquinavir	Protease Inhibitor	Inhibit function of HIV protease, preventing proper HIV protein processing[267]	Available for clinical use	Effective against both HIV-1 and HIV-2[495]. Point mutations at Leu90 results in resistance[496].
Indinavir	Protease Inhibitor	Inhibit function of HIV protease, preventing proper HIV protein processing[267]	Available for clinical use	Point mutations at Met46, Val82, Ile84 results in resistance[496].
Ritonavir	Protease Inhibitor	Inhibit function of HIV protease, preventing proper HIV protein processing[267]	Available for clinical use	Long-term treatment with Ritonavir has been associated with multiple mutations in the HIV-1 protease gene[497]
Nelfinavir	Protease Inhibitor	Inhibit function of HIV protease, preventing proper HIV protein processing[267]	Available for clinical use	Point mutations at Leu90 results in resistance[496].
Lopinavir	Protease Inhibitor	Inhibit function of HIV protease, preventing proper HIV protein processing[267]	Available for clinical use	Major resistance mutations detected[498]
Amprenavir	Protease Inhibitor	Inhibit function of HIV protease, preventing proper HIV protein processing[267]	Available for clinical use	Strongly effected by the protease mutation I50V[499]
Fosamprenavir	Protease Inhibitor	Inhibit function of HIV protease, preventing proper HIV protein processing[267]	Available for clinical use	Point mutations at Ile50 and Ile84 results in resistance[496].
Atazanavir	Protease Inhibitor	Inhibit function of HIV protease, preventing proper HIV protein processing[267]	Available for clinical use	Major resistance mutations detected[498]
Tipranavir	Protease Inhibitor	Inhibit function of HIV protease, preventing proper HIV protein processing[267]	Available for clinical use	Minimal cross-resistance with Tipranavir and other PIs have been observed[500]. Major resistance mutations have been detected[498].
Darunavir	Protease Inhibitor	Inhibit function of HIV protease, preventing proper HIV protein processing[267]	Available for clinical use	Major resistance mutations have been detected[498] however they occur at a much slower rate than that observed for other protease inhibitors[501]

11.3 Primers used in rtPCR

Table 14: Primers used in HIV DNA quantitation

Primer Name	Forward	Reverse
GAPDH DNA	5'-ATCATCCCTGCCTCTACTGG-3'	5'-GTCAGGTCCACCACTGACAC-3'
Early HIV DNA	5'-TTAGACCAGATCTGAGCCTGGGAG-3'	5'-GGGTCTGAGGGATCTCTAGTTACC-3'
Late HIV DNA	5'-TGTGTGCCCGTCTGTTGTG-3'	5'-GAGTCCTGCGTCGAGA-3'
2-LTR Circle DNA	5'-CCCTCAGACCCTTTTAGTCAGTG-3'	5'-TGGTGTGTAGTTCTGCCAATCA-3'

Table 15: Primers used in HIV mRNA splice variant analysis

Primer Name	Forward	Reverse
HIV Gag RNA	5'-AGTAAGAATGTATAGCCCTACCAGCAT-3'	5'-CTTAGAGTTTTATAGAACCGGTCTACATAGTC-3'
Unspliced RNA	5'-GACGCTCTCGCACCCATCTC-3'	5'-CTGAAGCGCGCACGGCAA-3'
Single Spliced RNA	5'-GGCGGCGACTGGAAGAAGC-3'	5'-CTATGATTACTATGGACCACAC-3'
Multi-spliced RNA	5'-GACTCATCAAGTTTCTCTATCAAA-3'	5'-AGTCTCTCAAGCGGTGGT-3'

11.4 Genscript Serpin B1 sequences

Serpin B1 379 Amino Acid Sequence (Mass 42.742 kDa):

1 MEQLSSANTR FALDLFLALS ENNPAGNIFI SPFSISSAMA MVFLGTRGNT
51 AAQLSKTFHF NTVEEVHSRF QSLNADINKR GASYILKLAN RLYGEKTYNF
101 LPEFLVSTQK TYGADLASVD FQHASEDARK TINQWVKGQT EGKIPELLAS
151 GMVDNMTKLV LVNAIYFKGN WKDKFMKEAT TNAPFRLNKK DRKTVKMMYQ
201 KKKFAYGYIE DLKCRVLELP YQGEELSMVI LLPDDIEDES TGLKKIEEQL
251 TLEKLHEWTK PENLDFIEVN VSLPRFKLEE SYTLNSDLAR LGVQDLFNSS
301 KADLSGMSGA RDIFISKIVH KSFVEVNEEG TEAAAATAGI ATFCMLMPEE
351 NFTADHPFLF FIRHNSSGSI LFLGRFSSP

Post-translational ubiquitylation sites

Post-translational Phosphorylation sites

Post-translational Acetylation sites

Serpin B1 Nucleotide Sequence including restriction sites in vector:

```
     GCAGAGCTCG TTTAGTGAAC CGTCAGATCG CCTGGAGACG CCATCCACGC
     CGTCTCGAGC AAATCACTTG GCAGTCTAGC GGACCTCTGC GGTAGGTGCG

     TGTTTTGACC TCCATAGAAG ATTCTAGAGC CACCATGGAG CAGCTGAGCT
     ACAAAACTGG AGGTATCTTC TAAGATCTCG GTGGTACCTC GTCGACTCGA
                                        M  E  Q  L  S  S

2301 CCGCTAACAC CAGGTTCGCA CTGGACCTGT TTCTGGCCCT GTCTGAGAAC
     GGCGATTGTG GTCCAAGCGT GACCTGGACA AAGACCGGGA CAGACTCTTG
       A  N  T  R  F  A  L  D  L  F  L  A  L  S  E  N

2351 AATCCCGCTG GCAATATCTT CATTTCCCCT TTTTCTATCT CTAGTGCCAT
     TTAGGGCGAC CGTTATAGAA GTAAAGGGGA AAAAGATAGA GATCACGGTA
     N  P  A  G  N  I  F  I  S  P  F  S  I  S  S  A  M

2401 GGCTATGGTG TTCCTGGGCA CACGCGGGAA CACTGCCGCT CAGCTGAGTA
     CCGATACCAC AAGGACCCGT GTGCGCCCTT GTGACGGCGA GTCGACTCAT
      A  M  V  F  L  G  T  R  G  N  T  A  A  Q  L  S  K

2451 AGACCTTCCA CTTTAATACA GTGGAGGAAG TCCATAGCAG GTTTCAGTCC
     TCTGGAAGGT GAAATTATGT CACCTCCTTC AGGTATCGTC CAAAGTCAGG
       T  F  H  F  N  T  V  E  E  V  H  S  R  F  Q  S

2501 CTGAACGCAG ATATCAACAA GCGCGGAGCC AGCTACATTC TGAAGCTGGC
     GACTTGCGTC TATAGTTGTT CGCGCCTCGG TCGATGTAAG ACTTCGACCG
     L  N  A  D  I  N  K  R  G  A  S  Y  I  L  K  L  A

2551 TAACCGACTG TACGGCGAGA AAACCTATAA TTTCCTGCCT GAATTTCTGG
     ATTGGCTGAC ATGCCGCTCT TTTGGATATT AAAGGACGGA CTTAAAGACC
      N  R  L  Y  G  E  K  T  Y  N  F  L  P  E  F  L  V

2601 TGTCCACTCA GAAGACCTAT GGCGCAGACC TGGCCTCCGT CGATTTCCAG
     ACAGGTGAGT CTTCTGGATA CCGCGTCTGG ACCGGAGGCA GCTAAAGGTC
       S  T  Q  K  T  Y  G  A  D  L  A  S  V  D  F  Q

2651 CACGCCTCTG AGGATGCTCG AAAAACCATC AACCAGTGGG TGAAAGGGCA
     GTGCGGAGAC TCCTACGAGC TTTTTGGTAG TTGGTCACCC ACTTTCCCGT
     H  A  S  E  D  A  R  K  T  I  N  Q  W  V  K  G  Q

2701 GACAGAGGGA AAGATTCCAG AACTGCTGGC TAGCGGCATG GTCGACAATA
     CTGTCTCCCT TTCTAAGGTC TTGACGACCG ATCGCCGTAC CAGCTGTTAT
      T  E  G  K  I  P  E  L  L  A  S  G  M  V  D  N  M

2751 TGACAAAGCT GGTGCTGGTC AACGCCATCT ACTTCAAAGG GAATTGGAAG
     ACTGTTTCGA CCACGACCAG TTGCGGTAGA TGAAGTTTCC CTTAACCTTC
       T  K  L  V  L  V  N  A  I  Y  F  K  G  N  W  K

2801 GATAAGTTTA TGAAGGAGGC AACCACAAAC GCCCCCTTCC GGCTGAATAA
     CTATTCAAAT ACTTCCTCCG TTGGTGTTTG CGGGGGAAGG CCGACTTATT
     D  K  F  M  K  E  A  T  T  N  A  P  F  R  L  N  K

2851 GAAAGACAGA AAAACTGTGA AGATGATGTA CCAGAAGAAA AAGTTTGCCT
     CTTTCTGTCT TTTTGACACT TCTACTACAT GGTCTTCTTT TTCAAACGGA
      K  D  R  K  T  V  K  M  M  Y  Q  K  K  K  F  A  Y
```

```
2901 ACGGGTATAT TGAGGATCTG AAGTGCAGGG TGCTGGAACT GCCTTATCAG
     TGCCCATATA ACTCCTAGAC TTCACGTCCC ACGACCTTGA CGGAATAGTC
       G  Y  I   E  D  L   K  C  R  V   L  E  L   P  Y  Q

2951 GGAGAGGAAC TGAGTATGGT CATCCTGCTG CCAGACGATA TTGAGGACGA
     CCTCTCCTTG ACTCATACCA GTAGGACGAC GGTCTGCTAT AACTCCTGCT
     G  E  E  L   S  M  V   I  L  L   P  D  D  I   E  D  E

3001 ATCAACCGGG CTGAAAAAGA TCGAGGAACA GCTGACACTG GAGAAACTGC
     TAGTTGGCCC GACTTTTTCT AGCTCCTTGT CGACTGTGAC CTCTTTGACG
      S  T  G   L  K  K  I   E  E  Q   L  T  L   E  K  L  H

3051 ATGAATGGAC TAAGCCCGAG AACCTGGATT TCATTGAAGT GAATGTCTCC
     TACTTACCTG ATTCGGGCTC TTGGACCTAA AGTAACTTCA CTTACAGAGG
       E  W  T   K  P  E   N  L  D  F   I  E  V   N  V  S

3101 CTGCCTCGGT TTAAACTGGA GGAATCTTAT ACACTGAACA GTGACCTGGC
     GACGGAGCCA AATTTGACCT CCTTAGAATA TGTGACTTGT CACTGGACCG
     L  P  R  F   K  L  E   E  S  Y   T  L  N  S   D  L  A

3151 CAGACTGGGC GTGCAGGATC TGTTCAATTC AAGCAAGGCA GACCTGAGTG
     GTCTGACCCG CACGTCCTAG ACAAGTTAAG TTCGTTCCGT CTGGACTCAC
      R  L  G   V  Q  D  L   F  N  S   S  K  A   D  L  S  G

3201 GAATGTCAGG CGCCCGGGAC ATCTTCATTT CAAAAATCGT GCACAAGAGC
     CTTACAGTCC GCGGGCCCTG TAGAAGTAAA GTTTTTAGCA CGTGTTCTCG
       M  S  G   A  R  D   I  F  I  S   K  I  V   H  K  S

3251 TTTGTGGAGG TCAACGAGGA AGGCACTGAA GCAGCCGCTG CAACTGCTGG
     AAACACCTCC AGTTGCTCCT TCCGTGACTT CGTCGGCGAC GTTGACGACC
     F  V  E  V   N  E  E   G  T  E   A  A  A  A   T  A  G

3301 GATTGCAACC TTTTGTATGC TGATGCCAGA GGAAAATTTC ACCGCCGATC
     CTAACGTTGG AAAACATACG ACTACGGTCT CCTTTTAAAG TGGCGGCTAG
      I  A  T   F  C  M  L   M  P  E   E  N  F   T  A  D  H

3351 ATCCCTTTCT GTTTTTCATT AGGCATAACT CATCTGGCAG CATCCTGTTC
     TAGGGAAAGA CAAAAAGTAA TCCGTATTGA GTAGACCGTC GTAGGACAAG
       P  F  L   F  F  I   R  H  N  S   S  G  S   I  L  F

                                 BamHI  NotI
3401 CTGGGGAGGT TCAGTAGTCC ATGAGGATCC GCGGCCGCAA GGATCTGCGA
     GACCCCTCCA AGTCATCAGG TACTCCTAGG CGCCGGCGTT CCTAGACGCT
     L  G  R  F   S  S  P   *   Frame 2

3451 TCGCTCCGGT GCCCGTCAGT GGGCAGAGCG CACATCGCCC ACAGTCCCCG
     AGCGAGGCCA CGGGCAGTCA CCCGTCTCGC GTGTAGCGGG TGTCAGGGGC
```

11.5 Functions of antiproteases

Table 16: Summary of antiproteases' chromosomal loci and functions

Common Name	Alternate Names	Accession Number	Chromosome Locus	Primary Functions	Endopeptidase Inhibitory Activity	Additional Information
A2ML1	Alpha-2-macroglobulin-like protein 1, C3 and PZP-like-alpha-2 macroglobulin domain containing protein 9	A2ML1_HUMAN	12p13.31	Inhibitor of chymotrypsin, papain, thermolysin, subtilisin A and exhibits partial inhibition of elastase	Serine	Acute Phase response protein
Cystatin A	Cystatin-AS, Stefin-A	CYTA_HUMAN	3q21.1	Role in desmosome-mediated cell-cell adhesion in epidermi, cystein-type endopeptidase inhibitor	Cysteine	None
Cystatin B	CPI-b, Liver thiol proteinase inhibitor, Stefin-B	CYTB_HUMAN	21q22.3	Inhibitor of cathepsins L,H and B	Cysteine	None
Elafin	Elastase-specific inhibitor, Peptidase inhibitor 3, protease inhibitor WAP3, skin-derived antileukoproteinase, WAP four-disulfide core domain protein 14	ELAF_HUMAN	20q13.12	Skin-derived neutrophil and pancreatic elastase-specific inhibitor	Serine	None
Serpin A1	Alpha-1-antitrypsin, alpha-1-protease inhibitor, alpha-1-antiproteinase	A1AT_HUMAN	14q32.13	Inhibitor of elastase, plasmin, thrombin, trypsin, chymotrypsin and plasminogen activator, has proteolytic activity against insuling and plasmin	Serine	Acute phase respones protein, C-36 fragment reversibly binds chymotrypsin and inhibits elastase and inhibits HIV binding to host cell
Serpin A3	Alpha-1-antichymotrypsin, cell-growth-inhibiting gene 24/25 protein	AACT_HUMAN	14q32.13	Inhibitor of capthespin G and mast cell chymase, which convert angiotensin-1 to angiotensin-2	Serine	Acute phase respones protein
Serpin A5	Plasma serine protease inhibitor, Acrosomal serine protease inhibitor, Plasminogen activator inhibitor 3, Protein C inhibitor	IPSP_HUMAN	14q32.13	Inhibitor of plasminogen and kallikrein activity	Serine	Present in seminal plasma and has large role in spermatogenesis, fusion of sperm to egg and sperm motility
Serpin A7	Thyroxine-binding globulin, T4-binding globulin	THBG_HUMAN	Xq22.3	Major thryroid hormone transport protein	Serine	None
Serpin A8	Angiotensinogen	ANGT_HUMAN	1q42.2	Component of renin-angiotensin system, regulates blood pressure, body fluids and electrolytes, activates NF-kB and ERK1/ERK2 cascades	Serine	Angiotensinogen is cleaved into 8 chains, all with unique functions aside from the primary functions

Serpin B1	Leukocyte elastase inhibitor, Monocyte/neutrophil elastase inhibitor, Peptidase inhibitor 2	ILEU_HUMAN	6p25.2	Inhibitor of neutrophil protease elastase, cathepsin G, proteinase-3, chymase, chymotrypsin kallikrein-3 and granzyme H	Serine	None
Serpin B2	Plasminogen activator inhibitor 2	E9PDK7_HUMAN	18q21.33	Negative regulator of apoptosis, serine-type endopeptidase activity	Serine	None
Serpin B3	Protein T4-A, Squamous cell carcinoma antigen 1	SPB3_HUMAN	18q21.33	Papain-like cystein protease inhibitor modulating tumor cell specific immune response, inhibitor of UV-induced apoptosis through suppression of c-Jun NK(2)-terminal kinase (JNK1)	Cysteine and Serine	Postively regulates cell migration and proliferation
Serpin B4	Leupin, Peptidase inhibitor 11, Squamous cell carcinoma antigen 2	SPB4_HUMAN	18q21.33	Protease inhibitor, modulates immune response against tumor cells, protection from NK cell-mediated cytotoxicity	Serine	None
Serpin B5	Maspin, Peptidase inhibitor 5	SPB5_HUMAN	18q21.33	Tumor Suppressor, blocks growth, invasion and metastatic properties of mammary tumors	None	Does not undergo S to R conformational change common to serpins; does not exhibit serine protease inhibitory activity
Serpin B6	Cytoplasmic antiproteinase, Peptidase inhibitor 6, Placental thrombin inhibitor	SPB6_HUMAN	p625.2	Inhibitor of cathepsin G, Kallikrein-8 and thrombin.	Serine	None
Serpin B9	Cytoplasmic antiproteinase 3, Peptidase inhibitor 9	SPB9_HUMAN	6p25.2	Granzyme B inhibitor, protection from NK cell mediated cytotoxicity	Cysteine and Serine	Cellular response to estrogen
Serpin B10	Bomapin, Peptidase inhibitor 10	SPB10_HUMAN	18q21.33	May regulate protease activity during hematopoiesis and TNF-induced apoptosis	Serine	None
Serpin B12	None	SPB12_HUMAN	18q21.33	Inhibitor of trypsin and plasmin	Serine	None
Serpin B13	HaCaT UV-repressible serpin, Headpin, Peptidase inhibitor 13, Proteinase inhibitor 13	SPB13_HUMAN	18q21.33	Negative regulation of keratinocyte apoptosis, role in proliferation/differentitation of keratinocytes	Cysteine and Serine	None
Serpin C1	Antithrombin-III	ANT3_HUMAN	1q25.1	Regulates blood coagulation pathway. Inhibitor of thrombin, matriptase-3/TMPRSS7, Factors IXa, Xa and Xia	Serine	Inhibitory activity is enhanced in the presence of heparin; Serpin C1 is the most important serine protease inhibitor in plasma, regulates blood coagulation.

Serpin D1	Heparin cofactor 2, Heparin cofactor II, Protease inhibitor leuserpin-2	HEP2_HUMAN	22q11.21	Inhibitor of thrombin, chymotrypsin, chemotactic for monocytes and neutrophils	Serine	None
Serpin F1	Pigment epithelium-derived factor, Cell proliferation-inducing gene 35 protein, EPC-1	PEDF_HUMAN	17p13.3	Induces neuronal differentiation in retinoblastoma cells, inhibitor of angiogenesis	None	Does not undergo S to R conformational change common to serpins; does not exhibit serine protease inhibitory activity
Serpin F2	Alpha-2-antiplasmin, Alpha-2-plasmin inhibitor	A2AP_HUMAN	17p13.3	Inhibitor of plasmin, trypsin, matriptase-3/TMPRSS7 and chymotrypsin	Serine	Acute phase respones protein
Serpin G1	Plasma protease C1 inhibitor	IC1_HUMAN	11q12.1	Regulates complement activation, blood coagulation, fibrinolysis and generation of kinins. Inhibitor of chymotrypsin and kallikrein	Serine	Acute phase response protein
SLPI	Antileukoproteinase, BLPI, HUSI-1, Mucus proteinase inhibitor, Protease inhibitor WAP4, Secretory leukocyte protease inhibitor, Seminal proteinase inhibitor, WAP four-disulfide core domain protein 4	SLPI_HUMAN	20q13.12	Inhibitor of trypsin, chymotrypsin, elastase, cathepsin G, negative regulator of viral genome replication	Serine	Is acid stable, may be associated with protection against elastase-mediated damage within the oral and other mucosal compartments

All information was obtained from www.genecards.org[631]

11.6 Cytokine and chemokine general and HIV-specific functions

Table 17: Cellular source and functions of specific cytokines and chemokines

Cytokine/ Chemokine	Cellular Source	General Function	HIV-specific Function
IL-1α	• MDMs • Monocytes • B cells • Endothelial cells • Fibroblasts	• Pro-inflammatory	• Up-regulated following infection of MDMs with HIV. • Suppressed following ARV treatment • Enhances HIV replication within IL-2 stimulated cells
IL-1β	• MDMs • Monocytes • B cells • Endothelial cells Fibroblasts	• Pro-inflammatory	• Up-regulated following infection of MDMs with HIV. • Suppressed following ARV treatment • Enhances HIV replication within IL-2 stimulated cells[171,502]
IL-2	• $CD4^+$ T lymphocytes • $CD8^+$ T lymphocytes	• Stimulates proliferation of activated T cell[431,503] • Stimulates cytotoxic effects of $CD8^+$ T and NK cells[431] • Stimulates B cell and monocyte functions[431]	• Expression is down-regulated following *in vitro* HIV infection of $CD4^+$ T cells[504,505] • Down-regulation of IL-2 is one of the first immunological dys-functions associated with HIV infection[506] • Correlated with $CD4^+$ T cell counts in HIV infected individuals[507] • Potent HIV inducer in PBMCs through post-transcriptional mechanism[508] • Also induces $CD8^+$ T cells to suppress HIV replication and inhibits HIV-induced apoptosis[509] • Used in combination HAART therapy to increase the total CD4 T cell counts without an increase in viral load[510,511]
IL-4	• Activated $CD4^+$ T cells NK cells • Mast cells • Basophils	• Stimulates B cell activation/differentiation • Stimulates secretion of IgG1 and IgE • Stimulates T cell activation • Stimulates MHC II expression on B cells and macrophages[431,512]	• Up-regulation of IL-4 is associated with *in vivo* HIV-infection • IL-4 is up-regulated in HIV-infection and becomes significantly depleted following ARV treatment[513] • Stimulates HIV replication within monocytes and MDM[514]
IL-6	• T cells • B cells • Macrophages • Fibroblasts • Endothelial cells	• Pro-inflammatory in response to bacterial and viral infections • B cell stimulation • Monocyte differentiation • Stimulates production of IL-4 [431,515]	• Up-regulated following infection of MDMs, $CD4^+$ T cells, B cells with HIV, specifically during early stages of infection[431,516,517] • Acts synergistically with TNF-α in up-regulating the transcription of NF-κβ[171]
IL-7	• Epithelial cells • Keratinocytes • DCs • Follicular DCs • Lymphocytes	• Induces growth and expansion of immature and mature B cells [518] • Stimulates immature thymocyte (T cell precursors) survival and expansion[519] • Necessary for the development and maintenance of thymus-derived γδ T cells[520] • Induces rearrangement of TCR[521]	• Significantly increases HIV replication within PBMCs both *in vitro*[522] and within the PBMCs of asymptomatic HIV-positive individuals[523] • Treatment with IL-7 results in increased activation and expansion of HIV-specific CTL responses[524] • Increases HIV transmission through prevention of apoptosis of HIV-1 infected cells[525].

IL-8	• Macrophages • T lymphocytes • Neutrophils • Endothelial Cells	• Pro-inflammatory and antimicrobial • Chemotactic for T lymphocytes, NK cells, basophils and neutrophils[526]	• Up-regulated in MDMs following *in vitro* HIV-infection as well as within the serum and bronchoalveolar fluids within HIV infected individuals[527-529]
IL-10	• Activated T lymphocytes • Activated B lymphocytes • Monocytes • Macrophages • Keratinocytes • pDCs	• Inhibits T cell proliferation through inhibition of T_H1 cytokines (IL-2, IFN-γ) • Inhibits macrophage activation Inhibits pro-inflammatory cytokine production (IL-1, IL-6, IL-8, IL-12, TNF-α)[431]	• Increased expression occurs in MDM and monocytes following HIV infection *in vitro* as well as within PBMCs and bronchoalveolar fluids from HIV-infected individuals at all stages of infection[513,530] • Decreases HIV-1 replication within macrophages through modulation of the expression of TNF-α and IL-6[531]
IL-12	• Macrophage • DCs • T cells • Monocytes	• Cell-mediated immunity against pathogens through up-regulation of IFN-γ and regulating CTL and macrophage function	• IL-12 production is up-regulated during HIV infection resulting in increased production of IFN-γ by NK cells and T cells within *in vitro* assays[431,532] • IL-12 is under-expressed within PBMCs of HIV infected individuals compared to uninfected[431,533] • Inhibits HIV-induced apoptosis and within MDMs infected with HIV-1, decreases reverse transcriptase activity[508,534]
IL-13	• T lymphocytes • Dendritic Cells	• Regulates B cell, monocyte, DCs and fibroblast function • Required for IgE [431,535,536]production • Induces MHC II expression on APCs • Regulates inflammation through inhibition of pro-inflammatory cytokines/chemokines[535-537]	• Lower levels of IL-13 are produced in PBMCs from HIV-infected individuals[538] • Correlates positively with $CD4^+$ T cell count and correlates negatively with HIV viral load[538,539] • Strong inhibitor of HIV replication within MDMs • Inhibits infectivity and virus production by down-regulating CCR5 expression on MDMs[538]
IL-15	• DCs • Macrophages • Monocytes • Keratinocytes • Fibroblasts • Nerve cells	• Regulation of T cell and NK cell activation and proliferation [540,541]	• Enhances NK cell and T cells anti-HIV activity[542,543] • Is associated with increased HIV replication[544]
IL-16	• $CD4^+$ T lymphocytes • Mast cells • Eosinophils	• Chemoattractant for T cells, monocytes and eosinophils • Regulates T cell function through enhancing expression of IL-2 receptor and HLA-DR	• Natural ligand for CD4 receptor and thus acts as a competitive inhibitor for HIV[545] • Significant down-regulation of IL-16 expression is present in individuals with progressed HIV/AIDS[546]
IL-17	• T_H17 cells • NKT cells • γδ T cells	• Regulation of pro-inflammatory responses[547] • Associated with allergic immune responses resulting in airway remodelling[547] • Significantly up-regulates mucosal tissue inflammation during extracellular bacteria and fungi infections[548,549] • Up-regulates neutrophil migration[547]	• Expression is up-regulated in both CD4+ and CD4- T cells following HIV-1 infection, via interactions with Tat[550,551] • T_H17 cells are systematically depleted and exhibit dysfunction following HIV-infection[552]
IP-10	• Monocytes • mDCs • Endothelial cells	• Chemoattractant for monocytes/macrophages, T cells, NK cells and DCs[553]	• Up-regulated following HIV infection[554] • Decreases T cell function in

	• Fibroblasts		HIV-infected individuals[555] • Reduced level of IFN- γ expression within PBMCs infected with HIV[555] • Significantly associated with HIV viral load[554]. Induces viral replication within PBMCs and MDMs[556]
IFN-α	• Leukocytes • pDCs	• Anti-viral • Stimulate CTL-mediated killing through increased MHC I expression • Stimulate NK cell cytotoxic function • Inhibits cellular proliferation	• Up-regulated in HIV-infected individuals with cerebral-HIV (dementia)[557] • Inhibits HIV replication through suppression of both RT and transcription of integrated provirus[172] • Correlates with immune activation and has thus been implicated in HIV-1 pathogenesis[173] • Plasma levels correlate inversely with CD4 T cell count and positively with HIV-1 RNA plasma levels as well as with CD38 expression on CD8+ T cells[173]
IFN-β	• Fibroblasts • Lymphocytes • pDCs • Monocytes	• Anti-viral • Inhibits viral replication • Inhibits cellular proliferation	• Up-regulated following HIV infection of monocytes and MDMs[558] • p24 levels increase upon blocking of IFN-β with antibodies[559,560] • Inhibitor of HIV within MDMs[559] • Plasma levels correlate inversely with CD4 T cell count and positively with HIV-1 RNA plasma levels as well as with CD38 expression on CD8+ T cells[173]
IFN-γ	• Activated T lymphocytes • Activated NK cells • Monocytes • pDCs	• Stimulates T cell and B cell proliferation • Activates macrophages and neutrophils phagocytic functions • Increases MHC II expression • Decreases IL-4 expression	• Expression decreases with progressed disease state[507] • Correlates with patient CD4 counts and inversely correlates with viral load[507] • Inhibits HIV entry through down-regulating expression of CD4, however it up-regulates expression of CCR5 an CCR3 (co-receptors used by R-tropic viruses) • Has been identified as inhibitory against HIV within MDMs and monocytes, resulting in decreased p24 levels and HIV-1 DNA/mRNA levels[431,561] • Within U937 cells IFN-γ has been shown to enhance the enhancing effect of TNF-α on HIV replication[562]
GM-CSF	• Activated T lymphocytes • Endothelial cells • Macrophages • Fibroblasts	• Required for granulocyte/macrophage progenitor cell proliferation, survival, differentiation[563] • Inhibits neutrophil migration	• Decreased expression following HIV infection of MDMs[564] • Conflicting data exists for GM-CSF's role in HIV infection however the majority of studies suggest that it inhibits HIV replication within MDMs[438] through binding to the β-chain of the GM-CSF receptor

M-CSF	• Fibroblasts • Endothelial cells	• Stimulates growth and proliferation of cells within the macrophage lineage	• Up-regulated following infection with HIV[565] • Increases HIV-susceptibility of MDMs by up to 400-fold. • Treatment of MDMs with M-CSF results in increased entry and replication of HIV, potentially through up-regulating the expression of CD4 and CCR5W[566]
TNF-α	• Monocytes • Macrophages • T lymphocytes • B lymphocytes • NK cells • Neutrophils • Microglial cells	• Pro-inflammatory • Involved in immune response against bacterial pathogens	• Expression is increased following HIV infection[567] • HIV production is decreased, CD4 depletion is reduced and NF-κβ activation is decreased following blocking of TNF-α with antibodies[568] • Inducer of HIV replication within T cells and macrophages through activation of NF-κβ[408,569,570]. • Pro-HIV action is amplified in the presence of IFN-γ[562] • An inhibitory role preventing efficient entry into MDMs has been reported when TNF-α is added to cultures prior to HIV infection[571]
TNF-β	• T lymphocytes • B lymphocytes	• Pro-inflammatory	• Enhanced expression from CTLs is evident following HIV infection[567] • An inhibitory role preventing efficient entry into MDMs has been reported when TNF-α is added to cultures prior to HIV infection[571]
RANTES	• T lymphocytes • Macrophages • Monocytes • Mast cells • Fibroblasts	• Natural ligand for CCR5 • Chemotactic for leukocytes • Role in hematopoesis[572]	• Increased expression by $CD4^+$ T cells following R- and X-tropic HIV infection[573] • Increased expression by MDMs and $CD8^+$ T cells following HIV-1 infection resulting in chemotactic effects and activation of T cells[574,575] • Inhibitor of only R-tropic HIV-1 virus entry, uncoating and replication in macrophages, PBMCs and microglial cells [575-578] • Down-regulates CCR5 expression[579]
MIP-1α	• T lymphocytes • Macrophages • Monocytes • Mast cells • Fibroblasts	• Natural ligand for CCR5 • Chemotactic for leukocytes • Role in hematopoesis[572]	• Increased expression by $CD4^+$ T cells following R-tropic HIV infection[573] • Increased expression by MDMs following HIV-1 infection resulting in chemotactic effects and activation of T cells[574,575] • Inhibitor of only R-tropic HIV-1 virus entry, uncoating and replication in macrophages, PBMCs and microglial cells [575,577,578] • Down-regulates CCR5 expression[579]
MIP-1β	• T lymphocytes • Macrophages • Monocytes	• Natural ligand for CCR5 • Chemotactic for leukocytes • Role in hematopoesis[572]	• Increased expression by $CD4^+$ T cells following R-tropic HIV infection[573]

	• Mast cells • Fibroblasts		• Increased expression by MDMs following HIV-1 infection resulting in chemotactic effects and activation of T cells[574,575] • Inhibitor of only R-tropic HIV-1 virus entry, uncoating and replication in macrophages, PBMCs and microglial cells [575,577,578] • Down-regulates CCR5 expression[579]
MIP-3 α	• Neutrophils • Epithelial • Monocytes • Lymphocytes	• Chemoattractant for lymphocytes, DCs and mildly for neutrophils[580]	• Directly inhibits both X-tropic and R-tropic HIV viruses[581]
MCP-1	• Endothelial • Fibroblasts • Epithelial • Monocytes • Microglial • Smooth muscle	• Chemoattractant and activator of monocytes/macrophages, memory T cells, basophils, NK cells and immature DCs[582]	• Tat-induced up-regulation of MCP-1 expression during HIV infection [583] • Plasma levels of MCP-1 correlate with HIV viral load[584] • Inhibits HIV bidning to CCR2 and CCR5 receptors[585]
MCP-3	• Endothelial • Fibroblasts • Epithelial • Monocytes • Astrocytes	• Chemoattractant and activator of monocytes/macrophages[582]	• Inhibits X-tropic HIV viruses within PBMCs[586] • Inhibits activation of CCR5 by MIP-1β thereby acting as a natural antagonist for CCR5[456]
MDC	• B cells • Macrophages • MDMs • Activated NK cells • $CD4^+$ T cells	• Chemoattractant for monocytes, DCs and NK cells	• Suppresses HIV activity • Treatment with MDC results in increased %CD4 T cells [587]
Fractalkine	• Endothelial • Epithelial • Microglial	• Chemoattractant for monocytes, microglia cells and NK cells • Induces IFN-γ production by NK cells[588]	• Protects neurons from HIV-1 Tat-induced dendritic loss and death[589]
GRO	• Epithelial • Endothelial	• Chemoattractant and activator of neutrophils and basophils	• Expression is up-regulated following stimulation with X-tropic and R-tropic HIV viruses[590] • GRO stimulates HIV-1 replication in both MDMs and T lymphocytes[590]
TGF-β	• CD8 T lymphocytes • Monocytes • pDCs • B cells • Macrophages • Epithelial • Endothelial • Produced by most cell types	• Regulate cellular proliferation, growth, differentiation and chemotaxis • Immunosuppressive	• Suppresses HIV replication within MDMs[591] • Expression is up-regulated by Tat following HIV infection[592]. • Suppresses both B and T lymphocyte anti-HIV specific responses[593]

11.7 Cohort consent forms
11.7.1 Nairobi, Pumwani cohort

Comprehensive Studies of Mechanisms of HIV Resistance in Nairobi, Kenya

Majengo, Kibera, Korogocho and MCH Pumwani Research Clinics

Patient Information and Consent Form

This information will be communicated orally in English, Swahili or other Kenyan dialect of potential participant's preference.

Investigators:
Dr. Charles Wachihi, University of Nairobi, tel. 714851, PO Box 19676, Nairobi, Kenya
Dr. Joshua Kimani, University of Nairobi, tel. 714851, PO Box 19676, Nairobi, Kenya
Dr. Jessie Kwatampora University of Nairobi, tel. 714851, PO Box 19676, Nairobi, Kenya
Dr. Samson Barasa University of Nairobi, tel. 714851, PO Box 19676, Nairobi, Kenya
Dr. Walter Jaoko, University of Nairobi, tel. 714851, PO Box 19676, Nairobi, Kenya
Dr. T. Blake Ball University of Manitoba, 730 William Ave. Winnipeg, MB, Canada
Dr. Francis Plummer University of Manitoba, 730 William Ave. Winnipeg, MB, Canada
Dr. Joanne Embree, University of Manitoba, 730 William Ave. Winnipeg, MB, Canada
Dr. Keith R. Fowke, University of Manitoba, 730 William Ave. Winnipeg, MB, Canada
Dr. Rupert Kaul, University of Toronto, 1 King's College Toronto, ON, Canada

Background information

The University of Nairobi and its collaborators from Canada have been working for many years to fight the epidemics of AIDS and other sexually transmitted infections that we are facing in Kenya. This basic science research program is conducting studies to determine the relationship between immunity and susceptibility to sexually transmitted infections (STI) with the goal of developing vaccines or treatments for STIs. You are being asked to participate in this study because you are:

a) at a very high risk of acquiring an STI or are already infected with an STI or

b) at a low risk of acquiring an STI; or

c) the relative of a person in group a) or b).

The purpose of this research program is to determine if there are factors that could protect individuals from acquiring sexually transmitted infections (STI) especially HIV. It is important to keep free of other sexually transmitted diseases, as the presence of these infections may increase your risk of becoming infected with HIV. If you have an STI, you should seek treatment for it as quickly as possible. However, sometimes you may have an STD and not know it, because you may not have any symptoms and thus advised to visit the clinic monthly for free check ups.

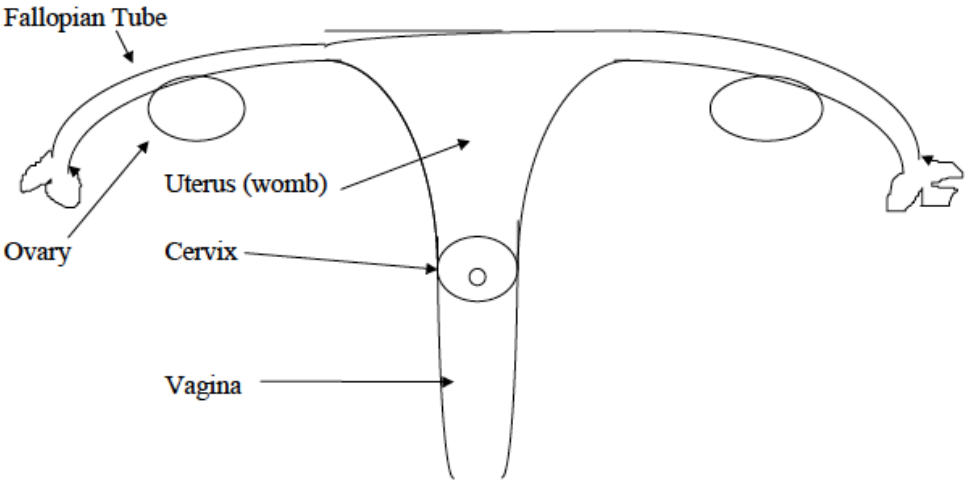
Why Is This Study Being Done?

This study is being done to find out why some people are more or less likely to get the Human Immunodeficiency Virus (HIV), the virus that causes AIDS. There is more and more evidence that the immune system in some people is able to protect them against infection with HIV. Since most people get HIV through sexual exposure to an HIV infected partner, the first contact with the virus occur in the genital tract, the vagina and cervix in women. We know from some of our previous work that some women, who seem to be protected against HIV, have a special type of immune

Date___ /___ /___ **(dd/mm/yy)** **Initials** _____ **Study #** ___ ___ ___

response that it not present in women who get HIV. The purpose of this study is to try to find out the targets of this immune response in the vagina, uterus and cervix and to try to find out what is special about the immune system of these few individuals. This work may be helpful in eventually making a vaccine for HIV.

To help you understand what is involved in the study a drawing of the vagina, cervix and uterus of the female genital tract is shown below.



How Many People Will Take Part in the Study

About 3000 participants mainly women will take part in this study.

What Is Involved in the Study?

You have been invited to voluntarily participate in this study because all are at risk of becoming infected with STDs and HIV. Some sexual behavior especially among sex workers or those who use sex as an income generating activity exposes those involved or their partners to a higher risk of contracting HIV. If you now agree to participate in the study, you will first be given additional counseling and information on HIV risk reduction. Those who are sex workers will be given additional counseling, advised on appropriate STIs prevention strategies and requested to consider leaving prostitution. If a sex worker and you choose not to leave prostitution at this time you will be asked to return to the clinic every month for free check ups. Again, the results of these tests will be ready after one week or less, and you will be informed of the results and given the correct treatment if you have an infection. You will also be encouraged to come to the clinic for examination and treatment at any other time that you feel ill. If you forget to return to the clinic for one of your scheduled visits, a clinic staff member will contact you by phone, SMS or send one of your friends to remind you of the missed appointment. All study participants will also be encouraged to either retest for HIV or recheck their CD4/CD8 profiles depending on HIV infection status every three months. In addition, we will store specimens from your blood for future studies of the genes involved in resistance and susceptibility to HIV and other infections.

Clinic visits

Date___ /___ /___ **(dd/mm/yy)** **Initials** _____ **Study #** ___ ___ ___

First visit and semi-annual visits (All study participants)

1. We will ask you general questions about your life, about problems you are having, and about your sexual history.
2. The doctor will examine your body, including your female parts.
3. Swab and washing from your vagina to look for germs and to collect samples for studying your immune response.
4. Swab from your cervix to look from germs and to collect samples for studying your immune response.
5. A thin plastic tube will be placed in your cervix (opening to your womb) to get some of the mucous your cervix makes.
6. Urine to look for germs.
7. Three tablespoons of blood will be taken for testing syphilis, and HIV and for studying your immune response. We will inform you of your results at your one month visit. We also will test your spouse for the HIV virus free of charge if he/she wishes.

Monthly visits (Those in sex work or engaged in high risk sexual behaviors)

1. Questions will be asked about you, and what problems you are having.
2. If you have any complaints the doctor will examine your body, including your female parts.
3. Every third month all study participants will be encouraged to either retest for HIV or recheck their CD4/CD8 profiles depending on individuals HIV infection status.

Follow-up visits (All study participants)

1. You will be asked to return 3 to 7 days after every visit to be given you laboratory results.
2. You will be treated for new infections, free of charge.

How Long Will I Be in the Study?

The study will last 5 years. Although we would appreciate if you stayed in the study for the entire period you may choose to leave the study at any time without any penalty to you.

What Are the Risks of the Study?

Risk of blood and cervical collection

This study requires the use of your blood. In order to get the blood we will need to insert a needle into a vein in your arm so that the blood can be removed. There will be some pain associated with the needle stick but this will be only for a short period of time. There may be some bruising around the needle site and, although we will sterilize the site to minimize infection, there is a very minimal risk of infection at the site. There is also some discomfort associated with taking specimens from your cervix.

HIV test

Non-physical risks:

1. If you are HIV positive, learning so may cause you to become depressed. We will counsel you about your HIV test results if you are negative or positive. If you are HIV positive, we will refer you to another clinic for care and treatment. We will also test your husband or boyfriend for the HIV virus if he wants.

Risks of taking antibiotics / Antiretrovirals

Date___ /___ /___ **(dd/mm/yy)** **Initials** _____ **Study #** ___ ___ ___

If we find that you have an STD or AIDS we will provide you with the appropriate treatment. With any drug there is some potential for side effects. For the antibiotics/ antiretrovirals you might receive, the following side effects are possible.

Very likely:

1. Sick to your stomach
2. Headache
3. Metallic taste in mouth
4. Diarrhea
5. If a woman - infection of your vagina by yeast (a white discharge with itching). If this happens, we will give you medicine to put inside your vagina to treat the yeast infection.

Less likely but serious:

1. Less than 1 person in 100 will have a severe allergic reaction to one of the antibiotics/ antiretrovirals.

Are There Benefits to Taking Part in the Study?

The benefits that you will get from this study are that you will be examined regularly, and if you are found to have an STD or AIDS, you will receive appropriate and effective medication. Medical care will also be provided for other illnesses that you might have. You will also be informed about what you are suffering from, and you will be informed about the future implications of these STDs and of HIV.

What about Confidentiality?

Efforts will be made to keep your personal information confidential. We will record your information only by a special number assigned to you. The number will only be known to the clinic staff and yourself.

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as: the researchers, members of the local and international ethics teams and the National Institutes of Health in the United States of America. The research results will be published, but your identity will remain secret.

What Are My Rights as a Participant?

Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. Leaving the study will not result in any penalty or loss of benefits to which you are entitled. If the participation in the study results in you becoming ill, the study team will provide you with medical care for the problem for free.

Although you will not be paid to participate in the study, you will be offered a small payment of two hundred shillings (KSh 200) **for the first and every sixth month clinic visit to** compensate you for your transportation to the clinic and any other expenses you might incur.

We will also provide you with any new information and findings from the study that may affect your health, welfare, or willingness to stay in this study.

All information that is obtained will be kept strictly confidential, and your identity will not be known, except to those providing your medical care.

Date___ /___ /___ **(dd/mm/yy)** **Initials** _____ **Study #** ___ ___ ___

At the end of every year, we will be holding baraza's at the different clinics to give progress reports and share any new findings from the study with all members of the different clinics.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

For questions about the study or a research-related injury, call or contact Drs. Wachihi, Kwatampora, Barasa or any one of the researchers named above at the Medical Microbiology Annex at the University of Nairobi

For questions about your rights as a research participant, contact Professor Bhatt, who is the chairperson of the Ethical Review Committee at the University of Nairobi, by calling 725452, or make an appointment to see her in the Department of Medicine, at the University of Nairobi.

Statement of Consent:
If you agree to participate in the study, please sign below.

I, _____, have read or have had read to me, the consent form for the above study and have discussed the study with _____.

I understand that the following (check the box only if you fully understand and agree with each statement):

☐ the goals of this research program are to study resistance and susceptibility to sexually transmitted infections

☐ enrolment is completely voluntary and I can withdraw from the study at any time

☐ blood, cervical and vaginal specimens will be required for this study and may be used for genetic studies

☐ any blood specimens previously collected may be used for this study

☐ a portion of my blood, cervical and vaginal specimens will be stored for future studies of the genes involved in resistance and susceptibility to HIV and other infections.

I am willing to participate in the study.

Name of Study Participant_____

Signature/Thumb print: _____ Date: _____

For clinic staff:

I, _____, have explained the nature and purpose of the above study to

_____

Name of Clinic Staff: _____

Signature: _____ Date: _____

Assigned Study Number / Clinic Number ____ ____ ____

NB: All study participants will be issued with a copy of this information and consent forms

Date___ /___ /___ **(dd/mm/yy)** **Initials** _____ **Study #** ___ ___ ___

Standards of Medical Care for Participants in the Research Clinics

This document outlines the standard of medical care for all participants in the Majengo, MCH Pumwani, Kindred, Kibera and Korogocho cohorts, regardless of HIV-1 serostatus. It should be emphasized that any member of the said cohorts may freely decline to take part in any cohort substudy, and that this decision will in no way affect their access to this standard of care. All care outlined is provided free of charge, thereby significantly improving health care access and outcomes for all members of the cohorts... The nature of the medical care will vary depending on HIV-1 serostatus of the participants, as outlined below.

1. **General medical care for all participants, regardless of HIV-1 status.**
    - HIV and STD prevention services: provision of the male condom, and peer-based and clinic-based counseling regarding safer sexual practices.
    - Family planning services as directed in the *Kenyan National Family Planning Guidelines*
    - Rapid and effective treatment of sexually transmitted diseases in accordance with the *Kenya National Guidelines for the Syndromic Management of Sexually Transmitted Diseases*
    - Medical care for acute and chronic illnesses, both infectious and non-infectious
    - Access to diagnostic testing in haematology, biochemistry, infectious diseases, immunology, radiology
    - Prompt referral for specialist consultation and hospitalization when indicated
2. **Management of Opportunistic Infections in HIV-1 Infected Participants.**
    - **Primary Prophylaxis:** Trimethoprim-Sulphamethoxazole (Septrin): all participants with a CD4+ T cell count $<200/mm^3$, for prevention of PCP, toxoplasmosis and bacterial infections (bacterial pneumonia, bacteremias, some bacterial diarrhoea), according to *National AIDS/STD Control Program (NASCOP) Guidelines*
    - **Secondary Prophylaxis:** Septrin: offered to all participants regardless of CD4+ T cell count after an episode of PCP, toxoplasmosis, or severe bacterial infection. Fluconazole: provided for secondary prevention of Cryptococcus

Treatment

- Herpes. simplex/Herpes zoster infection: acyclovir
- Candidiasis (oral, esophageal, vaginal): nystatin, clotrimazole, Fluconazole
- Tuberculosis (pulmonary or extra pulmonary): referral to National TB Programme
- Toxoplasmosis: referral for inpatient therapy
- Cryptococcus: referral for inpatient therapy
- PCP: Septrin (with prednisolone, if severe)
- Kaposi's Sarcoma: ARV and referral to Clinical Oncologist

3. **Antiretroviral therapy.**

Antiretroviral therapy rollout in Kenya is supported and directed by NASCOP and The Ministry of Health. Kenya is a recipient of ARVs and infrastructure support through the *Presidents Emergency Plan for AIDS Relief (PEPFAR)* a US government international development initiative.

- ARV drugs and infrastructure support has been secured by the University of Manitoba from NASCOP/PEPFAR and CDC PEPFAR to provide HIV basic and ARV care for **all** cohorts members who are eligible as per the *"Guidelines to Antiretroviral Drug Therapy in Kenya"* (NASCOP-2002). Such medical treatment and its requisite follow-up, integrated with the above standard of care, will also be provided at no cost.

Date___ /___ /___ **(dd/mm/yy)** **Initials** _____ **Study #** ___ ___ ___

11.7.2 Winnipeg Women's Clinic cohort

PARTICIPANT CODE:_____

Participant Information and Consent Form

Title of Study: Examining Cervicovaginal Lavage and Cervical Cells from Local Manitoban Women to Better Understand the Transmission of HIV-1

Investigators:

- Dr. Terry Blake Ball, University of Manitoba, Department of Medical Microbiology, 1015 Arlington Street, Room H3440, Winnipeg, MB.
- Dr. Francis A. Plummer, University of Manitoba, Department of Medical Microbiology, 1015 Arlington Street, Room T2440, Winnipeg, MB.
- Dr. Helen Pymar, Obstetrician Gynecologist, Women's Hospital, Health Sciences Centre, WS021-735 Notre Dame Ave, Winnipeg, MB.
- Dr. Jennifer Chan, Family Physician, Women's Hospital, Health Sciences Centre, WR035-735 Notre Dame Ave, Winnipeg, MB.
- Dr. Adam Burgener, University of Manitoba, Department of Medical Microbiology, 1015 Arlington Street, Room, Winnipeg, MB.
- Lindsay Aboud, University of Manitoba, Department of Medical Microbiology, 507 Basic Medical Sciences Building, 745 William Avenue, Winnipeg, MB.

Introduction:

You are being asked to participate in a study as a healthy volunteer which involves the collection of 2mLs (0.4tsp) of cervical mucus and cervical cells, both of which the doctor can access during your routine pelvic exam, for research purposes. Please take your time in reading the description of this study and do not hesitate to ask any questions if you do not understand or are unclear about any aspect of the study. Should you decide to participate you are free to withdraw from the study at any time. If you decide to participate in this study, you will be asked to fill out a questionnaire containing questions about your general health, including menstrual cycle, pregnancy history as well as sexual history. These questions are all designed to give the researchers additional information to help them better complete their study, however if you at any point feel uncomfortable answering a question, you have the right to omit those specific questions. The description of the study below is designed to inform you of all the uses of the samples we obtain.

Purpose of this Study:

The goal of this study is to gain a better understanding of the natural environment in the female genital tract by examining cervical-vaginal lavage (CVL). We will determine how CVL alone, or in combination with semen, effect HIV-1 transmission / infection while also defining how some of the key factors identified as anti-HIV proteins of CVL are regulated during the different phases of the menstrual cycle.

Study Procedure:

During your scheduled regular gynecological exam, your physician will obtain cervical mucus and cervical cell samples. This is done by washing the inside of the vaginal vault with 2ml of room temperature sterile PBS (water-based salt solution). Once this fluid has gathered at the base of the vagina it will be collected using a plastic pipette (dropper) and transferred to a culture tube. The entire procedure will take no more than 2-3 minutes. Immediately after collection the samples will be labeled with a laboratory code and stored on ice for transport to the laboratory.

Also, part of your regular pelvic exam involves the collection of cells from your cervix/vagina using a brush and swab. Normally after smearing these instruments on a slide the physician would throw them away. For the purposes of this study, the physician will instead place the swab / brush into a separate tube and then on ice so that the lab can remove the remaining cells from the swab and brush for other studies. Once transported to the lab all

samples will be frozen at -70ºC until use in the experiments. Upon completion of the study any remaining samples will be discarded. Once the study is completed all personal information/documents will be properly destroyed so as to protect your privacy.

When you agree for the first time to be a part of this study we will ask if you are interested in being contacted for future samples. This study is designed in such a way that provides an opportunity to collect more than one sample from the same individual at two or more time points (at upcoming pelvic exam appointments), and as such requires us to obtain some personal identifying information from you, including name, age, ethnicity, contact information. Your name and contact information will only be accessed by the project coordinator or the principal investigator should you be contacted for future sample collection.

Risks and Discomfort:

This study is designed to be as non-invasive as possible and minimize potential discomfort. The physician will be collecting samples from within the vaginal and ectocervix (portion of cervix facing into the vagina) and will therefore only require a wash of the inside of the vagina. There may be a small amount of discomfort during the procedure; however this will be no more uncomfortable than a regular pelvic exam (Pap smear).

There is a chance that some of the questions (in the questionnaire) pertaining to your past pregnancy history or sexual history, may cause you to become emotionally upset. You are asked to answer the questions to the best of your ability however if there are things that are too difficult for you to discuss you have the right to omit these questions. If there is a significant portion of the questions left unanswered, leading to lack of important information, making it hard to use your samples in the study, you may be removed from the study and informed of this decision by the study coordinator before the doctor collects the samples from you.

Benefits:

By participating in this study you will be contributing to the understanding of the natural environment of the female genital tract during HIV-1 infection that may lead to the development of an effective microbicide (substance that will reduce rate of infection by HIV). Although there may not be any direct benefit to you from participating in this study, we hope that the information obtained from this study will benefit other people in leading to an effective preventative measure against HIV-1.

Any publications in scientific journals related to this study will be made available to you through the physician involved in this study. If you wish to be notified by email of any publications please include your personal email on the study questionnaire.

Confidentiality:

The consent forms, containing identifying information, will be locked in a filing cabinet, in a locked office at the research facility and will be kept in this location for the duration of the study. The project coordinator and principal investigator will be the only two people with access to these files, which means that not even the physician will be viewing any of these personal documents. However, the University of Manitoba Health Research Ethics Board may review laboratory records related to the study for quality assurance purposes. Upon collection, your cervical vaginal lavage/fluid samples will be given an arbitrary laboratory code for identification that will only be linked to your indentifying information on one computer. This computer will be password locked and the spreadsheets will be inaccessible to anyone other than the research coordinator and the principal investigator. For laboratory use, the laboratory code will be the only form of identification. Information such as age, ethnicity and phase of menstrual cycle may be used when analyzing or publishing research data

Voluntary Participation / Withdrawal from the Study:

Your decision to take part in this study is 100% voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision not to participate or to withdraw from the study will not affect

your care at this medical centre in any way. If the study staff feels that it is in your best interest to withdraw you from the study they will remove you without your consent.

Medical Care for Injury Related to the Study:

In the case of injury or illness resulting from this study, necessary medical treatment will be available to you at no cost.

You are not waiving any of your legal rights by signing this consent form, and you are not releasing the investigator or the sponsor from their legal and professional responsibilities.

Additional Comments:

Please remember that your participation in this study is completely voluntary and that you may refuse to participate or withdraw at anytime by contacting one of the researchers listed in the "questions" section of this form. This will not affect any aspect of your physician's exam, assessment or further treatment.

Questions:

You are free to ask any questions that you may have about the research study or the procedure that is involved in obtaining CVL samples. If you should have additional concerns once you leave the medical centre today or if at any point you wish to withdraw from the study you may contact the principal investigator directly, Dr. Terry Blake Ball ([redacted]. You are also free to contact the University of Manitoba Health and Research Ethics Board at 789-3255 with any concerns you may have.

Consent Form:

MEDICAL CLINIC ADDRESS_____ PHYSICIAN'S NAME_____

PARTICIPANT CODE:_____

PARTICIPANT NAME: _____

Please fill out the following form should you decide to participate in this study.

I, _____(please print), have read or have had read to me, the "Participation Information and Consent Form" for the above study and have discussed the study with _____(study coordinator). I have had my questions answered by them in a language that I understand. The risks and benefits have been explained to me. I believe that I have not been unduly influenced by any study team member to participate in this research study by any statements or implied statements. I understand that my participation in this study is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study. Finally, I understand that information regarding my personal identity will be kept confidential. I authorize release of any of my records that relate to this study to the University of Manitoba Research Ethics Board for quality assurance purposes.

By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

I agree to be contacted for future follow-up in relation to this study Yes_____ No_____

If Yes Please fill in your contact information:
PERMANENT ADDRESS_____
(Street number, Street Name, City, Province, Postal Code)

CONTACT PHONE #: (____)_____

If you would like to have scientific journal publications, relating to this study, sent to you via email please provide your email here: _____

Signature: _____ **Date:** _____
(day/month/year)

For Staff Only:

PARTICIPANT CODE:_____

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has knowingly given their consent.

Printed Name: _____ **Date;_____**
(day/month/year)

Signature:_____

Role in the study:_____

Alpha Numeric Code: _____

11.7.3 Winnipeg local donor cohort (University of Manitoba)

Control Samples for Immune Studies of HIV Donor # _____

Participant Information and Consent form

Title of study: Control Samples for Immune Studies of HIV

Investigators:
Dr. Keith R. Fowke, University of Manitoba, Department of Medical Microbiology, 539-730 William Ave. Winnipeg, MB, Canada tel. (204) 789-3818 fax (204) 789-3926.

Introduction:
You are being asked to participate in a basic science research program for which the studies have been reviewed by the research ethics board of the University of Manitoba. Please take your time in reading and listening to the description of this study. You are encouraged to ask any questions you may have to the study coordinator that is conducting this interview. You may take your time to make your decision about participating in this research study and you may discuss it with your regular doctor, friends, and family before you make your decision. If you do not understand any words being used please ensure that you ask for clarification from the staff. If you decide to join this study, you are free to withdraw from it at any time.

Purpose of the Studies: HIV is the virus that causes AIDS. The research studies being performed by Dr. Fowke's and Dr. Ball's group are being done to increase our understanding of HIV infection, in particular, why some people fight off the infection better than others. The goal of our research is to understand the mechanisms of defects in the immune system incurred in HIV infection, and to determine the role of the immunity in preventing HIV infection. To accomplish these objectives, we need to develop methods to assess the immune response. The purpose of these studies is to provide normal healthy samples for ongoing immunological and genetic studies.

The objective is to obtain fresh blood from normal healthy donors. The white blood cells will be separated from your blood and frozen or used fresh. Your cells could be used to measure normal immune response against HIV, or they could be used to measure normal immune responses in general, and they could also be used as a cell source for cultivation for HIV in the lab. Some of your blood cells may be made into permanent cell lines that grow continuously in the test tube. In a few instances, the immune response in some donors blood is readily detectable. These cells may be kept alive by making cell-lines, and used to test whether our experimental system is working properly. These cell lines will be kept and used only by the researchers participating in the study. These cells and your blood cells may also be used as a source of genetic material and used in studies of genetic factors involved in susceptibility to infectious agents, such as HIV.

Study Procedures:
Following the reading of this information if you choose to participate in this study you will sign this informed consent form. A date will then be arranged for you to come back and donate 3 to 6 tablespoons (50 -100 mls) of blood. You may donate blood more than twice a month and donations must be at least two weeks apart. The study coordinator, a certified laboratory medical technologist, will draw the blood, code it and give it to the lab staff for use in the research work. Your blood **may** be tested for the presence of HIV using an experimental in-house test. This test is done if we discover some HIV specific cellular responses while conducting our research. If your blood sample tests positive using our experimental in-house HIV test you will be contacted and advised to seek medical attention. If you request support in indentifying a physician, we will assist you in that process. If you tested positive for HIV infection, no additional blood sample will be requested for these studies. As a participant in these studies, you will not be able to receive any of the data directly relating to the study of your blood. However, so that you can know what your blood is being used for we would be glad to share with you the compiled data from a number of control donors such as yourself. Any publications arising from these studies will be made available to you. The samples collected will only be used in research studies that are approved by the Research Ethics Board at the University of Manitoba. All samples will be held for a period not to exceed 10 years.

Potential Risks and Discomforts:
This study requires the use of your blood. In order to get the blood we will need to insert a needle into a vein in your arm so that the blood can be removed. There will be some pain associated with the needle stick but this will be only for a short period of time. There may be some bruising around the needle site and, although we will sterilize the site to minimize infection, there is a very minimal risk of infection at the site.

When you donate your blood for genetic testing or research, you are sharing genetic information, not only about yourself, but also about biological (blood) relatives who share your genes or DNA.
Risks to insurability and employability could result from unintended disclosure of data associated with the specimens or generated from the analysis. For example: There is a potential risk of unintended disclosure of confidential information to parties outside the research context that might affect your ability to get insurance or a job. However, these risks are quite remote since appropriate confidentiality measures aree taken to protect any information about your health that is revealed by your DNA sample.

You should be aware that genetic information cannot be protected from disclosure by court order. Due to the rapid pace of technological advances, the potential future use of genetic information is unknown and therefore the potential future risks also are unknown.

Benefits:
By participating in this study, you will be providing information to the study researchers on how the immune system interacts with infectious agents such as HIV. We hope the information learned from this study will benefit individuals exposed or infected with such agents. There will not be a direct medical benefit to you for participating in this study.

Costs:
There will be no costs to you.

Payment for participation:
To compensate you for any inconvenience incurred by the donation, you will receive $2 for each tube of blood you donate.

Confidentiality:
Information gathered in this research study may be published or presented in research reports; however, your name will not be used or revealed. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Organizations that may inspect and /or copy your research records for quality assurance and data analysis include the University of Manitoba Research Ethics Board.

Voluntary Participation/Withdrawal from the Study:
Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw at any time. Your decision to not participate or to withdraw from the study will not affect any aspect of your association with the Department of Medical Microbiology or the University of Manitoba.

If at any time you wish to withdraw the samples related to you from the studies, the samples that you provided with the ID code assigned to you will be properly destroyed and disposed of by the study coordinator.

Medical Care for Injury Related to the Study:
In the case of injury or illness resulting from this study, necessary medical treatment will be available at no cost to you.

Questions:

If you have any questions during or after the study about your treatment or your rights as a research participant you are encouraged to direct them to the study principal investigator Dr. Keith Fowke, (204) 789-3818, 539-730 William Ave. You are also free to direct any comments or concerns you can to the University of Manitoba Research Ethics Board at 789-3389.

Do not agree to this study unless you have had a chance to ask question and have received satisfactory answers to all of your questions.

Control Samples for Immune Studies of HIV Donor # _____

Statement of Consent:
If you agree to participate in the study, please sign below.

I, _____, have read or have had read to me, the consent form for the above study and have discussed the study with _____.

I understand that the following (check the box only if you fully understand and agree with each statement):

- ☐ the goals of this research program are to study the immune response in normal individuals such as yourself and compare that to those infected and exposed to HIV.
- ☐ enrolment is completely voluntary and I can withdraw from the study at any time
- ☐ I am in good health and do not have any known immunodeficiency
- ☐ blood specimens will be required for this study and they will be used fresh or stored for later use
- ☐ my sample may be tested by an experimental in-house HIV assay. I will be advised to seek clinically proven HIV-test from my attending physician if the in-house HIV assay result is positive for my blood sample.
- ☐ I agree that my blood specimens, and any stored specimens, may be used for the generation of cell lines and for studies of the genetics of susceptibility to infectious agents
- ☐ I agree that genetic testing specifically concerned with susceptibility or resistance to HIV infection may be done on the blood drawn on for this study. Yes No
- ☐ I agree that my blood will be stored for up to 10 years Yes No

I am willing to participate in the study.

Name of Study Participant_____

Signature: _____ Date: _____

Contact Information: Telephone: Work: _____ Cell: _____

e-mail _____

For staff:

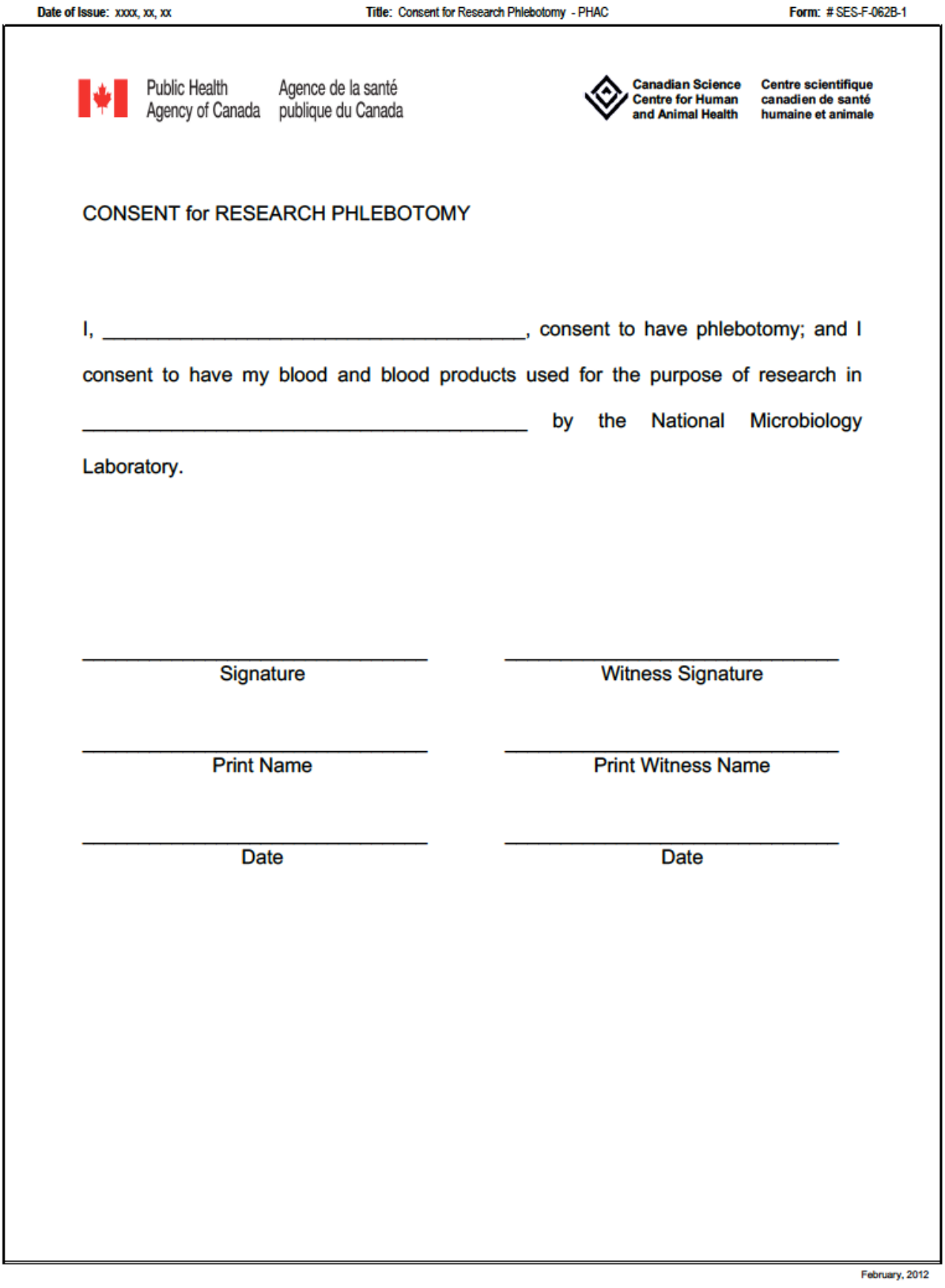
I, _____, have explained the nature and purpose of the above study to _____

Name of Staff: _____

Signature: _____ Date: _____

Assigned Study Number ____ ____ ____

11.7.4 Winnipeg local donor cohort (Public Health Agency of Canada)



Date of Issue: xxxx, xx, xx **Title:** Consent for Research Phlebotomy - PHAC **Form:** # SES-F-062B-1

Public Health Agency of Canada / Agence de la santé publique du Canada

Canadian Science Centre for Human and Animal Health / Centre scientifique canadien de santé humaine et animale

CONSENT for RESEARCH PHLEBOTOMY

I, _____, consent to have phlebotomy; and I consent to have my blood and blood products used for the purpose of research in _____ by the National Microbiology Laboratory.

_____ Signature

_____ Witness Signature

_____ Print Name

_____ Print Witness Name

_____ Date

_____ Date

February, 2012

11.8 Permissions for reuse of published journal figures

11.8.1 Permission for Figure 7

NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS

Mar 13, 2016

This is a License Agreement between Lindsay Aboud ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3827370939065
License date	Mar 13, 2016
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Genetics
Licensed content title	Human genetics and disease: Serpinopathies and the conformational dementias
Licensed content author	David A. Lomas, Robin W. Carrell
Licensed content date	Oct 1, 2002
Volume number	3
Issue number	10
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 1. Conformational transition of serpin family members
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Defining the HIV neutralizing activity of antiproteases within the female genital tract and evaluating the HIV inhibitory mechanism of Serpin B1
Expected completion date	May 2016
Estimated size (number of pages)	400
Total	0.00 CAD
Terms and Conditions	

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this

11.8.2. Permission for Figure 8, Figure 10, and Figure 11

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Mar 14, 2016

This Agreement between Lindsay Aboud ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	3764300601000
License date	Dec 08, 2015
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	American Journal of Reproductive Immunology
Licensed Content Title	The Role of Serpin and Cystatin Antiproteases in Mucosal Innate Immunity and their Defense against HIV
Licensed Content Author	Lindsay Aboud,Terry Blake Ball,Annelie Tjernlund,Adam Burgener
Licensed Content Date	Oct 31, 2013
Pages	12
Type of Use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	3
Original Wiley figure/table number(s)	Figure 1- The intracellular and extracellular role of serpins against inflammation and HIV infection Figure 3- The intracellular and extracellular role of cystitis against inflammation and HIV infection Figure 6- Hypothesis of protective mechanisms offered by antiproteases against inflammation and HIV infection at mucosal surfaces
Will you be translating?	No
Title of your thesis / dissertation	Defining the HIV inhibitory mechanism of Serpin B1 and evaluating the HIV neutralization activity of antiproteases within the female genital tract
Expected completion date	Mar 2016
Expected size (number of pages)	440
Requestor Location	
Billing Type	
Billing Address	

11.8.3 Permission for Figure 9

From: **Christine Foyer**
Subject: Re: Reuse of a figure from 2013 Agronomy article
Date: March 16, 2016 at 3:51 AM
To: Lindsay Aboud
Cc: Karl Kunert

CF

Dear Lindsay

Thank your for your message and the information about your dissertation. I am happy to give you my permission to re-use the figure that you have mentioned, as long as the origin of the figure is correctly cited in the figure legend. I have copied this reply to my co-authors for information.

Good luck with your dissertation

Christine

From: Lindsay Aboud
Sent: 14 March 2016 06:58
To: Christine Foyer
Subject: Reuse of a figure from 2013 Agronomy article

Hello Dr. Foyer,

I am a PhD student from the University of Manitoba, and am currently finishing the writing of my dissertation, in which I would like to reuse a figure from one of your articles. I had previously contacted the journal of Agronomy for permission to reuse the figure, however they informed me that since the journal is OpenAccess, it would be more appropriate for me to petition you directly for permission. The specific details are as follows:

Journal: Agronomy

The figure was published in the 2013 issue 3(3): 550-570.

The title of the article is: **The cysteine protease-cysteine protease inhibitor system explored in soybean nodule development.**

Publication authors: Barend Juan Vorster, Urts Schluter, Magdeleen du Plesses, Stefan van Wyk, Matome Eugene Makgopa, Ignatious Ncube, Marian Dorcas Quain, Karl Kunert and Christine Helen Foyer

Figure for reuse: **Figure 2. Cystatin binding to the active site of a cysteine protease**

I would be using this figure in the introduction of my doctoral thesis entitled: **Defining the HIV neutralizing activity of antiproteases within the female genital tract and evaluating the HIV inhibitory mechanism of Serpin B1**

The thesis will be submitted to the Department of Medical Microbiology at the University of Manitoba. Once accepted and published this thesis will be made available to the general public on the university website and will be printed in hardcopy.

If you could please provide with me any information that would allow me to complete the process of attaining formal permission for the use of this figure, I would very much appreciate it.

Thank you so much for your time

Lindsay Aboud
PhD Candidate
University of Manitoba

“I alone cannot change the world, but I can cast a stone across the waters to create many ripples.” ~Mother Teresa